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ORIGINAL ARTICLE

mTOR and ERK regulate VKORC1 expression in both hepatoma cells and hepatocytes which influence blood coagulation

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Abstract

Deficiency of γ-glutamyl carboxylation of coagulation factors, as evidenced by the elevated level of Des-γ-carboxyl prothrombin (DCP), is a common feature in hepatocellular carcinoma patients. Additionally, treatment of cancer patients with mTOR inhibitors significantly increases hemorrhagic events. However, the underlying mechanisms remain unknown. In the present study, Vitamin K epoxide reductase complex subunit 1 (VKORC1) was found to be significantly down-regulated in clinical hepatoma tissues and most tested hepatoma cell lines. In vitro investigations showed that VKORC1 expression was promoted by p-mTOR at the translational level and repressed by p-ERK at the transcriptional level. By exploring Hras12V transgenic mice, a hepatic tumor model, VKROC1 was significantly down-regulated in hepatic tumors and showed prolonged activated partial prothrombin time (APTT). In vivo investigations further showed that VKORC1 expression was promoted by p-mTOR and repressed by p-ERK in both hepatoma and hepatocytes. Consistently, APTT and prothrombin time were significantly prolonged under the mTOR inhibitor treatment and significantly shortened under the ERK inhibitor treatment. Conclusively, these findings indicate that mTOR and ERK play crucial roles in controlling VKORC1 expression in both hepatoma and hepatocytes, which provides a valuable molecular basis for preventing hemorrhage in clinical therapies.

Keywords Hepatocellular carcinoma · Coagulopathy · VKORC1 · mTOR · ERK

Abbreviations

APTT	Activated partial prothrombin time
DCP	Des-γ-carboxyl prothrombin
ERK	Extracellular signal-regulated kinase
GGCX	Gamma-glutamyl carboxylase
HCC	Hepatocellular carcinoma
mTOR	Mammalian target of rapamycin
NGS	Next-generation sequencing
P	Hepatic tumor-adjacent normal liver tissues
PT	Prothrombin time
Ras-Tg mice	Hras12V transgenic mice
T	Hepatic tumor tissues
TF	Tissue factor
VK	Vitamin K
VKDB	Vitamin K deficiency-related bleeding
VKORC1	Vitamin K epoxide reductase complex subunit 1
Wt	Wild-type mice or normal liver tissues of wild-type mice

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Novelty and impact

1. The disorders of coagulation factors are important factor for clinical coagulopathy of HCC patients.
2. Hepatic tumor is an independent factor for coagulopathy of Hras12V transgenic mice.
3. VKORC1 is significantly decreased in hepatoma cells at both mRNA and protein levels.
4. mTOR and ERK pathways control the expression level of VKORC1 in both hepatoma cells and hepatocytes, which determined the activated partial prothrombin time (APTT) and prothrombin time (PT) in Hras12V transgenic mice.

Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer and the third leading cause of cancer death worldwide [1]. The most common complication with HCC patients is coagulopathy [2, 3] which increased morbidity and mortality in patients undergoing abdominal surgery [4]. HCC patients with coagulopathy are usually recognized as the results of cirrhosis [5] from which over 80% HCC raised [6]. Nevertheless, whether HCC is an independent factor for coagulopathy is rare investigated and remains largely unknown.

The Vitamin K (VK) cycle, essential for the γ-glutamyl carboxylation of coagulation factors II, VII, IX and X for their normal biological functions [7], plays crucial roles in coagulation and is the key target for clinical anti-coagulation therapy. The unequivocally identified vital enzymes in the VK cycle are VKORC1 and gamma-glutamyl carboxylase (GGCX) [8]. Evidence from animal models has shown that knocking out VKORC1 or GGCX in mice induces bleeding and death [9, 10]. Specially, Des-γ-carboxyl prothrombin (coagulation factor II) (DCP) is a well-known marker for HCC [11] which indicates the disorders of VK cycle and therefore the deficiency of γ-glutamyl carboxylation of coagulation factors. However, the roles of VKORC1 or GGCX in HCC-associated disorders of VK cycle and coagulopathy have not been indicated.

Independent of multiple risk factors [12], numerous studies have shown that hyperactivation of the Ras/Raf/MEK/ERK and PI3 K/AKT/mTOR pathway frequently occurs in human hepatocarcinogenesis and is associated with an aggressive tumor phenotype and poor prognosis in human HCC [13–15]. In addition, the interaction between the two pathways may play an important role in hepatocarcinogenesis [16]. Accordingly, drugs targeting these pathways represent current main therapy strategies and promising directions for future therapies [17]. Notably, clinical reports have shown that treatment of HCC with sorafenib (a multi-kinase inhibitor) alone or in combination with everolimus (an mTOR inhibitor) significantly increased the frequency of hemorrhagic events [18–21]. However, hemorrhagic toxicity was not observed in patients treated with selumetinib (AZD6244, a MEK inhibitor) [22]. Intriguingly, other cancer patients with normal liver functions and received everolimus also frequently present with hemorrhage [23–27]. By contrast, hemorrhagic toxicity was only reported in endometrial cancer patients (1 in 54) receiving selumetinib [28]. These evidences indicate the possible roles of the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways in clinical coagulopathy regardless of patient complexity. However, the molecular basis is still unknown.

In the present study, we firstly identified that the disorders of coagulation factors are an independent factor for clinical coagulopathy of HCC patients. Then, the expression of VKORC1 in human HCC tissues and cell lines was detected and the effects of ERK and mTOR pathways on the expression of VKORC1 were investigated in vitro. By exploring Hras12V transgenic mice, a hepatic tumor model, we further determined the in vivo mechanism that the ERK and mTOR regulate blood coagulation by controlling VKORC1 expression in hepatocytes and hepatoma cells. These evidences provide a valuable molecular basis for preventing hemorrhage and DCP production in clinical molecular-targeted therapies.

Materials and methods

Patients

The clinical data and 5 matched pairs of HCC and adjacent tissues were collected from the First Affiliated Hospital of Dalian Medical University. The liver and HCC tissues for immunohistochemical analysis were obtained from Alenabio Biotechnology Co., Ltd., and authorized by US Biomax. All procedures we followed on human experimentation were in accordance with the ethical standards of the First Affiliated Hospital of Dalian Medical University Medical Ethics Committee and with the Helsinki Declaration.

Detection of APTT, PT, Ca^{2+} concentration, platelet count in mice blood samples

APTT and PT assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The Ca^{2+} concentration in serum was detected by Automatic Biochemical

Analyzer (Celercare® VI) (Tianjin, China). The platelet count was analyzed by YODER Hemastar3 (France).

Experimental animals, sampling and histopathological examination

The animal experiments and histopathological examination procedures for animal handling and tissue sampling were conducted in compliance with protocols approved by the Animal Care and Use Committee of Dalian Medical University. Hras12V transgenic males [29] and C57BL/6J wild-type males were used for experimental treatments. The samples of the wild-type normal liver tissues (Wt) and hepatic tumor-adjacent normal liver tissues (P) and hepatic tumor tissues (T) of the transgenic mice were removed and flash-frozen in liquid nitrogen. The remaining tissues were used for histopathological analysis as previously described [29]. The Wt, P and T tissues were confirmed by morphological diagnosis and used for subsequent procedures.

Differential gene expression analysis by next-generation sequencing (NGS)

Hepatic tissues from 5 wild-type mice and 5 Hras12V transgenic mice were chosen for total RNA extraction. The total RNA samples from the same group were mixed equivalently to generate three composite samples, i.e., Wt, P and T. Differential gene expression was detected by NGS analysis.

RT-qPCR

The RT-qPCR analysis was performed as previously described [30], and the primers used are shown in Table S1.

Western blotting

The Western blot analysis was performed as previously described [29]. The primary and secondary antibodies used were as follows: anti-VKORC1 (ab176118), anti-GGCX (ab197982) and anti-TF (ab151748) from Abcam, Cambridge, UK; anti-phospho-p44/42 MAPK (#4370) and anti-phospho-mTOR (#5536) from Cell Signaling, Frankfurt, Germany; and HRP-conjugated goat anti-rabbit IgG antibodies from Santa Cruz, CA.

Animal treatment

Hras12V transgenic mice were treated with either AZD6244 (120 mg/kg) intragastrically or rapamycin (30 mg/kg) intraperitoneally twice a day for 10 days. The control transgenic mice were treated with the corresponding solvents. The animals were killed at 3 h after the last dose of AZD6244 or rapamycin, the liver and body weights were recorded, and the tissues and blood samples were harvested for analysis.

Cell culture and treatment

The cell lines were cultured in DMEM containing 10% FBS in 5% CO_2 at 37 °C. Huh7 and MHCC97L cells were treated with AZD6244 (4 μmol/L) or rapamycin (100 nmol/L) for 48 h. HCCLM3 and HepG2 cells were treated with AZD6244 (20 μmol/L) or rapamycin (100 nmol/L) for 48 h. The cells were treated with the same concentrations of solvent (DMSO) for 48 h as controls for every group.

Immunohistochemistry

VKORC1 expression was determined by immunohistochemistry on archived paraffin-embedded sections of tissues (LV803 tissue array, http://www.alenabio.com/tissue-arrays) using the UltraSensitive™ S-P (Mouse/Rabbit) IHC Kit (KIT-9720; Maixin Biotechnology Development Co. Ltd.). A mouse anti-human monoclonal VKORC1 primary antibody (ab176118, Abcam) and biotinylated goat anti-rabbit/mouse polyclonal IgG secondary antibody were used. The staining intensity was scored by a pathologist.

Statistical analysis

The data were presented as mean ± SEM. Differences in means were analyzed by Student's t test. The statistical analysis for the IHC staining intensity score was performed by Kruskal–Wallis H test. P values < 0.05 were considered significant.

Results

Disorders of coagulation factors are important risk factors for coagulopathy of HCC patients

Prolonged coagulation time in HCC patients is a widespread problem [31], and several risk factors have been indicated, such as thrombocytopenia or disorders of coagulation factors [32, 33]. To confirm the symptoms and the causes, the commonly used clinical indexes such as platelet count, APTT, PT, concentration of Ca^{2+} and fibrinogen collected from 120 HCC patients were analyzed (Table S2). In our investigation, the platelet count of only 21(18.42%) HCC patients was under the lower limit (Table S2). Moreover, several investigations have reported that the risk of serious bleeding does not occur until the count less than 20,000 platelets per microliter and mild bleeding sometimes occurs when the count is less than 50,000 platelets per microliter [33, 34]. According to these criterions, only 6.14% of the patients are

less than 50,000 platelets per microliter and none of them are less than 20,000 (Table S2). The results indicate that thrombocytopenia may not be the reason for coagulopathy of those HCC patients.

Additionally, consistent with previous reports, our investigation showed that the incidence of prolonged APTT and/or PT in the HCC patients was 62.5% (75/120) (Fig. 1a). While no significant difference was detected in the concentration of Ca^{2+} and fibrinogen, two commonly used clinical indexes could affect APTT/PT (Fig. 1b, c). As the APTT/PT is determined by coagulation factors, these results indicate that disorders of coagulation factors may be the important risk factors for coagulopathy, but not Ca^{2+} and fibrinogen.

Decreased VKORC1 expression is a common event in the human HCC tissues and cell lines

As the rate-limiting enzymes VKORC1 and GGCX of Vitamin K (VK) cycle are essential for the γ-glutamyl carboxylation of coagulation factors for their normal biological functions, the expression levels of these enzymes were verified in human HCC tissues. First, 5 pairs of HCC tissue samples and their adjacent non-cancer tissue samples obtained by clinical surgery were examined (Table S3). Interestingly, VKORC1 expression was significantly decreased at both mRNA and protein levels in HCC tissues compared to adjacent non-cancer tissues (Fig. 2a, b). For GGCX, although there was no obvious difference between HCC tissues and adjacent non-cancer tissues at the mRNA levels, 3 out of 5 HCC tissues showed higher protein levels compared to their adjacent non-cancer tissues (Fig. 2a, b). It indicates that VKORC1 may play a key role in the disorders of γ-glutamyl carboxylation of coagulation factors in HCC tissues, but not GGCX.

Second, an LV803 tissue array including 24 liver tumor tissues (19 HCC tissues and 5 cholangiocellular carcinoma (CCC) tissues) and 48 non-tumor (NT, normal or tumor-adjacent) tissues was used to detect VKORC1 expression by immunohistochemical analysis. Positive VKORC1 staining was only detected in hepatocytes and liver tumor cells, but not in negative controls, tumor mesenchymal and inflammatory cells, confirming the reliable performance of the VKORC1 antibody in the immunohistochemical analysis (Fig. 2c; Fig. S1-2). As expected, the staining intensity scores of VKORC1 were significantly lower in HCC tissues ($n=19$) compared to NT tissues ($n=48$) ($P=0.003$) (Fig. 2c; Fig. S2; Table 1; Table S4). Moreover, there was no difference between cirrhosis tissues ($n=16$) and non-cirrhosis tissues ($n=32$) ($P=0.563$) in NT tissues (Table 1). These results indicate that VKORC1 expression was significantly decreased in HCC tissues.

In addition, the human HCC cell lines (MHCC97L, Huh7, HepG2, HCCLM3, MHCC97H and PLC/PRF/5) were explored to detect the mRNA and protein abundances of VKORC1 and GGCX, with primary human hepatocytes from HCC patients as controls. Consistently, VKORC1 was down-regulated at both the mRNA and protein level in all HCC cell lines compared to primary human hepatocytes except MHCC97L, in which no change was observed at the mRNA level (Fig. 3a, c). GGCX was overexpressed at both mRNA and protein levels in all cell lines (Fig. 3b, c), which is consistent with 3 clinical HCC tissue samples at protein levels (Fig. 2b). These results indicate that VKORC1 may be the central factor related to an ineffective VK cycle in HCC cells.

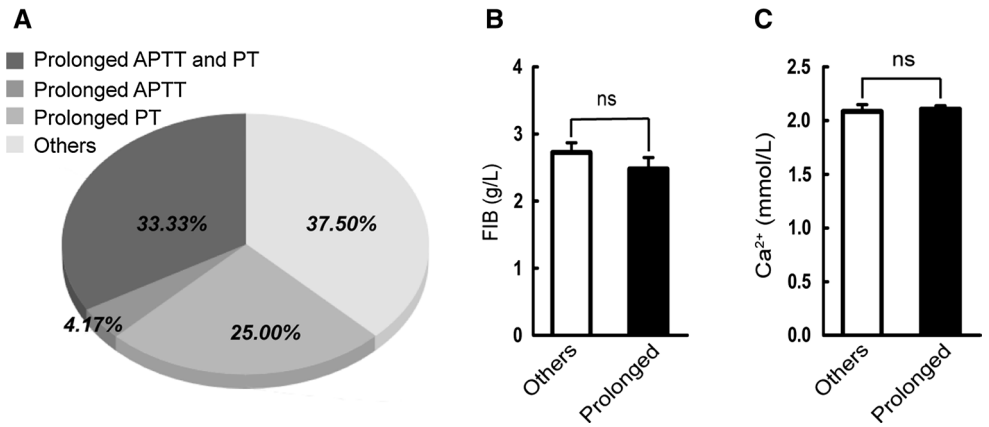

Fig. 1 Disorders of coagulation factors are important factor in the majority of coagulopathic HCC cases. The clinical data of 120 HCC patients collected from the First Affiliated Hospital of Dalian Medical University during 2002–2016 were analyzed. According to APTT and PT, patients were divided into two groups, prolonged: prolonged APTT and/or PT group ($n=75$) and others: normal APTT/PT group ($n=45$). **a** The proportion of patients with prolonged APTT and/or PT in 120 HCC patients. The plasma fibrinogen (**b**) and concentration of Ca^{2+} (**c**) in the blood of patients in the two groups. The data are expressed as mean ± SEM. $P<0.05$ is considered to be significant. ns, nonsignificant difference. The detailed clinical data are shown in Table S2

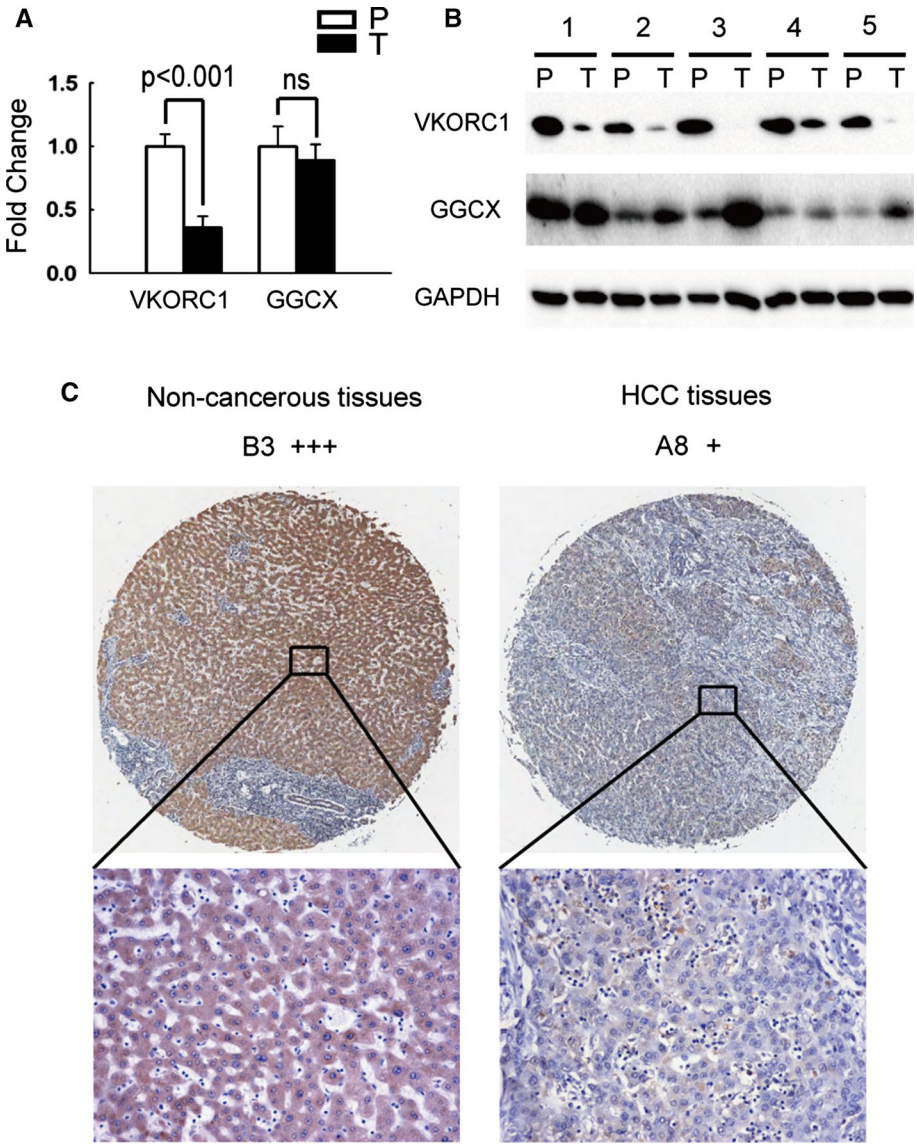

Fig. 2 The expression levels of VKORC1 are down-regulated in human HCC tissues. **a** The mRNA levels of VKORC1 and GGCX in HCC tissues and paired tumor-adjacent tissues were detected by RT-qPCR. The mRNA levels of genes were normalized to 18S RNA. The data are expressed as mean ± SEM ($n = 5$). $P < 0.05$ is considered to be significant. ns, nonsignificant difference. **b** The protein levels of VKORC1 and GGCX were detected by Western blotting. GAPDH was used to ensure equal protein loading. The numbers 1–5 indicate separate individual patients. P: tumor-adjacent tissues; T: HCC tissues. (C) The representative immunohistochemical images show that the VKORC1 staining intensity was down-regulated in hepatocellular carcinoma tissues (the right panels) compared to non-cancerous tissues (the left panels). (upper) ×20; (lower) ×200. The symbols above the images represent the positions and staining intensity scores of the samples in the LV803 tissue array. The detailed LV803 tissue array information is shown in Fig. S2 and Tables S3, 4

Table 1 The VKORC1 expression grade in HCC and cirrhosis tissues

Parameters	Case number	VKORC1 expression			*P* value
		Low +	Middle ++	High +++	
Cancer					
HCC	19	3 (15.8%)	16 (84.2%)	0 (0%)	0.003
NT[a]	48	3 (6.3%)	28 (58.3%)	17 (35.4%)	
Cirrhosis					
Cirrhosis	16	0 (0%)	10 (62.5%)	6 (37.5%)	0.563
Non-cirrhosis	32	3 (9.4%)	18 (56.3%)	11 (34.3%)	

[a]Normal or tumor-adjacent

The expression of VKORC1 is repressed by ERK and promoted by mTOR in human HCC cells

The human HCC cells showed over-activated ERK and mTOR compared to primary human hepatocytes (Fig. 3c). To investigate the possible roles of the ERK and mTOR pathways in regulating VKORC1 expression in human HCC cells, MHCC97L, Huh7, HepG2 and HCCLM3 cells were treated with ERK and mTOR inhibitors separately or in combination. When p-ERK was inhibited, the mRNA and

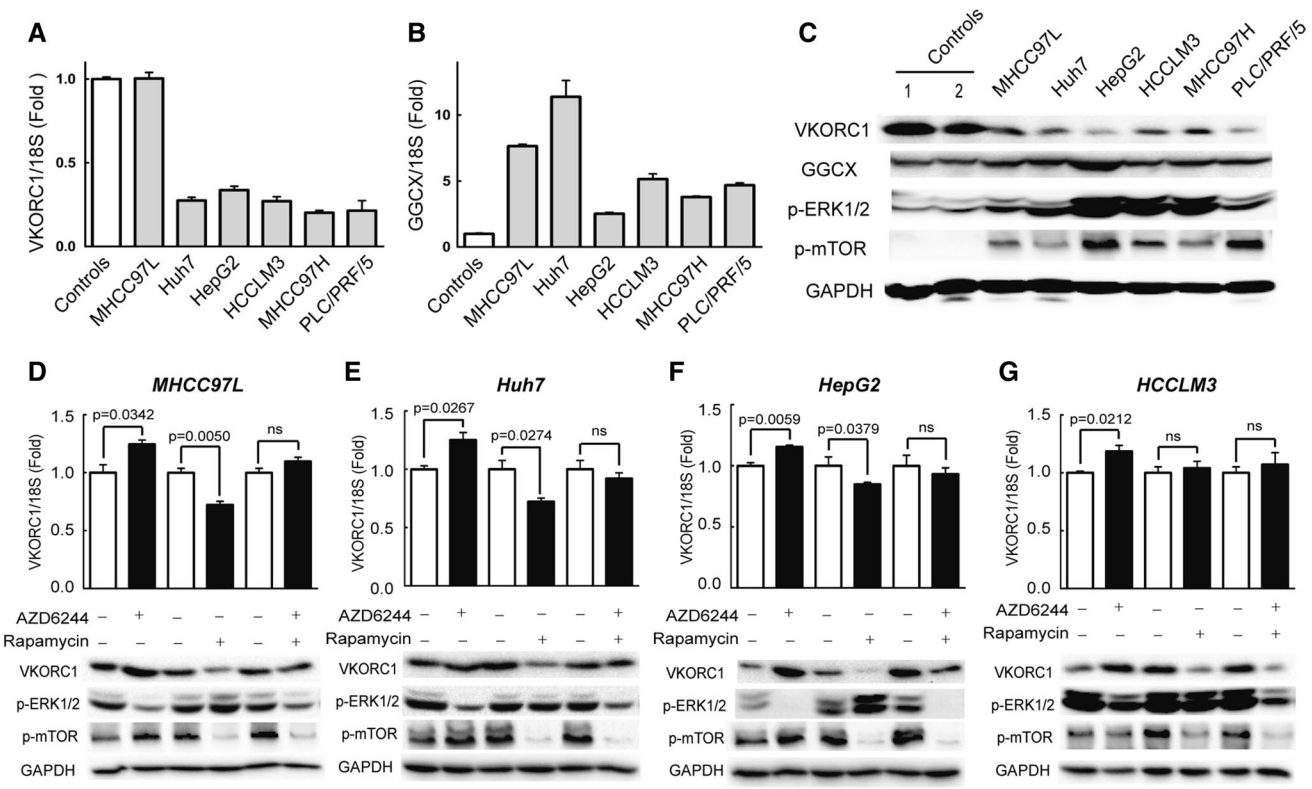

Fig. 3 VKORC1 expression is regulated by the ERK and mTOR pathways in human HCC cells. The mRNA levels of VKORC1 (**a**) and GGCX (**b**) in human primary human hepatocytes and the human HCC cell lines MHCC97L, Huh7, HepG2, HCCLM3, MHCC97H and PLC/PRF/5 were detected by RT-qPCR. **c** The protein levels of VKORC1, GGCX and the activation levels of ERK and mTOR were detected by Western blotting. Human primary hepatocytes of two separate individuals were used as the controls. The cells were treated with the ERK inhibitor AZD6244 and the mTOR inhibitor rapamycin independently or in combination for 48 h. The mRNA levels of VKORC1 in MHCC97L (**d**), Huh7 (**e**), HepG2 (**f**) and HCCLM3 (**g**) cells were detected by RT-qPCR (up panels). Correspondingly, the protein levels of VKORC1 and the activation levels of p-ERK, p-mTOR in MHCC97L (**d**), Huh7 (**e**), HepG2 (**f**) and HCCLM3 (**g**) cells were detected by Western blotting (down panels). GAPDH was used to ensure equal protein loading. The mRNA levels of genes were normalized to 18S RNA. The data are expressed as mean ± SEM of two independent experiments. $P < 0.05$ is considered to be significant. ns, nonsignificant difference

protein expression of VKORC1 was significantly increased compared to untreated cells (Fig. 3d–g). Conversely, when p-mTOR was inhibited, the mRNA and protein levels of VKORC1 were significantly decreased compared to untreated cells except in HCCLM3 cells, which exhibited no change at the mRNA level (Fig. 3d–g). When p-ERK and p-mTOR were inhibited simultaneously, VKORC1 exhibited no significant change at the mRNA level but was obviously down-regulated at the protein level in all cells (Fig. 3d–g). These results indicate that the VKORC1 expression is repressed by ERK and promoted by mTOR in human HCC cells.

In addition, when p-ERK was inhibited, p-mTOR levels decreased in HCCLM3 cells but increased in the other cells (Fig. 3d–g). When p-mTOR was inhibited, p-ERK levels were elevated in MHCC97L and HepG2 cells (Fig. 3d, f), but no change was observed in the other cells (Fig. 3e, g). These results indicate that the interaction between ERK and mTOR is complicated and this may be due to the complex pathogenic mechanisms in human hepatocarcinogenesis.

Coagulation defects are characteristic features in Ras-Tg mice carrying hepatic tumors

To further confirm the underlying mechanisms in vivo, Hras12V transgenic (Ras-Tg) mice, a hepatic tumor model induced by hepatocyte-specific expression of *Hras12V* oncogene, were explored (Fig. 4a, b). Firstly, we investigated whether Ras-Tg mice carrying hepatic tumors have coagulation defects. As expected, the results showed that APTT in 9-month-old Ras-Tg mice carrying hepatic tumors was significantly prolonged compared to non-transgenic mice (Fig. 4c). However, the PT of the mice has no significant change (Fig. 4d), and this may result from a high concentration of tissue factor (TF) secreted from tumor cells (Fig. S3A) [35, 36]. Additionally, coagulation defects did not

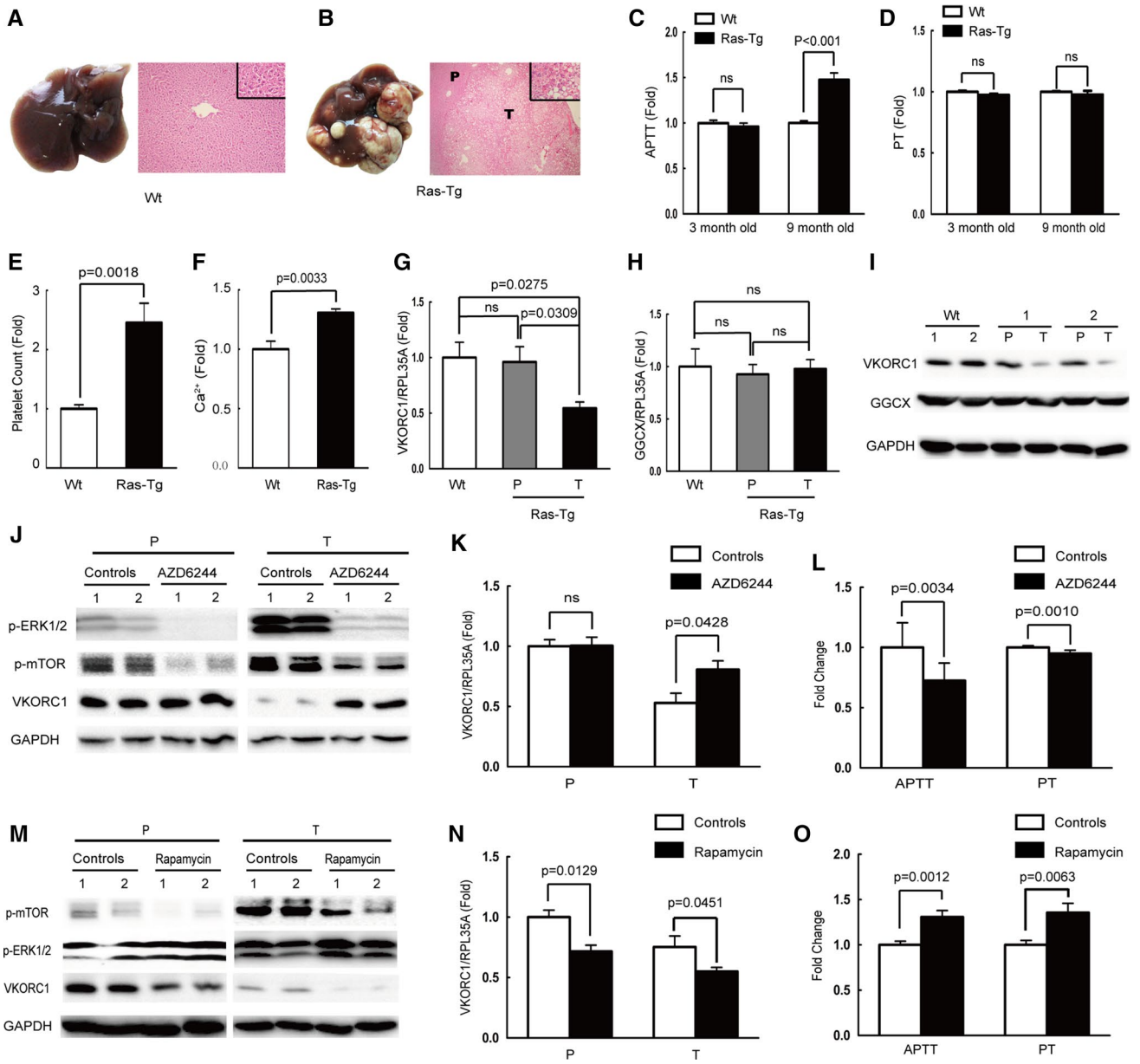

Fig. 4 mTOR and ERK contribute to the coagulation defects in Hras12V transgenic mice by controlling the expression of VKORC1. Representative liver stereogram images (left panel) and histopathological images (right panel) of a 9-month-old wild-type mouse (Wt) (**a**) and a 9-month-old Hras12V transgenic (Ras-Tg) mouse carrying multiple hepatic tumors (**b**). Histopathological images are of H&E-stained tissues. Magnifications: ×40; ×100 for the upper right corner. APTT (**c**) and PT (**d**) were detected in 3- and 9-month-old Wt and Ras-Tg mice ($n=8$). The platelet count (**e**) and the concentration of serum Ca^{2+} (**f**) were detected in the blood of 9-month-old Ras-Tg mice carrying hepatic tumors and Wt ($n=5$). The mRNA expression levels of VKORC1 (**g**) and GGCX (**h**) in the normal liver tissues of wild-type mice (Wt) and in peri-tumor tissues (P) and hepatic tumor tissues (T) of Ras-Tg mice were detected by RT-qPCR ($n=5$). **i** The protein levels of VKORC1 and GGCX were detected by Western blotting. After the 8-month-old Ras-Tg mice were treated with the ERK inhibitor AZD6244 for 10 days, the activation levels of ERK and mTOR and the protein levels of VKORC1 in peri-tumor tissues and hepatic tumor tissues were detected by Western blotting (**j**), and the mRNA levels of VKORC1 were detected by RT-qPCR (**k**). APTT and PT of mouse anti-coagulated plasma samples were also analyzed (**l**). After the 8-month-old Ras-Tg mice were treated with the mTOR inhibitor rapamycin for 10 days, the activation levels of ERK and mTOR and the protein levels of VKORC1, p-ERK and p-mTOR (**m**) and mRNA levels of VKORC1 (**n**), APTT and PT (**o**) were detected as described above. The samples obtained from the 8-month-old Ras-Tg mice treated with the corresponding AZD6244 or rapamycin solvents were used as controls ($n=5$). $P<0.05$ is considered to be significant. ns, nonsignificant difference. The data are expressed as mean ± SEM. The numbers 1–2 indicate separate individuals. The mRNA levels of genes were normalized to RPL35A. GAPDH was used to ensure equal protein loading

occur in 3-month-old Ras-Tg mice without hepatic tumors (Fig. 4c). These results indicate that coagulation defects are related to not only HCC patients but also HCC mice, and there may be similar mechanisms behind them. Specially, it indicates that HCC may be an independent cause for coagulation defects.

Reduced VKORC1 expression in hepatic tumors is coupled with coagulation defects in Ras-Tg mice

To investigate the mechanisms behind coagulation defects, coagulation-related factors were screened in the transgenic mice carrying hepatic tumors. In contrast to the coagulation defects, platelet counts and Ca^{2+} concentrations were significantly increased in the blood of transgenic mice and hence seem to contribute to blood clotting (Fig. 4e, f). This result indicates that platelets and Ca^{2+} may not be the dominant elements for hepatic tumor-related coagulation defects in Ras-Tg mice and this is consistent with the clinical phenotype of HCC patients (Fig. 1).

Also in contrast to coagulation defects, the mRNA levels of most coagulation-related genes were significantly up-regulated, while the mRNA levels of most anti-coagulation-related genes showed no significant changes in peri-tumor tissues (P) and hepatic tumor tissues (T) of Ras-Tg mice compared to liver tissues of non-transgenic mice (Wt), as detected by NGS analysis (Table S5). Additionally, GGCX showed no significant change at the mRNA and protein levels among P, T and Wt (Fig. 4h, i; Table S5). Although there were differences among the human and mouse HCC tissue samples and the in vitro HCC cell lines resulting from the different experimental systems, the unchanged or increased GGCX levels indicate that GGCX may not play an important role in the disorders of γ-glutamyl carboxylation of coagulation factors in HCC tissues. Taken together, these results indicate that the expression levels of coagulation-related genes, anti-coagulation-related genes and GGCX may not be the dominant factors for coagulation defects in Ras-Tg mice.

However, in accordance with the coagulation defects, VKORC1 was significantly down-regulated in T compared to P and Wt at both mRNA and protein levels (Fig. 4g, i; Table S5). These results indicate that down-regulation of VKORC1 in hepatic tumors may be the dominant factor contributing to coagulation defects in Ras-Tg mice.

ERK and mTOR pathways affect APTT and PT by regulating VKORC1 expression in Ras-Tg mice

ERK and mTOR were significantly activated in the T of Ras-Tg mice (Fig. S4). To investigate whether activation of ERK and mTOR contributes to the regulation of VKORC1 expression, we treated 8-month-old Ras-Tg mice with AZD6244 (an inhibitor of ERK) or rapamycin (an inhibitor of mTOR) for 10 days. AZD6244 treatment significantly reduced p-ERK and p-mTOR levels in P and T tissues compared to untreated controls, respectively (Fig. 4j). Interestingly, in AZD6244-treated T tissues, VKORC1 was significantly up-regulated at both the mRNA and protein level compared to untreated controls (Fig. 4j, k). In AZD6244-treated P tissues, VKORC1 exhibited no obvious change at the mRNA and protein levels compared to untreated P tissues (Fig. 4j, k). Consistently, APTT and PT were significantly shortened in AZD6244-treated mice compared to untreated mice (Fig. 4l).

Conversely, rapamycin treatment significantly reduced p-mTOR levels but significantly elevated p-ERK levels in both P and T tissues compared to untreated P and T tissues, respectively (Fig. 4m). Coupled with ERK activation, VKORC1 was significantly down-regulated at both the mRNA and protein level in both P and T tissues compared to untreated P and T tissues, respectively (Fig. 4m, n). Consistently, APTT and PT were significantly prolonged in rapamycin-treated mice compared to untreated mice (Fig. 4o). In addition, the protein levels of TF in plasma were obviously decreased in AZD6244-treated mice but had no change in rapamycin-treated mice compared to untreated mice (Fig. S3B). Taken together, the results indicate that reduced VKORC1 expression and prolonged APTT/PT were positively related to the p-ERK level but negatively related to the p-mTOR level. In addition, these data showed a clear interaction between the ERK and mTOR pathways in mouse hepatoma cells. In other words, ERK activates mTOR, which results in a feedback loop that represses the activity of ERK. Therefore, it suggests that ERK and mTOR are the main pathways regulating VKORC1 expression, which affects the coagulation status of Ras-Tg mice.

Discussion

HCC patients with coagulopathy are usually recognized as the results of cirrhosis [5] from which over 80% HCC raised [6]. Interestingly, the coagulopathy occurred in the Hras12V transgenic mice harboring hepatic tumors but without cirrhosis implies that HCC may be an independent factor in coagulopathy. VKORC1 has been identified as a key enzyme in the VK cycle and plays important roles in blood coagulation. Anti-coagulation drugs, such as warfarin, which targets VKORC1 to inhibit the carboxylation of coagulation factors, represent the main therapy strategy for long-term treatment to prevent thromboembolic events [37]. Previous animal experiments also showed that homozygous VKORC1-deficient mice developed normally until birth, while the knockout mice died within 2–20 days after birth

due to extensive bleeding, predominantly intracerebral hemorrhage and the bleeding resulted from a severe deficiency of γ-carboxylated clotting factors [9]. Fortunately, our findings, for the first time, showed that VKORC1 plays central roles in HCC-related coagulation defects. VKORC1 was significantly down-regulated in human HCC tissues, HCC cell lines and hepatic tumors of Ras-Tg mice at both the mRNA and protein level (Figs. 2, 3, 4). Consistently, APTT and PT were negatively related to VKORC1 levels in hepatic tumor tissues of Ras-Tg mice (Fig. 4). These results indicate that a low VKORC1 level may be the main cause for HCC-related coagulation defects.

Therefore, an investigation of the mechanisms involved in the regulation of VKORC1 expression will be important to clinical therapies for HCC patients-related coagulation defects. Activation of the ERK and mTOR pathways frequently occurs in HCC cells [14, 15, 38], and interactions between the ERK and mTOR pathways were observed [16, 39]. Interestingly, we found that the ERK and mTOR pathways play important roles in regulating VKORC1 expression. In hepatic tumors of Ras-Tg mice, VKORC1 expression was negatively related to the activation state of ERK (Fig. 4j). mTOR could up-regulate VKORC1 expression through its feedback repression on the activity of ERK (Fig. 4m). Consistently, APTT and PT were significantly shortened in AZD6244 (anti-ERK)-treated mice and significantly prolonged in rapamycin (anti-mTOR)-treated mice compared to untreated mice (Fig. 4l, o). Notably, in human HCC cells, VKORC1 is up-regulated at the mRNA and protein levels after AZD6244 treatment and is down-regulated at the protein level after rapamycin treatment (Fig. 3d–g). These data indicate that VKORC1 expression is repressed by ERK and promoted by mTOR in hepatoma cells.

Consistently, clinical reports have shown that HCC treatment with everolimus (a derivative of rapamycin, mTOR inhibitor) resulted in hemorrhagic adverse effects [18–21]. In contrast, hemorrhagic toxicity was not observed after selumetinib (AZD6244, MEK inhibitor) treatment [22]. Moreover, other cancer patients receiving everolimus frequently present with hemorrhage [23–27]. However, hemorrhagic toxicity was only reported in endometrial cancer patients (1 in 54) receiving selumetinib therapy [28]. These data indicate mTOR also regulates the coagulation functions of normal hepatocytes apart from hepatoma cells. In fact, in normal hepatocytes of transgenic mice, VKORC1 expression is also promoted by mTOR (Fig. 4m). These findings provide a valuable molecular basis for preventing hemorrhage in molecular-targeted therapy for not only HCC patients but also other cancer patients. Notably, clinical reports showed that hemorrhagic events were significantly increased after sorafenib treatment for HCC [18–21]. Sorafenib is a multi-kinase inhibitor and simultaneously suppresses the Raf/MEK/ERK and PI3 K/AKT/mTOR pathways [40, 41]. Interestingly, our data showed that simultaneous inhibition of the ERK and mTOR pathways substantially reduced the protein level of VKORC1, similar to the inhibition of mTOR alone in human hepatoma cells (Fig. 3d–g). These results indicate that mTOR may play primary roles in maintaining or promoting the protein level of VKORC1. These data offer a reasonable interpretation for why clinical hemorrhage occurs after sorafenib treatment alone or in combination with everolimus but rarely after selumetinib treatment.

Des-γ-carboxyl prothrombin (coagulation factor II) (DCP) has been universally found in the serum of HCC patients but not cirrhosis patients [42] and therefore is suggested to be an specific marker for the diagnosis of HCC and prediction of curative effects [43, 44]. Although the exact cause of DCP production in hepatoma cells still remains to be elucidated, the down-regulated VKORC1 expression in hepatoma cells but not hepatocytes of cirrhosis indicates its important roles in DCP production. In addition, DCP is suggested to be involved in cell proliferation and the migration of tumor cells [43, 45–47]. Due to the undoubted contribution of an ineffective VK cycle to DCP production [48], a significantly decreased VKORC1 level in HCC tissues (Fig. 2) is a predictable key risk factor for DCP production, and VKORC1 may, therefore, be a valuable target for HCC therapy by inhibiting DCP production.

GGCX is another important enzyme in the VK cycle for carboxylation of coagulation factors by consuming KH2 (Fig. 5). It has been reported that the exon 2 deletion form of GGCX may play roles in the decarboxylation of coagulation factors in several human HCC cell lines [49]. Unfortunately, we did not find the exon 2 deletion isoform of GGCX in HCC patients, Ras-Tg mice and most of the HCC cell lines (Fig. S5). We also did not detect decreased expression of GGCX in hepatoma cells or tissues (Figs. 2, 3, 4). Additionally, clinical Vitamin K administration significantly reduced DCP levels [50], which implies the normal function of GGCX. These data indicate that GGCX may not be the main cause of coagulation defects in HCC patients.

In conclusion, for the first time, our work shows that HCC is an independent factor for coagulopathy and the down-regulation of VKORC1 in hepatoma cells may be a cause factor for coagulopathy and DCP production in HCC patients (Fig. 5). Importantly, we found that the mTOR and ERK pathways play central roles in regulating VKORC1 expression in not only hepatoma cells but also normal hepatocytes, which provides a valuable molecular basis for preventing

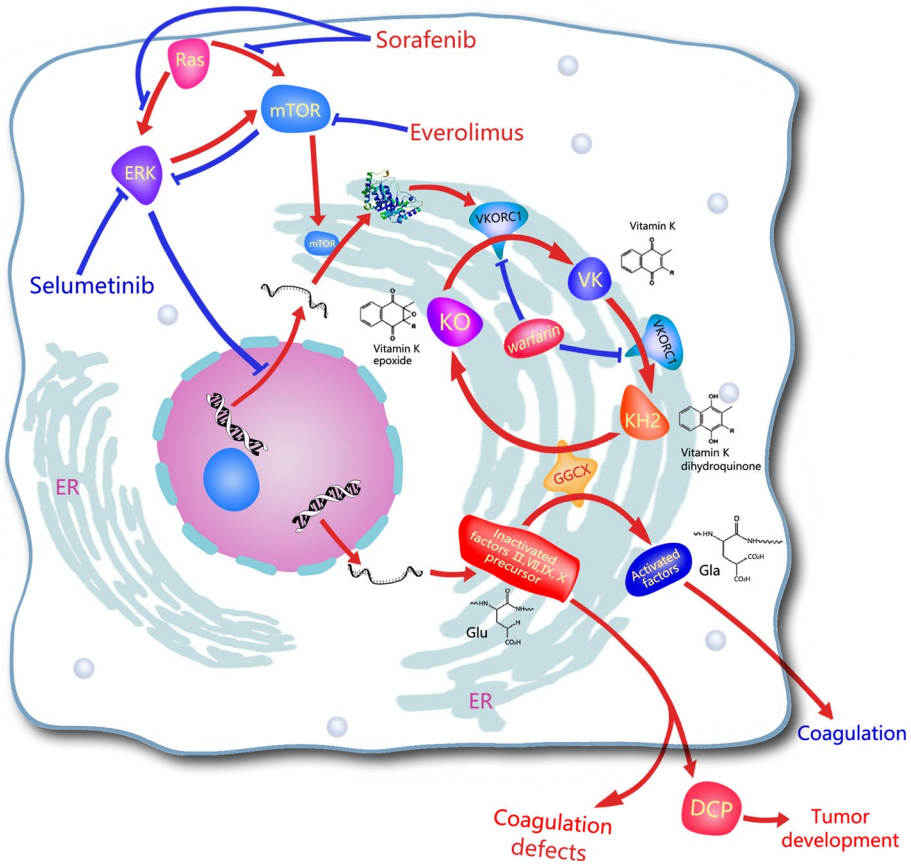

Fig. 5 Schematic diagram for HCC-related coagulation defects. The Ras/MAPK and mTOR signaling pathways have been found to be frequently activated in hepatocellular carcinoma (HCC). Activation of the Ras/ERK pathways stimulates the activation of mTOR, and phosphorylated mTOR represses ERK in a negative feedback loop. Phosphorylated ERK enters the nucleus and inhibits the transcription of the VKORC1 gene. Meanwhile, the activated mTOR promotes the translation of VKORC1 mRNA into protein. Due to the inhibition effects of ERK on the primary transcription step for the production of VKROC1 mRNA, the combined action of ERK and mTOR in HCC cells reduces the protein levels of VKORC1, which plays pivotal roles in the VK cycle by catalyzing the KO to VK and VK to KH2 in the endoplasmic reticulum (ER). Therefore, the factors II, VII, IX and X precursor cannot be carboxylated effectively to produce the activated mature forms that play central roles in blood coagulation processes. The uncarboxylated factors II, VII, IX and X precursors are released into the blood, reducing the level of functional factors and therefore causing coagulation defects and elevated DCP levels, which may further promote tumor development. HCC therapy strategies using ERK inhibitors (such as selumetinib) may improve the coagulation defects, while using an mTOR inhibitor (such as sorafenib or everolimus) may exacerbate the coagulation defects. Warfarin, an effective inhibitor of VKORC1, should not be used in cancer patients

hemorrhage production in molecular-targeted therapies for not only HCC but also other cancer patients (Fig. 5).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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