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Dysregulated KLF4 and Vitamin D Receptor Signaling Promotes Hepatocellular Carcinoma Progression

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Abstract

BACKGROUND & AIMS—Krüppel-like factor 4 (*KLF4*) is a putative tumor suppressor gene, however, the functional status and significance of KLF4 in hepatocellular carcinogenesis is unknown. In this study, we sought to determine the clinical significance and underlying mechanisms of its dysregulated signaling and biologic impact.

METHODS—We have used HCC tissue microarray and molecular biology and animal models to evaluate the activation and function of KLF4-Vitamin D Receptor (VDR) pathway in human HCC.

RESULTS—KLF4 protein expression was decreased or lost in primary HCC and, in particular, lymph node metastases when compared with that in normal liver. Moreover, loss of KLF4 expression in the primary tumors was significantly associated with poor survival, and also a prognostic marker. Consistently, most human HCC cell lines exhibited loss of or a substantial

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The authors disclose no conflicts.

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decrease in KLF4 expression. Promoter hypermethylation contributed to the decreased KLF4 expression. Enforced restoration of KLF4 expression resulted in MET, and marked inhibition of cell migration, invasion and growth *in vitro*, and significantly attenuated tumor growth and metastasis in animal models. Moreover, VDR is a direct transcriptional target of KLF4 and two KLF4-binding sites in the VDR promoter bound specifically to KLF4 protein. Increased VDR expression sensitized the inhibitory effects of Vitamin D on tumor growth.

CONCLUSIONS—The novel KLF4-VDR pathway plays a critical role in HCC development and progression and its deregulated signaling could be a promising new molecular target for designing novel preventive and therapeutic strategies to control this malignancy.

Keywords

VDR; KLF4; liver cancer; progression

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal human cancers worldwide and a significant health problem in the United States.[1,2] Conventional chemoradiation treatments have had little impact on the disease course.[1–3] The aggressive nature of this disease is reflected by the fact that it has a strong propensity for producing intra-hepatic and extra-hepatic metastatic disease and exhibiting intrinsic resistance to cytotoxic agents and radiotherapy.[1–3] Surgery, the only curative option, is not a viable option for most patients because of significant liver cirrhosis, or intra-hepatic and extra-hepatic metastatic disease that has spread to the lymph nodes, portal vein, or lungs.[1–3] The molecular changes leading to this invasive growth and metastasis are poorly understood. Identification and validation of causative genes and molecular pathways underlying HCC invasion and metastasis are critical for the rational development of improved strategies of diagnosis and prevention and treatment of this cancer.[1–3]

The past decade has seen an explosion of data providing global insight into genetic alterations and molecular profiles in large sets of HCC.[1,4–9] Most of these studies have shown a wide variety of genetic alterations and pronounced heterogeneity of gene expression profiles in individual HCC cases, reflecting the multiple etiologies of HCC and the long period of chronic inflammatory disease that fosters the accumulation of such genetic and epigenetic changes.[5,6] Established genetic events in HCC include loss of tumor suppressor gene function through a combination of inherent and/or acquired genetic and epigenetic events, including allele loss, mutation, and/or promoter hypermethylation. Although mutations of RB, CDKN2A, PTEN and CDH1 are rare in HCC patients, reduction of their expression and loss of their tumor suppressor functions appear to be caused by epigenetic silencing mechanisms.[6–9] These epigenetic mechanisms are also involved in the activation and overexpression of the Ras and Jak/Stat pathways in HCC.[1,8,9] As a consequence of the activation of cellular oncogenes or inactivation of tumor suppressor genes, various signaling pathways are deregulated in HCC subsets, such as the Wnt/β-catenin pathway.[1,2,9] However, key pathways and gene involvement in HCC pathogenesis remain to be elucidated.

Krüppel-like factor 4 (KLF4) is a potential tumor suppressor in patients with various cancers, including gastrointestinal cancers.[10–12] KLF4 is a zinc-finger transcription factor, and KLF4 mRNA expression is found primarily in postmitotic, terminally differentiated epithelial cells in organs such as the skin, lungs, and gastrointestinal tract.[13,14] However, the clinical relevance of KLF4 expression and function in HCC pathogenesis and impact on outcome are unknown. Also the molecular basis for the causal impact of KLF4 expression

and function on HCC pathogenesis in general and invasion and metastasis in particular is unknown.

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D(3) (VD3), has antiproliferation, anti-angiogenesis, pro-differentiation, anti-inflammation, proapoptosis, and immune regulation through binding to its nuclear receptor, vitamin D receptor (VDR) and other VDR independent mechanisms.[1,3,15–17] VD3 exhibits anti-tumor effects on several types of cancer,[1,3,18,19] suggesting that vitamin D deficiency and/or dysregulated VDR signaling may play an important role in cancer development and progression. However, little is known about the status of VDR and mechanisms of its dysregulation in HCC. Recent studies demonstrated that β-catenin interacts with both KLF4 and VDR.[20–23] However, whether expression of KLF4 and VDR is causally related and dysregulation of their signaling impacts HCC pathogenesis remain unknown.

In the present study, we demonstrated for the first time that KLF4 is an HCC tumor suppressor; its dysregulation is critical to lost expression of VDR and an enhanced invasive and metastatic phenotype of HCC. Furthermore, KLF4 is an important biomarker and a potential therapeutic target of HCC.

Materials and Methods

Detailed materials and methods are described in the Supplementary Methods.

Human tissue specimens and patient information

A commercially available tissue microarray (TMA) was used and patient information was provided by the manufacturers. The TMA sections (4 μm thick) were processed for immunostaining using a rabbit polyclonal antibody against human KLF4 (H-180 [1:500 dilution]; Santa Cruz Biotechnology) and a rabbit polyclonal antibody against human VDR (sc-13133 [1:200 dilution]; Santa Cruz Biotechnology). Use of human specimens was approved by proper institutional review boards.

Cell lines and culture conditions

The human HCC cell lines SK-Hep-1, Hep-3B, Hep-G2, SNU-182, SNU-387, SNU-398, SNU-423, SNU-449 and SNU-475 were purchased from the American Type Culture Collection (Manassas, VA). All of the cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Western blot analysis

Whole-cell lysates were prepared from the HCC cells and tissues as described previously.[13] Standard Western blot analysis was performed with an antibody against KLF4 (Santa Cruz Biotechnology) or against VDR (Santa Cruz Biotechnology) and a second anti-IgG antibody (Amersham Life Sciences). The membranes were then stripped and blotted with an anti-β-actin antibody (Sigma Chemical Co., St Loius, MO) and used as loading controls. The probe proteins were detected using an enhanced chemiluminescence system (Amersham Life Sciences) according to the manufacturer's instructions.

Transient transfection of HCC cells

To induce overexpression of KLF4 in SK-Hep-1 and Hep-G2 cells, the cells were infected with adenoviral KLF4 (Ad-KLF4) or enhanced green fluorescent protein (EGFP [Ad-EGFP]) as described previously.[10] For inhibition of KLF4 expression in these cells, they were transfected with a pool of KLF4 small interfering RNA (siRNA) oligonucleotides or

control siRNA oligonucleotides (Santa Cruz Biotechnology; 50 nmol/L). Also, cells treated with Oligofectamine reagent alone were included as mock controls.

Cell proliferation assay

SK-Hep-1 and Hep-G2 cells were seeded at 4×10^5 cells/well in six-well culture plates. Twelve hours later, the cells were incubated for 2 hours at 37 °C in a serum-free mediium or serum-free media with Ad-KLF4 or Ad-EGFP. After being washed with serum-free medium, the transduced cells were replenished with Dulbecco's minimal essential medium and incubated for 1 to 4 days. The cells were counted daily using trypan blue exclusion with a hemocytometer. The results were plotted as the mean ± standard deviation (SD) from three separate experiments with eight replicates per experiment for each experimental condition.

VDR promoter constructs, site-specific mutagenesis, and promoter activity analysis

A 1.241-kb VDR promoter was cloned into pGL3 basic luciferase reporter vectors. Site-specific mutagenesis of the VDR promoter was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primers used to generate the mutant vector were as follows: site 1, 5′-agcgcggaacagcttgtccataagccggccggaccaggt-3′ (sense) and 5′-acctggtccggccggcttatggacaagctgttccgcgct-3′ (antisense); and 2, 5′-tatgcctcaatttcccataatttcaccttgtcccct-3′ (sense) and 5′-gagggacaaggtgaaattatgggaaattgaggcata-3′ (antisense). This mutation was confirmed via DNA sequencing. The VDR promoter activity was normalized via cotransfection with a β-actin/Renilla luciferase reporter containing a full-length Renilla luciferase gene.[10] We quantified both firefly and Renilla luciferase activity using a dual-luciferase assay system (Promega, Madison, WI) 24 hours after transfection.

Animal Experiments

Female athymic nude mice were purchased from The JacksonLaboratory (Bar Harbor, ME. The mice were housed in laminarflow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. To produce subcutaneous tumors or experimental liver metastases, 1×10^6 cells in 100 μl of Hank's balanced salt solution were injected into the subcutis or ileocolic veins of nude mice. Mice were sacrificed 35 to 45 days after tumor implantation. Subcutaneous tumors were weighted and hepatic metastases were determined as described previously.[10] The animals were housed and maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with the current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and National Institutes of Health.

Statistical analysis

The two-tailed χ^2 test was used to determine the significance of the difference among the covariates. Survival durations were calculated using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival rates in the patient groups. A Cox proportional hazards model was used to calculate univariate and multivariate hazard ratios for the study variables. The KLF4 and VDR expression level, patient age, disease stage (American Joint Committee on Cancer system), and tumor differentiation and distant metastasis were included in the model. The significance of the *in vitro* data was determined using the Student *t*-test (two-tailed). In all of the tests, *P* values less than 0.05 were considered statistically significant. The SPSS software program (version 12.0; IBM Corporation, Armonk, NY) was used for statistical analyses.

Results

Clinical significance of loss of KLF4 expression in HCC

We first investigated the expression of KLF4 protein in 9 human HCC cell lines and 40 primary HCC and 9 normal liver specimens as well as 10 metastasis specimens in a TMA. We observed a drastic reduction of KLF4 expression in all human HCC cell lines (Fig. 1A) and human HCC tissues (Fig. 1B, Supplementary Table 1 & 2). We confirmed the presence of aberrant downregulation of KLF4 expression in HCC specimens using real-time PCR and Western blotting (Fig. 1C). Typically, KLF4-negative or -weak staining was observed in the cancer cells, but KLF4-positive staining in adjacent normal liver cells (Fig. 1Ciii). We further analyzed the relationship between clinicopathological features and KLF4 expression levels in HCC cases. KLF4 expression was positively correlated with tumor differentiation ($P = 0.003$) (Supplementary Table 1). In contrast, KLF4 expression was inversely correlated with overall survival rate (Supplementary Fig. 1). These findings strongly indicated that KLF4 expression plays a critical role in HCC development and progression and is a valuable biomarker of this disease.

Moreover, because the promoter region of *KLF4* contains typical CpG islands[10], we determined the DNA methylation status using methylation-specific PCR using genomic DNA extracted from surgically resected HCC specimens and matched normal liver tissues as well as from the HCC cell lines. All nine HCC cell lines and two gastric cancer cell lines and five tumors (Fig. 1D) exhibited hypermethylation in the exon 1 region of *KLF4*, whereas none of the matched adjacent normal tissue specimens had hypermethylation in the same region. Finally, we determined whether blockade of gene hypermethylation reactivates KLF4 expression in human HCC cells. Nine HCC cell lines were incubated in a medium alone or medium containing 5′-aza-2′-deoxycytidine (AZA), an inhibitor of DNA methyltransferase. The treatments increased KLF4 expression in 8 out of 9 cell lines as compared with their controls. Reactivation of the KLF4 gene was further confirmed by qPCR and Western blot analysis in SK-Hep-1 and Hep-3B cells (Fig. 1E) and other cell lines (Supplementary Figure 2). Therefore, promoter hypermethylation may contribute to the reduced KLF4 expression in a subset of liver cancer tissues and cell lines.

Inhibition of HCC cell growth in vitro and in vivo by restoration of KLF4 expression

To determine the role of KLF4 in HCC cell growth, we selected SK-Hep-1 and Hep-G2 cells with low KLF4 expression and restored KLF4 expression inhibited the growth of both cell lines (Fig. 2A & 2B), and inhibition of cell proliferation and induction of apoptosis (Supplementary Fig. 3). Morphologically, we found that increased KLF4 expression caused MET (Fig. 2C). In contrast, knockdown of KLF4 did the opposite (Supplementary Fig. 4). To determine the effect of KLF4 expression on tumor growth in vivo, SK-Hep-1 and Hep-G2 cells were injected subcutaneously into nude mice. Control SK-Hep-1 and Hep-G2 cells or SK-Hep-1 and Hep-G2 cells transduced with control Ad-EGFP produced large tumors, whereas SK-Hep-1 and Hep-G2 cells transduced with *KLF4* only produced small tumors (Fig. 2D & 2E).

To determine the effects of KLF4 expression on the metastatic potential of HCC cells, we injected SK-Hep-1 cells into the subcutis in mice in groups of 10. Control SK-Hep-1 and SK-Hep-1 cells transduced with control Ad-EGFP metastasized to the liver, whereas SK-Hep-1 cells transduced with *KLF4* only rarely produced liver metastases (Fig. 3A & 3B). Moreover, the SK-Hep-1 and Hep-G2 cells with similar treatments above were also injected intravenously via the ileocolic veins of groups of mice and liver metastases were determined 45 days after tumor cells injections. Consistently, enforced restoration of KLF4 expression suppressed human HCC cells metastasis (Fig. 3C & 3D).

Attenuation of the migratory and invasive ability of HCC cells by KLF4 restoration

Because KLF4 expression caused MET morphological change (Fig. 2C), we further determined whether the MET morphological change was consistent with changes of EMT biomarker and biologic behaviors. Clearly, restored KLF4 expression increased the expression of E-cadherin but decreased the expression of vimentin, and caused the morphological change from mesenchymal to epithelial transition in cultures (Fig. 4A) and xenograft tumors of HCC (Fig. 4B). Consistent with MET, restored KLF4 expression suppressed cell migration as determined by scratch assay and invasiveness as determined by invasion assay (Fig. 4C–4E), whereas knockdown of KLF4 did the opposite (Supplementary Fig. 4). Consistent with those in vitro findings, increased KLF4 expression suppressed the growth and metastasis of HCC cells.

Association of KLF4 underexpression with decreased VDR expression

To understand the molecular mechanisms underlying KLF4-mediated MET, we performed Western blot analyses of EMT-related biomarkers. As predicted, the increased expression of KLF4 led to the downregulation of Slug and Snail, while knockdown of KLF4 did the opposite (Fig. 5A). Strikingly, we found that increased KLF4 expression significantly upregulated VDR expression (Fig. 5A) and sensitized the cells to the inhibitory effects of VD3 (Fig. 5B).

Prior studies have shown that loss of KLF4 expression contributed to carcinogenesis, while the underlying mechanisms remain to explore.[12,14] It is unknown whether loss of KLF4 in fact is in part responsible for VDR underexpression and its potential impact on HCC pathogenesis. To that end, we first performed immunostaining for KLF4 and VDR in normal liver tissue and HCC specimens (Fig. 5C, Supplementary Tables 3 and 4). The levels of VDR and KLF4 expression in the primary tumors were lower than those in the normal liver tissue (Supplementary Tables 5 and 6). We observed a significant direct correlation between the levels of VDR and KLF4 expression (Fig. 5Cii). However, we were not able to observe a similarly significant direct correlation between the levels of KLF4 and VDR expression in tumor cell lines (Fig. 5Di). Additionally, the levels of VDR expression were not directly correlated with the sensitivities of cells to the inhibitory effects of VD3 treatment (Fig. 5Dii). The lack of significant correlations may be due to the fact that the majority of those cell lines expressed insignificant levels (very low) of KLF4 and VDR. However, the reduced expression of KLF4 correlated with poor survival (Fig. 5Ei), although no significant correlation was found between the expression of VDR and patient survival (Fig. 5Eii). These results indicated that reduced VDR expression was significantly associated with loss of KLF4 expression in human HCC tissues.

Positive regulation of VDR expression by KLF4

To further identify the molecular mechanisms of upregulation of VDR expression by KLF4, we generated a VDR promoter and mutations of VDR promoter in two KLF4-binding sites using site-specific mutagenesis, whereas mutations of the VDR promoter reporter in either or both KLF4-binding sites significantly decreased this activity (Fig. 6A). Transfection of KLF4 significantly increased and knockdown of KLF4 decreased the VDR promoter activity (Fig. 6B). KLF4-mediated transactivation of VDR promoter was consistently observed in various HCC cell lines (Fig. 6Biii). These results suggested that the KLF4-binding sites were positive regulatory elements in the VDR promoter.

Finally, to determine whether KLF4 directly interacts with the VDR promoter, we performed a ChIP assay using specific PCR primers (Fig. 6Ai). The predicted-size DNA fragments were amplified from the chromatin precipitates of the cells using anti-KLF4 antibodies but not control IgG (Fig. 6C), the expression levels of KLF4 directly affected the

binding (Fig. 6Ciii). These results clearly suggested that endogenous KLF4 bound to the VDR promoter in HCC cells and that enforced KLF4 expression transcriptionally activates VDR gene and sensitizes tumor cells to the growth inhibitory effect of VD3.

Discussion

In this study, we discovered five lines of evidence supporting a critical role for loss of KLF4 in HCC pathogenesis. First, KLF4 expression was drastically reduced in both HCC cell lines and tissues, and the hypermethylation of its promoter was evident. Second, loss of KLF4 expression correlated with HCC progression. KLF4-negative staining of HCC cells could be used to identify a greatly increased risk of recurrence and metastasis in patients. Third, restored KLF4 expression suppressed the proliferation, invasion and metastasis of human HCC cells, and KLF4 caused MET of HCC cells. Fourth, KLF4 expression directly correlated with VDR expression in human HCC specimens, and KLF4 induced VDR expression in HCC cells. Fifth, KLF4 directly regulated the expression of the VDR gene at the transcriptional level via binding to the VDR promoter. Therefore, loss of KLF4 expression during the initiation and development of HCC contributed to reduced VDR expression and activation; the latter may contribute to HCC pathogenesis and aggressive biology.

Numerous studies have indicated the importance of KLF4 expression in the oncogenesis of several malignancies, including GI cancer.[10–14] In the present study, we observed the loss of KLF4 staining in primary HCC, particularly the metastasis specimens. We also found that reduced KLF4 expression was significantly correlated with advanced cancer biology, and poor patient survival duration. Therefore, this is the first evidence that KLF4 could be used as a novel biomarker for outcome in patients with HCC after surgery. This means that at the time of initial diagnosis of HCC, KLF4 expression could be used not only to design optimal, individualized treatment but also to distinguish patients who would benefit from close monitoring after surgery from those who would not.

KLF4 gene functions in a variety of contexts, including cell cycle regulation.[24,25] However, the precise roles of KLF4 in carcinogenesis remain to be elucidated. Herein we provide evidence indicating that KLF4 expression may affect the proliferation, migration, and invasiveness of HCC cells. Moreover, our results also suggested that KLF4 is a positive regulator of VDR, whereas VDR signaling has multiple antitumor effects, including negative regulation of cell cycle progression by directly binding to the promoter region and activating the expressions of p21 and p27,[26–28] which are two critical cell cycle regulators and also the direct downstream target genes of KLF4.[24,25] The induction of p21 and p27 could lead to suppression of cyclins (D1, E and A) and cyclin dependent kinases 2 and 4 [29] and produce antiproliferative effect mainly attributable to cell cycle arrest at G0/G1[30] in many types of cancer, including HCC cells.[31] The antitumor activities of VD3-VDR have been demonstrated in a preclinical model and in a clinical trial.[20,32] These promising results suggest that VD3, especially its analog has a potential to be developed as a new therapeutic regimen for HCC. Given the crosstalk between VDR and KLF4 signaling, it is likely that activation of both KLF4 and VD3-VDR signaling pathways may produce synergistic antitumor effect, which awaits further experimental validation.

The mechanisms responsible for VDR underexpression in malignances are currently unclear. A recent study showed that promoter methylation-mediated silencing of expression of the functional variants of VDR may contribute to reduced expression of downstream effectors of the VDR pathway in breast cancer.[33] On the other hand, Snail can negatively regulate vitamin D receptor expression,[34,35] while aberrant overexpression of Snail has been found in HCC.[36,37] It has been reported that KLF4 silencing increased Snail expression, whereas

overexpression of KLF4 led to a significant reduction of Snail expression.[38] Mechanistically, VDR can interact with β-catenin and control its nuclear content and target gene expression in colorectal cancer,[21,22] Interestingly, KLF4 directly interacts with the C-terminal transactivation domain of β-catenin and inhibits Wnt/β-catenin signaling in intestinal tumors.[23] In the present study, we found that KLF4 could bind directly to the VDR promoter and cause transcriptional activation of VDR. Thus, KLF4 can mediate VDR gene expression via both direct transcriptional activation and attenuation of the Wnt/β-catenin and snail signaling pathways. It warrants further investigations on how the interactions among Wnt/β-catenin, Snail and KLF4 regulate the expression and function of VDR and contribute to HCC carcinogenesis. Moreover, our data clearly showed that an aberrant hypermethylation of KLF4 promoter and drastic loss of its protein expression cause significant reduction of VDR expression in human HCC. All these results suggest that loss of KLF4 expression and overexpression of Snail may contribute the underexpression of VDR in HCC. Therefore, improved understanding of the across talk between KLF4 and VDR signaling could lead to the identification of potential therapeutic targets for designing novel effective therapies for HCC. Finally, our recent study has shown the existence of alternative splicing variants of KLF4 in other tumor types. It warrants further investigation into the alterations of KLF4 isoforms expression and their biological and clinical significance.[13]

In summary, this study provided critical insight into the role of this novel KLF4-VDR signaling pathway in HCC progression by downregulating the proliferation, migration, invasion, and metastatic potential of tumor cells. The frequent loss of KLF4 expression, which causes deregulated VDR signaling in human HCC cells, highlights its potential as a novel biomarker for this cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

ChIP	chromatin immunoprecipitation
DMEM	Dulbecco’s modified Eagle’s medium
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
HCC	hepatocellular carcinoma
H&E	hematoxylin and eosin
KLF4	Krüppel-like factor 4

MOI	multiplicity of infection
PCR	polymerase chain reaction
RT	reverse transcriptase
TMA	tissue microarray
VD3	1,25-dihydroxyvitamin D(3)
VDR	vitamin D receptor

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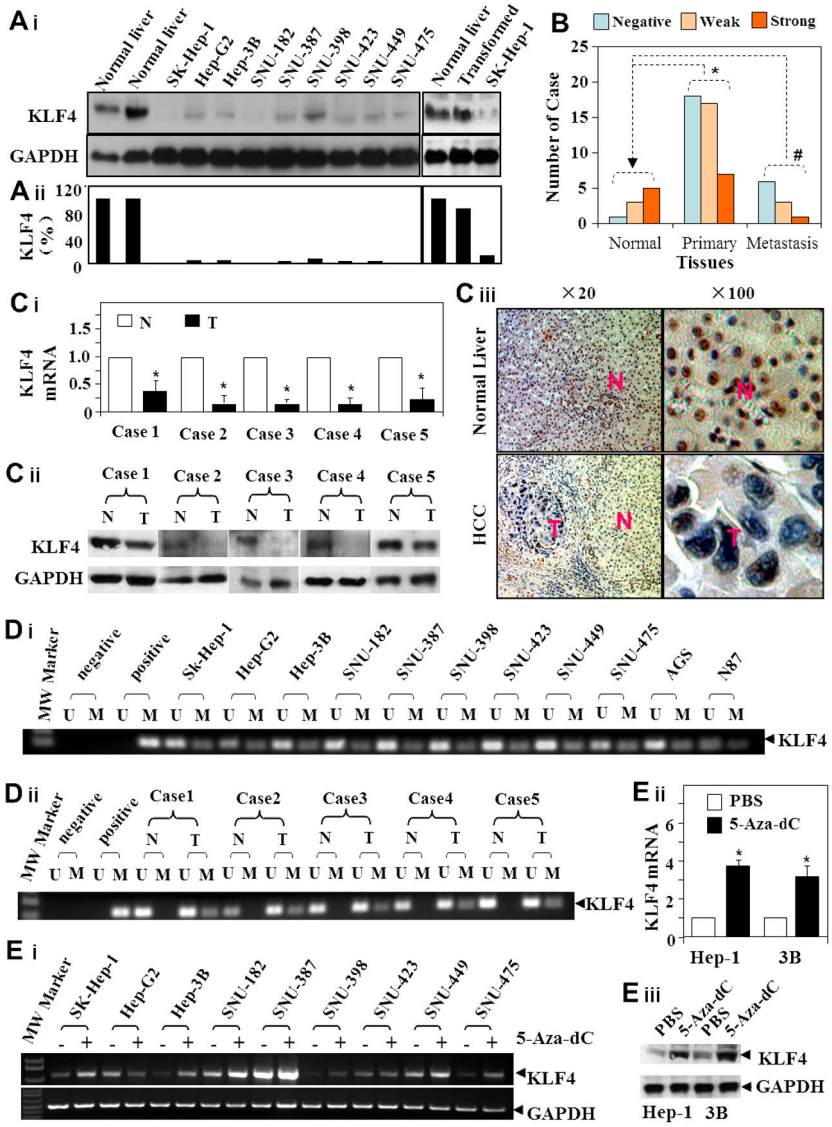

Figure 1.

KLF4 expression in HCC cells and tissues. ***A***, Total protein lysates were harvested from a panel of HCC cell line cultures and normal liver tissues and KLF4 protein expression was measured by Western blot analysis (***A***i). The relative KLF4 expression was expressed as a ratio between KLF4 and GAPDH (normal tissues were given 100%, ***A***ii). ***B***, KLF4 immunostaining of HCC TMA sections. Pearson's two-tailed χ^2 test showed a significant statistical difference in KLF4 protein expression among the three tissue types (*P<0.05; #P<0.05). ***C***, KLF4 mRNA (qPCR, ***C***i) and protein (Western blot, ***C***ii) expression in HCC tissues. Representative photos of KLF4 protein expression in HCC were shown (***C***iii). The majority of the adjacent normal liver tissue cells (N) were strongly positive for KLF4 expression, whereas HCC cells (T) were negative. ***D***, Genomic DNA extracted from HCC cell lines (***D***i), HCC tissues (T) and the matched normal liver tissues (N, ***D***ii). Methylation-specific PCR was performed using primers specific for the unmethylated (U) or methylated (M) *KLF4* exon 1 region in the genomic DNA. ***E***, Reactivation of KLF4 expression. Cells were cultured in the presence of 5-AZA-CdR (1μM) for 3 days. Total

RNA was extracted and KLF4 expression was measured by RT-PCR (***E***i), qPCR (***E***ii) and Western blot (***E***iii).

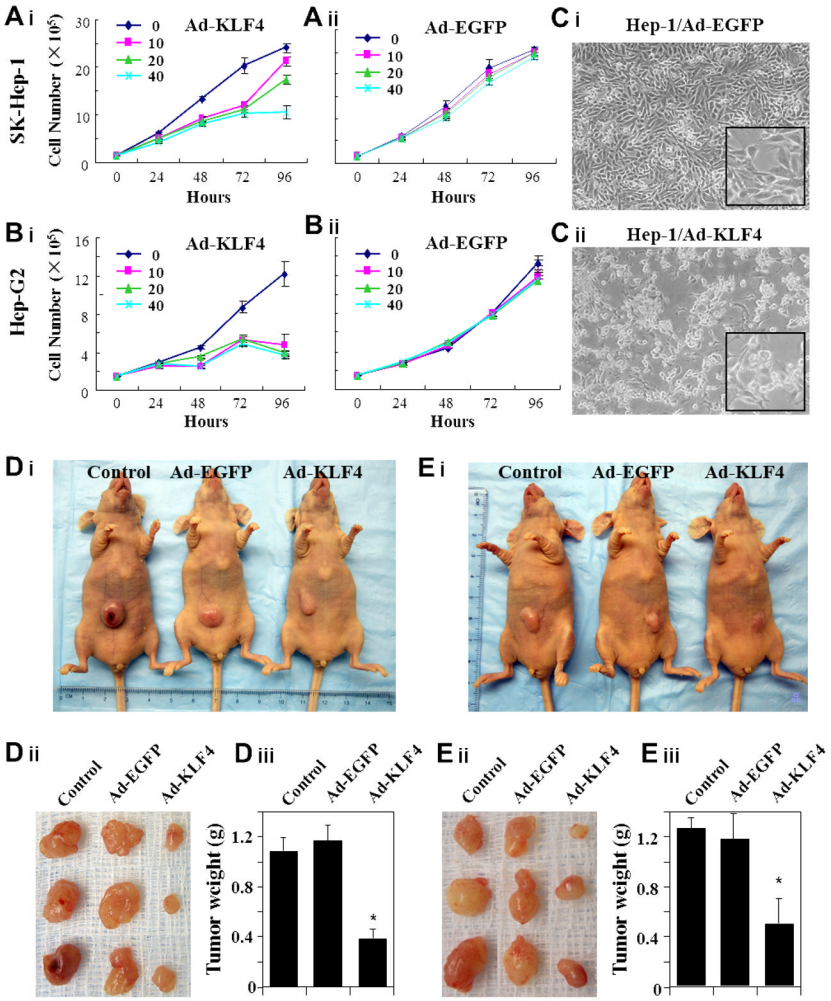

Figure 2.

The effect of KLF4 expression on HCC growth *in vitro* and *in vivo*. ***A, B***, & ***C***, *In vitro* growth. SK-Hep-1 and Hep-G2 cells were transduced with Ad-KLF4 or a control Ad-EGFP for 24 to 96 hrs. Cell growth was determined by cell counting. Representative photos of SK-Hep-1 cell morphology changes were shown. ***D*** & ***E***, *In vivo* growth. SK-Hep-1 (***D***) and Hep-G2 (***E***) cells were transduced with 10 MOI of Ad-KLF4 or a control Ad-EGFP and injected into the subcutis of groups of mice (n=10). Tumors were harvested 45 days after tumor injections and weighed. Shown from each group were representative mouse with tumors (***D***i & ***E***i), three gross tumors (***D***ii & ***E***ii), and mean (±SD) tumor weights (***D***iii & ***E***iii). This representative experiment was one of two with similar results. **P*<0.05.

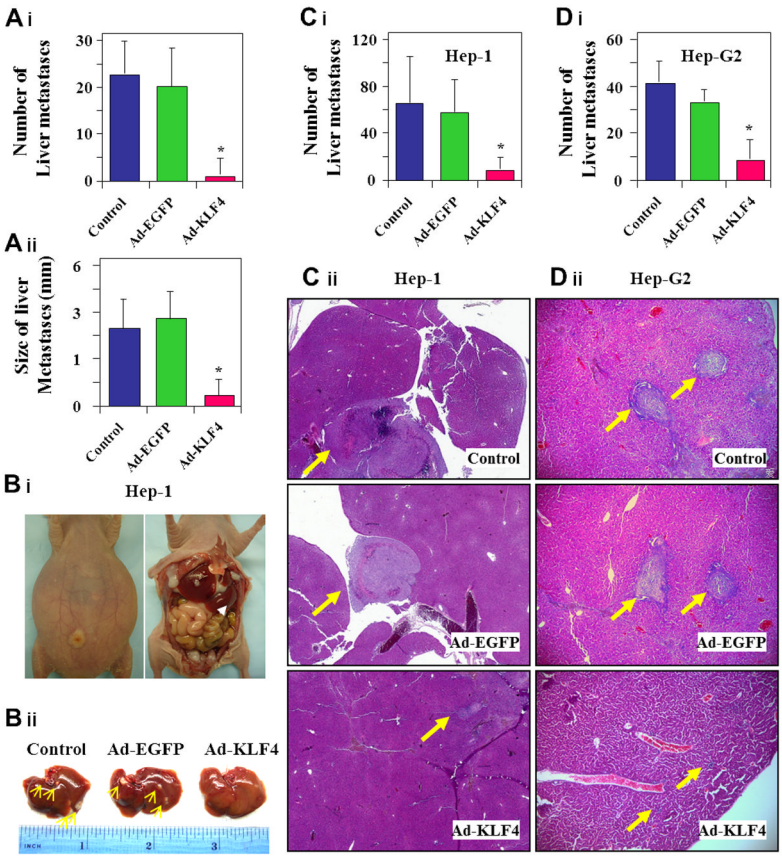

Figure 3.

Inhibition of HCC metastasis by KLF4. SK-Hep-1 and Hep-G2 cells with mock transduction, control Ad-EGFP, or Ad-KLF4 were implanted in the subcutis of nude mice (n=10). Numbers and sizes of liver metastases were determined (***A***i & ***A***ii: SK-Hep-1). Also shown were a representative mouse with a subcutaneous tumor and liver metastases (yellow arrows, ***B***i) and gross liver metastases (white arrow) from the groups injected with SK-Hep-1 cells (***B***ii). The cells were also injected intravenously via the ileocolic veins of groups of mice (n=10) and liver metastases were determined 45 days after tumor cells injections. Numbers of liver metastases were determined (***C***: SK-Hep-1). Also shown were representative photos of liver micrometastases (yellow arrows) (***D***: Hep-G2).

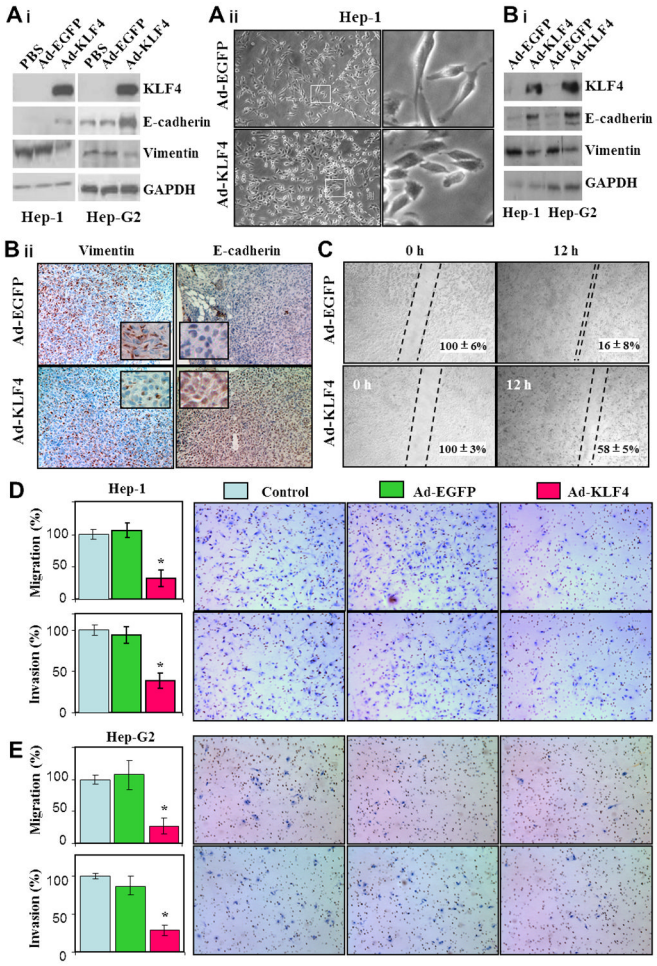

Figure 4.

Inhibition of HCC cell migration and invasion by KLF4. ***A***, SK-Hep-1 and Hep-G2 cells were transduced with 10 MOI of Ad-KLF4 or a control Ad-EGFP. Total protein lysates were harvested from the cultures and expression of KLF4 protein and EMT markers was measured by Western blot analyses (***A***i) and morphological changes were shown for SK-Hep-1 cells (***A***ii). ***B***, Total protein lysates were harvested from the HCC xenograft tumors and expression of KLF4 protein and EMT markers was measured by Western blot analyses (***B***i) and immunostaining (***B***ii). Gap-closing (***C***), migration (***D***), and invasion (***E***) of tumor cells were determined as described in Materials and Methods. The untreated cell cultures were given arbitrary migration and invasiveness percentages of 100%. Representative photos of cell migration and invasion were shown. $*P < 0.001$ in a comparison of the KLF4–treated group with the control and Ad-EGFP–treated groups. This representative experiment was one of three with similar results.

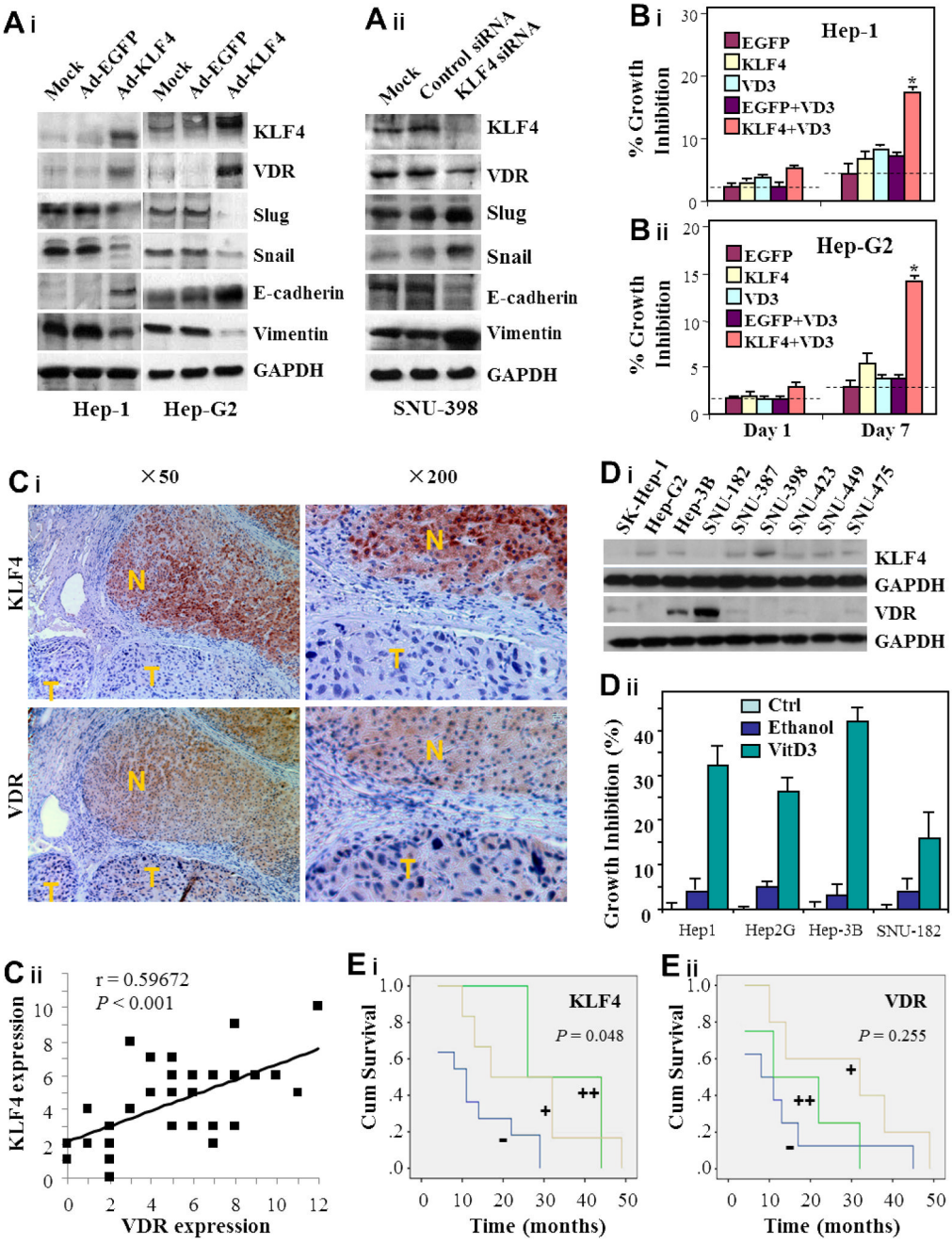

Figure 5.

Direct association of VDR expression with KLF4 expression. ***A***, SK-Hep-1 and Hep-G2 were transduced with Ad-KLF4 (KLF4) or control Ad-EGFP (EGFP) at a 10 MOI (***A***i), or KLF4 siRNA (***A***ii) and incubated for 24 h. Total protein lysates were harvested and VDR and KLF4 expression was determined using Western blotting. ***B***, Sensitivity of HCC cells to VD3. ***C***, Three sets of consecutive TMA sections were prepared for immunostaining using specific antibodies against VDR and KLF4. Representative photos of VDR and KLF4 protein expression in HCC and adjacent normal liver cells (original magnification: 50× for left panels; 200× for right panels, ***C***i). The VDR expression levels directly correlated with the KLF4 expression levels (Pearson χ^2 test, $P < 0.001$) in primary HCC (***C***ii). ***D***, KLF4 and

VDR protein expression levels in HCC cells were determined by Western blot analysis (***D***i). Growth inhibition of HCC cells by VD3 ($P < 0.001$, *t* test, ***D***ii). ***E***, The survival rates were estimated using the Kaplan-Meier method: the KLF4 expression and patient survival (***E***i, rates in patients with KLF4-negative primary tumors were significantly worse than those in patients with strong KLF4-positive primary tumors); and the VDR expression levels and patient survival (***E***ii, no significant difference among different groups).

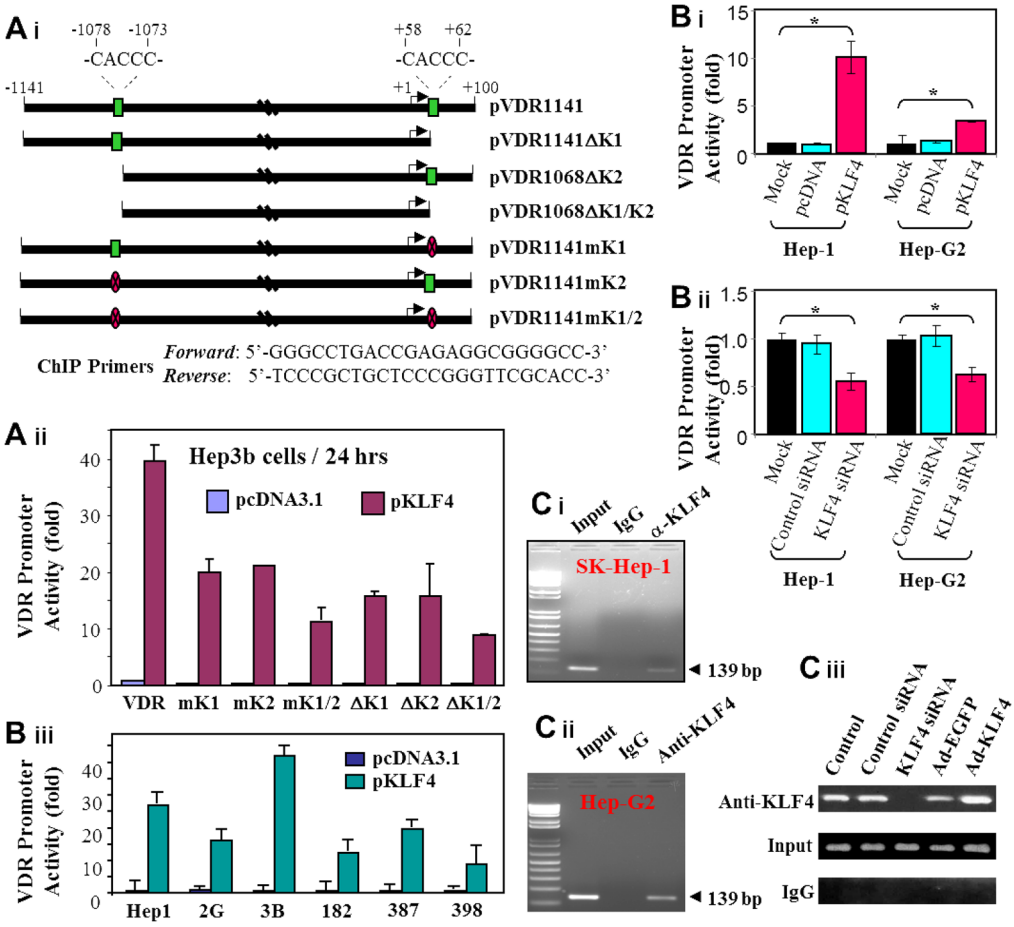

Figure 6.

Upregulation of VDR expression by KLF4 in HCC cells. ***A***, Schematic structure of the VDR promoter reporter and its deletion- and point-mutation reporters, and sequences of ChIP primers were shown (***A***i). The VDR promoter reporter and mutations of it in KLF4-binding site 1 (K1), KLF4-binding site 2 (K2), or both (K1/2) were transfected into Hep-3B cells in triplicate. The relative VDR promoter activities were measured 24 h after transfection, and the activities in the treated groups were expressed as the fold of that in their respective control groups (***A***ii). ***B***, The VDR promoter reporter was transfected into SK-Hep-1 and Hep-G2 cells in triplicate with pKLF4 and mock transfection or transfection with pcDNA3 were used as controls (***B***i), or KLF4 siRNA (***B***ii). Similarly, VDR promoter activities were determined in various HCC cell lines (***B***iii). ***C***, Chromatin was extracted from Hep-1 (***C***i) and Hep-G2 (***C***ii) cells, or Hep-G2 cells treated with KLF4 siRNA or Ad-KLF4 (***C***iii). A ChIP assay was performed using a specific anti-KLF4 antibody and oligonucleotides flanking the VDR promoter regions containing putative KLF4-binding sites. The nucleotide positions and sequences of the PCR forward and reverse primers flanking those sites in the ChIP assay are described in Materials and Methods.