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Downregulation of KLF6 is an early event in hepatocarcinogenesis, and stimulates proliferation while reducing differentiation

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Abstract

Background/Aims—Hepatocellular carcinoma (HCC) has the most rapidly rising cancer incidence in the US and Europe. The KLF6 tumor suppressor is frequently inactivated in HCC by loss-of-heterozygosity (LOH) and/or mutation.

Methods—Here we have analyzed 33 HBV- and 40 HCV-related HCCs for mRNA expression of wildtype KLF6 (wtKLF6) as well as the KLF6 variant 1 (SV1), a truncated, growth-promoting variant that antagonizes wtKLF6 function. The HCV-related tumors analyzed represented the full histologic spectrum from cirrhosis and dysplasia to metastatic cancer.

Results—Expression of *KLF6* mRNA is decreased in 73% of HBV-associated HCCs compared to matched surrounding tissue (ST), with reductions of ~80% in one-third of the patients. *KLF6* mRNA expression is also reduced in dysplastic nodules from patients with HCV compared to cirrhotic livers ($p < 0.005$), with an additional, marked decrease in the very advanced, metastatic

stage ($p < 0.05$). An increased ratio of KLF6SV1/wt KLF6 is present in a subset (6/33, 18%) of the HBV-related HCCs compared to matched ST. Reconstituting KLF6 in HepG2 cells by retroviral infection decreased proliferation and related markers including cyclin D1 and beta-catenin, increased cellular differentiation based on induction of albumin, E-cadherin, and decreased alpha fetoprotein.

Conclusions—We conclude that reduced KLF6 expression is common in both HBV- and HCV-related HCCs and occurs at critical stages during cancer progression. Effects of KLF6 are attributable to regulation of genes controlling hepatocyte growth and differentiation.

Keywords

Hepatocellular carcinoma; Alternative splicing; KLF6SV1; Dysplasia; E-cadherin

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death in the world with an increasing incidence in the US, mainly due to HCV, HBV and alcoholic liver disease [1]. While new therapeutic strategies have significantly improved survival for tumors detected at early stages, the majority of patients are still diagnosed at an advanced stage and their prognosis remains poor [2]. These findings highlight the need for improved diagnosis and treatment of HCC.

Many molecular pathways have been implicated in the pathogenesis of HCC, which usually develops in a diseased liver with chronic hepatitis and/or cirrhosis [3]. Hepatocyte turnover is markedly increased in this setting, leading to clonal selection of cells with growth advantage through the accumulation of genetic and epigenetic events disrupting key regulatory pathways. These events include chromosomal instability, activation of oncogenes, as well as inactivation of tumor suppressor genes through either mutation or promoter methylation [3]. Typically, HCC associated with viral hepatitis occurs in a step-wise progression from dysplastic nodules to early, advanced and very advanced stages [4]. Little is known about the specific regulatory derangements occurring at each stage of the cancer's development, although a clearer understanding of these events could lead to advances in diagnosis, treatment and defining prognosis.

KLF6 is a tumor suppressor gene that is functionally inactivated in several types of cancer, including HCC, through a range of mechanisms. Inactivation by loss of heterozygosity (LOH) and/or mutation occurs in prostate cancer [5,6], colorectal carcinoma [7], astrocytic glioma [8], nasopharyngeal carcinoma [9] and gastric cancer [10]. Furthermore, there is decreased KLF6 expression in primary lung cancer [11] and prostate cancer cell lines [5] and gene silencing through promoter hypermethylation has been reported in esophageal cancer cells [12]. Recently, a unique mechanism of *KLF6* inactivation has been identified, involving the generation of KLF6 alternatively spliced isoforms that antagonize the tumor suppressing functions of the full-length, wtKLF6 protein [13,14].

We and others have previously established KLF6's role in HCC by demonstrating frequent loss and/or mutation in the gene [15,16], but stage-specific changes in KLF6 biology during hepatocarcinogenesis have yet to be characterized. Here we investigate the functional deregulation of KLF6 in HCC by assessing its mRNA levels in HBV- and HCV-related tumors, and thereby establish a role for KLF6 in hepatocyte growth and differentiation. Furthermore, we have defined the behavior of KLF6 in the initiation and progression of HCC in HCV-related disease from pre-malignant lesions through successive histological stages of the tumor.

2. Methods

2.1. Patient sample sets

2.1.1. HBV—HCC—HCC samples from 33 patients and matched surrounding tissue (ST), as well as 10 control livers (CL), were obtained, all with the approval of the Institutional Review Board (IRB) of all institutions involved, as described previously [17]. These were mostly HBV-related HCC, all with Edmondson's grade II or III. ST were either cirrhotic or non-cirrhotic patient samples.

2.1.2. HCV—An HCV-related sample set in which histological progression was carefully characterized was analyzed for *KLF6* mRNA expression. Samples were collected from three institutions: Mount Sinai Medical Center, New York, USA; Hospital Clinic, Barcelona, Spain; and INT, Milan, Italy, all with IRB approval. We included patients with HCV-related HCC undergoing surgical liver resection or liver transplantation. Samples were excluded from the study if patients had evidence of co-infection with HBV, alcohol abuse, NASH (non-alcoholic steatohepatitis), hemochromatosis, as well as previous treatment, whether loco-regional or systemic. Control patient samples included 10 patients undergoing resection for benign conditions including hemangioma, focal nodular hyperplasia or cystadenoma. Control tissue was derived from other liver segment distal from the lesion.

Collected samples were placed within one hour of resection/transplantation in liquid nitrogen or *RNAlater*[™] (Ambion). Explanted livers were sectioned into 5 mm slices and evaluated for the number and size of lesions, degree of differentiation, as well as vascular invasion. Pathological staging was made according to a consensus agreement between two blinded expert pathologists. Distinction between dysplastic nodules and HCC was made according to the International Working Party Criteria [18]. The HCV sample set included the following histological groups: 10 control patient samples (C); 10 cirrhosis (Ci) found in HCC patients; 18 dysplastic nodules (Dys) including low grade and high grade dysplastic nodules; and 40 HCC patient samples including: 20 early stages (10 'very early' HCC—well-differentiated tumors less than 2 cm in diameter; 10 'early' HCC within Milan criteria); 10 'advanced' HCC—large HCC with microvascular invasion or satellites and/or poorly differentiated tumors; 10 very advanced HCC ('AA HCC')—with evidence of macrovascular invasion or extrahepatic metastases.

2.2. RNA extraction

RNA was isolated from the HBV-related sample set as previously reported [17]. The HCV-related sample set RNA was extracted using Trizol reagent[™] followed by column purification (Qiagen). RNA was assessed for quality by agarose electrophoresis and accepted for analysis only if the 28S/18S ratio was >0.7.

Cell line RNA was extracted using RNeasy[™] mini kit (Qiagen) and treated with DNase (Qiagen). A total of 1 µg of RNA was reverse transcribed per reaction using first strand complementary DNA synthesis with random primers (Promega).

2.3. Quantitative real-time PCR (QRT-PCR)

In HBV-related patient samples SYBR green-based QRT-PCR was performed on an ABI PRISM 7900HT Sequence Detection System. The following primers were used: wtKLF6 forward 5' CGG ACG CAC ACA GGA GAA AA 3' and wtKLF6 reverse 5' CGG TGT GCT TTC GGA AGT G 3'; variant 1 (Var 1) forward 5' CCT CGC CAG GGA AGG AGA A 3' (with wtKLF6 reverse); GAPDH forward 5' CAA TGA CCC CTT CAT TGA CC 3' and GAPDH reverse 5' GAT CTC GCT CCT GGA AGA TG 3'; Cyclin D1 forward 5' CGA TTT CAT TGA CAC TTC C 3' and cyclin D1 reverse 5' AGT CTG GGT CAC ACT

TGA TC 3'; E-cadherin forward 5' CAA AGT GGG CAC AGA TGG TGT G 3' and reverse 5' CTG CTT GGA TTC CAG AAA CGG 3'; albumin forward 5' CTT TTC TCT TCT GTC AAC CCC AC 3' and reverse 5' TGA AGA TAC TGA GCA AAG GCA ATC 3'; beta catenin forward 5' CAT CTG TGC TCT TCG TCA TCT GA 3' and reverse 5' CAG GGT GCC ATT CCA CGA 3'. All experiments were done in triplicate and normalized to GAPDH.

Taqman QRT-PCR was performed on the HCV-related sample set using Taqman gene expression assay Hs00154550 m1 (Applied Biosystems). All data were normalized to 18S rRNA.

The difference between cycle number for wtKLF6 and 18S was calculated and results were expressed in fold changes compared to 18S rRNA, log2 transformed. Results were confirmed in a subset of samples by QRT-PCR using SYBR green.

2.4. Cell culture and retroviral infection

HepG2 cells were obtained from American Tissue Culture Collection (ATCC, Manassas, VA) and were cultured as described [15]. Retroviral infection with wildtype KLF6 was performed as previously described [19].

2.5. Proliferation assay

Proliferation was assessed by measuring ³H-labeled thymidine incorporation into DNA. A total of 300,000 cells were plated in a 12-well dish and assessed at 24, 48 and 72 h [15].

2.6. Western blot analysis and antibodies

Whole cells were lysed in RIPA buffer at 72 h after plating, and the lysate was sonicated and pelleted by centrifugation. The supernatant was denatured, and 40 µg was separated on a polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blotting of the membranes was performed using rabbit polyclonal antibody to KLF6 (R-173); goat polyclonal to beta-actin (I-19); mouse monoclonal to cyclin D1 (A-12); mouse monoclonal to E-cadherin (67A4) and mouse monoclonal to beta-catenin (E-5) (all from Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibody to albumin was also used (A6684, Sigma-Aldrich, Saint Louis, MI).

Enhanced chemiluminescent images of immunoblots were analyzed by scanning densitometry and quantified with the BIOQUANT NOVA imaging system (BIOQUANT NOVA PRIME Measurement Software). Values represent fold change compared to the control following normalization to actin.

2.7. Statistical analysis

Comparison between groups was done by using the Student's t test or the Mann-Whitney's test for continuous variables, and the Fisher's exact test for categorical variables. Correlations between two variables were assessed by Pearson's correlation coefficient. *p* values equal to or smaller than 0.05 were considered significant. The calculations were done by the SPSS package (SPSS 10.0, Inc. 1989–1995, Chicago, Illinois).

3. Results

3.1. Reduced KLF6 mRNA expression in HCC from patients with HBV, compared to surrounding tissue (ST)

KLF6 mRNA levels were evaluated in 33 HBV-related HCCs and matched surrounding tissue (ST) patient samples, in addition to 10 control livers (CL). This specific subset of

patient samples was previously used to identify gene signatures associated with patient survival [17]. In that study, consistent downregulation of the *KLF6* mRNA was also noted in the microarray analysis comparing HCCs to surrounding tissue [17]. Based on these findings, we performed QRT-PCR on a subset of these same samples to validate the earlier array results. Our data using real-time PCR primers specific for wtKLF6 (full length) correlated well with the array data, with a linear regression coefficient of $R^2 = 0.86$, and a p value of 0.0001 (Pearson) (Fig. 1A).

Overall, *KLF6* mRNA expression in HCCs from patients with HBV was significantly reduced compared to both CL and matched ST (p values of 0.001 and 0.0001, respectively) (Fig. 1B). *KLF6* mRNA expression in HCC was significantly reduced in 57% and 73% of patient samples compared to CL and matched ST, respectively (Fig. 1C and D). In contrast, there was no difference in *KLF6* mRNA levels between cirrhotic and non-cirrhotic livers (data not shown). In about one-third of the tumor samples wtKLF6 levels were reduced by more than 80% compared to either matched surrounding tissue or CL, suggesting inactivating events in addition to LOH that may account for this decrease. Of note, there was no correlation between reduced *KLF6* mRNA levels and survival subclasses identified by Lee et al. [17]. Nevertheless, reduced *KLF6* mRNA levels were significantly associated with tumors larger than 5 cm ($p = 0.01$).

Increased *KLF6* alternative splicing was recently identified in prostate cancer [13,14]. These splice forms antagonize full length KLF6 tumor suppression and promote growth in prostate cancer cells [13]. Three alternative splice forms have been described, all of which contain most of the N terminal activation domain but deleting all or part of the C-terminal DNA binding domain. KLF6 splice variant 1, KLF6-SV1, is the most biologically active of the three KLF6 splice forms identified to date and is overexpressed in prostate cancer [14].

We evaluated the KLF6 splicing ratios (SV1/ wtKLF6) in the HBV- and HCV-related HCCs. In the HBV-related sample set, 6 of 33 patients (18%) displayed an increased splicing ratio in HCC compared to ST (patients 5, 9, 15, 16, 22, and 28) (Fig. 2A). These patients were not distinguishable by prognostic subclass [17], tumor stage or size, compared to patients with no increase in splicing ratio. In the HCV-related sample set, there was no increased splicing ratio in any of the histological groups that was statistically significant. Nevertheless, there was a trend towards increased splicing ratio in very early HCC: splicing ratio of 0.05 in very early HCC compared to 0.03 in dysplastic nodules (p value of 0.059), and compared to 0.033 in all other histological stages of HCC (p value of 0.077) (Fig. 2B).

3.2. KLF6 mRNA expression is significantly decreased in the preneoplastic stage of HCV-related hepatocarcinogenesis

To establish that KLF6 dysregulation was not confined to patients with HBV, we explored the potential role of KLF6 in HCCs associated with HCV infection, as well as investigating its behavior in early stages of tumor formation. *KLF6* mRNA expression was significantly reduced in the dysplastic samples compared to cirrhosis ($p = 0.0001$) and decreased even further in the very advanced HCCs ($p = 0.03$) (Fig. 3). In addition, non-metastatic HCC and very advanced HCC both had decreased levels compared to cirrhosis ($p = 0.0001$ for both), and very advanced were also decreased compared to control liver ($p = 0.004$). Moreover, there was a significant reduction in wtKLF6 mRNA levels in 4/9 HCC patients compared to their matched ST (Supplemental Data) and this decrease correlated with advanced tumor stage ($p = 0.025$). Together, these data indicate that *KLF6* mRNA downregulation is an early event in the development of HCC, and further downregulation of wtKLF6 expression occurs at a very late stage.

3.3. KLF6 inhibits growth and increases differentiation in HepG2 cells

HepG2 cells have very low levels of KLF6, similar to those detected in patient-derived tumors. In order to study the role of KLF6 in hepatocyte growth and differentiation, HepG2 cells stably expressing KLF6 were generated by retroviral infection (Fig. 4A). *KLF6* mRNA levels were increased 10-fold and the protein 5-fold compared to cells infected with the empty vector (pBabe) (Fig. 4A, left panel). Stable overexpression of KLF6 in these cells decreased proliferation by 40% (p value < 0.0005) when compared to cells expressing empty vector [15] (Fig. 4A, right panel).

In many tissues growth arrest and differentiation are intricately linked, such that rapidly growing cells are typically less differentiated [20]. Therefore, we next explored whether inhibition of hepatocellular growth by KLF6 was associated with increased differentiation, as assessed by analysis of several growth-related (cyclin D1 and beta catenin) and differentiation-related transcripts (albumin, transthyretin (TTR)), as well as alpha fetoprotein (AFP), a marker of the undifferentiated, oncofetal state of hepatocytes. In addition, E-cadherin was assessed as a marker for differentiation, as well as growth inhibition [21]. The effect of KLF6 overexpression on these genes is summarized in Table 1. In HepG2 cells expressing retroviral-derived KLF6, beta-catenin mRNA and protein were decreased by 20% ($p < 0.05$) and 35%, respectively (Fig. 4B), along with decreases in *cyclin D1* mRNA and protein (20% ($p < 0.005$) and 80%, respectively) (Fig. 4B). In contrast, alpha fetoprotein was decreased by 40%, with evidence of increased *TTR* mRNA by 3.7-fold (not shown). *E-cadherin* mRNA and protein were increased (4-fold ($p < 0.05$) and 4.3-fold) (Fig. 4C), as well as albumin mRNA and protein (5-fold ($p < 0.05$) and 3.3-fold, respectively). In the KLF6 overexpressing cells, co-localization of beta-catenin and E-cadherin protein to the cell membrane was established by immunocytochemistry (Supplemental Data). These data suggest that KLF6 stimulates both hepatocyte growth suppression and differentiation, and that reconstitution of wt KLF6 decreases cellular proliferation and promotes cellular differentiation in HepG2 cells.

We next determined whether the growth suppression and increased differentiation produced by KLF6 in HepG2 cells correlated with similar changes in human HCCs. Among these target genes, there was a strong correlation between mRNA levels of *KLF6* and *E-cadherin* ($R^2 = 0.66$, $p < 0.0001$). A non-significant trend was found in the correlation between *KLF6* and *TTR* ($R^2 = 0.334$, $p = 0.057$).

4. Discussion

We have previously shown that KLF6 is involved in HCC by LOH and mutation [15]. Here we demonstrate that *KLF6* mRNA levels are reduced in the majority of tumors compared to matched surrounding tissue in HBV-related HCC. In addition, we demonstrate a very early decrease in *KLF6* mRNA levels in HCV-related preneoplastic lesions compared to expression levels in cirrhotic livers, with an even further decrease in metastatic tumors, compared to focal HCC. The data provide a more comprehensive picture of KLF6 dysregulation in HCC, and point to a particularly critical decrease in *KLF6* mRNA associated with the transition from dysplasia to carcinoma. Combined with our previous LOH data, the findings indicate that multiple mechanisms of functional inactivation of the KLF6 tumor suppressor gene occur in the majority of HCCs.

Our previous study demonstrated LOH and/or mutation in ~50% of HCCs compared to matched non-tumor liver [15] but did not quantify mRNA levels. Here we report decreased *KLF6* mRNA expression in ~75% of HCC samples compared to surrounding tissue in samples from patients with HBV. Moreover, about one-third of patient samples had levels reduced by more than 80% compared with matched ST. These findings are not likely to be

accounted for solely by LOH, since in principle loss of a single allele should lead to a ~50% decrease. Thus, additional mechanisms underlying KLF6 downregulation may be present in HCCs, including decreased transcription due to promoter hypermethylation or increased mRNA turnover. Of these two possibilities, there is already evidence for *KLF6* promoter methylation in esophageal cancer [12]. In addition, when compared to CL some HCC patient samples displayed only a moderate reduction of KLF6 expression. This suggests potential epigenetic events apart from inactivation of one or both alleles, including promoter methylation, a prospect that needs further exploration.

We report here a correlation between decreased KLF6 expression in the tumors and increased tumor size, which complements our previous finding of KLF6 LOH correlated with larger tumors [15]. Liver transplantation is the curative treatment for HCC and the outcome of patients who are candidates for this treatment is comparable to those who undergo liver transplantation for non-cancerous etiologies [22]. The criteria for transplant candidacy rely heavily on the size of the tumor, with cutoffs of a single lesion of less than 5 cm or 3 lesions less than 3 cm each. Beyond this size, a tumor is thought to have already spread and the outcome of these patients is poorer following transplantation [23]. As we demonstrate here, KLF6 correlates with poorer outcome. Therefore, analysis of KLF6 may offer a molecular approach to complement the clinical parameters in pre-transplant assessment.

In the HBV sample set, the HCC patient samples were of Edmondson grades II and III, and did not reflect the full spectrum of carcinogenesis in HBV-related HCC. Therefore, in order to establish the temporal sequence of KLF6 dysregulation, we analyzed an independent sample set of HCV-related HCC in which histological stages were well defined. The data indicate that *KLF6* mRNA expression is abruptly downregulated in dysplastic nodules, a pre-malignant lesion, and is further reduced in metastatic tumors. Thus, KLF6 deregulation appears to occur sequentially in hepatocarcinogenesis: first, in the initiation of cancer, in which reductions in *KLF6* mRNA expression may play a role in rendering hepatocytes more sensitive to oncogenic or environmental insults, and then with further reduction in the very late stages, when tumors become invasive. Similarly, while KLF6 dysregulation occurred in both HBV and HCV-related tumors, it is uncertain whether the same pathways are disrupted by reduction of KLF6 in each of these etiologies, as the pathways of neoplasia may differ between these two viral diseases [3,4].

Our group has recently established an association between increased alternative splicing of KLF6, and enhanced prostate cancer risk and tumor growth [13,14]. However, in HBV-related HCC, increased splicing of KLF6 was evident in only a small fraction of the HBV-patient samples and did not correlate with previously identified prognostic subclasses [17] (Fig. 2A). In contrast, in the HCV-related HCCs, there was a trend towards an increased splicing ratio in very-early HCC compared to either other stages of HCC, or compared to pre-malignant stages (Fig. 2B). This finding contrasts with other tumors including prostate and ovarian cancers, where increased KLF6 splicing is evident almost universally in the tumors compared to non tumorous tissue [14,24]. Given the relatively small number of HCC samples analyzed in each stage, further studies are merited to determine whether increased splicing ratio plays a significant role in sharply defining the presence of cancer.

In parallel with analysis of KLF6 dysregulation in HCC, we have explored its biological activity in the hepatoblastoma cell line, HepG2. This cell line offers the advantage of very low endogenous levels of KLF6, comparable to that seen in HCC, so that effects of increased ectopic KLF6 can be readily discerned. In this system, ectopic expression of KLF6 in a range similar to that of normal cells leads to growth inhibition and enhanced differentiation, as reflected in decreased mRNA levels of *cyclin D1* and *beta catenin*

associated with induction of *albumin*, *E-cadherin*, and *TTR* mRNAs, with decreased AFP expression. It is uncertain if each of these genes represents a direct transcriptional target of KLF6, except for E-cadherin, which interacts directly with the *KLF6* promoter [25].

Several lines of evidence implicate cyclin D1 in control of hepatocyte growth and HCC. For example, transgenic mice overexpressing cyclin D1 develop HCC within 17 months of age [26], while cyclin D1 expression in human HCC patient samples has been correlated with a poorer prognosis [27]. Of note, the reduction in cyclin D1 protein following retroviral KLF6 expression appears to be greater than the corresponding suppression of cyclin D1 mRNA (Fig. 4B); this difference may reflect the potential post-translational effect of KLF6 by sequestration of cyclin D1 protein [19].

We demonstrate that overexpression of KLF6 downregulates *beta-catenin* mRNA and protein, associated with decreased proliferation and increased expression of differentiation markers. These findings add to a substantial body of data implicating beta-catenin/Wnt signaling in HCC. In early liver development, beta-catenin is upregulated and its nuclear localization correlates with cell proliferation [28]. In adult cells, beta-catenin regulates cell-cell adhesion, and is localized to the submembranous compartment of the cell (reviewed in [29,30]). In this setting the protein is localized to the hepatocyte membrane, while nuclear staining is rare [28]. Inappropriate reactivation of beta-catenin is observed in HCC, in part as a result of mutations [31]. Beta-catenin overexpression is apparent in the early stages of HCC, whether cytoplasmic or nuclear, while correlation with poor survival is noted in the later, more de-differentiated stages [32].

Our data have identified a close correlation between wtKLF6 and E-cadherin levels in HCC samples. KLF6 also markedly upregulates *E-cadherin* mRNA and protein, and elicits a similar effect to E-cadherin itself on hepatocellular proliferation and differentiation. E-cadherin is a candidate tumor suppressor gene located at the 16q22 locus that is frequently lost in HCC [33]. E-cadherin, along with the adenomatous polyposis coli (APC) gene, is frequently hypermethylated in HCC, associated with poorer survival [34,35]. Cadherins can regulate the balance between hepatocyte differentiation and proliferation [21] and E-cadherin is upregulated in HCC cell lines by agents that induce differentiation, including sodium butyrate and interferon alpha [36].

Mounting data indicate a generalized role of KLF6 in cellular differentiation, a conclusion further reinforced by its effects on HepG2 cells in this study. In these cells, KLF6 promotes expression of differentiation genes, including upregulation of albumin and TTR, and downregulation of AFP. Albumin and TTR are well-characterized markers of differentiated hepatocytes [37,38], while AFP is a marker of the oncofetal or undifferentiated state. Moreover, increased serum AFP in HCC patients has been associated with histologically less differentiated state in some [39], but not all [40] studies. Similarly, in other tissues KLF6 contributes to corneal development [41], promotes adipogenesis [42], and is required for normal hematopoiesis and vasculogenesis during development [43].

In summary, deregulation of KLF6, either through reduced expression, alternative splicing, inactivating mutation and/or LOH is an almost universal event in HCC. The biological importance of a tumor suppressor may rely not solely on the absolute amount of growth inhibition it confers, but also on the stage it is perturbed in a tumor's development. In particular, reduced expression occurs at a very early stage in HCV-related tumorigenesis, and is associated with development of larger tumors. Reconstitution of KLF6 in a hepatoblastoma line not only inhibits growth, but also induces a more differentiated phenotype. Collectively, the data reinforce the contribution of KLF6 to normal hepatic differentiation, and the importance of KLF6 deregulation in hepatic carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

KLF6	Kruppel like factor 6
HCC	hepatocellular carcinoma
HBV	hepatitis B virus
HCV	hepatitis C virus
LOH	loss of heterozygosity
wtKLF6	wildtype KLF6
SV1	splice variant 1

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.jhep.2006.10.012.

Fig. 1.

(A) Correlation of wtKLF6 mRNA levels between microarray and real time analyses in HBV-related HCCs. Wildtype *KLF6* mRNA levels were assessed by QRT-PCR using the SYBR green method in 33 HBV-related HCCs and ST, and normalized to *GAPDH* mRNA. The same patient samples were independently assessed for *KLF6* mRNA by microarray using Cy3 and Cy5 dyes [17]. The results in both methodologies demonstrate very high internal consistency with a correlation coefficient (R^2) of 0.86, and p value = 0.0001 (Pearson). (B) Wildtype KLF6 levels in HBV-related HCC are reduced compared to control livers (CL) and surrounding tissue (ST). Evidence of decreased expression of wtKLF6 in HCC when compared to CL (** p value < 0.005) as well as ST (** p value < 0.0005). mRNA values were normalized to *GAPDH* mRNA, log 2 transformed and represent means of three independent PCRs. (C) wtKLF6 mRNA levels are reduced in 57% of HBV-related tumors compared to CL. Wildtype *KLF6* mRNA levels in patient tumor samples were compared to unrelated pooled control livers (CL) from 10 patients. For each patient, mRNA levels in the tumor are represented as fold change compared to CL. Fifty-seven percent of patient tumor samples demonstrate significantly decreased expression of wtKLF6 mRNA in the tumor relative to CL. In 11 of 33 (33%) patients *KLF6* mRNA levels were decreased by at least 80%. Values shown are means of three different PCRs (* p value < 0.05). (D) wtKLF6 levels are reduced in 73% of HBV-related tumors compared to matched ST.

Wildtype KLF6 levels in patient tumor samples were compared to paired matched ST. In each pair, mRNA levels in the tumor are represented as fold change compared to ST. 73% of patient tumor samples demonstrate decreased expression of wtKLF6 in the tumor relative to matched ST. In 10/33 (30%) patients, *KLF6* mRNA expression was decreased by at least 80%. Values shown are mean of three different PCRs. All reached statistical significance except for patient samples 4, 18, 21 and 22 (**p* value < 0.05, ** *p* value < 0.005, *** *p* value < 0.0005). [This figure appears in colour on the web.]

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Fig. 2.

(A) Increased KLF6 splicing ratio in the tumors compared to ST in HBV-related HCC. The splicing ratio, *SV1*/wtKLF6, reflects the expression of the biologically potent *SV1* mRNA relative to the expression of full-length *KLF6* mRNA, as assessed by QRT-PCR. A relative ratio between the tumor and the ST of >1 reflects increased splicing in the tumor. In 6 of 33 patients (patients 5, 9, 15, 16, 22, and 28) there was an increased splicing ratio in HCC compared to matched ST. (B) No increase in KLF6 splicing in any histological group in HCV-related HCC patient samples. Mean splicing ratio in each histological group was assessed by QRT-PCR as indicated. There was no significantly increased splicing in any HCV-associated histological group analyzed. There was, however, a trend towards increased splicing in very early HCC. [This figure appears in colour on the web.]

Fig. 3. wtKLF6 levels are abruptly reduced in dysplastic nodules, and further reduced in advanced, metastatic stages of HCV-related HCC. Wildtype KLF6 levels in HCV-related HCC patient samples were assessed by QRT-PCR using the Taqman method. The result for each patient is represented by a dot in a scatter plot. Patient samples are divided according to histological diagnosis as follows: control, non diseased liver (C); cirrhosis (Ci); dysplastic nodules (Dys); HCC including very early, early and advanced stages (HCC); very advanced HCC, including macrovascular invasion or extrahepatic metastases (AA HCC). Starting from the Dys stage, there is significant reduction in wtKLF6 levels compared to Ci. In the AA HCC stage, there is an additional significant reduction in wtKLF6 levels compared to HCC. mRNA values are normalized to 18S and are log 2 transformed.

Fig. 4.

(A) Stable expression of wtKLF6 in HepG2 cells by retroviral infection decreases cell proliferation. (Left panel) Following retroviral infection, HepG2 cells stably expressing wtKLF6 display a 10-fold increase in wtKLF6 mRNA, and 5-fold increase in protein levels compared to infection with empty vector (pBabe). *** p value < 0.0005. mRNA values are expressed as fold change compared to GAPDH and protein levels are normalized to actin by densitometry. (Right panel) As assessed by ^3H -labeled thymidine incorporation, stable expression of wtKLF6 in HepG2 cells results in a 40% decrease in proliferation, compared to empty vector. *** p value < 0.0005. (B) HepG2 cells expressing wtKLF6 display decreased expression of beta-catenin and cyclin D1 mRNAs and proteins. Increased expression of wtKLF6 in HepG2 cells resulted in decreased beta-catenin (left) and cyclin D1 (right) mRNAs (20% and 30%) as well as their protein levels (40% and 78%, respectively). mRNA levels were normalized to GAPDH and expressed as fold change compared to the empty vector control; protein levels were normalized by densitometry to actin. * p value < 0.05; ** p value < 0.005. Of note, HepG2 cells carry a deletion in beta-catenin gene, and when examined by Western, 2 different bands are observed, a wildtype form (97 kDa) and a truncated or predominant form (75 kDa). Both bands are reduced as a result of KLF6 over-expression. (C) HepG2 cells expressing wtKLF6 display increased mRNA expression of E-cadherin and albumin. Increased expression of wtKLF6 in HepG2 cells resulted in increased

E-cadherin (left) and albumin (right) mRNAs (4- and 5-fold, respectively) and protein (4.3- and 3.3-fold, respectively). mRNA levels were normalized to GAPDH and expressed as fold change compared to the empty vector control (pBabe); protein levels were normalized by densitometry to actin. * p value < 0.05.

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Table 1

Changes in mRNA and protein levels of hepatocellular growth- and differentiation-related genes following KLF6 expression in HepG2 cells

	Fold change in mRNA compared to empty vector	Fold change in protein compared to empty vector
KLF6	10	5.2
Beta-catenin	0.8	0.64
Cyclin D1	0.7	0.22
E-cadherin	4	4.3
Albumin	5	3.3

Summary of the data shown in Fig. 4A–C. Following retroviral infection, expression of growth and differentiation-related genes was analyzed by QRT-PCR and Western blot, normalized to actin protein, each repeated at least three times. *KLF6* mRNA expression is increased 10-fold and KLF6 protein increased 5-fold following KLF6 expressing retroviral infection, compared to cells infected with the empty vector (pBabe-puro). This results in reductions in *beta-catenin* and *cyclin D1* mRNAs by 20% and 30% respectively, and their proteins decreased by 36% and 78%, respectively. In addition, there was a ~4-fold increase in *E-cadherin* mRNA and protein, a 5-fold increase in *albumin* mRNA and a ~3-fold increase in albumin protein.