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Enhanced hepatocarcinogenesis in mouse models and human HCC by coordinate KLF6 depletion and increased mRNA splicing

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Abstract

Background—*KLF6-SV1* (*SV1*), the major splice variant of *KLF6*, antagonizes the KLF6 tumor suppressor by an unknown mechanism. Decreased KLF6 expression in human hepatocellular carcinoma (HCC) correlates with increased mortality, but the contribution of increased SV1 is unknown. We sought to define the impact of SV1 on human outcomes and experimental murine hepatocarcinogenesis, and to elucidate its mechanism of action.

Results—In HCV-related HCC, an increased ratio of *SV1/KLF6* within the tumor was associated with more features of more advanced disease. Six months after a single injection of diethylnitrosamine (DEN), *SV1* hepatocyte transgenic mice developed more histologically advanced tumors, whereas *Klf6*-depleted mice developed bigger tumors compared to the *Klf6*Δl(+/+) control mice. Nine months after DEN, *SV1* transgenic-mice with *Klf6*-depletion had the greatest tumor burden. Primary mouse hepatocytes from both the *SV1* transgenic animals and those with hepatocyte-specific *Klf6* depletion displayed increased DNA synthesis, with an additive effect in hepatocytes harboring both *SV1* over-expression and *Klf6* depletion. Parallel results were obtained by viral SV1-transduction- and depletion of Klf6 through adenovirus-Cre infection of primary *Klf6*Δl(+/+) hepatocytes. Increased DNA synthesis was due to both enhanced cell proliferation and increased ploidy. Co-IP studies in 293T cells uncovered a direct interaction of transfected *SV1* with *KLF6*. Accelerated KLF6 degradation in the presence of *SV1* was abrogated by the proteasome inhibitor MG132.

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Conclusion—Increased SV1/KLF6 ratio correlates with more aggressive HCC. In mice, an increased SV1/KLF6 ratio, generated either by increasing SV1, decreasing KLF6, or both, accelerates hepatic carcinogenesis. Moreover, SV1 binds directly to KLF6 and accelerates its degradation. These findings represent a novel mechanism underlying the antagonism of tumor suppressor gene function by a splice variant of the same gene.

Keywords

hepatocellular carcinoma; tumor suppressor; proteasome; alternative splicing; transgenic mice

Introduction

Hepatocellular carcinoma (HCC) is comprised of several molecular subclasses(1). We previously reported that allelic loss of the KLF6 tumor suppressor enhances chemical carcinogenesis in mice, and the molecular signatures of the resulting tumors closely mimicked aggressive human HCC(2). KLF6 belongs to the family of Krüppel-like factors, which are characterized by the presence of Cys2/His2 zinc finger motifs in their carboxy-terminal domains, which confer binding to GC/GT-rich sequences in gene promoter and enhancer regions(3). KLF6 regulates cellular pathways that inhibit tumor cell proliferation, migration, angiogenesis and invasion(2, 4-7), while enhancing apoptosis(8) and differentiation(9). Reduction of *KLF6* mRNA expression in HCCs due to chronic HBV(10) and HCV(2, 10) is frequent, and correlates with advancing stage; moreover, extremely low *KLF6* mRNA levels are linked to reduced survival(2).

KLF6 activity in human cancer can be attenuated by loss of heterozygosity(5, 11-14), somatic mutation(11, 12) and promoter methylation(15). Additionally, alternative splicing of *KLF6* into an antagonistic splice form, SV1, is increased in HCC(10, 16) and other cancers(9, 17-19). Specifically, ratios of *SV1/KLF6* in tumors from HBV(10)-and HCV(2, 10, 16)-related HCCs are increased compared to surrounding tissues.

SV1, the major *KLF6* splice variant, lacks the DNA binding domain, is proproliferative and facilitates tumor invasion by antagonizing the transactivation of p21 and E-cadherin by KLF6(5, 6). SV1 also displays pro-apoptotic caspase activity and accelerates degradation of the anti-apoptotic protein NOXA(20, 21). Moreover, silencing of SV1 in ovarian cancer models decreases invasiveness and angiogenesis, with reduced VEGF protein(9).

Mechanisms driving splicing of *KLF6* and accounting for its antagonism of full length KLF6 are largely unknown. Activation of the *Ras* oncogene stimulates *KLF6* splicing, which promotes proliferation(15, 22). The specific ratio of SV1/KLF6 appears to regulate proliferative and tumorigenic activity, but it is unclear whether the effect is due solely to increased SV1, decreased KLF6 or both. Accordingly, in this study we have first established the clinical relevance of an increasing ratio of *SV1/KLF6* as a predictor for HCV-associated HCC behavior, and then modeled the key features of KLF6 dysregulation in human HCC using mouse models, including loss of KLF6 expression through hepatocyte-specific deletion, increased SV1 through hepatocyte-specific transgene expression, and a combination of the two defects. These findings confirm KLF6 dysregulation in human HCC and provide novel insights into this tumor suppressor gene's regulation and impact on hepatocarcinogenesis.

Materials and Methods

Human Data

We analyzed *SVI*- and *KLF6* mRNA levels in 149 HCV infected human liver samples covering the entire hepatocarcinogenic spectrum: normal liver (n=9), cirrhosis (n=9), dysplastic nodules (n=27), very early HCC (n=16), early (n=17), advanced HCC (n=51) and very advanced HCC (n=20) as previously described(2). *SVI*-/*KLF6* mRNA expression ratio was further correlated with clinic and pathological variables in a subset of 55 HCC patients. Real time PCR was performed as previously described(2).

Mice

Klf6^{fl/+} mice provided by Genentech were bred with Albumin-Cre mice(23). The TTR-flag-humanSVI-PolyA construct was cloned with a 3-fragment recombination into the pcDNA6.2/V5-pL destination vector using the MultiSite Gateway Pro system from Invitrogen. The construct was injected into *Klf6*^{fl/+} fertilized eggs. The resulting *SVI Klf6*^{fl/+} mice were bred with *AlbCre Klf6*^{fl/+} mice. Male *Klf6*^{fl/+}-, *AlbCre Klf6*^{fl/+}-, *SVI Klf6*^{fl/+}- and *SVI AlbCre Klf6*^{fl/+} mice were injected with 5mg/kg body weight diethylnitrosamine (Sigma, #N0258) i.p. at 2 weeks of age. Tumors were measured macroscopically and analyzed microscopically as previously described(2).

Primary hepatocytes

Primary hepatocytes were isolated by in situ perfusion with Liberase™ (Roche 05-401-119-001)(2). Twelve hours later, either AdenoCre- or LacZ-expressing control virus was added at a concentration of 10 multiplicity of infection. Twenty-four hours later, media was replaced with a lentivirus expressing pBabe- or pBabe-KLF6. After 12 hours, fresh virus-containing media was added and the cells were collected 24 hours later.

Incorporation of ³H-thymidine was used to measure DNA synthesis(5). Hepatocytes were trypsinized and counted 5-, 24- and 48 hours after isolation, and the number of nuclei per hepatocyte were counted in triplicate by ImageJ64 in ten 10x fields of isolated primary hepatocytes, from all four mice lines.

For cell cycle analysis, approximately 10⁶ hepatocytes were suspended in 0.5ml PBS, fixed with 4.5ml of ice cold 70% ethanol, stained with PI solution (propidium iodide, RNaseA, PBS), strained through polystyrene cell strain tubes, incubated in the dark for 20 minutes at room temperature and FACS analysis performed with Calibur cell sorter.

Immunohistochemistry

PCNA-immunostaining was performed using Sodium Citrate 10mM, pH6.0, Dako Kit Envision System HRP labeled Polymer, anti mouse (Dako K4000) and the sc-56 α -PCNA antibody.

Cell Culture

293T and HUH7 cells were cultured in DMEM+GlutaMAX™-1 GIBCO 31985 with 10%FBS and transfected with Lipofectamine 2000 (invitrogen 11668-019), according to the manufacturer's instructions. Cells were transfected with *pCI-neo-GFP*, *pCI-neo-FLAG-KLF6*, *pCI-neo-FLAG-SVI*, a p21 luciferase promoter(5), and Renilla luciferase vector (Promega, WI) as internal control.

Protein was collected in RIPA Buffer with added protease- (Roche Complete Mini 04693124001) and phosphatase-inhibitors (Thermo Scientific #78428), and the following antibodies were used: α -KLF6 (sc7158), α -FLAG (Sigma, F7425), α -calnexin (ab75801),

α -p21 (sc397), α -Cyclin B1 (sc752). For co-immunoprecipitation studies, protein was collected in CoIP Buffer (50 mM Tris, 150 mM NaCl with PI 1:10, PPI 1:100, PMSF 100 μ M 1:100) 24 hours after transfection. 500 μ g of protein was incubated with 40 μ l of washed FLAG agarose beads (Sigma Red ANTI-FLAG M2 Affinity Gel F246) overnight. Beads were washed with PBS, 20 μ l of 2 $\times$ sample buffer was added per sample and an immunoblot with α -KLF6 (sc7158) was performed.

RNA was collected, reverse transcribed, and amplified as previously described(23). The following primers were used: mCyclophilin F/R(CAGACGCCACTGTCGCTTT/TGTCTTTGGAACCTTGTCTGCAA), hKLF6 F/R(CGGACGCACACAGGAGAAAA/CGGTGTGCTTTTCGGAAGTG), SV1 F/R(CCTCGCCAGGGAAGGAGAA/CGGTGTGCTTTTCGGAAGTG) Cycloheximide (10 mg/ml) was added 24 hours after co-transfection, and protein collected at 0, 15, 30 and 60 minutes. MG132 (Sigma C2211) was added (5 μ M) 4 hours prior to adding the Cycloheximide.

Co-transfection p21 luciferase- and Renilla-plasmids was performed as described(5). Cells were washed with PBS twice and 30 μ l of Lysis Buffer (Promega passive Lysis Buffer 5x, #E194A) was added per well.

Statistical analysis

Data are expressed as mean $\pm$ SEM or SD. Student's t-test, ANOVA, Mann-Whitney and Chi-square (3-way contingency table) were calculated to compare experimental groups. Differences were considered as statistically significant if $p < 0.05$. Analyses were conducted using R statistical package and SPSS® software (version 14) for the human data (2), and Graph Pad prism for the remaining data.

Results

Correlation of *SV1/KLF6* ratio in HCV-associated hepatocellular carcinoma with aggressive clinical features

The *SV1/KLF6* mRNA splicing ratio is increased in 18% of HBV-associated(10) and 0-76% of HCV-positive(10, 16) HCCs. Here we analyzed the *SV1/KLF6* mRNA splicing ratio in liver tissues from 149 HCV-positive patients with progressive stages of HCV-associated liver disease(2). The splicing ratio was significantly increased in HCC samples compared to non-tumoral tissues, including normal liver ($p=0.03$), cirrhotic liver ($p=0.01$), or dysplastic nodules ($p<0.001$). In addition, the ratio linearly increased with progressive stages of HCC ($p<0.001$) (Figure 1a). This finding raised the possibility that increased *KLF6* splicing might contribute to tumor behavior or clinical outcomes.

We examined whether the *SV1/KLF6* mRNA ratio was correlated with features of more advanced disease. Accordingly, we correlated *SV1/KLF6* mRNA ratio with clinical and pathological variables in a subset of 55 HCCs, from whom these data were available. Increased *SV1/KLF6* ratio was significantly associated with larger tumors ($p=0.04$) and vascular invasion ($p=0.01$) (Figure 1b). *KLF6* splicing ratio was not correlated with survival, however (data not shown). These findings are consistent with earlier reports in prostate, lung(21), ovarian(20) and pancreatic(19) cancers, where SV1 has been correlated with more aggressive disease.

Hepatocyte-specific *SV1* transgenic mice have more advanced tumors 6 months after DEN treatment

To clarify the role of an increased *KLF6* splicing ratio in hepatocarcinogenesis, we generated mice with different *SV1/KLF6* ratios by first crossing double floxed *Klf6* mice

(*Klf6^{fl}/+*) with albumin-Cre transgenic animals (*AlbCre*) (23, 24). Then, we generated hepatocyte-specific human SV1-expressing mice in a *Klf6^{fl}/+* background (*SV1 Klf6^{fl}/+*). Hepatocyte-specificity was conferred by using the transthyretin (*TTR*) (25) promoter. These mice were then bred with the *AlbCre Klf6^{fl}/+* mice. Altogether, this breeding strategy yielded four lines of mice: 1) *Klf6^{fl}/+* mice (used as controls) with endogenous *Klf6*; 2) *AlbCre Klf6^{fl}/+* mice with hepatocyte specific *Klf6* depletion and no SV1; 3) *SV1 Klf6^{fl}/+* mice with hepatocyte-specific *SV1* over-expression and endogenous *Klf6*, and; 4) *SV1 AlbCre Klf6^{fl}/+* mice with hepatocyte-specific *SV1* over-expression on a background of *Klf6* depletion. All mice appeared phenotypically normal (Suppl. Figure 1A,B) with normal liver architecture and no spontaneous tumorigenesis. Hepatocyte-specific over-expression of SV1 and endogenous KLF6 levels were validated in the *SV1 Klf6^{fl}/+* transgenics by immunoblot and immunostaining (Suppl. Figure 1C,D). Of note, due to an inverted distal LoxP site *AlbCre Klf6^{fl}/+* mice have a partial *Klf6* depletion and are effectively hypomorphic rather than complete knockouts (DeSavage, F, personal communication; see Suppl. Figure 1C, 1D).

To assess the propensity of each of these four mouse lines towards hepatocarcinogenesis, animals were injected with a single i.p. dose (5mg/kg) of diethylnitrosamine (DEN) at 2 weeks of age (2), and tumor development was assessed at 3, 6 and 9 months, and compared to non-transgenic littermates. At three months there were no macro- or microscopically visible tumors. At six months 57% (4/7) of the *Klf6^{fl}/+* controls had microscopic tumors compared to 83% (5/6) in the *SV1 Klf6^{fl}/+* transgenics and 100% of both the *AlbCre Klf6^{fl}/+* (13/13) and the *SV1 AlbCre Klf6^{fl}/+* (7/7) animals (n.s.) (Supplemental Table 1). Tumors were significantly larger in the *AlbCre Klf6^{fl}/+* mice ($p < 0.05$) (Figure 2A, 2B) and contained a significantly higher tumor grade in the *SV1 Klf6^{fl}/+* transgenics ($p < 0.05$) and even more so in *SV1 AlbCre Klf6^{fl}/+* mice ($p < 0.005$) (Figures 2C, 2D) at 6 months after DEN injection. These findings indicate that both *Klf6* depletion and *SV1* over-expression independently promote tumorigenesis after DEN-treatment, by increasing size and advancing the histologic grade of the tumors.

Hepatocyte-specific SV1 transgenic mice, in combination with Klf6 depletion, have a greater tumor burden and heavier livers 9 months after DEN

The tumorigenic activities of *Klf6* depletion and/or *SV1* over-expression at 6 months were more clearly evident at 9 months, with both more (Figure 3A ($p < 0.001$), Figure 3B ($p < 0.05$)) and larger tumors (Figure 3C ($p < 0.05$)) in *SV1 AlbCre Klf6^{fl}/+* mice, resulting in a significantly greater tumor burden (Figure 3D) and heavier livers ($p < 0.01$) (Suppl. Figure 2) than *Klf6^{fl}/+* controls. Interestingly this trend was also observed in both *Klf6* depleted mice (*AlbCre Klf6^{fl}/+*) and in *SV1* transgenic mice (*SV1 Klf6^{fl}/+*), but only the combination of both defects led to an additive and significant effect, reinforcing the contribution of the *SV1/Klf6* ratio in hepatocarcinogenesis.

Both SV1 over-expression and Klf6 depletion increase proliferation in primary hepatocytes

To determine whether increased tumorigenesis arose from altered cellular growth we analyzed primary hepatocytes from the four transgenic mouse models. We also explored the impact of acute, rather than chronic *Klf6* depletion, by treating primary hepatocytes from *Klf6^{fl}/+* mice with either LacZ- or AdenoCre virus to deplete *Klf6* in culture, and additionally treating cells with either pBabe- or pBabeSV1 lentivirus to over-express *SV1* in order to recapitulate the phenotypes of the four different mouse lines. This approach yielded a consistent *Klf6* mRNA depletion of ~50% and ~5-fold over-expression of *SV1*, as assessed by quantitative PCR (Suppl. Figure 3 A-C).

Primary hepatocytes from both chronic- (*AlbCre Klf6fl*(+/+)) and acute (*Klf6fl*(+/+) & adenoCre virus) *Klf6* depleted hepatocytes displayed a significant increase in DNA synthesis, based on ³H-thymidine incorporation assay (Figure 4A, *p*<0.05; Figure 4B, *p*<0.05). Although *SV1* over-expression in hepatocytes both from *SV1* transgenic animals and pBabeSV1-infected *Klf6fl*(+/+) hepatocytes only displayed a trend toward increased ³H-thymidine incorporation, the combination of *Klf6* depletion and *SV1* over-expression led to an additional and significant increase in DNA synthesis in both models (Figure 4A, *p*<0.05; 4B, *p*<0.0005). These findings correlated with a significantly increased hepatocyte count from either *SV1 Klf6fl*(+/+) transgenics 24 hours after isolation (*p*<0.05), and of *AlbCre Klf6fl*(+/+) (*p*<0.05) or *SV1 AlbCre Klf6fl*(+/+) hepatocytes (*p*<0.02) 48 hours after isolation (Figure 4C). Thus, *Klf6* depletion increases proliferation, which is further augmented when SV1 is simultaneously over-expressed.

SV1 over-expression and *Klf6* depletion increase ploidy in primary hepatocytes

To examine if *SV1 over-expression* or *Klf6* depletion had an impact on cell cycle distribution, we performed FACS analysis of primary hepatocytes. There was a significant increase of cells with 4N DNA, but without any measurable differences in S-phase distribution (Figure 5A) in *SV1* over-expressing- and/or *Klf6* depleted cells, compared to *Klf6* wild-type hepatocytes. This corresponded with very few PCNA positive hepatocytes in untreated hepatocytes of all four murine models (Suppl. Figure 4A-B). The increased number of 4N cells was consistent with an increase in cell ploidy, which was confirmed by nuclear quantification per cell (Figure 5B, 5C).

The control mice had significantly more cells with a single nucleus at baseline compared with the *SV1* transgenic- or *Klf6* depleted hepatocytes. Forty-eight hours after isolation there was a shift towards higher ploidy of the hepatocytes in all groups. This shift was significant for hepatocytes from *SV1 AlbCre Klf6fl*(+/+) mice (*p*<0.05) (Figure 4B). The increased ploidy is DEN-independent, as it was observed in hepatocytes isolated from untreated mice and in age-matched animals between 10-12 weeks of age.

Cyclin B1, which is a key regulator of G2/M transition, was consistently downregulated in SV1-transgenic and *Klf6*-depleted hepatocytes indicating reduced G2/M transition in the *SV1*- transgenic and *Klf6*-depleted hepatocytes (Suppl. Figure 5).

SV1 binds to KLF6 and inhibits its transcriptional activity (p21)

We next examined how SV1 antagonizes KLF6 tumor suppressor function. To do so, we tested the hypothesis that SV1 might physically sequester KLF6 to prevent it from transactivating growth-inhibitory genes. 293T cells were transfected with either *FLAG-SV1* or *FLAG-KLF6*. Co-immunoprecipitation using α-FLAG beads, followed by immunoblotting with a KLF6 polyclonal antibody that detects both KLF6 and SV1 protein was performed. In the cells transfected with *FLAG-SV1*, endogenous KLF6 was recovered and conversely, in the cells transfected with *FLAG-KLF6*, SV1 was recovered, documenting physical interaction between SV1 and KLF6 (Figure 6). To date, Ras-induced SV1 has been shown to decrease KLF6 promoter occupancy(16, 22). We confirmed the antagonistic function of SV1 to KLF6 by assessing the impact of SV1 on the transcriptional activity of KLF6 in cell culture using the p21 promoter, a well validated direct transcriptional target of KLF6(5). In both 293T (Figure 7A), and HUH7 cells (Figure 7B), transfection of *FLAG-SV1* alone did not affect p21 activity whereas co-transfection of *FLAG-SV1* with *FLAG-KLF6* significantly reduced the increase in p21-luciferase activity induced by *FLAG-KLF6* alone (*p*<0.05).

Similar to transfected cells, p21 protein levels were decreased in primary hepatocytes from our *AlbCre Klf6^{fl/+}*- and *SV1 AlbCre Klf6^{fl/+}* mice in comparison to the *Klf6^{fl/+}* hepatocytes with endogenous *Klf6* (Figure 7C). Moreover, in primary hepatocytes from *Klf6^{fl/+}* mice transduced with either AdenoCre virus or pBabe- or pBabeSV1 lentivirus respectively, *Klf6* depletion was also associated with a decrease in p21 protein levels (Figure 7D). These findings confirm p21 as a transcriptional target of KLF6 and establish a bona fide antagonistic function of SV1.

SV1 accelerates the degradation of KLF6

Because SV1 is localized primarily in the cytoplasm(26) and can interact with KLF6, we hypothesized that SV1 antagonizes KLF6 by accelerating its degradation in the cytoplasm. To test this hypothesis, increasing amounts of *FLAG-SV1* cDNA were co-transfected with a constant amount of *FLAG-KLF6*. This led to an SV1-dependent decrease in KLF6 protein in both 293T (Figure 8A), and HUH7 cells (Figure 8C), without affecting *KLF6* RNA levels (not shown), which was quantified in four 293T immunoblots (Figure 8B, $p < 0.05$).

To assess if decreased KLF6 protein was due to accelerated degradation of KLF6, 293T cells were co-transfected with either *pCI-neo-GFP+FLAG-KLF6* or *FLAG-SV1+FLAG-KLF6*, and protein was collected 24 hours after transfection and at 0, 15, 30, and 60 minutes after adding cycloheximide (Figure 8D-E). In the presence of SV1, KLF6 protein levels at baseline were significantly lower (Figure 8E, $p < 0.05$) and KLF6 degradation was accelerated 60 minutes after adding cycloheximide (Figure 8E, $p < 0.005$). The accelerated degradation was inhibited by adding the proteasomal inhibitor MG132 (Figure 8D, 8F), indicating that SV1 accelerates the proteasomal degradation of KLF6. These findings were reproducible in primary hepatocytes isolated from *Klf6^{fl/+}* control mice compared to *SV1 Klf6^{fl/+}* transgenics (Figure 8F). The findings establish that SV1 antagonizes KLF6 at least in part by accelerating its degradation.

Discussion

Our findings demonstrate that the *SV1/KLF6* mRNA ratio is significantly increased in patients with HCV-associated HCC relative to surrounding tissue, and correlates with features of more aggressive disease. This observation is consistent with findings in gastric(18), lung(21), prostate(27), and pancreatic(19) cancers, where increased splicing has been associated with worse patient outcomes. Consistent with these findings, KLF6 depletion in mouse models resulted in significantly increased tumor size, whereas hepatocyte-specific over-expression of SV1 led to more advanced tumor grade, consistent with findings of higher grade tumors associated with an increased *SV1/KLF6* ratio in both ovarian(9) and pancreatic(19) cancers. The increased tumorigenesis after DEN resulted in a higher tumor burden, with both larger and more numerous tumors in mice with hepatocyte specific *Klf6* depletion and *SV1* over-expression 9 months after DEN treatment, supporting a role of increased *KLF6* splicing in hepatocarcinogenesis.

The proliferative effect of an increased *SV1/KLF6* ratio was directly validated in freshly isolated primary hepatocytes from four different mouse lines and in *Klf6^{fl/+}* hepatocytes in which *Klf6* was depleted or *SV1* was over-expressed in culture. Here, *Klf6* depletion was associated with significantly increased DNA synthesis and cell number, and was further enhanced by additional *SV1* over-expression. However, hepatocyte-specific *SV1* over-expression alone was not sufficient to significantly affect cell count. Together, these findings suggest that the loss of KLF6 and increased SV1 confer separate, complementary effects on tumor propensity, with hepatocyte-specific *Klf6* depletion driving enhanced proliferation, whereas hepatocyte specific *SV1* over-expression largely provokes an

increased tumor grade. Of equal importance, the ratio of *SV1/KLF6*, and not just *Klf6* depletion or *SV1* over-expression alone, is additive in promoting tumorigenesis.

Additionally, whereas *KLF6* mRNA levels in HCV-associated liver disease decreased progressively and significantly from non-cirrhotic to cirrhotic liver tissue, with a further significant decrease in dysplastic liver tissue(2), the ratio of *SV1/KLF6* mRNA did not change between normal, cirrhotic and dysplastic liver tissue. It increased significantly from dysplastic to very early HCC, with a further significant increase in advanced and very advanced HCC. This finding suggests that a decrease of *KLF6* precedes onset of splicing, and that splicing coincides with malignant transformation. This finding is consistent with evidence of increased *KLF6* splicing only in malignant tissues from gastric(18) and pancreatic(19) cancers. In aggregate, the findings indicate that reduction in *KLF6* mRNA and increased *SV1* expression occur independently and sequentially, ultimately accelerating the development of HCC when both are present. The association of onset of *KLF6* splicing and malignant transformation, as well as the inhibition of tumor progression by *siSV1* in ovarian(9), gastric(18), and lung(28) cancer models underscore the importance of *KLF6* splicing in hepatocarcinogenesis, and reinforces its antagonism as a potential therapeutic target, possibly by antagonizing Ras(16) or other drivers of *KLF6* splicing.

The distinct phenotypes between *SV1* over-expressing mice after DEN-treatment and those with *Klf6* depletion indicate that in addition to *KLF6*-dependent functions, *SV1* likely has *KLF6*-independent functions, although little is known about these activities. Since *SV1* has been localized to the nucleus as well as cytoplasm(22), one possibility is that *SV1* functions as a transcriptional co-factor. Alternatively, *SV1* might bind to other proteins and influence their degradation and/or cytoplasmic-nuclear partitioning.

There is a growing recognition that functional antagonists of tumor suppressors may contribute to cancer progression, including those of p53(29), or cell cycle checkpoint kinases like Chk2(30). Interestingly, for both p53 and Chk2, heterodimerization with their splice variants is essential for their antagonistic function(29, 30) and can have an impact on cellular localization(29). Whereas *SV1* binds to *KLF6*, and can increase the degradation of *KLF6* by the proteasome, it is uncertain whether this interaction is required for *SV1*'s tumor promoting activity, or if *KLF6*-*SV1* heterodimerization affects cellular localization. Finally, both *SV1* over-expression as well as *Klf6* depletion in hepatocytes each increases cell ploidy, implying a role of *SV1*- and *KLF6* in G2/M cell cycle checkpoint regulation.

Our findings in HCC confirm *KLF6* splicing as a mechanism to inactivate *KLF6* full length and further confirm findings in a growing list of tumors in which splicing is enhanced in cancer, and in which an increased *SV1/KLF6* ratio has been associated with poorer outcome. In vivo cancer models employing siRNA to knock down *SV1*, for example in ovarian(9), lung(28) and gastric(18) cancers, emphasize the therapeutic potential of blocking *SV1* and justify efforts to elucidate mechanisms of *KLF6* splicing regulation in hopes of developing splicing antagonists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

ALT	alanine transaminase
AST	aspartate transaminase
DEN	diethyl nitrosamine
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
LOH	loss of heterozygosity

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SV1/KLF6 ratio – Tumor size

SV1/KLF6 ratio – Vascular invasion

Figure 1. SV1/KLF6 ratio is increased in HCV-associated hepatocellular carcinoma and is associated with aggressive clinical behavior

The SV1/KLF6 mRNA ratio in human HCV-associated liver disease samples is significantly increased in HCC compared to non-tumoral tissues ($p < 0.001$; N: normal liver (n=9), Ci: cirrhosis (n=9), D: dysplastic nodules (n=27), VE: very early HCC (n=16), E: early HCC (n=17), A: advanced HCC (51), AA: very advanced HCC (n=20). SV1/KLF6 ratio was significantly higher in larger tumors (0.09 vs 0.13, $p = 0.04$) and in HCC with vascular invasion (0.07, 0.12, $p = 0.01$, Figure 1B).

Figure 2. Hepatocyte specific *Klf6* depleted mice have larger tumors, and *SV1* transgenic mice have more progressed tumors than controls after 6 months of DEN treatment

*AlbCre Klf6*fl(+/+) mice have significantly larger tumors than the *Klf6*fl(+/+) controls 6 months after DEN treatment ($p < 0.05$) (2A, 2B). *SV1 Klf6*fl(+/+) mice have tumors with higher grading. This is even more pronounced in *SV1 AlbCre Klf6*fl(+/+) animals ($p < 0.0001$) (2C). 2D shows representative H&E images of *Klf6*fl(+/+) livers 6 months after DEN treatment with vaguely nodular parenchyma with large cell changes (grade 0-1) (i) and tumor cells with hyperchromatic nuclei and increased nuclear/cytoplasmic ratio with microvascular invasion in *SV1 AlbCre Klf6*fl(+/+) livers (grade2) (ii) .

Figure 3. Hepatocyte specific SVI transgenic mice with *Klf6* depletion have a greater tumor burden than *Klf6* wildtype control mice 9 months after DEN treatment

SVI AlbCre Klf6^{fl/fl} mice have significantly more (3A ($p < 0.001$), 3B ($p < 0.05$)) and larger tumors (3C ($p < 0.05$)) resulting in a significantly higher tumor load (3D) than *Klf6*^{fl/fl} controls 9 months after DEN treatment.

Figure 4. SVI over-expression and *Klf6* depletion increase proliferation in primary hepatocytes
 In primary hepatocytes isolated from *Klf6*fl(+/+) mice with induced acute *Klf6* depletion through adenoCre virus infection ex vivo, ³H-thymidine incorporation is increased significantly ($p < 0.05$), with an additional effect when cells are treated with both adenoCre- and pBabeSV1-lentivirus ($p < 0.05$) (4A). Correspondingly hepatocytes from *AlbCre* *Klf6*fl(+/+) ($p < 0.05$), *SVI* *Klf6*fl(+/+) ($p = 0.09$) and *SVI* *AlbCre* *Klf6*fl(+/+) ($p = 0.0005$) have more ³H-thymidine incorporation than *Klf6*fl(+/+) control cells (4B). The cell count of *SVI* *Klf6*fl(+/+) hepatocytes 24 hours after isolation ($p < 0.05$)- and of *AlbCre* *Klf6*fl(+/+) ($p < 0.05$) and *SVI* *AlbCre* *Klf6*fl(+/+) ($p < 0.02$) mice 48 hours after isolation was significantly higher than that of *Klf6*fl(+/+) control hepatocytes (4C).

Figure 5. *SV1* over-expression and *Klf6* depletion increase ploidy in primary hepatocytes

FACS analysis of freshly isolated hepatocytes from *AlbCre Klf6*fl(+/+), *SV1 Klf6*fl(+/+) and *SV1 AlbCre Klf6*fl(+/+) mice shows a significant increase of hepatocytes with 4N as opposed to 2N when compared to *Klf6*fl(+/+) hepatocytes (5A) ($p < 0.05$) without a corresponding increase in S-phase (5A). Ploidy in hepatocytes from *AlbCre Klf6*fl(+/+), *SV1 Klf6*fl(+/+) and *SV1 AlbCre Klf6*fl(+/+) animals compared to *Klf6*fl(+/+) controls is significantly increased 5 hours-, and even more pronounced 48 hours after hepatocyte isolation. At 48hours after isolation there were significantly more hepatocytes with 3 or 4 nuclei as compared to 5 hours after isolation in *SV1 AlbCre Klf6*fl(+/+) hepatocytes ($p < 0.05$). 5C shows representative phase contrast microscopic images of hepatocytes from *Klf6*fl(+/+) hepatocytes 5 hours (aⁱ) and 48 hours (aⁱⁱ) and from *SV1 Klf6*fl(+/+) again at 5-

(bⁱ) and 48 hours (bⁱⁱ) after hepatocyte isolation. The arrows point out hepatocytes with one nucleus. “*” indicates a hepatocyte with 3 nuclei.

Figure 6. SV1 physically binds to KLF6

In 293T cells transfected with either empty *pCI-neo*, *FLAG-KLF6* or *FLAG-SV1* cDNA Immunoprecipitation was performed with α -FLAG, followed by an immunoblot with α -KLF6. In cells transfected with *FLAG-KLF6*, SV1 was pulled down and in cells transfected with *FLAG-SV1*, KLF6 was pulled down.

Figure 7. SV1 inhibits KLF6 transcriptional activity

Transfection of *FLAG-KLF6* significantly increases p21-luciferase activity in 293T-(7A, $p < 0.05$) and HUH7 cells (7B, $p < 0.01$). This increase is inhibited when *FLAG-SV1* is co-transfected (7A, $p < 0.05$) (7B, $p < 0.05$). *FLAG-SV1* alone does not affect p21 luciferase activity (7A,B). Similarly in isolated primary hepatocytes from *Klf6* depleted mice (*AlbCre Klf6*^{fl/fl}), *SV1 AlbCre Klf6*^{fl/fl} versus *Klf6*^{fl/fl} controls (7C) as well as hepatocytes from *Klf6*^{fl/fl} mice treated with adenoCre- versus lacZ control virus (7D) p21 protein levels were decreased.

Figure 8. SV1 accelerates KLF6 degradation

Co-transfecting increasing amounts of *FLAG-SV1* to a constant amount of *FLAG-KLF6* leads to a decrease in KLF6 protein levels in 293T- (8A) and HUH7 cells (8C). 8B shows the immunoblot quantification in 293T cells ($p < 0.05$) ($n = 4$). 293T- (8D) and HUH7 cells (8F) were co-transfected with either *FLAG-KLF6* + *pCI-neo-GFP* or *FLAG-KLF6* + *FLAG-SV1* and protein was collected 0, 15, 30 or 60 minutes after adding cycloheximide. Co-transfection of *FLAG-SV1* accelerated the degradation of KLF6 in both 293T cells (8D). This degradation can be inhibited by adding the proteasome inhibitor MG132 (8D). The quantification of 6 immunoblots in 293T cells (8E) shows lower KLF6 protein levels at timepoint 0 ($p < 0.05$) and accelerated KLF6 degradation ($p < 0.005$) in the presence of SV1 as seen at timepoint 60 minutes after cycloheximide (8E). Corresponding can be observed in protein from primary hepatocytes of *Klf6* fl(+/+) versus *SV1 Klf6* fl(+/+) mice (8F).