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SIRT6 dependent genetic and epigenetic alterations are associated with poor clinical outcome in HCC patients

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Abstract

Sirtuin 6 (SIRT6) is a member of the sirtuin family of NAD-dependent deacetylases. Genetic deletion of Sirt6 in mice results in a severe degenerative phenotype with impaired liver function and premature death. So far the role of SIRT6 in development and progression of hepatocellular carcinoma is unknown. We first investigated SIRT6 expression in 153 primary human liver cancers, normal and cirrhotic livers using microarray analysis. SIRT6 was significantly downregulated in both cirrhotic livers and cancer. A Sirt6 knock out (KO) gene expression signature was generated from primary hepatocytes isolated from three week old Sirt6-deficient animals. Sirt6-deficient hepatocytes showed upregulation of established HCC-biomarkers Alpha-fetoprotein (Afp), Insulin-like growth factor 2 (Igf2), H19 and Glypican-3 (Gpc3). Furthermore decreased SIRT6 expression was observed in hepatoma cell lines that are known to be apoptosis insensitive. Re-expression of SIRT6 in HepG2 cells increased apoptosis sensitivity to CD95-stimulation or chemotherapy treatment. Loss of Sirt6 was characterized by oncogenic changes including global hypomethylation as well as metabolic changes including hypoglycemia and increased fat deposition. The hepatocyte-specific Sirt6-KO signature had prognostic impact and was enriched in patients with poorly differentiated tumors with high AFP levels as well as recurrent disease. Finally, we could demonstrate that the Sirt6-KO signature possessed a predictive value for tumors other than HCC, i.e. breast and lung cancer.

Conclusion—Loss of SIRT6 induces epigenetic changes which may be relevant to chronic liver diseases and HCC development. Downregulation of SIRT6 and genes dysregulated by loss of SIRT6 possess oncogenic effects in hepatocarcinogenesis. Our data demonstrate that deficiency in one epigenetic regulator predisposes a tumorigenic phenotype which ultimately has relevance for outcome of HCC and other cancer patients.

Keywords

liver cancer; SIRT6; molecular pathogenesis; gene expression profile; comparative genomics

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Introduction

Hepatocellular Carcinoma (HCC) is the most deadly consequence of the majority of chronic liver diseases.(1) While vaccination programs in several Asian countries effectively control incidence rates over recent decades, incidences in several Western countries and Japan steadily increased, mainly due to constant elevation of hepatitis C infections.(2) Moreover, predisposing risk factors for HCC development such as alcohol and metabolic diseases exhibit alarmingly increasing trends in the Western world. Among these metabolic syndrome and non-alcoholic fatty liver disease (NAFLD) are of particular interest due to predicted raise in prevalence and high numbers of HCCs without underlying cirrhosis. (3, 4) Although considerable efforts to unravel genetic determinants of liver cancer have been made over the last decades, the exact pathogenesis remains to be elucidated and significantly varies between the different etiologies. In nonalcoholic steatohepatitis (NASH) patients, the molecular changes are highly associated with the development of insulin resistance.(4) However, besides etiological differences a common phenotypic hallmark feature of the majority of HCCs is the so called inflammation-fibrosis-cancer axis, orchestrated by a complex interplay of different cell types and molecular features.(5)

SIRT6 is a member of the evolutionarily conserved sirtuin family of NAD(+)-dependent protein deacetylases and is involved in the regulation of glucose metabolism, triglyceride synthesis, and fat metabolism.(6–8) Sirt6-deficient animals present with early lethality due to profound abnormalities including hypoglycemia and premature aging.(9, 10) Moreover, conditional disruption of Sirt6 in hepatocytes leads to increased glycolysis, triglyceride synthesis, reduced beta oxidation, and, ultimately, to fatty liver formation. Further, specimens from steatotic human livers show significantly lower levels of SIRT6 than control tissues, indicating a prominent role of SIRT6 in liver homeostasis.(11) A well known mechanism in expediting the inflammation-fibrosis-cancer sequence is the activation of NF- κ B.(12) Although the regulation of NF- κ B is complex, epigenetic modulation of NF- κ B activation, e.g. by histone deacetylation, is well characterized.(8, 13) Recently, it was demonstrated that SIRT6 is a key component of histone H3 lysine 9 activity and plays a prominent role in the regulation of NF- κ B signaling during inflammation, stress response and aging.(14, 15)

Over the last decade comparative functional genomics have been repeatedly and successfully employed to reproduce molecular features of human hepatocellular cancers using appropriate mouse models. This approach significantly contributed to a better understanding of the molecular features of HCC and led to the discovery of novel therapeutic targets.(16–18) Given the importance of SIRT6 in hepatocyte function and homeostasis of liver metabolism, we applied comparative and integrative genomics to determine the role of SIRT6 in human hepatocarcinogenesis. As a result, we could demonstrate a stepwise reduction of SIRT6 levels from pre-neoplastic stages of hepatocarcinogenesis to human HCCs as well as an association of SIRT6 signaling with the outcome of liver and other cancers. The Sirt6-deficient microarray gene expression signature we generated from isolated hepatocytes of three week old Sirt6^{-/-} mice showed significant upregulation of known HCC biomarkers. Using Western blot and qRT-PCR analysis, the expression of these biomarkers was validated in Sirt6^{-/-} mouse hepatocytes and human hepatoma cell lines. Further, re-expression of SIRT6 in HepG2 cells restored sensitivity to apoptotic stimuli. Global transcriptomic analyses confirmed the prominent role of Sirt6 signaling in regulation of key hepatocyte functions such as cell cycle, metabolism, and oxidative stress response. On the molecular level, genetic loss of Sirt6 caused changes in the methylation pattern of affected livers leading to a metabolic and pro-oncogenic phenotype. Together, our results indicate a clinical significance of SIRT6 and disrupted SIRT6 signaling during liver carcinogenesis.

Materials and Methods

Isolation of primary mouse hepatocytes

Mice of the strain 129-Sirt6^{tm1Fwa/J} were obtained from the Jackson Laboratory and interbred to obtain mice homozygous for the Sirt6^{tm1Fwa} allele. Hepatocytes from Sirt6^{-/-} and Sirt6^{+/+} mice were isolated from mouse livers by hepatic portal vein perfusion as described in (Teufel et al., J Hep 2010).(19) Mice were kept in the central laboratory animal facility (ZVTE) of the Johannes Gutenberg University. Blood glucose levels were measured in whole blood with the device Accu-Chek® Sensor (Roche).

Global DNA methylation analysis

For genomic DNA preparation, tissues were lysed at 37°C overnight in a buffer containing 75mM NaCl, 30 mM EDTA, 0.5% SDS and 250 µg/mL Proteinase K (pH 8.0). After addition of NaCl to a final concentration of 2M, the lysate was centrifuged for 20 min at 10,000 rpm. Genomic DNA was precipitated, washed with 70% Ethanol, air-dried and resuspended in TE buffer. The global DNA methylation status of livers from Sirt6^{-/-} and Sirt6^{+/+} mice was determined using the colorimetric MethylFlash Methylated DNA Quantification Kit (Epigentek Inc.) according to manufacturer's instructions.(20) Relative quantification of 100 ng genomic DNA was performed on an ELISA plate reader at 450 nm. All investigations were performed in triplicates using 2 independent replicates.

Microarray analysis

Total RNA from isolated hepatocytes was extracted using Absolutely RNA® Miniprep Kit (Agilent Technologies) following the instructions of the manufacturer. RNA quantity was estimated using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Gene expression microarrays were performed using Affymetrix GeneChip® Mouse Genome 430 2.0 arrays. The arrays were deposited at EMBL-EBI (accession number: E-MTAB-1477). Arrays were normalized based on mean intensity values across the chips. Changes in expression levels were calculated based on log2 ratio. Publically available microarray data (GEO accession number: GSE21965)(11) was downloaded from GEO, processed and analyzed using BRB ArrayTools V3.3.0 software package 3 (Biometric Research Branch, National Cancer Institute). Samples were normalized using SAM and differentially expressed genes were identified at a nominal p-Value 0.05. Unsupervised cluster analysis was performed using Cluster and TreeView programs.(2) Only genes with a fold change 2 were included in the analyses. Functional classification and network analysis were performed using Ingenuity Pathway Analysis tool (Ingenuity Systems Inc.) and the GeneGo microarray tool.

Patients, Databases and Statistical analysis

Microarray data from 139 HCC samples(21) were used for the survival analysis according to the SIRT6 signatures. SIRT6 expression was investigated in a sub-contingent of 53 HCC tumor specimens.(22) Oncomine Cancer Microarray database (<http://www.oncomine.org>) was used to study gene expression of the SIRT6 signature in human hepatocellular carcinoma and conduct a meta-analysis for the predictive value of the SIRT6 signature in more than 40 different cancer types. Expression values of tumor samples were log-transformed, median-centered and standard deviation was normalized to one per array before comparison to their normal tissue counterparts as described recently.(23) Statistical analysis was performed using Student's *t*-test or ANOVA as indicated. *P*-values 0.05 were considered statistically significant. Results are presented as mean ± SD or mean ± SEM as indicated. Univariate and multivariate analysis were performed using CHI² Test or Cox

Proportional Hazard Regression, respectively. For the multivariate analyses only significant variables with sufficient data points were included.

RESULTS

Downregulation of SIRT6 in human hepatocellular carcinomas

To investigate the relevance of SIRT6 for primary human HCC, we first used publically available gene expression data of liver cancer patients from the Oncomine Cancer Microarray database.⁽²³⁾ A significant reduction of SIRT6 expression was revealed in cirrhotic livers and HCC specimens (p-values: <0.001) compared to levels observed in non-cirrhotic livers (Figure 1A). Confirming these findings a downregulation of SIRT6 in HCC tissues compared to non-diseased normal livers was also observed in around 45% (24/53) using independent gene expression data from our recently published cohort of 53 human HCCs (Figure 1B, upper panel) (22). Consistently, around 42% (16/38) of the tumor samples showed SIRT6 levels below the median center of the expression data of all samples (normalized expression units < 0) of patient samples analyzed in Figure 1A (Figure 1B, lower panel). These data indicate a stepwise reduction of SIRT6 in both pre-malignant and malignant stages of hepatocarcinogenesis.

SIRT6 Gene Expression Signature

To investigate the gene expression pattern deregulated by SIRT6 loss, we established a SIRT6 knock-out gene expression signature. To obtain a hepatocyte specific transcriptome analysis, we isolated primary mouse hepatocytes from WT and Sirt6-deficient livers at 3 weeks of age. Gene expression levels were then quantified using genome-wide mouse microarrays from Affymetrix for comprehensive covering of changes occurring as a result of Sirt6 loss. Only genes for which expression was significantly altered in Sirt6-null hepatocytes (Signal Log Ratio (SLR)>1 and filtered for absent calls) were included as part of the Sirt6 signature. The resulting Sirt6 signature contained 1615 probe IDs representing 1241 genes (Supplemental Table 1). Eighteen of the most deregulated targets were further validated using qRT-PCR (Supplemental Figure 1) overall demonstrating a high concordance ($P < 0.001$; $r = 0.85$).

Next, we investigated in more detail functional enrichment of these genes in different networks and signaling pathways by using Ingenuity Pathway Analysis and the GeneGo microarray analysis tools. The two most significant pathway map folders were related to cell cycle and its regulation and cholesterol/bile acid homeostasis (Table 1). Dysregulated pathways also included tissue remodeling and wound repair, lipid biosynthesis and immune system response as well as nuclear receptor signaling. Additional map folders with a significant number of genes affected by the loss of Sirt6 were involved in mitogenic signaling, cell differentiation, DNA-damage response, and apoptosis. Further, canonical pathways and signaling resembling NF- κ B and IGF signaling were consistently activated in Sirt6 deficient hepatocytes (Supplemental Figure 2).

The analyses suggested that loss of Sirt6 predisposes hepatocytes for oncogenic transformation. To validate the results, we performed qRT-PCR and Western Blot analyses of selected HCC-marker genes in serum samples and isolated hepatocytes from WT and Sirt6-deficient animals (Figure 2). For these studies we examined Afp, Igf2, H19, and Gpc3 as well-established HCC biomarkers which we found to be upregulated in our microarray analysis. Consistently, these genes were more abundantly expressed in Sirt6 knock-out hepatocytes compared to WT littermates. Afp and Igf2 were readily detectable on Western blots of serum, and in the case of Afp, in hepatocytes from Sirt6 knock-out mice (Figure 2B). Also, the recently reported H19-derived miRNA-675 was elevated in hepatocytes of

knock-out animals (Figure 2B, right panel). These results confirm that key oncogenic molecules associated with hepatocarcinogenesis are affected by the loss of Sirt6 signaling; thus strengthening the validity of the results from the microarrays. We next characterized a series of human hepatoma cell lines for SIRT6 expression in comparison to that of the series of HCC biomarkers (Figure 3). SIRT6 was consistently downregulated in comparison to primary human hepatocytes in all hepatoma cell lines examined. AFP was upregulated in all cell lines compared to primary hepatocytes. IGF2 was upregulated in all cell lines except PLC/PRF/5 cells. H19 was increased in Hep3B only. Together these results suggest that the deregulation of SIRT6 and genes in the SIRT6 signature can at least in part be recapitulated in established hepatoma lines.

We next compared our microarray results with publicly available microarray data from whole liver tissue with conditional deficiency of Sirt6 in hepatocytes at 2 and 8 months (GEO accession number: GSE21965).(11) A total of 3909 genes were differentially expressed between WT and Sirt6-deficient livers. From these, 329 genes overlapped with our identified Sirt6 knockout signature (26.5%), indicating a high grade of concordance within Sirt6 signaling. In accordance with the previous studies, the overlapping 329 genes were functionally involved in lipid metabolism and cholesterol synthesis, hepatic cholestasis, oxidative stress response and hepatocellular cancer development; thus independently confirming the probable involvement of SIRT6 in the affected pathways. Consistently, the major associated signaling pathways centered around NF- κ B signaling, metabolism and differentiation. Interestingly, the previously reported association with proliferation, cell death and hepatocyte function as well as inflammatory signaling and tissue remodeling was less pronounced, potentially due to the confounding signaling of other cell types in whole liver tissues in contrast to isolated hepatocytes, overall warranting our approach. Together these data reveal that genetic loss of Sirt6 causes massive changes in essential hepatocyte functions such as cellular metabolism, stress response, differentiation and proliferation and are predisposing Sirt6 deficient animals to chronic liver diseases.

Expression of SIRT6 in human hepatoma cell lines increases apoptosis

Resistance or insensitivity to chemotherapy is one of the hallmarks of hepatocellular carcinomas. To analyze the effect of SIRT6 on apoptosis, we expressed SIRT6 in HepG2 hepatoma cells and studied the functional consequences. Transfection resulted in high expression of SIRT6 (Figure 4A). Furthermore while SIRT6 expression did not lead to a change in cell proliferation, a significant increase in apoptosis sensitivity, both mediated by CD95-stimulation (Figure 4B), as well as in response to chemotherapeutic drugs was observed (Figure 4C–D). These results suggest that loss of SIRT6 contributes to the resistance against cell death in tumor cells and supports a role for SIRT6 in suppressing the development of tumors in the liver.

SIRT6 Signature is associated with Clinical Outcomes in Liver and other Cancers

To test the clinical significance of the SIRT6 knockout signature for human hepatocellular cancers, we used a comparative genomic approach(17) and integrated the generated SIRT6 signature with our previously published gene expression dataset from 139 human HCC (21) (Figure 5A) based on the expression of 958 orthologous genes. Hierarchical clustering analysis successfully identified two distinct subtypes concordant with previously published prognostic subtypes of HCC.(21) Further, Kaplan-Meier plots and log-rank statistics revealed a significant ($P < 0.001$) association with shortened mean survival time (306,7 days vs. 1611,2 days) among these two identified subclasses (Figure 5B). As an independent prognostic factor, we also compared the recurrence between the subgroups of HCC. In accordance with poor prognosis demonstrated by survival analysis, a significant ($P < 0.015$) association to a shorter time to recurrence (703 days vs 1520 days) could be demonstrated

for tumors with disrupted SIRT6 signaling (Figure 5C, Supplemental Table 2). Notably, when we compared the expression of the SIRT6 signature in the human HCCs around 182 genes (around 15%) significantly differed between both subclasses. These genes again were significantly associated with the prognosis of patients overall indicating that the these tumor retains a core SIRT6 signature ($P < 0.001$; not shown).

Furthermore, to evaluate the clinical significance of the SIRT6 knockout signature in molecular classification of HCC, we then compared the distribution of several clinical and pathological variables of the two subclasses using an univariate analysis (Table 2). The two subtypes of HCC were comparable with respect to individuals' gender, presence of cirrhosis in surrounding tissues, tumor size and stage, and vascular invasion. In contrast, a significant association with patient age, overall survival and recurrence, and Edmondson grade could be found. Further, the two subclasses differed with respect to plasma AFP levels which confirms the results of our microarray analyses. Interestingly, while distribution of HCV-positive and -negative patients was similar among the two subgroups, a significant higher proportion of HBV positive patients was found in the poor prognosis cluster. Notably, the significant association to overall survival remained present using a multivariate analyses ($P = 0.0157$; hazard ratio: 1.9273; CI (95%): 1.1351 to 3.2724).

Moreover, both GSEA and Oncomine meta-analysis suggested that the SIRT6 signature was significantly associated with cancer development, progression and clinico-pathological features in several different tumor entities other than liver cancer, suggesting prognostic relevance of the SIRT6 signature for cancers other than HCC (Supplemental Table 3 and Supplemental Figure 3). Thus, the SIRT6 signature is characterized by an unfavourable patient outcome with reduced survival and aggressive tumor phenotype in liver and other cancers.

Sirt6 deficiency causes a metabolic phenotype and leads to hypomethylation of liver tissue

To support the idea that Sirt6 loss is creating a pro-cancer environment in the liver, we investigated whether other changes playing a role in tumorigenesis are observed in the Sirt6-deficient livers. SIRT6 plays a major role in the epigenetic regulation by modulating chromatin function.(24) Genetic loss of Sirt6 leads to genomic instability, metabolic defects and degenerative pathologies with aging-associated degenerative phenotypes.(9, 25) Animals with Sirt6 deficiency die within 3–4 weeks of age. The observed phenotypic changes are predominantly caused by profound changes in the regulation of cellular metabolism. Consistent with this phenotype Sirt6^{-/-} animals show a significantly reduced level of blood glucose ($p\text{-Value} < 0.001$) in comparison to control animals already at three weeks of age (Figure 6A). Additionally, increased hepatic fat deposition was already observed in young homozygous animals with Sirt6 deficiency (Figure 6B). Similar observations have recently been demonstrated in a hepatocyte specific model of conditional Sirt6 deficiency when the animals were 3–4 months old.(11)

Aberrant methylation has been reported in HCCs where global methylation was decreased while local CpG promoter methylation increased.(26) Given the importance of DNA methylation for liver specific gene transcription, differentiation and essential hepatocyte functions as well as the known interplay between histone modifications and DNA methylation, we next assessed the level of global DNA methylation in Sirt6^{-/-} and control livers. In agreement with the dominant role of Sirt6 in epigenetic regulation, significantly reduced levels of DNA methylation were present in Sirt6-deficient animals (Figure 5C). Taken together, these results indicate that genetic loss of Sirt6 induces epigenetic changes and disruption of the liver homeostasis leading to a metabolic phenotype; both are associated with progression to cancer.

Discussion

Aging is an established risk factor for cancer; but the mechanisms and genes involved are just beginning to be unraveled. The dramatic phenotype of Sirt6-deficient mice indicates a critical role for SIRT6 in physiological processes involved in aging. We used an integrative genomic approach to investigate the importance of the longevity gene Sirt6 in chronic liver diseases and progression to hepatocellular carcinoma. We provide evidence that loss of SIRT6 in hepatocytes results in a pro-cancer milieu by deregulating a suite of genes, including known HCC-biomarkers, which contribute to this phenotype. This is supported by our finding that disruption of Sirt6 leads to global hypomethylation and causes metabolic changes consistent with a pro-oncogenic phenotype. Comparison of 139 HCC gene expression profiles with the SIRT6-deficient signature revealed significant association with disease progression and recurrence. Furthermore, comparisons with publicly available data sets of other tumor types revealed that SIRT6 may be involved in other tumor types since the signature is also linked to the clinical outcome of these cancer patients. Consistently, a recent study confirms the crucial role of SIRT6 in cancer metabolism leading to poor prognosis of colorectal and pancreatic cancer patients.(27) Further, the global gene expression changes observed in hepatocytes devoid of Sirt6 support the essential role of SIRT6 for liver homeostasis by maintaining the hepatic epigenome. Our results shed light on SIRT6 as a potential tumor suppressor since its loss results in an oncogenic phenotype that is associated with poor clinical outcome of human liver cancer patients. Mechanistically, genetic loss of SIRT6 causes resistance against cell death (Figure 4) a key mechanism in cancer development and progression.(28) Conversely, re-expression of SIRT6 in HepG2 cells partly rescued its onco-suppressive function by restoring apoptosis sensitivity mediated by CD95 stimulation as well as chemotherapy treatment. These results extend a recent finding that suggest a critical involvement of SIRT6 in the early phase of hepatocarcinogenesis.(29)

Already at three weeks of age the genetic loss of Sirt6 causes profound alterations in the liver including hepatic metabolism. These changes involve the progressive accumulation of fat in Sirt6-deficient hepatocytes as well as dramatic disruption of insulin homeostasis resulting in significantly increased glycolysis.(4, 10, 11) Our analysis revealed upregulation of HCC biomarker genes in livers of three week old mice with Sirt6 deficiency even though these mice show no overt tumors. Upon comparing the Sirt6 levels to the biomarker expression levels in primary human hepatocytes and several established human hepatoma cell lines, we found a surprising congruency between the Sirt6 KO livers and the human hepatoma cell lines. These results are in line with the dominant role of SIRT6 as regulator of essential hepatocyte functions and support a role of modulating SIRT6 for the prevention of liver diseases.

Our global transcriptome analyses confirmed that the disruption of SIRT6 in hepatocytes leads to activation of multiple key signaling pathways with known association to liver diseases including hepatocarcinogenesis.(30). This includes activation of genes important for proliferation (Cyclins A, A2, B1–2, D1–2, CDC20, CDC34, CDK1, CDK4, Casein kinase I) and several members of the mitogen-activated protein kinase members (MAP3K1, MAP3K8, MAP4K4) known to play a role for HCC proliferation, survival and differentiation.(31, 32) Additionally, other key molecules affected by the loss of SIRT6 were involved in malignancy-associated metabolic abnormalities of cholesterol and bile acid homeostasis (CYP2B6, CYP2C18, CYP2C44, CYP2F1, CYP2J2, CYP2J5, CYP2J9, CYP3A4, CYP4A22, CYP4F12, CYP51A1), as well as lipid biosynthesis and regulation. In addition SIRT6 loss influences chemoresistance drug transporters (ABCB11, ABCB1B, ABCG8) (33, 34) and oxidative stress regulation (GSTM1, GSTM2, GSTM4, GSTM5, GSTM6, GSTM3) further underlining the essential role of SIRT6 for maintaining

hepatocyte stress defense. Importantly, we also demonstrate that SIRT6 deficiency causes aberrant growth receptor signaling (EGFR, PDGFR) and IGF2 expression. The role of IGF2 in many human cancers as well as HCC is well recognized. Activation of IGF2 is observed in around 30% of human HCCs.(35) Recently, activation of IGF signaling was demonstrated in a subclass of HCC with poor clinical outcome (referred to as “proliferation class”).(36) This study further showed that modulation of IGF signaling provides a promising target for therapeutic strategies in HCC. Together, these results indicate that loss of the aging-related gene Sirt6 might establish a link between key molecules involved in metabolism, inflammation and steatosis lead to the development of chronic liver diseases and HCC formation.

Another key finding of the study is the disruption of the hepatic epigenome caused by the loss of SIRT6 signaling. Compelling evidence indicates a causal role of aberrant epigenetic regulation for the development of a variety of cancers including HCC.(37) Epigenetic changes of the inflamed and chronically diseased liver microenvironment are supposed to be early promoters of oncogenic transformation in HCC. Therefore, epigenetic mechanisms might tie genomic alterations with environmental influences in the liver.(38) It is well known that different epigenetic alterations cause activation of signals from the microenvironment leading to cellular proliferation, disruption of the hepatic metabolism and ultimately in cancer initiation and progression. A multistep disruption of the hepatic epigenome leading to allelic imbalances has recently been confirmed in HBV mediated HCC.(39) Importantly, global hypomethylation could be associated with poor clinical outcome in HCC patients.(26) Consistent with this, we observed a stepwise reduction of SIRT6 from pre-neoplastic stages of hepatocarcinogenesis to fully malignant HCC. Furthermore, disruption of Sirt6 was associated with significantly reduced global DNA methylation in mouse livers. Thus, our results highlight the importance of Sirt6 in maintaining the hepatic epigenome and demonstrate that disruption of its function is frequently observed during hepatocarcinogenesis. Further, our results point towards the potential of modulating this pathway in a clinical setting to complement existing treatment strategies and owing to the promise of epigenetic therapies in HCC, this may be an important addition(22). Finally, to further support the role of SIRT6 for hepatocarcinogenesis we performed integrative transcriptomic analyses of SIRT6 signaling in authentic primary HCC. Similar to previously generated prognostic signatures(30) (such as MET and TGF- β) our integrative strategy uncovered two distinct subclasses of HCC patients based on the molecular features of SIRT6 signaling. These distinct subclasses showed significant differences in biological properties as well clinical outcome underlining the clinical relevance of SIRT6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

SIRT6	Sirtuin 6
HCC	Hepatocellular Carcinoma

NAFLD	Nonalcoholic fatty liver disease
IGF	Insulin-like growth factor
NF-κB	Nuclear factor kappa B
MAPK	Mitogen-activated protein kinase
GSEA	Gene set enrichment analysis
NASH	Nonalcoholic steatohepatitis
Gpc3	Glypican 3
RPII	RNA Polymerase II
AFP	Alpha Fetoprotein

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Figure 1. SIRT6 expression in human hepatocarcinogenesis

A) Oncomine Cancer Microarray database was used to assess SIRT6 levels during hepatocarcinogenesis. SIRT6 expression was compared in primary human HCC specimens (n=38), normal tissue (n=10) and cirrhosis (n=58). Whiskers plots represent min-max normalized expression units. (P-value <0.001) (B) Expression levels of SIRT6 in primary liver cancer from our recently published HCC database in comparison to normal liver (n=47, Lee et al.) (upper panel) and from the Oncomine database (n=38, Mas et al.(40) (lower panel) are shown. Only specimens with detectable expression values for SIRT6 are displayed.

Figure 2. Expression of potential Sirt6 target genes

(A) Gene expression of Afp, Igf2, H19 and Gpc3 was analyzed in *Sirt6*^{-/-} in comparison to *Sirt6*^{+/+} hepatocytes by qRT-PCR. Experiments were performed in triplicates. (B) Western blot analysis of Afp and Igf2 in serum (upper) and from isolated hepatocytes (lower) of 4 different mice confirmed preferential activation of both genes in *Sirt6*^{-/-}. β -tubulin was used as loading control. H19 derived miR-675 in hepatocytes of *Sirt6*^{-/-} mice is elevated (right panel). *Sirt6*^{+/+} mice were labeled as WT1-WT3, *Sirt6*^{-/-} mice were labeled as KO1-KO3.

Figure 3. SIRT6 expression in human hepatoma cell lines

Gene expression analyses of SIRT6, AFP, IGF2 and H19 was analyzed in 4 hepatoma cell lines by qRT-PCR. Primary human hepatocytes (ph-Hep) were used as a reference. Data obtained from triplicate PCRs relative to expression level of ph-Hep.

Figure 4. Re-expressing SIRT6 increases apoptosis sensitivity

HepG2 cells were transfected with SIRT6 or control vectors. Effective transfection demonstrated by Western blotting (A). Cells were treated with 100ng/ml Anti-Apo-1 (B) or cultured in the presence of 1mg/ml or 10mg/ml Doxorubicin (C) or Daunorubicin (D) for 24h. Viability was measured by Celltiter glow. All experiments were performed in 4 replicates. Graphs are represented as % of control cells. Statistical analyses were performed using a Students t-Test (Anti-Apo-1) or ANOVA (Doxorubicin and Daunorubicin). (P-values: * <0.05 ; ** <0.01 ; *** <0.001).

Figure 5. SIRT6 knockout signature is associated with outcome in liver cancer

Prognostic implication of the SIRT6 knockout signature was determined using gene expression microarray data from 139 HCC patients (21). (A) Hierarchical cluster analysis based on the SIRT6 knockout (red) and wildtype (green) signature. Clustering was performed using Euclidean distance and average linkage analyses. Bars under cluster tree represent integrated cell fractions and overlap with previously generated HCC subclasses (subtype A, B, HB, and HC). (B) Kaplan-Meier plots of overall survival ($n=110$) and (C) recurrence ($n=63$) of the HCCs with SIRT6 knockout (red) and wildtype (green) signature (log-rank Mantel-Cox test).

Figure 6. Loss of Sirt6 is associated with hypomethylation and metabolic changes

(A) Sirt6^{-/-} animals show reduced blood glucose levels at 21 days of age (P-value <0.001). (B) H&E staining of SIRT6^{+/+} and SIRT6^{-/-} mice (upper pannel). Increased deposition of fat in hepatocytes of Sirt6^{-/-} mice was demonstrated by oil red staining (lower pannel). (C) Global DNA methylation in Sirt6^{-/-} in comparison to Sirt6^{+/+} was determined using a colorimetric quantification by specifically measuring levels of 5-methylcytosine (5-mC) (P-value <0.01). Experiments were performed in 4 independent matched sample pairs. The graph shows the mean from 3 technical replicates $\pm$ SD.

Table 1
Top signaling pathways from the Sirt6 knockout signature as determined by GeneGo analysis

The expression signatures were generated using isolated primary mouse hepatocytes from WT and Sirt6-deficient livers at 3 weeks of age. The resulting Sirt6 knockout signature contained 1241 genes. Shown are the top functional networks identified by GeneGO.

#	Map folders	pValue	Ratio
1	Cell cycle and its regulation	4.57E-15	69/444
2	Cholesterol and bile acid homeostasis	5.20E-10	66/528
3	Tissue remodeling and wound repair	1.47E-05	56/554
4	Lipid Biosynthesis and regulation	2.46E-05	43/393
5	Immune system response	3.72E-05	81/925
6	Nuclear receptor signaling	7.51E-05	64/698
7	Mitogenic signaling	1.51E-04	53/560
8	Cell differentiation	1.73E-04	78/922
9	DNA-damage response	2.40E-04	37/354
10	Apoptosis	2.99E-04	71/834
11	Hematopoiesis	5.09E-04	29/264
12	Vascular development (Angiogenesis)	9.37E-04	48/532
13	Xenobiotic Metabolism and its regulation	1.30E-03	31/306
14	Oxidative stress regulation	2.42E-03	51/600
15	Estrogen signaling	3.79E-03	28/287
16	Protein synthesis	4.48E-03	29/304
17	Inflammatory response	1.08E-02	49/617
18	Androgen signaling	1.74E-02	21/224

Table 2
Univariate Analysis of the Sirt6 signature to clinicopathological features in 139 HCCs

Values are presented as % positive for the corresponding feature.

	Cluster 1 (n=(%))	Cluster 2 (n=(%))	p-value
Subtype A	49 (98)	12 (14)	5.85E-21
Age >60	15 (30)	42 (51)	0.020043
AFP levels (>300)	24 (63)	24 (40)	0.025452
HBV positive	31 (78)	31 (47)	0.001987
Grade>2	40 (80)	38 (46)	0.000104
Survival	7 (15)	30 (47)	0.000527
Recurrence	20 (91)	24 (54)	0.003135
ALD	2 (5)	15 (23)	0.015914
Size (>5cm)	26 (63)	39 (64)	0.957307
HCV	5 (13)	16 (27)	0.141492
Cirrhosis	27 (54)	39 (48)	0.47293
Invasion	13 (69)	14 (50)	0.210026
Stage >2	12 (75)	38 (73)	0.878814
Male Gender	37 (74)	60 (72)	0.829696