Inhibition of the cell death pathway in non-alcoholic steatohepatitis (NASH)-related hepatocarcinogenesis is associated with histone H4 lysine 16 deacetylation

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Abstract

Hepatocellular carcinoma (HCC) is one of the most aggressive human cancers and its incidence is steadily increasing worldwide. Recent epidemiological findings have suggested that the increased incidence of HCC is associated with obesity, type 2 diabetes mellitus, and nonalcoholic steatohepatitis (NASH); however, the mechanisms and the molecular pathogenesis of NASH-related HCC are not fully understood. In order to elucidate the underlying mechanisms of the development of NASH-related HCC, we investigated the hepatic transcriptomic and histone modification profiles in Stelic Animal Model (STAM) mice, the first animal model of NASH-related HCC to resemble the disease pathogenesis in humans. The results demonstrate that the development of NASH-related HCC is characterized by progressive transcriptomic alterations, global loss of histone H4 lysine 20 trimethylation (H4K20me3), and global and gene-specific deacetylation of histone H4 lysine 16 (H4K16). Pathway analysis of the entire set of differentially expressed genes indicated that the inhibition of cell death pathway was the most prominent alteration and this was facilitated by persistent gene-specific histone H4K16 deacetylation. Mechanistically, deacetylation of histone H4K16 was associated with down-regulation of lysine acetyltransferase KAT8, which was driven by over-expression of its inhibitor nuclear protein 1 (Nupr1). The results of the present study identified a reduction of global and gene-specific histone H4K16 acetylation as a key pathophysiological mechanism contributing to the development of NASH-derived HCC, and emphasized the importance of epigenetic alterations as diagnostic and therapeutic targets for HCC.

Statement of implication: Histone H4K16 deacetylation induces silencing of genes related to the cell death inhibition occurs during the development of NASH-related HCC.
Introduction

Hepatocellular carcinoma (HCC) is the sixth most common human cancer (1) and the third leading cause of cancer-related deaths in the world (2). In the United States, the incidence of HCC has greatly increased over the past two decades (3,4) and it is expected to continue rising over the next 20 years (2). HCC commonly arises in humans with chronic liver disease caused by well-identified etiological factors, including chronic hepatitis B and C viral infections, chemical exposure, and excessive alcohol consumption. In addition to these well-characterized risk factors, the results from comprehensive epidemiological studies have demonstrated that nonalcoholic steatohepatitis (NASH), an advanced form of nonalcoholic fatty liver disease (NAFLD), is becoming a major risk factor of HCC in the United States (5,6).

NAFLD, a liver component of metabolic syndrome represented by several related liver disorders ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis (7,8), is a major health problem and a leading cause of chronic liver disease worldwide (8). Uncomplicated hepatic steatosis is generally considered to be a benign form of NAFLD and has a favorable outcome. HCC is a rare complication of NAFLD (8); however, a recent comprehensive meta-analytic study of a total 8,016,928 individuals with NAFLD in North America demonstrated that approximately 30% of NAFLD individuals diagnosed with simple steatosis will develop NASH (8), a condition that poses a considerably greater risk of progressing to HCC (6,8) with the incidence being 5.29 cases of HCC/1000 NASH patients/year (8).

The development of HCC, in general, and NASH-related HCC, in particular, is a complex process of dysregulated cellular and molecular “hallmarks-of-cancer” events driven by genetic and epigenetic abnormalities (9,10). While the role of sequential accumulation and natural progression of well-characterized histomorphological changes in the NASH-related liver
Carcinogenesis is well established, the pathophysiological mechanisms underlying the disease development are less well understood. Uncovering the molecular basis of the pathogenesis NASH-related HCC is critical for effective disease prevention and treatment. Although investigating the molecular mechanisms of NASH-related HCC using human samples is desirable, this is frequently unfeasible and typically very complex. Additionally, analysis of human samples provides only a snap-shot of molecular alterations, rather than uncovering molecular changes linked to the progression of disease. In contrast, relevant animal models that resemble the development of HCC in NASH patients may largely compensate the limitations of human-only studies.

Based on these considerations, the goal of the present study was to investigate and identify key molecular events associated with the development of HCC associated with NASH. To achieve this goal, we investigated molecular alterations in liver during the development of HCC using Stelic Animal Model (STAM) mice, the first animal model of NASH-related liver carcinogenesis resembling disease development in humans (11,12).

Materials and Methods

Mouse model of NAFLD-derived carcinogenesis and liver samples

The Stelic Animal Model (STAM) of NASH-derived HCC is the first mouse model to depict the sequential evolution of clinical and pathomorphological features of the development of HCC in diabetes-associated NASH patients (11,12). Briefly, 2-day-old male C57BL/6J mice were injected with streptozotocin (200 μg/mouse). Starting from 4 weeks of age, the mice were continuously fed a high-fat diet (HFD-32; Clea, Tokyo, Japan) during the duration of the study. Control mice were maintained on standard animal chow for the duration of the study. At 6 weeks (steatotic stage) the livers of STAM mice became pale yellow in color and showed the deposition of triglycerides that gradually decreased at later stages.
Accumulation of F4/80+ macrophages was detectable starting from 6 weeks; however, inflammatory foci were not observed this time. A granular surface, an increase of ER-TR7+ fibroblasts, and pericellular fibrosis around the central veins were observed in the livers at 12 weeks (NASH-fibrotic stage) and 20 weeks (full-fledged HCC stage). All mice developed HCC. The complete pathomorphological description of the STAM NASH-related hepatocarcinogenesis model has been described by Fuji et al. (11). Liver tissue samples from male C57BL/6J STAM mice at steatotic (6 weeks), NASH-fibrotic (12 weeks), and full-fledged HCC (20 weeks) stages of liver carcinogenesis and liver samples from age-matched C57BL/6J mice were purchased from the Stelic Institute & Co., Inc. (Tokyo, Japan).

In order to compare the STAM mice data with another mouse model of NASH, we used the gene expression data and liver samples from male C57BL/6J mice fed a choline- and folate-deficient (CFD) diet for 12 weeks, which caused the development of NASH with uniform morphological features of human NASH (13; GSE96936).

RNA extraction and gene expression analysis using microarray technology

Total RNA was extracted from liver tissue samples (n = 4/group/treatment) using miRNeasy Mini kits (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Gene expression profiles in the mouse livers (n = 4/group/time interval, except n = 3/STAM mice at the 6-week time point) were determined using whole genome SurePrint G3 Mouse Gene Expression v2, 8x60K microarrays (Agilent Technologies, Santa Clara, CA) that include complete coverage of RefSeq coding transcripts. Sample labeling and microarray processing were performed as detailed in the manufacturer’s protocol. The hybridized slides were scanned with an Agilent SureScan DNA Microarray Scanner (Agilent Technologies) at 3 µm resolution. The resulting images were analyzed by determining the Cy3 fluorescence intensity of all gene spots (features) on each array using the Agilent Feature Extraction Software (Version 11.5). The raw data were then uploaded into the ArrayTrack database (14). The median fluorescence intensity of all the
pixels within one feature was taken as the intensity value for that feature. The raw intensity values were then normalized using 75 percentile channel scaling normalization within ArrayTrack. Heat maps were generated and unsupervised hierarchical clustering and principal component analyses were conducted using ArrayTrack. Benjamini-Hochberg adjusted p-values (15) were calculated and an adjusted p value cut-off of 0.05, and a fold-change threshold of 2.0, were used to compare significantly altered gene transcripts at each time point. The microarray gene expression data were deposited in the NCBI's Gene Expression Omnibus (GEO) database (accession number GSE83596).

Functional analysis of differentially expressed genes

The lists of differentially expressed genes in the liver of STAM mice at 6, 12, and 20 weeks, containing gene identifiers and corresponding expression values, were uploaded into the Ingenuity Pathway Analysis (IPA, version 28820210; Ingenuity, Qiagen). The “core analysis” function included in the software was used to interpret the differentially expressed genes and categorize by specific diseases, biological functions, and gene networks. For each molecular pathway, a p value was calculated based on a right-tailed Fisher’s exact test that determined whether a pathway was overrepresented by calculating overlap of the predicted genes in a given pathway with experimental significantly differentially-expressed genes (16). The IPA activation z-score algorithm for “diseases and functions” was used to determine the predictive status of molecular pathway, and the values of z-score were visualized as a Heat map. Only those pathways with a p value below the threshold of p < 0.05 and having > 3 representative genes in the data set were considered significant. Additionally, the IPA Knowledge Base was used to generate and visualize the gene network interactions with specific biological functions at each time point.

Quantitative reverse transcription-PCR
Total RNA (2 μg) was reverse transcribed using random primers and High Capacity cDNA
Reverse Transcription kits (Life Technologies, Grand Island, NY) according to the
manufacturer’s protocol. cDNA was analyzed in a 96-well plate PCR assay format using a
QuantStudio™ 7 Flex Real-Time PCR System (Life Technologies). Each plate contained
experimental genes and a housekeeping gene (TATA box binding protein; Tbp). All primers for
the gene expression analysis were obtained from Life Technologies and are listed in
Supplementary Table 1. The relative amount of each mRNA transcript was determined using
the 2^−ΔΔCt method (17). Template negative controls were run on each plate. All samples were
analyzed in triplicate; the values are expressed as the mean of three runs.

**Western blot analysis**

Whole liver tissue lysates or total acid histone extracts containing equal quantities of
proteins were separated by 7-15% SDS-PAGE and transferred to PVDF membranes. The
levels of tumor necrosis factor (TNF), FAS ligand (FASL), tumor necrosis factor receptor
superfamily, member 9 (CD137), soluble form of tumor necrosis factor receptor superfamily,
member 1a (sTNFR1), CD44, caspase 3, caspase 8, lysine methyltransferase 5B (KMT5B),
lysine acetyltransferase 8 (KAT8; hMOF), and sirtuin 1 (SIRT1), the trimethylation status of
histones H3 lysine 4 (H3K4me3), H3 lysine 9 (H3K9me3), H3 lysine 27 (H3K27me3), H3 lysine
79 (H3K79me3), H4 lysine 20 (H4K20me3), and the acetylation status of histones H3 lysine 9
(H3K9ac), H3 lysine 27 (H3K27ac), and H4 lysine 16 (H3K16ac) were determined by Western
blot analysis as described previously (18). The primary antibodies and their dilutions used for
Western blotting are listed in Supplementary Table 1. IRDye 800CW-labeled anti-rabbit or
IRDye 680RD-labeled anti-mouse secondary antibodies (LI-COR Biosciences, Lincoln, NE)
were used for visualization. Fluorescence was measured using the Odyssey CLx Infrared
Imager (LI-COR Biosciences). The images were quantified using ImageStudio 4.0 Software (LI-
COR Biosciences). To control for equal loading, the relative amount of the protein of interest
was normalized by staining of the membranes with REVERT Total Protein Stain (LI-COR Biosciences), or by using the total H4 immunostaining signal (Supplementary Figure S1).

**Chromatin immunoprecipitation and qPCR**

Chromatin immunoprecipitation (ChIP) was performed using a Chromatin Immunoprecipitation Assay kit (EMD Millipore Corporation, Billerica, MA) according to the manufacturer's protocol. The liver tissue lysates were incubated with anti-H4K16ac (Cell Signaling Technology), anti-H4K20me3 (Abcam, Cambridge, MA). As a negative control, liver tissue lysates were incubated with normal rabbit IgG (EMD Millipore Corporation) primary antibodies, and for a positive control for H4K16ac immunoprecipitation reaction, primers for promoter region of GAPDH gene were analyzed by qPCR (data not shown). The optimized primers used for ChIP-qPCR are listed in Supplementary Table 1. The results were normalized to the amount of input DNA and presented as fold change for each DNA in liver of STAM mice relative to age-matched control mice.

**Statistical analyses**

Results are presented as mean ± SD, n = 4. Significant differences, p <0.05, between groups were evaluated using an unpaired 2-tailed Student's t-test. When necessary, the data were natural log transformed before conducting the analyses to maintain a more equal variance or normal data distribution. Values of p < 0.05 were considered significant.

**Results**

**Global gene expression alterations in the livers of STAM mice**

In order to determine the underlying mechanisms of NASH-related liver carcinogenesis, hepatic transcriptomic profiles in steatotic (6 weeks), NASH-fibrotic (12 weeks), and full-fledged HCC (20 weeks) liver samples were investigated. The results of high-throughput whole genome
Microarray analyses showed progressive changes in the liver transcriptome during the development of HCC (Figure 1). Unsupervised hierarchical clustering showed that each stage of the carcinogenic process could be distinguished by its hepatic gene expression profile (Figure 1A). Principal component analysis showed the tight clustering of samples within each group, and clear, distinct separation of control mice from mice in experimental groups (Figure 1B).

To identify genes that were differentially expressed between the control group and each experimental group, Benjamini-Hochberg adjusted p-values were calculated. Genes that passed 2-fold change criterion and had Benjamini-Hochberg adjusted p-value < 0.05 were considered statistically significant. A total of 373, 1730, and 2253 of differentially expressed genes were detected in the livers of STAM mice at 6, 12, and 20 weeks of liver carcinogenesis, respectively (Figure 1C, Supplementary Table 2). Despite differences in the number of differentially expressed genes at each time point, 110 genes were differentially expressed in common at each of the three time points (Figure 1C). Also, should it be noted that nearly 30% of the differentially expressed genes at 6 weeks were carried forward at 12 and 20 weeks.

**Progression of NASH-related liver carcinogenesis is associated with evasion of apoptosis**

Gene set enrichment analysis of the differentially expressed genes at different stages of liver carcinogenesis (Figure 2A) revealed significantly altered pathways, including the inhibition of apoptosis and activation of hepatic stellate cells (HSC), cell movement, carbohydrate synthesis, and hepatic steatosis, among which the inhibition of apoptosis and the activation of HSC were the most significant, especially in NASH-fibrotic and HCC stages (12 and 20 weeks, respectively). A gene network interaction analysis identified markedly down-regulated \textit{Tnf} and up-regulated \textit{Cd44} as the central genes associated with the inhibition of apoptosis and the activation of HSC (Figure 2B and 2C). This was further confirmed by Western blot analysis showing a substantial reduction in members of the TNF superfamily (TNF, sTNFR1, FASL, and...
CD137 proteins) in the livers of STAM mice, with the lowest values being detected in HCC (20 weeks; Figure 3A). In contrast, the levels of \textit{Cd44} transcript and CD44 protein in the livers of STAM mice were substantially greater than in the age-matched control mice at 6, 12, and 20 weeks (Figure 3B).

Considering the involvement of TNF, sTNFR1, FASL, and CD137 proteins (19-22) and \textit{Cd44} (23,24) in the regulation of apoptosis, the levels of caspase 8 and caspase 3, key mediators of apoptotic cell death (25,26), were investigated. Figure 3C shows a decrease in the level of caspase 8 and, especially, the executioner caspase 3, during the entire hepatocarcinogenic process, which corresponded to the results of the gene expression pathway analysis (Figure 2A). No difference in the level of cleaved caspase 3 or cleaved caspase 8 was found at any time point (Supplementary Figure S2).

\textbf{Loss of histone H4K20 trimethylation and deacetylation of H4K16 during liver carcinogenesis}

To elucidate mechanisms that may be associated with profound alterations in gene expression in the livers of STAM mice, especially the progressive decrease in the ratio of up-regulated to down-regulated genes (Figure 1C), the status of post-translational covalent histone lysine modifications, including histone H3K4, H3K9, H3K27, H3K79, and H4K20 trimethylation and histone H3K9, H3K27, and H4K16 acetylation, was examined. These histone lysine modifications play a fundamental role in the regulation of gene expression and chromatin assembly and organization, and are frequently found to be altered in cancer (27).

While no unilateral changes or only sporadic changes were found in the extent of trimethylation of histones H3K4, H3K9, H3K27, and H3K79 or acetylation of histones H3K9 and H3K27 (Supplementary Figure S3), the development of NASH-associated HCC in STAM mice was characterized by a time-dependent and persistent reduction in the level of histone H4K20me3 and H4K16ac (Figure 4A) that were explored further. To investigate the mechanism
of loss of H4K20me3 and H4K16ac in the livers of STAM mice, the expression of histone modifying genes and the level of proteins encoded by those genes were investigated. Besides an increase in the expression of Kmt1a at 20 weeks, no changes in the expression of the chromatin-modifying genes Kmt5b (Suv420h1), Kmt5c (Suv420h2), Kat8, Sirt1, Kdm5a, Phf8, Sirt2, Sirt6, and Hdac1 were found in the livers of STAM mice as compared to control mice (Figures 4B and 4C, and Supplementary Figure S4). Considering the fact that gene expression does not always correlate with the level of encoded proteins (28), the levels of histone H4K20me3 methyltransferase KMT5B (29), histone H4K16 acetyltransferase KAT8 (30), and histone H4K16 deacetylase SIRT1 (31) were analyzed by Western blotting. No changes in the level of KMT5B and SIRT1 were found in the livers of STAM mice (data not shown). In contrast, the level of histone H4K16 acetyltransferase KAT8 was reduced by 33%, 43%, and 51% in the livers of STAM mice at 6, 12, and 20 weeks of liver carcinogenesis (Figure 4D).

Several lines of evidence have attributed a decrease of KAT8 to the induction of stressor protein nuclear protein 1 (NUPR1). In particular, it has been demonstrated that NUPR1 binds to MSL1, a component of histone H4K16 acetylating complex hMOF-MSL, and inhibits its activity (32-34). Therefore, the status of the expression of Nupr1 was investigated in the livers of STAM mice. The level of the Nupr1 transcript was greatly (3.3-, 9.3-, and 64.7-times) increased during the progression of the hepatocarcinogenic process (Figure 4E).

Gene-specific histone H4K20 trimethylation and H4K16 acetylation alterations in HCC

To determine the functional consequences of the observed loss of histone H4K20me3 and histone H4K16ac, two common epigenetic hallmarks of cancer (35), in liver carcinogenesis, the status of gene-specific histone H4K20me3 and H4K16ac was investigated in greater detail. Among the 21 differentially-expressed cell death-related genes (Figure 5A), only two down-regulated genes, Fgfr2 and Egfr, exhibited significant changes in gene-specific histone
H4K20me3 (Figure 5B). In contrast, the extent of histone H4K16ac at the gene promoters (Figure 5C) was significantly decreased in 14 down-regulated genes, and increased in one up-regulated gene (Cdkn2a).

Gene-specific histone H4K16 acetylation and gene expression alterations in STAM livers

One of the outstanding questions in the field of epigenetic cancer research is a lack of conclusive information about the causative or consequential link between epigenetic and gene expression alterations in carcinogenesis (36). Therefore, to elucidate the role of histone H4K16 deacetylation and gene expression in the development of NASH-related HCC, we evaluated the status of gene-specific histone H4K16ac and gene expression of the same 21 differentially-expressed in HCC cell death-related genes in the NASH-fibrotic liver tissues samples. While there was significant histone H4K16 deacetylation of 11 genes in the NASH-fibrotic liver tissues samples, only the expression of histone H4K16-deacetylated Tnf and Aatk genes was significantly reduced, while the expression of other histone H4K16-deacetylated genes was not changed or, in case of Tnfrsf10b, even increased (Supplementary Figure S5). Interestingly, there was a substantially similar pattern of gene-specific histone H4K16ac at 12 and 20 weeks, with more changes reaching significance at 20 weeks. This suggests that gene-specific histone H4K16 deacetylation may precede gene expression alterations and may play a causative role in the inhibiting gene expression.

Epigenetic alterations in a choline- and folate-deficient (CFD) model of NASH

In order to investigate if molecular alterations found in the livers of STAM mice also exist in another mouse model of NASH, we investigated the expression of Nupr1 and Kat8 genes, the protein level of KAT8 histone H4K16 acetyltransferase, gene expression of the same 21 differentially-expressed cell death-related genes, and the status of their gene-specific histone H4K16ac in the livers of male C57BL/6J mice fed a CFD diet. We observed similar pattern of
changes: up-regulation of the Nupr1 transcript, no changes in the expression of the Kat8 gene, and significant reduction in the level of KAT8 protein in mice subjected to the CFD model of NASH (Supplementary Figure S6). Furthermore, 7 genes exhibited histone H4K16 deacetylation in their promoters that correlated with reduced gene expression (Supplementary Figure S6), and 6 histone H4K16-deacetylated genes were in common with those in the livers of STAM mice at the NASH-fibrosis stage (Supplementary Figure S5).

Discussion

NASH-related liver carcinogenesis is a complex pathological process that involves dysregulation of multiple cellular and molecular pathways driven by genetic and epigenetic alterations that enable tumor development. Much is known about molecular aberrations in HCC tumor tissue; however, these alterations provide only a snapshot of molecular abnormalities in HCC rather than uncover the role and mechanism of their dysregulation in the hepatocarcinogenic process. To understand better the molecular pathogenesis of liver tumor development, transcriptomic and histone modification profiles were investigated at different stages of tumor development using the STAM mouse model of NASH-related HCC.

In this study, we demonstrate that NASH-related liver carcinogenesis is characterized by profound global transcriptomic changes and loss of histone H4K20me3 and H4K16ac. Previously, Fraga et al. (35) described the loss of histone H4K20me3 and histone H4K16ac as a common epigenetic feature of human cancer. The results of our study showed that these histone modification changes occurred at the preneoplastic stages and persisted during the entire hepatocarcinogenic process. This indicates the significance of histone H4K20me3 demethylation and histone H4K16 deacetylation in the development of HCC. Analysis of gene expression changes revealed a progressive decrease in the ratio between up-regulated and down-regulated genes, suggesting that mechanisms inhibiting gene transcription were activated
during the development of HCC. Additionally, pathway analysis categorized fundamental molecular pathways associated with the development of HCC. In particular, among the dysregulated pathways for the entire differentially expressed set of genes, inhibition of cell death was the most prominent alteration, in terms of significance and expression changes. Accumulated evidence has established a fundamental role of inhibition of apoptosis in liver carcinogenesis (19,37) The inhibition of the extrinsic apoptotic pathway during the development of NASH-related HCC may be explained, in part, by a profound decrease in the protein levels of TNF superfamily members, the most prominent inducers of hepatocyte programmed cell death (19-22), and an increase of CD44, one of the major mediators of resistance to apoptosis (23,24) and a marker of hepatic stellate and progenitor cells (38,39). This suggestion is supported by our finding of a tight network interaction between genes related to the cell death inhibition and hepatic stellate cell activation during the development of NASH-derived HCC.

Despite the fact that an inhibition of apoptosis during cancer development and progression is considered as one of the “hallmarks of cancer”, the mechanisms responsible continue to be the subject of intense research. Several different mechanisms may be responsible for the evasion of programmed cell death during carcinogenesis. In the present study, we report a new finding that the inhibition of apoptosis in liver carcinogenesis may be attributed, at least in part, to down-regulation of critical genes mediated by deacetylation of histone H4K16. This was evidenced by a persistent gene-specific histone H4K16 deacetylation in NASH-fibrotic and HCC livers. This finding of significant histone H4K16ac as a gene-expression regulating mark is in a good agreement with the report by Taylor et al. (40), who demonstrated that histone H4K16ac is a marker of active gene enhancers, and a recent study of Wang et al. (41) that showed an association between increased promoter histone H4K16ac and over-expression of the Per2 gene. Furthermore, our observation of histone H4K16-mediated inhibition of cell death-associated genes corresponded to previous findings that genome-wide histone H4K16
deacetylation is associated with the down-regulation of autophagy-related genes and inhibition of autophagy-dependent cell death (42), which is, along with apoptosis, one of the major types of cell death (26).

The results of this study indicated that, mechanistically, deacetylation of histone H4K16 may be initiated by a NUPR1-mediated inhibition of histone acetylase KAT8. NUPR1 is a small 82 amino acid polypeptide that is highly expressed in several types of human cancer (43). The over-expression of NUPR1 disrupts cell cycle progression, inhibits programmed cell death, affects chromatin accessibility, and activates TGF-β signaling (43). Recent comprehensive studies have identified over-expression of NUPR1 as a key effector of liver carcinogenesis (44-46). In particular, over-expression of NUPR1 has been reported in primary human HCC samples (45-46), non-tumor adjacent tissues (46), and was continuously up-regulated with tumor progression (45). With respect to the pathogenesis of HCC, the over-expression of NUPR1 was associated with several key cancer characteristics, including inhibition of cell death (44), cancer cell migration and invasiveness (44-46), and chemoresistance (46). The involvement of NUPR1 in the acquisition of these cancer phenotypic features is facilitated by its role in the regulation of several molecular pathways, e.g., NUPR1-mediated histone H4K16 acetylation, NUPR1-granulin, NUPR1-SMAD4, and NUPR-RUNX2 (34, 44-46), among which NUPR1-mediated histone H4K16 acetylation is of special interest. A number of studies have demonstrated the involvement of NUPR1 in the regulation of histone H4K16 acetyltransferase KAT8 (hMOF) via binding to MSL1, a component of hMOF-MSL complex, and inhibiting its activity (32-34). In addition to histone H4K16 acetylation, KAT8 also acetylates non-histone proteins, especially the p53 tumor suppressor protein, the acetylated form of which is critical for activation of pro-apoptotic genes (47). This is another mechanism by which the inhibition of KAT8 might contribute to cancer-related evasion of apoptosis. Several reports have demonstrated the
down-regulation of KAT8 in human primary breast (48), colorectal (49), and renal cell carcinomas (49). Furthermore, KAT8 is significantly down-regulated in human HCC (50).

In summary, the results of the present study underline the important role of histone H4K16 deacetylation, in general, and gene-specific H4K16 deacetylation-mediated silencing of critical cancer-related genes, in particular, in the development of NASH-related HCC. Additionally, the mechanism leading to histone H4K16 deacetylation was associated with the NUPR1-facilitated inhibition of KAT8, as evidenced by a tight link between histone H4K16 deacetylation, over-expression of Nupr1, and down-regulation of lysine acetyltransferase KAT8 in the absence of SIRT1 up-regulation. These results emphasize the role of epigenetic alterations in the hepatocarcinogenic process and suggest their importance as attractive diagnostic and therapeutic targets for HCC.

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References


Figure legends

Figure 1. Whole-genome microarray analysis of gene expression in the livers of control mice and STAM mice subjected to NASH-derived hepatocarcinogenesis. (A) Heat maps and unsupervised hierarchical cluster analysis of log-normalized intensities illustrating differences in global hepatic gene expression profiles between control mice and STAM mice at steatosis (6 weeks), NASH-fibrosis (12 weeks), and full-fledged HCC (20 weeks) stages of NASH-related liver carcinogenesis. Color bar identifies high-expressed (red) and low-expressed (blue) genes. (B) Principal component analysis (PCA) illustrating transcriptomic differences in gene expression in the livers of control mice and STAM mice subjected to NASH-derived hepatocarcinogenesis. (C) Venn diagram illustrating number of differentially expressed genes at 6, 12, and 20 weeks in the livers of STAM mice subjected to NASH-derived hepatocarcinogenesis.

Figure 2. Summary of affected molecular pathways in the livers of control mice and STAM mice subjected to NASH-derived hepatocarcinogenesis. (A) Pathway analysis of differentially expressed genes related to biological functions in STAM mice at different stages of NASH-related liver carcinogenesis. Z-scores were calculated using IPA to predict activation (orange) or inhibition (blue) of biological functions. (B and C) Molecular networks of differentially expressed cell death-associated genes in mice subjected to the STAM model of hepatocarcinogenesis at 12 and 20 weeks. Genes in red color are up-regulated and genes in green color are down-regulated.

Figure 3. Level of TNF, sTNFR1, FASL, CD-137, CD44, caspase 8, and caspase 3 in the livers of control mice and STAM mice subjected to NASH-derived hepatocarcinogenesis. (A) Level of TNF, sTNFR1, FASL, CD-137, (B) Cd44 transcript and CD44 protein, and (C) caspase 8 and caspase 3 in the livers of control mouse and STAM mice subjected to NASH-
derived hepatocarcinogenesis. The results are presented as an average fold change in the level of each protein in the livers of STAM mice relative to that in the age-matched control mice, which were assigned a value 1. Values are mean ± SD, n = 4. * Denotes a significant (P < 0.05) difference from the age-matched control group. Representative Western blot images of 2 different samples from each group are shown.

**Figure 4. Levels of histone H4K20me3 and H4K16ac and chromatin modifying genes and proteins in the livers of control mice and STAM mice subjected to NASH-derived hepatocarcinogenesis.**

(A) Level of global histone H4K20me3 and H4K16ac, (B) expression of lysine methyltransferases \textit{Kmt5b} and \textit{Kmt5c}, (C) expression of lysine acetyltransferase \textit{Kat8} and lysine deacetylase \textit{Sirt1}, (D) protein level of KAT8 (arrow), and (E) expression of the \textit{Nupr1} gene in the livers of control mice and STAM mice subjected to NASH-derived hepatocarcinogenesis. The results are presented as an average fold change in expression of each gene and protein in the livers of STAM mice relative to that in the age-matched control mice, which were assigned a value 1. Values are mean ± SD, n = 4. * Denotes a significant (P < 0.05) difference from the age-matched control group. Representative Western blot images of 2 different samples from each group are shown.

**Figure 5. Gene expression and gene-specific histone modifications in the promoter region of cell death-related genes in the control livers and HCC.**

(A) Gene expression, (B) gene-specific histone H4K20me3, and (C) H4K16ac in the promoter region of cell death-related genes in HCC. The results of chromatin immunoprecipitation are presented as an average fold change of immuno-precipitated DNA in the livers of age-matched control mice and HCC normalized to input DNA. Values are mean ± SD, n = 4. * Denotes a significant (P < 0.05) difference from the age-matched control group.
Figure 1

A

B

C

STAM, 6 weeks
Total 373
316 up-regulated
57 down-regulated
Ratio: 5.5

STAM, 12 weeks
Total 1730
1323 up-regulated
407 down-regulated
Ratio: 3.2

STAM, 20 weeks
Total 2253
1078 up-regulated
1175 down-regulated
Ratio: 0.91
Figure 2

A

STAM, 6 weeks

STAM, 12 weeks

STAM, 20 weeks

Apoptosis
HCS activation
Carbohydrate synthesis
Cell movement
Hepatic steatosis

Activation z-score

-2.030 2.883

B

STAM, 12 weeks

C

STAM, 20 weeks
Figure 3

A

**TNF**

<table>
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<tr>
<th>6 weeks</th>
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**sTNFR1**

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**FASL**

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**CD137**

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<td>Control</td>
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B

**Cd44**

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C

**Caspase-8**

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**Caspase-3**

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<tr>
<td>0.8</td>
<td>0.5</td>
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</tbody>
</table>
Figure 4

A

H4K20me3

Control STAM

6 weeks 12 weeks 20 weeks

Fold change

Time, weeks

H4K16ac

Control STAM

6 weeks 12 weeks 20 weeks

Fold change

Time, weeks

B

Kmt5b

Kmt5c

Fold change

Time, weeks

C

Kat8

Sirt1

Fold change

Time, weeks

D

KAT8

KMT5B

SIRT1

6 weeks 12 weeks 20 weeks

Fold change

Time, weeks

E

Nupr1

Fold change

Time, weeks

* Denotes statistically significant difference from control.
Figure 5

A

Gene expression at 20 weeks

B

H4K20 trimethylation – 20 weeks

C

H4K16 acetylation – 20 weeks

Fold change

Tnfrsf10b Cdkn2a Trp53 Cyld Sdhb Cebpa Acvr2b Tnfrsf10 Nox4 Fgfr2 Fgfr4 Egfr Apc Cxcl12 Hgf Foxo4 Cdh1 Notch1 Esr1 Tnf Aatk

Fold change

Tnfrsf10b Cdkn2a Trp53 Cyld Sdhb Cebpa Acvr2b Tnfrsf10 Nox4 Fgfr2 Fgfr4 Egfr Apc Cxcl12 Hgf Foxo4 Cdh1 Notch1 Esr1 Tnf Aatk

Fold change

Tnfrsf10b Cdkn2a Trp53 Cyld Sdhb Cebpa Acvr2b Tnfrsf10 Nox4 Fgfr2 Fgfr4 Egfr Apc Cxcl12 Hgf Foxo4 Cdh1 Notch1 Esr1 Tnf Aatk
Inhibition of the cell death pathway in non-alcoholic steatohepatitis (NASH)-related hepatocarcinogenesis is associated with histone H4 lysine 16 deacetylation

Aline de Conti, Kostiantyn Dreval, Volodymyr Tryndyak, et al.

Mol Cancer Res  Published OnlineFirst May 16, 2017.

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