Published in final edited form as:

Cancer Lett. 2017 April 10; 391: 152–161. doi:10.1016/j.canlet.2017.01.028.

A single non-synonymous *NCOA5* variation in type 2 diabetic patients with hepatocellular carcinoma impairs the function of *NCOA5* in cell cycle regulation

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Abstract

Type 2 Diabetes (T2D) is a risk factor for hepatocellular carcinoma (HCC). We have previously described that haploinsufficiency of nuclear receptor coactivator 5 (*NCOA5*) is a genetic defect linking glucose intolerance to HCC. Here we report identification and characterization of a single nucleotide variation (T445A) in *NCOA5*, causing an amino acid Thr to Ala substitution, in adjacent non-tumorous liver tissues derived from patients with concurrent HCC and T2D. By using Tet-On inducible expression cells, we show that ectopic expression of *NCOA5*wt suppressed proliferation of HCC cells via induction of G2/M arrest, while ectopic expression of *NCOA5*T445A had a significantly lesser effect compared to ectopic expression of *NCOA5*wt. Furthermore, ectopic expression of *NCOA5*wt increased the occurrence of DNA damage and cell senescence, whereas expression of *NCOA5*T445A partly lost this activity. Xenograft tumor model analysis demonstrated that ectopic *NCOA5*wt expression reduced HCC tumor growth and the T445A variation impairs its tumor growth inhibitory function. Collectively, our data show that the T445A variation impairs the ability of *NCOA5* to inhibit growth of HCC, suggesting that this variation may have potential to increase susceptibility to HCC comorbid with T2D.

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Conflicts of Interest

The authors declare no conflict of interest.

Keywords

NCOA5; Hepatocellular carcinoma; Type 2 diabetes; Tumor suppressor; SNV

1. Introduction

The incidence of liver cancer has increased more than three times since 1980 and was increased by 2.3% per year in the United States from 2008 to 2012 according to the estimation by American Cancer Society. In 2016, about 39,230 new liver cancer cases will be diagnosed and about 27,170 liver cancer mortalities are expected in the United States. The majority of primary liver cancer is hepatocellular carcinoma (HCC), a heterogeneous disease [1]. Although molecular mechanism underlying HCC remains largely unknown, HCC is generally believed to be initiated and/or promoted by various genetic and environmental risk factors, leading to accumulation of genetic alterations acting as driver genes during the process of carcinogenesis [2]. While the environmental risk factors comprise of chronic hepatitis viral (B and C) infection, alcohol consumption and aflatoxin exposure, genetic risk factors include monogenic risk factors such as α_1 -antitrypsin deficiency resulting from mutations in the SERPINA1 genes and glycogenic storage disease type I due to mutations in the G6Pase gene, as well as polygenic risk factors such as autoimmune hepatitis, nonalcoholic fatty liver disease (NAFLD) and T2D [1; 3; 4; 5]. It was proposed that individuals carrying genetic variations in an allele or a combination of alleles in HCC-associated genes might have a higher susceptibility to HCC development [2]. Indeed, a number of genetic variations in genes, including PAPSS1 gene, cytokine and DNA repair genes as well as a germline deletion of APOBEC3B gene, have been found to increase susceptibility to HCC with hepatitis B viral infection [6; 7; 8]. In addition, several single nucleotide polymorphisms (SNPs) in genes including EGF, PNPLA3 and TM6SF2 PNPLA3 rs738409 C > G polymorphism were identified to be associated with the risk of development of HCC or non-alcoholic fatty liver disease associated HCC, respectively [9] [10]. However, genetic variations in genes associated with the increased risk for development of HCC comorbid with T2D have not been identified.

Nuclear receptor coactivator 5 (NCOA5) has been known to mediate transcriptional regulation in cells through interacting with several nuclear receptors including estrogen receptors, and LXR [11; 12; 13]. The importance of NCOA5 in HCC development was first revealed experimentally in a genetically-engineered mouse model [14; 15; 16]. Heterozygous deletion of *Ncoa5* gene was shown to increase IL-6 expression in the liver, leading to hepatic inflammation, glucose intolerance, hepatic steatosis and HCC development in mice, which can be partially decreased, but not completely prevented, by either heterozygous or homozygous deletion of IL-6 gene [14]. Consistent with the tumor suppressing role shown in the mouse, decreased expression of NCOA5 has also been found in about 44% of HCC specimens that were derived from Chinese HCC patients with most of them being HBV-positive [14]. Moreover, deletions and mutations in the NCOA5 gene were reported in various types of human cancers (cBioPortal for Cancer Genomics and ICGC Data Portal). Particularly, somatic NCOA5 mutations were identified in 0.61–9.30% of HCC samples from several cohorts of patients in ICGC Data Portal (Supplementary table 1). As

NCOA5 function seem to be vital to normal cell growth and development, naturally occurring sequence variations or mutations in the NCOA5 gene may result in aberrant function of NCOA5, thereby impacting its tumor suppression and potentially increasing susceptibility to HCC in humans. However, the effects of *NCOA5* single nucleotide variations (SNVs) or mutations on the function of NCOA5 in human HCC have not yet been investigated. In this report, we show that ectopic expression of wild type NCOA5 (NCOA5wt) induces G2/M cell cycle arrest and inhibits tumor formation in nude mice and a single non-synonymous *NCOA5* variation identified in liver tissues from patients with concomitant HCC and T2D reduces this effect.

2. Materials and Methods

2.1 Human HCC samples and sequence

Ten formalin fixed-paraffin embedded (FFPE) human liver specimens from patients with or without T2D, HCC, HCC comorbid with T2D or metastatic cancers in the liver and one fresh human liver specimen from a patient with metastatic pancreatic cancer in the liver were collected from consenting patients after hepatectomy in Massachusetts General Hospital (MGH), Boston. Four fresh human liver specimens from patients with HCC or HCC comorbid with T2D were collected from consenting patients after hepatectomy in Nanfang Hospital, Southern Medical University, China. The experimental procedures were approved by IRB of MGH, the Research Ethics Committee of Southern Medical University or the Biomedical and Health Institutional Review Board of Michigan State University, respectively. RNAs were extracted from FFPE liver adjacent noncancerous and tumor specimens with Qiagen's RNeasy Kit and Qiagen's RNeasy FFPE Kit, respectively. Then mRNAs were reversely transcribed to cDNAs with Invitrogen's SuperScript III Reverse Transcriptase. *Ncoa5* cDNA was amplified by specific primers to gain ~500 bp fragments. The PCR products were run in agarose gel and purified by Zymoclean Gel DNA Recovery Kit. The PCR products were subsequent for Sanger sequencing. The primers used for PCR and sequencing are as follows, hNCOA5-F 5'-CTGCTACACCCACTCCATCTGC-3'; hNCOA5-R1 5'-TTAGCTTCAGTAATGCCTCTG-3'; hNCOA5 R5 5'-TCTGTGCTGTGGTCTGGCTAAC-3'.

2.2 Plasmids and cell lines

pCR2.1-HA-NCOA5wt and pCR-2.1-HA-NCOA5T445A were generated by cloning NCOA5wt and NCOA5T445A cDNAs with the sequences encoding HA-tagged at C-terminal ends into pCR2.1 at EcoR1 site. pRevTRE-HA-NCOA5wt and pRevTRE-HA-NCOA5T445A were constructed by subcloning DNA fragments encoding HA-NCOA5wt or HA-NCOA5T445A from PCR2.1-HA-NCOA5wt or PCR2.1-HA-NCOA5T445A into pRevTRE (Clontech) at SalI and HindIII sites. pRevTet-On plasmid was purchased from Clontech Laboratories and then transfected into PLC/PRF/5 cells to constructed PLC/PRF/5 Tet-on cell line. HepG2 Tet-on advanced cell line was purchased from Clontech Laboratories (Catalog No. 630932) and cultured following the supplier's guidelines. PLC/PRF/5 and HepG2 cell lines were purchased from American Type Culture Collection and were recently authenticated by ATCC Cell line authentication service using Short Tandem Repeat (STR) analysis.

2.3 Retroviral transfection

pRevTet-On retroviruses containing rtTA-expressing vector and pRevTRE, pRevTRE-HA-NCOA5wt or pRevTRE-HA-NCOA5T445A retroviruses were produced by transfecting plasmids into the packaging Phoenix cell line with Fugene 6. PLC/PRF/5 Tet-on stable cell line was generated by infecting PLC/PRF/5 cells with pRevTet-On retroviruses according to the manufacturer's instruction (Clontech) and was selected for 2 weeks with G418 (400ug/ml) and then maintained in culture with half concentration. pRevTRE-Luc was transiently transfected into PLC/PRF/5 Tet-on cells and doxycycline (Dox) was used to induce expression of luciferin. The PLC/PRF/5 Tet-on cells were successfully induced by 1ug/ml Dox by subjected to test the luciferase activity. The Dox induced double-stable HepG2 and PLC/PRF/5 cells were generated by infecting HepG2 Tet-on and PLC/PRF/5 Tet-on cells with the viral supernatants (empty vector, HA-NCOA5wt and HA-NCOA5T445A) and selected for 7 days by adding hygromycin (400ug/ml for both cell lines) then maintained in culture by using 200ug/ml. The G418 and hygromycin resistant stable cells were confirmed by Western blot analysis. All the induced HepG2 and PLC/PRF/5 cells were cultured in DMEM with 10% Tet system approved FBS (Clontech, 631106).

2.4 Cell proliferation MTT assay

For 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, five thousand HepG2 or PLC/PRF/5 cells were plated onto 96 wells plate. The medium were changed and 1ug/ml Dox was added to the Dox+ groups at the next day to induce the expression of HA-NCOA5wt or HA-NCOA5T445A. After induction with Dox for 1, 3, 5 days, the MTT assay was performed as described [17].

2.5 Cell cycle assay

HepG2 and PLC/PRF/5 cells were plated into 6cm dishes and treated the following day with or without Dox (1ug/ml) for 48 hours and then harvested by trypsinization. After centrifugation, cell cycle assay was performed as described (propidium iodide Sigma P4170) and at least 10,000 cells were analyzed each time in triplicate experiments.

2.6 Cell senescence assay for β -Galactosidase

2×10^5 cells were plated onto 6-well plates and supplemented with Dox the next day. For the β -Galactosidase staining, cells were stained using Senescence β -Galactosidase Staining Kit (CST, #9860) on the fifth day after cell plating as described by the manufacture's protocol. The β -Galactosidase positive cells were examined and counted under a regular phase contrast microscope (200 $\times$) for the development of blue color. Results were expressed as the percentage of β -Galactosidase positive cells to total cells.

2.7 Western blots

Proteins were extracted from HepG2 and PLC/PRF/5 cells with or without induction by Dox and blotted with primary antibodies NCOA5 (1:1000; ab70831, Abcam), β -actin (1:1000; sc-47778, Santa Cruz Biotechnology), P21 (F-5, 1:200; sc-6246, Santa Cruz Biotechnology), Cyclin B1 (1:1000; cst-12231, Cell Signaling Technology) HA-tag (1:1000; cst-3724, Cell

Signaling Technology), respectively. All membranes were subsequently incubated for 1 hour with IRDye® 800CW Goat anti-Rabbit IgG (H + L) or 680RD Donkey anti-Mouse IgG (H + L). Corresponding protein bands were visualized by using Odyssey scanner and the Odyssey 2.1 software (LI-COR Biosciences, Lincoln, NE, USA).

2.8 Single-cell gel electrophoresis (comet assay)

DNA damage was determined by a Comet Assay Kit (Trevigen, Gaithersburg, MD, USA) in accordance with the manufacturer's protocol. HepG2 and PLC/PRF/5 cells were treated with Dox for 5 days. DNA was stained with SYBR® Green I Nucleic acid gel Stain (Invitrogen). The slides were analyzed using epifluorescence microscopy. The tail length and percentage of tail DNA were calculated using OpenComet software [18].

2.9 Tumor formation in nude mice

Single-cell suspensions of the induced PLC/PRF/5 cell lines with ectopic expression of HA-NCOA5wt, HA-NCOA5T445A or empty expressing vectors were trypsinized and collected. The cell viability was determined by trypan blue staining. Equivalent amounts of PLC/PRF/5 cells (1×10^7 cells per mice) were injected subcutaneously into the right flank of male BALB/c nude mice at 5–8 weeks of age. At 24 hours post-surgery, Dox (500ug/ml) was added to the drinking water. Tumorigenesis procedure was observed and determined by measuring tumor volume every 3 days using calipers. Tumor volume was calculated as follows: $V = (\text{width})^2 \times \text{length} \times 0.52$. All experimental procedures on mice were approved by the Michigan State University Institutional Animal Care and Use Committee, and performed in accordance with institutional and national guidelines.

2.10 Immunocytochemistry and immunostaining

After induction with Dox for 5 days, cells were subjected to immunofluorescence staining with primary antibody γ H2AX (pSer139, 1:150; NB100-79967, Novus), 53BP1 (1:500; NB100-304, Novus), ATM (pSer1981, 1:500; NB100-306, Novus) and secondary antibody anti-Rabbit IgG conjugated with Alexa Fluor 488 (A11034, Life Technologies), anti-Mouse IgG conjugated with Alexa Fluor 546 (A11036, Life Technologies) and DAPI (F6057, Sigma). Fluorescence micrographs were taken with a QImaging Micropublisher Camera under a Nikon Eclipse E600 microscope. Immunohistochemistry was performed in tumors derived from CON (n=3), NCOA5wt (n=2) or NCOA5T445A (n=3) cell group according to the protocol supplied by Vector Laboratories. PCNA (PC10, 1:100, sc-56, Santa Cruz Biotechnology) and γ H2AX (pSer139, 1:100) antibody were used. Quantification of IHC staining score of PCNA and γ -H2AX were performed in five random 200 $\times$ images per tumor slide.

2.11 Statistical analysis

All results were presented as means $\pm$ SEM or $\pm$ SD as indicated. The differences between groups were analyzed with one-way ANOVA and Student's *t*-test with two-tailed *P*-values of < 0.05 was considered statistically significant.

3. Results

3.1 A human non-synonymous single nucleotide (SNV) in NCOA5 is found in human HCCs comorbid with T2D

Next-generation sequencing analysis has revealed a great number of somatic mutations in driver and passenger genes in HCC [19; 20]. While the most frequent somatic mutations were found to affect several key cancer-related genes including TERT (~70%), CTNNB1 (~30%), and p53 (~30%), a large number of lower frequent somatic mutations were identified in genes involved in various regulatory pathways and cellular functions [21; 22; 23; 24]. Given the difficulty of obtaining a larger number of human HCC comorbid with T2D specimens, we sought to first examine somatic *NCOA5* mutations in publicly available cancer database. According to the International Cancer Genome Consortium (ICGC) data portal, there were 410 somatic *NCOA5* mutations identified in 10033 cancer specimens, 48 of which are in 1093 HCC specimens. Since the diabetic status were not annotated in those patients, it is not clear whether any of the *NCOA5* mutations are associated with HCC comorbid with T2D. To search for *NCOA5* mutations in HCC comorbid with T2D, we first examine the *NCOA5* cDNA sequences from eight liver specimens derived from four unrelated individuals who had HCC and four unrelated individuals who had HCC comorbid with T2D as well as seven control liver tissues from unrelated individuals who had metastatic cancers in the liver (Table 1). Among these control liver tissue specimens without HCC, two from individuals who had T2D. Intriguingly, a non-synonymous SNV (p.T445A, c. 46062707. A>G) in exon 8 of *NCOA5*, resulting in an amino acid Thr to Ala substitution at 445 position (T445A), was found in the adjacent noncancerous liver tissues from two out of four patients with HCC comorbid with T2D (Fig. 1A and Table 1). The same variation (T445A) was also found in a liver specimen from a T2D patient, but not in the liver tissue specimens from the other diabetic patient and patients who had neither HCC nor diabetes. Apparently, the same variation was previously reported in NCBI SNP database as rs77552828 (at Ch20. 44691346 T>C or G, p.T445A or P) with unknown allele frequency, but it was not found in the Exome Aggregation Consortium (ExAC). These results indicate that T445A is a rare SNV and might potentially impair the function of *NCOA5* in liver cells.

3.2 Ectopic expression NCOA5 suppresses proliferation of human HCC HepG2 and PLC/PRF/5 cells *in vitro* and T445A variation reduces the ability of NCOA5 to suppress cell proliferation

To begin evaluating whether T445A variation has a functional consequence, we used two HCC cell lines to investigate the effect of this *NCOA5* variation on its tumor suppressor function. Since stable cell lines with ectopically and constitutively expressed wild type *NCOA5* (*NCOA5*wt) have been difficult to establish due to its growth-suppressive effect, we first generated stable polyclonal HepG2 and PLC/PRF/5 cell lines with ectopic expression of HA-*NCOA5*wt or HA-*NCOA5*T445A using a lentivirus-based tetracycline inducible gene expression system. The levels of ectopic protein expression were characterized at various times following Dox induction using Western analyses (Fig. 1B and C). Ectopically-expressed proteins were specifically detected using an HA antibody, whereas both endogenous and ectopic *NCOA5*wt expressions were detected using a C-terminal *NCOA5* antibody. As shown in Figure 1B and 1C, the expression levels of HA-*NCOA5*T445A after

induction were significantly higher than the levels of endogenous NCOA5 in HepG2 (Fig. 1B) or PLC/PRF/5 cells (Fig. 1C), whereas HA-NCOA5wt were only moderately expressed in PLC/PRF/5 cells. We then compared the effects of HA-NCOA5wt and HA-NCOA5T445A on proliferation in a cell growth assay. As expected, a small amount of HA-NCOA5wt expression significantly inhibited proliferation of HepG2 and PLC/PRF/5 cells in the presence of Dox induction when compared to mock expressing controls (Fig. 1D and E). This inhibitory effect on cell growth was observed even in the absence of Dox induction, which is most likely due to a leaky expression of HA-NCOA5wt. In contrast, cell growth of HepG2 and PLC/PRF/5 cells containing HA-NCOA5T445A expressing vectors was not significantly different from that of control cells containing mock expressing vectors in the absence of Dox induction. By five days after Dox induction, growth of HepG2 and PLC/PRF/5 cells containing HA-NCOA5T445A expressing vectors was significantly inhibited when compared with that of cells containing mock expressing vectors. Nevertheless, their growth inhibition was significantly lesser in HA-NCOA5T445A expressing cells than in the HA-NCOA5wt expressing cells, even though the protein levels of HA-NCOA5T445A in cells were much higher than that of HA-NCOA5wt in cells. These results suggest that the T445A variation partially reduces the growth inhibitory function of NCOA5.

3.3 T445A variation impairs the inhibitory effect of NCOA5 on G2/M transition of HepG2 and PLC/PRF/5 cells *in vitro*

Since aberrant changes in cell cycle progression are well known to affect cell proliferation, flow cytometry was used to examine whether ectopic NCOA5wt expression inhibited cell proliferation through impeding the cell cycle progression and if the T445A variation affected this activity. Representative histograms and the results are showed in Figure 2 and Supplementary Figure 1. HepG2 and PLC/PRF/5 cells containing ectopic NCOA5wt expression exhibited a significantly increase in the percentage of cells in G2/M phase in the presence or absence of Dox induction as compared with mock control cells. In contrast, the percentage of HepG2 or and PLC/PRF/5 cells in G2/M phase containing HA-NCOA5T445A expressing vectors was significantly decreased compared to with cells containing HA-NCOA5wt expressing vectors. To confirm the results, we measured the expression of cyclin B1 in HepG2 cells, which could promote the G2/M transition of cells. Consistent with the observations of their effects on G2/M transition of cells, the expression of cyclin B1 was significantly reduced in HepG2 cells containing ectopic NCOA5wt expression, but less reduction was observed in HepG2 cells with ectopic NCOA5T445A expression (Fig. 2D). These data suggest that the T445A variation impairs the function of NCOA5 in the suppression of cell growth through reducing its inhibitory activity in G2/M transition.

3.4 The T445A variation impairs the effects of NCOA5 on DNA damage and cell senescence in HCC HepG2 and PLC/PRF/5 cells

Since cell senescence triggered by DNA damage is also a main reason for arresting cell cycle and inhibiting proliferation, senescence β -Galactosidase staining was used to test whether ectopic NCOA5wt expression increased cell senescence and if the T445A variation impaired this activity. As showed in Figure 3A and B, the percentage of cells positive for stain of senescence associated (SA)- β -Galactosidase, a commonly used marker of

senescence was significantly increased in both HepG2 and PLC-PRF 5 containing ectopic NCOA5wt expression compared with those cells containing a mock expressing vector. However, the increase in the percentage of (SA)- β -Galactosidase positive cells in HepG2 and PLC-PRF 5 containing ectopic NCOA5T445A expression was significantly lesser than that in cells containing ectopic NCOA5wt expression. Consistent with this observation, expression of p21, a senescence marker, was increased in cells with ectopic NCOA5wt after induction with Dox, but a relatively less increase was observed in cells with ectopic NCOA5T445A (Fig. 3C). To explore the role of NCOA5 in DNA damage response, we first examined the activation of DNA damage signaling by measuring γ -H2AX, 53BP1 and pATM foci formation in cells. In comparison to cells containing a mock expressing vector, a significantly increased amount of cells with double-staining of γ -H2AX (pSer139) and pATM or 53BP1 and pATM were observed in HepG2 and PLC/PRF/5 cells containing ectopic HA-NCOA5wt expression (Fig. 3D, E and Supplementary Fig. 2). Furthermore, the number of cells with double-staining of γ -H2AX (pSer139) and pATM foci or 53BP1 and pATM foci in HepG2 and PLC/PRF/5 cells containing HA-NCOA5T445A expression was significantly reduced compared to the cells containing HA-NCOA5wt expression. Next, comet assay was used to confirm that HepG2 and PLC/PRF/5 containing ectopic NCOA5wt expression had more cells with DNA damage than cells containing a mock expressing vector (Fig. 3C and supplementary Fig. 2). This finding suggests that NCOA5T445A variation may impair the function of NCOA5wt in the enhancement of DNA repair response, consistent with its effect on cell senescence and cell cycle arrest.

3.5 The T445A variation impairs the effect of NCOA5 on suppression of HCC tumor growth *in vivo*

To examine whether the effect of T445A variation on the role of NCOA5 in cell growth is recapitulated under a more physiological condition, PLC/PRF/5 cell stable lines were selected for the tumorigenicity study in nude mice as the original HepG2 cell line has lower tumorigenicity. PLC/PRF/5 cell lines with mock control, HA-NCOA5wt or HA-NCOA5T445A expressing vector were inoculated subcutaneously into the right flank of nude mice, respectively. Inducible expression of NCOA5wt or NCOA5T445A was achieved by feeding mice with Dox-containing water. The effect of HA-NCOA5wt and HA-NCOA5T445A on tumor growth was determined by measuring the volume of tumors every three days in the presence of Dox induction (Fig. 4). After 60 days, tumors with an average weight of 0.36 ± 0.04 g were detected in all six mice inoculated with cells containing a mock expressing vector (Fig. 4A and B), whereas only two of six mice with implanted cells containing ectopic expression of NCOA5wt grew detectable tumor masses with an average weight of 0.11 ± 0.005 g. In contrast, four of six mice implanted with cells containing ectopic expression of NCOA5T445A grew tumors with an average weight of 0.20 ± 0.04 g. Nevertheless, the average weight and volume of tumors formed by NCOA5T445A cells were significantly larger than that of tumors formed by ectopic NCOA5Twt expressing cells, even though they were smaller than those of control cells (Fig. 4C and D). Consistently, tumors cells with ectopic NCOA5T445A displayed a significant increase in the number of PCNA-positive cells when compared with tumor cells ectopically expressing NCOA5wt (Fig. 4E and F). NCOA5T445A cells also exhibited decreased γ H2AX staining compared to NCOA5Twt, but similar to those of mock controls cells (Fig. 4E and G), indicating an

impaired induction of γ -H2AX foci formation by ectopic NCOA5T445A expression. In addition, tumors cells with ectopic NCOA5T445A displayed a significant decrease in the number of cells positively stained with Sudan Black B, a cell senescence staining marker [25; 26], in comparison to tumor cells with ectopic NCOA5wt (supplementary Fig. 4). Collectively, these results suggest that ectopic NCOA5wt expression reduced HCC tumor growth, whereas T445A variation impairs the tumor growth inhibitory function of NCOA5.

4. Discussion

Until recent years, the genetic changes and molecular mechanisms leading to development of HCC comorbid with T2D have been largely unknown. Studies on a genetically-engineered mouse model recently suggested that NCOA5 haploinsufficiency is a genetic driver in the development of HCC comorbid with T2D in mice [14]. However, it has remained unknown whether there are SNVs in the NCOA5 gene predisposed to development of HCC and HCC comorbid with T2D. In the current study we identify and characterize a single amino acid change, NCOA5T445A, caused by a non-synonymous SNV in adjacent non-tumorous tissues of HCC specimens and a non-HCC liver tissue derived from Caucasian patients with T2D, which impairs the function of NCOA5 in cell cycle regulation. Results of this study suggest that T445A variation impairs the tumor suppression role of NCOA5 in HCC cells. We therefore hypothesize that the T445A SNV in NCOA5 may increase susceptibility to HCC and thus serves as a potential marker for predicting risk of hepatocellular carcinoma. In view of this possibility, establishment of a medically useful link between this NCOA5 variation and the risk of HCC comorbid with T2D and application of this study will require family case studies as well as significantly larger case control studies for different racial cohorts.

In this article, the activities of NCOA5wt and NCOA5T445A were investigated using Tet-On inducible expression HepG2 and PLC/PRF/5 HCC cell lines, which express wild type p53 or mutant p53, respectively. Slightly elevated levels of NCOA5wt in these cells were shown to result in cell cycle arrest, DNA damage response and senescence *in vitro* and *in vivo*, suggesting induction of cell cycle arrest and senescence by NCOA5wt as an intriguing mechanism by which NCOA5 acts as a tumor suppressor in hepatocytes. Interestingly, observed activities of NCOA5wt are somewhat similar to those of p53 that plays important roles in the regulation of cell cycle and DNA damage response [27; 28]. Previous studies have shown that p53 expression could induce gene transcription, cell cycle arrest, senescence and apoptosis dependent on the abundance of the p53 protein itself, cofactors and other contexts [29]. Given the fact that the common features of senescence comprise SA- β galactosidase activity, increased expression of DNA damage markers, increased expression of cell cycle inhibitors and reduced expression of cell proliferation markers, we speculate that increased expression of NCOA5wt results in cell cycle arrest and increased DNA damage response, leading to cell senescence.

The lack of detailed molecular mechanisms by which NCOA5 exerts its tumor suppressor function makes it difficult to explain how a single amino acid substitution results in such functional effects. NCOA5 has been shown to act as a coactivator or corepressor in context with nuclear receptors to regulate transcription of genes in HeLa cervical and MCF7 breast

cancer cells [11; 12]. Consistent with its corepressor activity, NCOA5 was shown to repress transcription of IL-6 in mouse livers [14]. Moreover, NCOA5 was recently reported to act as an LXR corepressor to inhibit Abca1 expression in macrophages [13]. We show that ectopic expression of NCOA5wt reduced the expression of CyclinB1 in HCC cells whereas NCOA5T44A expression had relatively less effect, implying that NCOA5 might directly or indirectly affect expression of genes related to cell cycle control, such as cyclin B1. In addition, we have tested the possibility that T445A impairs the ability of NCOA5 to repress IL-6 transcription. We co-transfected Raw 264.7 cells with the combination of NCOA5wt or NCOA5T445A with NF- κ B p65 and IL-6 reporter constructs with or without LPS treatment. (Supplementary Fig. 2). We observed that NCOA5T445A had significantly less inhibitory effect on p65-mediated transcriptional enhancement of the IL-6 promoter compared to NCOA5wt. As noted in Figure 1A, T445A presents in a relatively weak transcriptional repression domain located between amino acids 341 and 457 of NCOA5, even though it is not in the canonical repression or activation domain of NCOA5 [11]. Thus, it is reasonable to propose that an amino acid (T445A) change in NCOA5 might impair this transcriptional repression activity of NCOA5 in hepatocytes and macrophages, resulting in impairment in DNA repair response, cell cycle arrest and senescence, thereby increasing susceptibility to malignant transformation of liver cells. Future studies should explore the molecular mechanism by which T445A impairs the function of NOCA5 in regulation of transcription of specific genes, senescence and cell cycles.

In conclusion, this study expands on the promoting effect of NCOA5 deficiency on the development HCC and suggests a role of NCOA5 in the regulation of G2/M transition during cell cycle. Our study also implies an intriguing etiological link between T445ASNV and HCC comorbid with T2D and supports further studies on the validation of the association of this SNV with increased risk of HCC comorbid with T2D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank members of the H.X. lab for helpful discussions. This work was supported by National Cancer Institute (R01 CA188305) to H. Xiao, and the Joint Research Fund for Overseas Chinese, Hong Kong and Macao Scientists of the National Natural Science Foundation of China (Grant No. 81428017) to R. Luo. X. Liu and F. Liu were supported in part by fellowships from Southern Medical University, China. F. Chen was supported in part by Youth Fund of the National Natural Science Foundation of China (Grant No. 81502378). C. Yang was supported by National Institute of Health (1R01ES017777-01).

Abbreviations

SNVs	Single nucleotide variations
LPS	Lipopolysaccharide

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Highlights

Identification of a T445A variation in NCOA5 gene from patients with both HCC and T2D.

NCOA5 inhibits proliferation of HCC cells via promoting G2/M phase cell cycle arrest.

The T445A impairs the ability of NCOA5 to inhibit growth of xenograft tumors.

Figure 1. Ectopic expression of NCOA5T445A reduced the effect of NCOA5wt on proliferation of HepG2 and PLC/PRF/5 cells. (A) The schematic representation of NCOA5 gene and T445A variation. (B) and (C) the protein levels of endogenous NCOA5, HA-tag NCOA5wt and NCOA5T445A in HepG2 and PLC/PRF/5 cells before or after Dox induction. Cell lysates were Western blotted with the indicated antibodies. (D) and (E) Proliferation of HepG2 and PLC/PRF/5 cells containing empty vector (CON), HA-NCOA5wt or HA-NCOA5T445A

before and after Dox induced was analyzed using MTT assay. Error bars represent SD. *, P 0.05; **, P 0.01; NS, non-significant difference.

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Figure 2.

Ectopic expression of NCOA5T445A impaired the effect of NCOA5 on the induction of G2/M cell cycle arrest. After induction with Dox for 48 hours, HepG2 cells were subjected to PI staining and flow cytometry analysis. Quantification of the percentage of cells in G2/M is shown (n = 3; error bars, SEM). *P* values were calculated using an unpaired two-tailed *t* test (A) Representative cell cycle profiles of HepG2 cells. (B) and (C) Quantifications of indicated HepG2 cells with or without Dox induction. (D) The protein levels of Cyclin B1 in

HepG2 cells with or without Dox induction. Cell lysates were blotted with indicated antibodies. β -Actin was used as a control. *, P 0.05; **, P 0.01.

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Figure 3.

T445A SNV impaired the promoting effect of NCOA5 on cell senescence and DNA damage *in vitro*. (A) and (B) HepG2 and PLC/PRF/5 cells were induced with Dox for 5 days and then subjected to SA- β -Galactosidase staining. Representative images quantification of positively stained cells were shown. Black arrow indicated the positive cells. (C) The protein levels of P21 in HepG2 cells containing HA-NCOA5wt and HA-NCOA5T445A with or without Dox for 5 days. Cell lysates were blotted with indicated antibodies. (D) HepG2 and PLC/PRF/5 cells were induced with Dox for 5 days and immunofluorescence staining was

used for γ -H2AX pSer139 (green) and ATM pSer1981 (red) double-positive foci. (E) The number of cells with γ -H2AX and pATM foci positive was quantified. (F) HepG2 cells were induced with Dox for 5 days and comet assay was used to detect DNA damage. White arrows indicate cells with comet tail. (G) Quantification of the average length of tails in HepG2 cells. (H) Quantification of proportion of comet-bearing nuclei with >40% fragmented DNA in HepG2 cells. Error bars represent the mean $\pm$ SD. *, P 0.05; **, P 0.01; NS, non-significant difference.

Figure 4.

T445A SNV partially impaired the tumor growth effect of NCOA5 on PLC/PRF/5 cells in xenograft tumor models. (A) Representative gross morphology of the subcutaneous tumors in nude mice injected with PLC/PRF/5 cells (1×10^7 cells per mouse). (B) The number of tumors in nude mice injected with indicated PLC/PRF/5 (n=6 for each group). (C) Mean tumor weights in mice injected with indicated PLC/PRF/5 cells. Tumor weight was measured at the time of sacrifice. (D) Mean tumor volumes in mice injected with indicated PLC/PRF/5 cells. Volume of tumors were measured at the time of sacrifice. (E) H&E

staining and immunohistochemical staining of PCNA and γ -H2AX on tumor sections derived from each group were showed. (F) and (G) Quantification of IHC staining score of PCNA and γ -H2AX in tumors derived from CON(n=3), NCOA5wt (n=2), NCOA5T445A (n=3) cell group were compared. Error bars represent SEM. * $P<0.05$; ** $P<0.01$; NS, non-significant difference.

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Table 1

Clinical data and sequence analysis of FFPE or frozen liver specimens

Case	Race	Sex	Age	HBV Surface Ag	Sample Specification	Liver Disease	T2D	Sequence analysis (T445A)
1	Asian	Male	55	Positive	Frozen	HCC	No	No
2	Asian	Male	58	Negative	Frozen	HCC	No	No
3	Asian	Male	62	Negative	Frozen	HCC	T2D	No
4	Asian	Male	71	Positive	Frozen	HCC	T2D	No
5	Caucasian	Male	81	Negative	FFPE	HCC	T2D	T445A
6	Caucasian	Male	54	n/a	FFPE	HCC	T2D	T445A
7	Asian	Female	66	Positive	FFPE	HCC	No	No
8	Asian	Male	50	n/a	FFPE	HCC	No	No
9	Caucasian	Female	59	n/a	FFPE	Metastatic colorectal adenocarcinoma to the liver	No	No
10	Caucasian	Female	62	n/a	FFPE	Metastatic colonic adenocarcinoma to the liver	No	No
11	Hispanic	Female	41	n/a	FFPE	Metastatic colonic adenocarcinoma to the liver	T2D	No
12	Caucasian	Female	84	n/a	FFPE	Metastatic renal cell carcinoma to the liver	T2D	T445A
13	Caucasian	Male	67	n/a	FFPE	Metastatic colonic adenocarcinoma to the liver	No	No
14	Caucasian	Male	69	n/a	FFPE	Metastatic renal cell carcinoma to the liver	No	No
15	Asian	Female	42	Positive	Frozen	Metastatic neuroendocrine tumor to the liver	T2D	No