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Original Article

Vimentin acetylation is involved in SIRT5-mediated hepatocellular carcinoma migration

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Abstract: Sirtuin 5 (SIRT5) belongs to the sirtuin family of protein deacetylases and contributes to tumorigenesis and migration. However, the underlying molecular mechanism of SIRT5 in hepatocellular carcinoma (HCC) migration is not fully understood. Here we report that SIRT5 was significantly downregulated in HCC, based on analysis of RNA-seq data from the liver HCC dataset of The Cancer Genome Atlas (TCGA). In addition, as compared to adjacent non-tumor tissues, SIRT5 was also significantly downregulated in HCC tissues. *In vitro*, gain and loss-of-function studies were performed to evaluate the role of SIRT5 in epithelial-mesenchymal transition (EMT). Knockdown of SIRT5 promoted EMT, as indicated by the upregulation of Snail and downregulation of E-cadherin, whereas overexpression of SIRT5 decreased Snail and upregulated E-cadherin. Mechanistically, SIRT5 was found to bind to and deacetylate vimentin at lysine 120. Cell migration was enhanced by overexpression of either wild-type vimentin or acetylation mimetic vimentin (K120Q), whereas cell migration was inhibited by overexpression of the non-acetylation vimentin (K120R). Taken together, these findings indicated that downregulated SIRT5-mediated vimentin acetylation may be involved in the EMT in HCC. Better understanding of SIRT5 may lead to its clinical application as a biomarker for prognosis of prediction of prognosis, as well as a novel therapeutic target.

Keywords: Hepatocellular carcinoma, SIRT5, vimentin, acetylation, epithelial-mesenchymal transition

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide [1, 2]. Currently, surgical resection and/or liver transplantation are the best options for clinical treatment of HCC, although a high recurrence rate and poor prognosis is caused by metastasis [3]. Therefore, there is a critical medical need to unveil the pathogenesis of HCC that is involved in metastasis.

Sirtuins are class III histone deacetylases that have been shown to play important roles in regulating the life-span of yeast, worms, and flies [4]. Among them, Sirtuin 5 (SIRT5) has become a research hotspot because of its unique protein post-translational modification (PTM) activities and biological and physiological functions [5-7]. SIRT5 has been shown to regulate the urea cycle by deacetylating and activating carbamoyl phosphate synthase 1 [8]. It can also deacetylate FOXO3 to prevent apoptosis of lung epithelial cells [9]. Furthermore, SIRT5 can revert endotoxin-tolerance to a pro-inflammatory phenotype in macrophages by activating the NF-κB pathway [10]. Although aberrant expression and altered function of SIRT5 have been found in various cancers [11, 12], its role in HCC remains largely unknown.

Intermediate filaments (IFs) are major components of the cytoskeleton in eukaryotic cells [13]. Vimentin, a type III IF, is one of the most conserved and abundant proteins in the IF protein family. Vimentin expression in tumor cells has been recognized as a hallmark of epithelial-mesenchymal transition (EMT), and is associated with cell invasion and poor prognosis [14,

15]. Meng et al. reported that vimentin was involved in the process of EMT in HCC [16] and that its down-regulation inhibits EMT [17]. Similar to other IFs, vimentin undergoes various functionally important PTMs, such as phosphorylation, glycosylation, sumoylation, and ADP ribosylation to perform its functions [18]. Vimentin can be phosphorylated by Akt1 to promote tumor metastasis [19], and sumoylation of vimentin upon PIAS3 stimulation inhibits glioma cell migration [20]. However, whether other modifications of vimentin, such as acetylation, are involved in HCC EMT process in HCC remains unclear. Therefore, this study aimed to explore the role of SIRT5 and vimentin acetylation in HCC.

Materials and methods

Cell lines

THLE-3 (immortalized human liver cell line) and SNU449 (HCC cell line) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HCC cell lines HepG2 and BEL-7402 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). THLE-3 cells were cultured using a BEGM Bullet Kit (#CC3170, Lonza, Allendale, NJ, USA), according to manufacturer's instructions. SNU449 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO, Invitrogen Inc., Carlsbad, CA, USA). HepG2 and BEL-7402 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen Inc., Carlsbad, CA, USA).

Hepatocellular carcinoma samples

A total of 19 HCC samples and corresponding non-tumor tissues were collected, among which four pairs of samples were collected from the First Affiliated Hospital of Shantou University (Guangdong, China) and 15 pairs from the Shengjing hospital of China Medical University (Liaoning, China). All patients provided written informed consent and the study was approved by the Committee for Ethical Review of Research involving Human Subjects at the First Affiliated Hospital of Shantou University. The mRNA-Seq data of 423 HCC samples was obtained from The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov/publications/publicationguidelines).

RNA interference

Cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX (#13778150, Invitrogen Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. The cells were harvested for further analysis 48 h after transfection. The sequences of the siRNAs used in this study were as follows. Vimentin: 5'-GAGUCAAACGAGUACCGGAdTdT-3', (Ribobio Co., Guangzhou, China). SIRT5: 5'-CAGCAUCCCAGUUGAGAAAdTdT-3'; non-targeting (Scramble): 5'-UUCUCCGAACGUGUCACGUdTdT-3' (#SASI_Hs01_00108263, Sigma, St Louis, MO, USA).

Plasmid constructs and transfection

The cDNAs encoding human SIRT5 (NM_012241) or vimentin (NM_003380) were constructed by Genechem Co., Ltd. (Shanghai, China) and cloned into GV141 or GV366 eukaryotic expression vectors, respectively. Point mutants were generated using the Mut Express II Fast Mutagenesis kit V2 (Vazyme, Nanjing, China), according to the manufacturer's instructions. Successful mutation was confirmed by DNA sequencing. The plasmid encoding SIRT5 mutant H158Y was generated using the following primers: H158Y forward: 5'CTGGAGATCTATGGTAGCTTATTTAAAACTCGATGTACCTCTTGTGTGG3'; reverse: 5'TAAGCTACCATAGATCTCCAGAAGGTTCTTGGTGCCAGCCTTG3'. The plasmid encoding the vimentin mutant K120Q was generated using the following primers: K120Q forward: 5'TACATCGACCAGGTGCGCTTCCTGGAGCAGCAGAATAAGATCCTG3'; K120Q reverse: 5'GAAGCGCACCTGGTCGATGTAGTTGGCGAAGCGGTCATTCAGCTC3'. The plasmid encoding the vimentin mutant K120R was generated using the following primers: K120R forward: 5'TACATCGACCGGGTGCGCTTCCTGGAGCAGCAGAATAAGAT3'; K120R reverse: 5'GAAGCGCACCCGGTCGATGTAGTTGGCGAAGCGGTCATT3'. Candidate plasmids were transiently transfected into cells using FuGENE HD (#E2311, Promega, Madison, WI, USA) according to the manufacturer's instructions. After transfection, the cells were harvested at the indicated time points and subjected to further analysis.

Cell migration assays

Cell migration was assessed by a transwell assay. Briefly, HCC cells were transfected with the indicated siRNAs or plasmids; 48 h after

transfection, the cells were treated with mitomycin C (10 mg/ml) for 4 h [21]. Next, 6 × 10^4 cells were seeded in the upper chamber in 100 μl serum-free medium, while medium containing 10% FBS in the lower chamber served as chemoattractant. The SNU449 and 7402 cells were incubated for another 24 h, whereas HepG2 cells were incubated for another 48 h. The migrated cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai, China). Photographs were taken using an inverted microscope at 200 × magnification. Migrated cells were scored using images from five random fields.

Wound healing assay

HCC cells were seeded in six-well plates and transfected with indicated siRNA or plasmids. When the cells reached confluence, they were treated with 10 mg/ml mitomycin C for four hours [21], followed by wounding using a sterile plastic pipette. Then, the cells were washed twice with PBS to remove cellular debris. Finally, the wounded monolayer was subsequently maintained in conditioned media (CM) supplemented with 1% FBS for 24-48 h. Photographs were taken with a phase contrast microscope.

Western blot

Tissue and cell lysates were extracted using RIPA buffer supplemented with 1% protease inhibitor cocktail (Millipore, USA). Protein concentration was determined with a BCA protein assay kit (Beyotime, Shanghai, China). Equal amounts of protein were electrophoresed on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, USA). The membrane was incubated overnight at 4°C with SIRT5 (#8779, Cell Signaling, Danvers, MA, USA), E-cadherin (#14472, Cell Signaling, Danvers, MA, USA), SNAIL (#3879, Cell Signaling, Danvers, MA, USA), acetylated-lysine (#9681, Cell Signaling, Danvers, MA, USA), vimentin (#PA5-27231, Thermo Fisher Scientific, Waltham, MA, USA), or β-actin (#A1978 Sigma, St Louis, MO, USA) antibodies. Then, the membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Protein bands were developed on X-ray film. The intensity of bands was determined using ImageJ 2X software (National Institutes of Health, Bethesda, MD, USA). β-Actin was used as a loading control.

Immunofluorescence

The cells were grown on gelatin-coated coverslips and fixed with 4% paraformaldehyde. The cells were permeabilized with PBS containing 0.1% Triton-X 100 and blocked with PBS containing 5% bovine serum albumin, followed by an overnight incubation with primary antibody targeting SIRT5 (#sc-271635, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C. The next day, cells were washed with PBS and incubated with FITC-conjugated anti-mouse secondary antibody (#F2761, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1 h. Subsequently, the cells were washed three times with PBS and treated with primary antibody targeting vimentin (#PA5-27231, Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight, followed by a 1-h incubation with Cy3-conjugated anti-rabbit secondary antibody (#C2306, Sigma, St Louis, MO, USA) at room temperature. Slides were mounted using antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (#S36939, Thermo Fisher Scientific, Waltham, MA, USA) and imaged using a Zeiss confocal microscope. Images were analyzed by Zeiss LSM software.

Immunoprecipitation assay

Protein extraction and quantification were similar to western blot. A total of 500 μg of lysate was adjusted to a final volume of 1 ml with PBS. Then, 5 μg of the SIRT5 (#730109, Thermo Fisher Scientific, Waltham, MA, USA) or vimentin (#A5-27231, Thermo Fisher Scientific, Waltham, MA, USA) antibody, or corresponding IgG was added to the lysate and incubated at 4°C for 8 h. Next, 40 μl of protein A/G PLUS-agarose (#sc2336, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to each sample and the samples were kept on a rotator at 4°C overnight. The samples were then centrifuged at 500 g for 5 min at 4°C. Pellets were washed four times with PBS and then resuspended in 20 μl of loading buffer. Samples were subjected to electrophoresis on an 8% SDS-polyacrylamide gel and immunoblotted with appropriate antibodies against SIRT5 (#8779, Cell Signaling, Danvers, MA, USA) and vimentin (#PA5-27231, Thermo Fisher Scientific, Waltham, MA, USA).

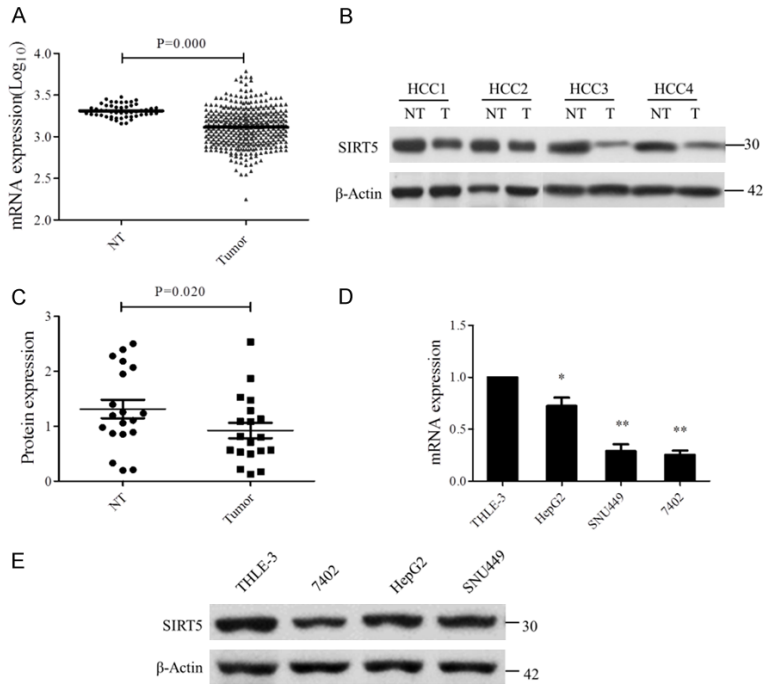


Figure 1. Decreased expression of SIRT5 in HCC tumor tissues. A. SIRT5 mRNA expression was downregulated in HCC tumor tissues (n = 374) as compared to the adjacent non-tumor tissues (n = 50). Results were calculated using the LIHC dataset from TCGA. B. Representative western blot images of SIRT5 in 19 paired HCC tumor tissues and adjacent non-tumor tissues are shown. C. Relative protein expression levels of SIRT5 in 19 paired HCC tumor tissues, as determined by densitometry. D, E. mRNA and protein levels of SIRT5 in one immortalized liver cell line (THLE-3) and three HCC cell lines were quantified by qPCR and western blot, respectively. Data are expressed as the mean ± standard deviation.

LC/MS-MS

SNU449 cells were transfected with siRNA targeting SIRT5 or scrambled siRNA. After 48 h of transfection, cells were harvested and extracted as described above. Soluble fractions were subjected to immunoprecipitation. Then, 40 µl of Protein A/G PLUS-Agarose was added and incubated for another 12 h at 4°C. The protein complex-containing beads were washed four times with PBS, then proteins were eluted, boiled in loading buffer (containing 1% SDS), and resolved by SDS/PAGE. Coomassie blue staining was used to visualize the protein bands. Protein bands were excised from the gel, fully trypsinized, and analyzed by reverse-phase LC/MS/MS (service provided by PTM BioLabs, Inc., China). The MS/MS data were processed by Proteome Discoverer 1.3.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (IBM, Armonk, NY, USA). Results are expressed as the means ± standard deviation of at least three independent experiments. Differences between two groups were analyzed by the independent samples t-test. Comparisons among groups were carried out using one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

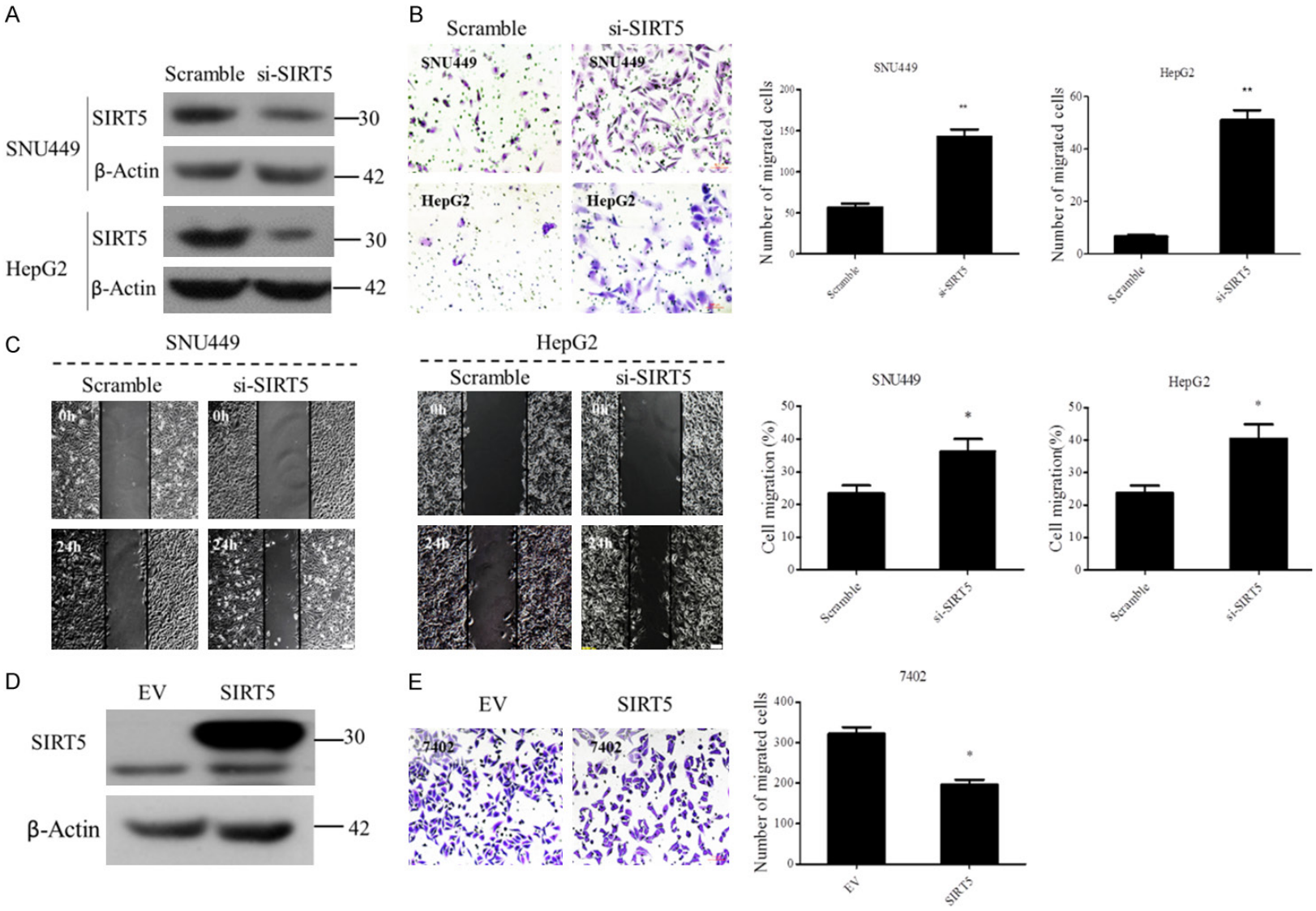
Results

SIRT5 is downregulated in human HCC tumor tissues

RNAseq data of 423 HCC patients were obtained from the TCGA liver hepatocellular carcinoma (LIHC) dataset. We analyzed the data and compared the expression of SIRT5 in 374 tumors with that in 50 adjacent non-tumor tissues (NT). As shown in **Figure 1A**, SIRT5 was significantly downregulated in HCC tissues ($P < 0.001$). Similarly, western blot results of 19 HCC tumor tissues and their paired adjacent NT tissues indicated that the protein levels of SIRT5 were also significantly decreased in HCC ($P = 0.012$) (**Figure 1B**, **1C**). Next, the expression of SIRT5 in three HCC cell lines and one immortalized liver cell line was tested by RT-PCR and western blot (**Figure 1D**, **1E**). The results showed that SIRT5 was downregulated in the three HCC cell lines compared with THLE-3 cells. BEL-7402 cells, which had the lowest expression level of SIRT5, were used for SIRT5 overexpression experiments, while SNU449 and HepG2 cells, which had relatively high levels of SIRT5, were used for SIRT5 silencing studies.

SIRT5 inhibits HCC cell migration

Given that SIRT5 was downregulated in HCC, we next examined its biological functions in cells. Expression of SIRT5 was knocked down or overexpressed in candidate cells (**Figure 2A**, **2D**). Transwell and wound healing assays showed that silencing SIRT5 increased the migratory ability of SNU449 and HepG2 cells (**Figure 2B**, **2C**). In contrast, ectopic expression of SIRT5 in BEL-7402 cells decreased their


A
Scramble si-SIRT5
SNU449
SIRT5
β-Actin
30
42
HepG2
SIRT5
β-Actin
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B
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si-SIRT5
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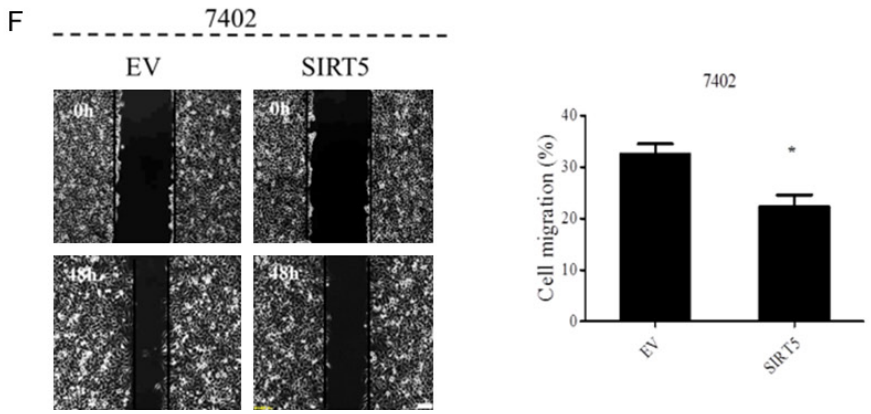


Figure 2. SIRT5 inhibits HCC cell motility. (A) Validation of SIRT5 knockdown in SNU449 and HepG2 cells by immunoblots. Representative pictures of the transwell migration assay in SIRT5-silenced liver cancer SNU449 and HepG2 cells (B) and SIRT5-overexpressing BEL-7402 cells (E). Cell number was determined using five randomly captured fields. Migration was normalized to control. Scale bar, 100 μm. Wound healing assay in SIRT5-silenced SNU449 and HepG2 cells (C) and SIRT5-overexpressing BEL-7402 cells (F). Scale bar, 100 μm. (D) Validation of SIRT5-overexpression in BEL-7402 cells by immunoblots. Data are shown as the mean ± standard deviation. $*P < 0.05$, $**P < 0.01$.

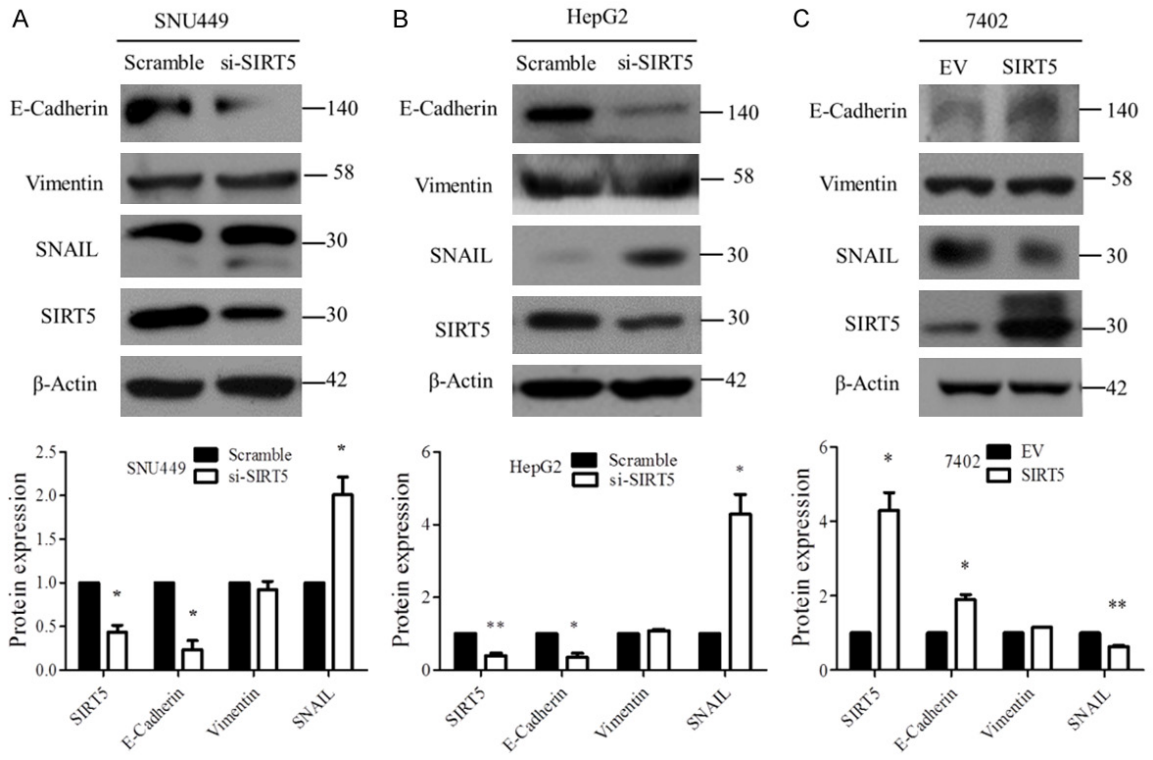


Figure 3. SIRT5 induces partial EMT. A. Upregulated Snail and downregulated E-cadherin and vimentin were observed in SIRT5-silenced SNU449 cells as compared to the control cells by western blot. B. Upregulated Snail, and downregulated E-cadherin and vimentin were observed in SIRT5-silenced HepG2 cells as compared to the control cells by western blot. C. Upregulated E-cadherin and vimentin, and downregulated Snail were observed in SIRT5-overexpressing BEL-7402 cells as compared to the control cells by western blot. *P < 0.05, **P < 0.01.

migratory ability as compared to the cells transfected with empty vector (EV) (**Figure 2E**, **2F**). Therefore, these findings indicated that SIRT5 inhibited HCC migration. Metastasis is known to be closely related to EMT. Therefore, we also examined the expression of EMT-related proteins [22]. As shown in **Figure 3A**, **3B**, silencing SIRT5 decreased the expression of E-cadherin in SNU449 and HepG2 cells, while the expression of SNAIL was increased. Conversely, the expression of E-cadherin was increased, while SNAIL was decreased upon SIRT5 overexpression in BEL-7402 cells (**Figure 3C**). However, the expression of vimentin was not affected by SIRT5 (**Figure 3A-C**). These findings suggest that SIRT5 is involved in the migration of HCC cells.

Vimentin is acetylated at lysine 120 and involved in SIRT5-mediated HCC migration in SNU449 cells

Several proteins have been shown to undergo various functionally important PTMs in SIRT5-KO liver tissue and mouse embryonic fibroblast cells (MEFs). Previous reports suggested that vimentin could be acetylated [18, 23]. Since SIRT5 has deacetylase activity, it was speculated that vimentin acetylation may be regulated by SIRT5. Since SIRT5 deacetylates its downstream proteins, we next sought to explore if vimentin acetylation contributed to SIRT5 function [7]. The localization of SIRT5 and vimentin was detected by immunofluorescence. As shown in **Figure 4A**, **4B**, SIRT5 and vimentin were co-localized in the cytoplasm of SNU449 and BEL-7402 cells. Furthermore, immunoprecipitation was performed to validate this phenomenon. The results show that SIRT5 co-precipitated with vimentin in HCC cells (**Figure 4C**, **4D**). Taken together, these results indicate that SIRT5 interacts with vimentin in HCC cells.

Next, we examined the acetylation level of vimentin in HCC cells. As indicated in **Figure 4E**, acetylated-vimentin levels were substantially increased in SIRT5-silenced cells. Then, we determined the precise residue that was deacetylated by SIRT5, using mass spectrometry to identify the acetylated sites. Vimentin was puri-

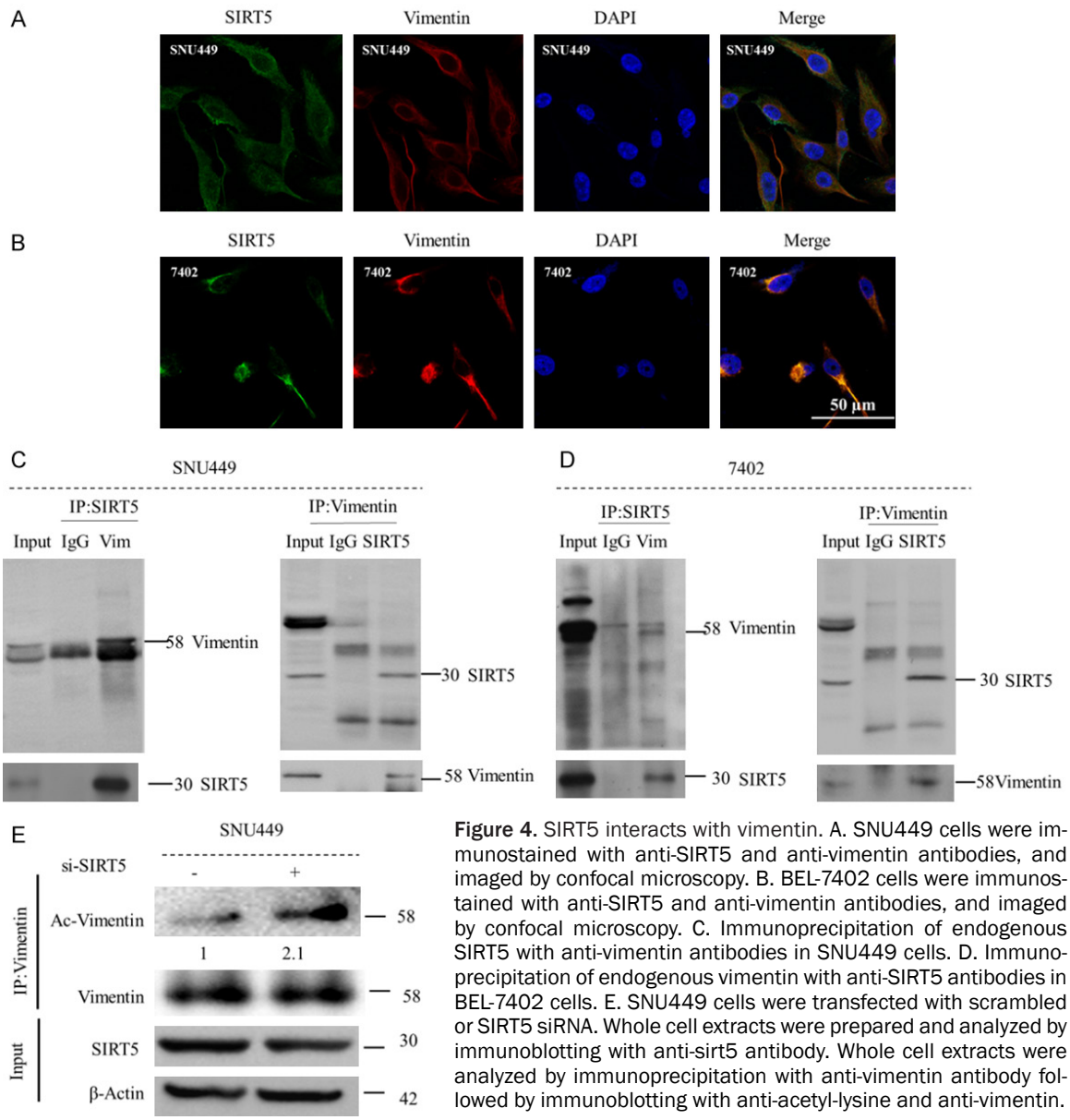
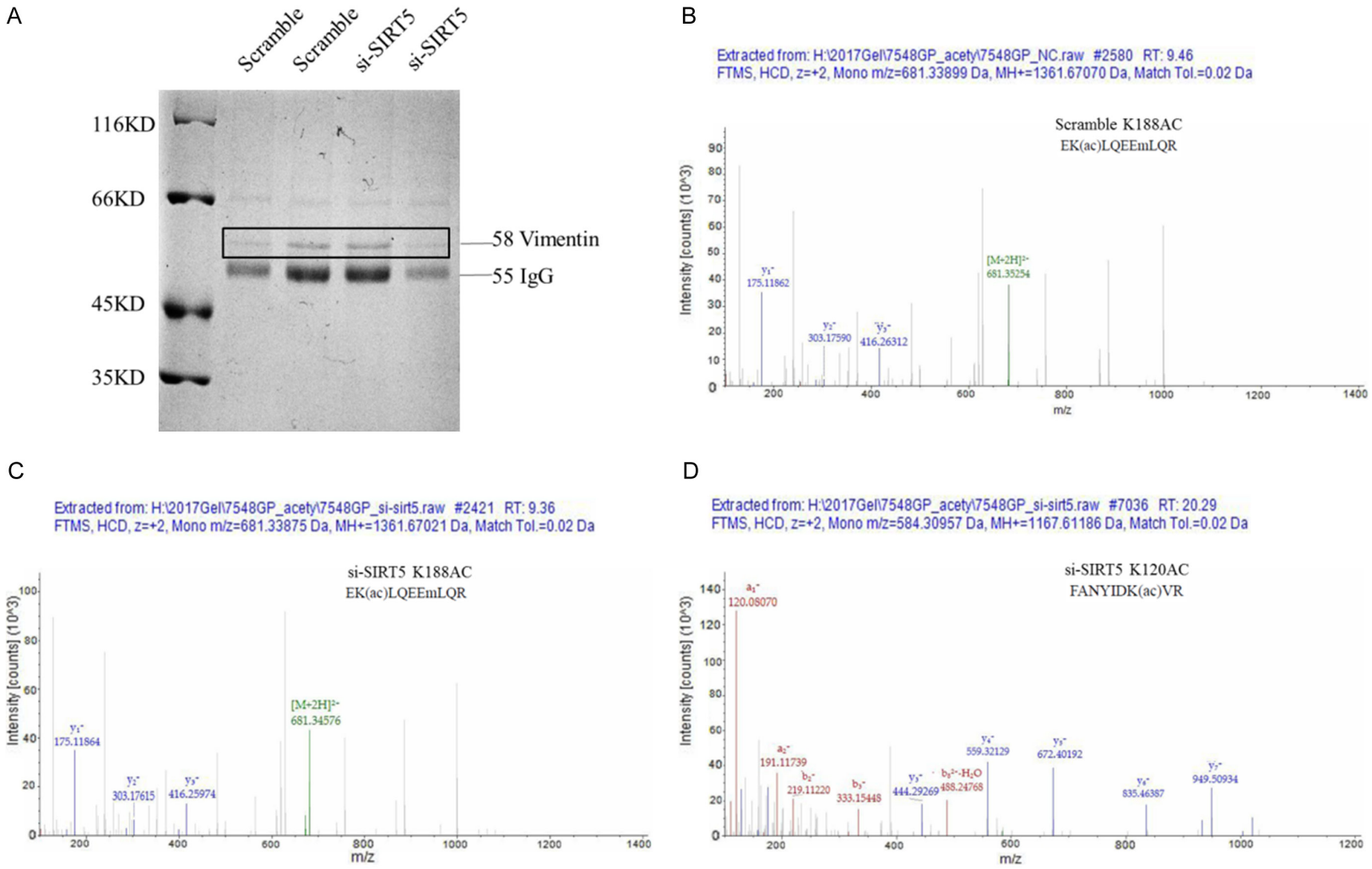


Figure 4. SIRT5 interacts with vimentin. A. SNU449 cells were immunostained with anti-SIRT5 and anti-vimentin antibodies, and imaged by confocal microscopy. B. BEL-7402 cells were immunostained with anti-SIRT5 and anti-vimentin antibodies, and imaged by confocal microscopy. C. Immunoprecipitation of endogenous SIRT5 with anti-vimentin antibodies in SNU449 cells. D. Immunoprecipitation of endogenous vimentin with anti-SIRT5 antibodies in BEL-7402 cells. E. SNU449 cells were transfected with scrambled or SIRT5 siRNA. Whole cell extracts were prepared and analyzed by immunoblotting with anti-sirt5 antibody. Whole cell extracts were analyzed by immunoprecipitation with anti-vimentin antibody followed by immunoblotting with anti-acetyl-lysine and anti-vimentin.

fied from SNU449 cells (**Figure 5A**). Compared to the negative control, the acetylation levels of vimentin at lysine 120 (K120) was increased in SIRT5-silenced SUN449 cells (**Figure 5B-F**). Taken together, these results demonstrate that vimentin may be a substrate for SIRT5, which deacetylates vimentin at K120.

Lysine acetylation functions by generating a site for specific recognition by cellular factors or by neutralizing positive charges. Lysine-to-arginine (K/R) substitution prevents acetylation, but maintains the same positive charge, thus mimicking the non-acetylated form of the original protein. In contrast, lysine-to-glutamine (K/Q) substitution mimics a constitutively acetylated form of the original protein by neutralizing the positive charge [24, 25]. Hence, HA-tagged vimentin (K120R) and HA-tagged vimentin (K120Q) were generated. The results revealed that both wild-type and K120Q vimentin transfection individually promoted cell migration, but K120R vimentin decreased cell migration (**Figure 6A**). To further determine if SIRT5 was involved in the invasion of HCC, a Flag-tagged SIRT5 mutant (H158Y) was generated. Ectopically expressed wild-type SIRT5, but not catalytically impaired mutant SIRT5 (H158Y), was able to revert the enhanced migration of resuting from etopic overexpression of wild-type vimentin in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FTMS, HCD, z=+2, Mono m/z=681.33899 Da, MH+=1361.67070 Da, Match Tol.=0.02 Da
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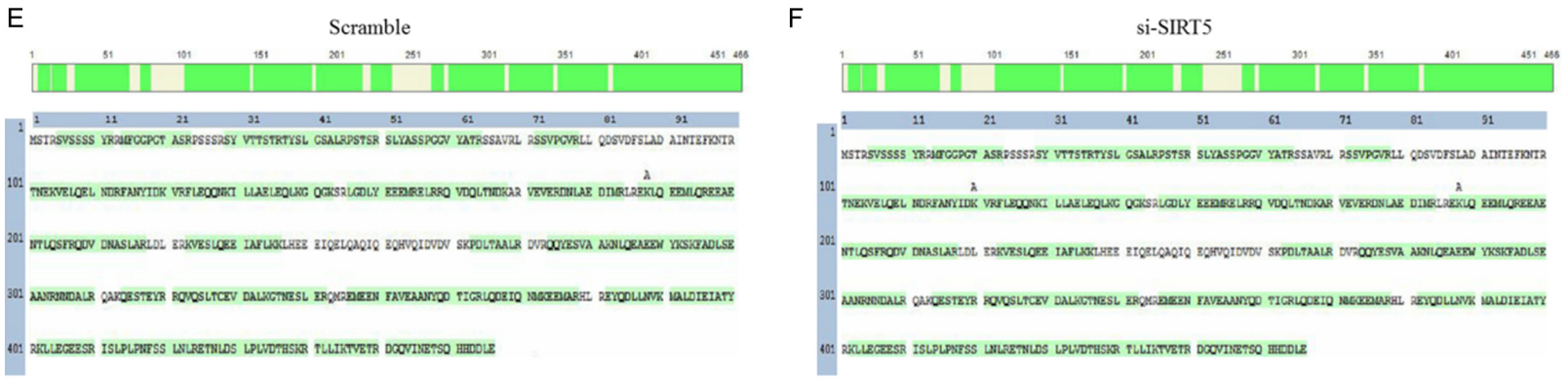

Figure 5. SIRT5 deacetylates vimentin at Lys120. (A) Proteins were immunoprecipitated with anti-vimentin antibody from total lysates of SNU449 cells transfected with scrambled or SIRT5 siRNA. Then, the proteins were fractionated by 10% SDS-PAGE and stained with Coomassie brilliant blue. (B-D) The MS/MS spectra showing the identification of K120ac in vimentin. The b and y ions indicate peptide backbone fragment ions, which contain the N and C termini, respectively. (E, F) Acetylated lysine residues of vimentin identified from MS/MS data are denoted by the letter (A).

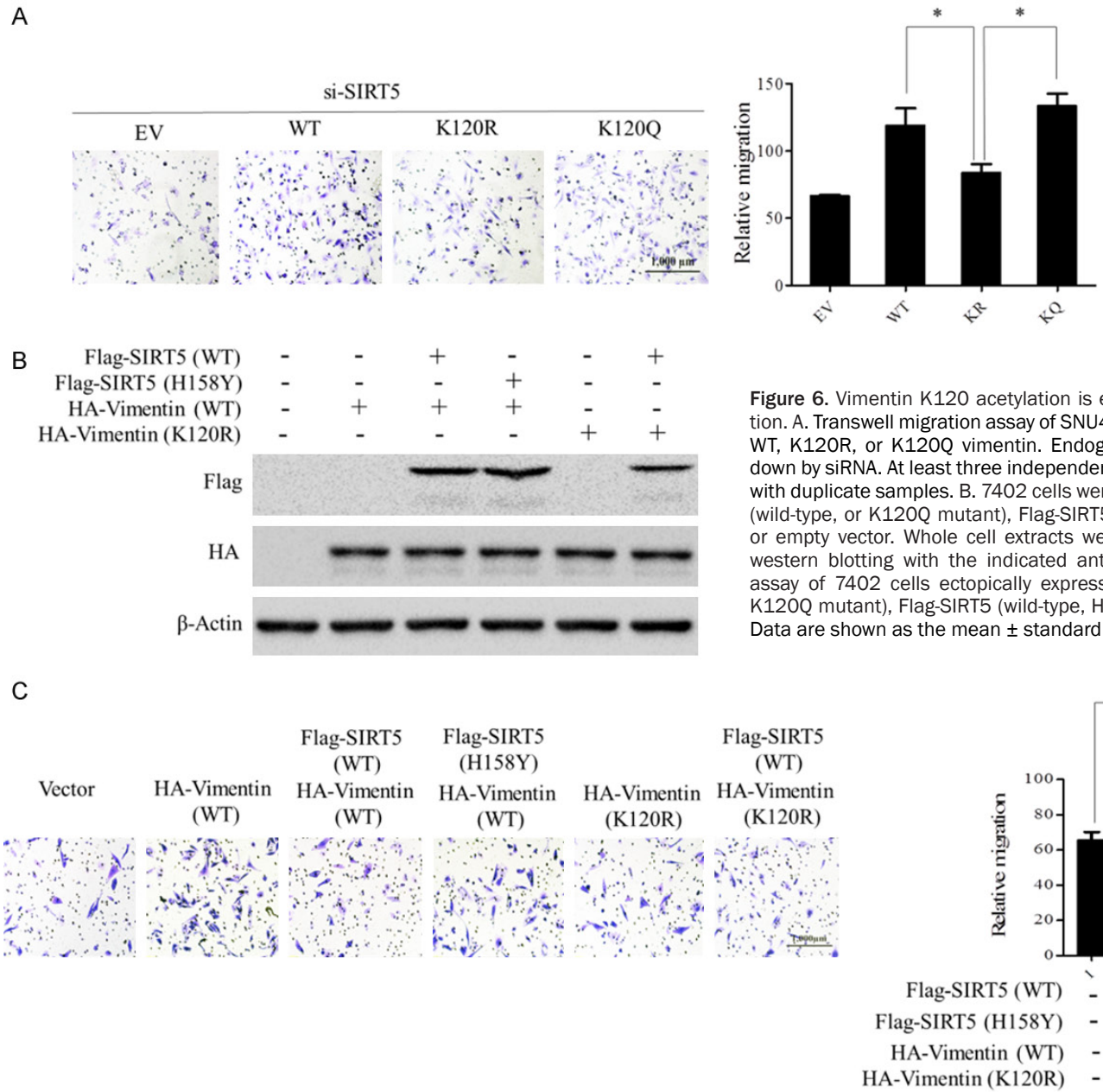

Figure 6. Vimentin K120 acetylation is essential for cancer cell migration. A. Transwell migration assay of SNU449 cells ectopically expressing WT, K120R, or K120Q vimentin. Endogenous vimentin was knocked-down by siRNA. At least three independent experiments were performed with duplicate samples. B. 7402 cells were transfected with HA-vimentin (wild-type, or K120Q mutant), Flag-SIRT5 (wild-type, or H158Y mutant) or empty vector. Whole cell extracts were prepared and subjected to western blotting with the indicated antibodies. C. Transwell invasion assay of 7402 cells ectopically expressing HA-vimentin (wild-type, or K120Q mutant), Flag-SIRT5 (wild-type, H158Y mutant) or empty vector. Data are shown as the mean ± standard deviation. *$P < 0.05$.

7402 cells. Similarly, ectopically expressed wild-type SIRT5 had no effect on the migration of ectopically expressing vimentin-K120R 7402 cells (**Figure 6B**, **6C**). These data suggest that vimentin K120 acetylation is likely responsible for the increase in HCC cell migration following SIRT5 knockdown.

Discussion

This study showed that SIRT5 expression was decreased in HCC tumor tissues as compared to NT hepatic tissues, and acetylation of vimentin at K120 may be the key modulator for this process. This is the first report on vimentin as a candidate protein for SIRT5 in HCC.

EMT is thought to be an important mechanism contributing to the migration and metastasis of epithelial-derived cancers [26, 27]. In the present study, SIRT5 is shown to suppress EMT in HCC, by inducing Snail downregulation and E-cadherin upregulation. Furthermore, the expression of SIRT5 is low in HCC cells, which was consistent with a previous study [28]. However, the role of SIRT5 in cancer cells remains controversial. For instance, SIRT5 plays a tumor suppressive role in gastric cancer [29], but has been found to be an oncogene in other cancer types [30-33]. This study showed that SIRT5 expression is decreased in HCC tumor tissues, as compared to adjacent hepatic tissues, and acetylation of vimentin at K120 may play a key role in the effects of SIRT5. This is the first report on vimentin as a candidate protein for SIRT5.

In the present study, a direct physical interaction was demonstrated between SIRT5 and vimentin. PMTs of vimentin have been shown to play a role in various cancer cells, but the impact of SIRT5 on vimentin remains unknown. Our LC/MS/MS analysis revealed that vimentin acetylated at K120. Overexpression of the vimentin (K120R) mutant in SNU449 cells reduces migration. This finding is consistent with previous research that links the acetylation of cytoskeletal proteins to cancer cell migration [34]. Additionally, this is the first report to show that vimentin is a downstream target of SIRT5, and its acetylation plays vital roles in HCC migration. However, further studies are needed to validate the role of vimentin acetylation in other cancer types.

In summary, this study suggests that SIRT5 is downregulated in HCC. Vimentin deacetylation at K120 by SIRT5 is involved in HCC metastasis. Further investigations are warranted to explore the interaction between SIRT5 and vimentin in other cancer cells.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

SIRT5, Sirtuin 5; DAPI, 4',6-Diamidino-2-phenylindole; EMT, Epithelial-mesenchymal transition; HCC, Hepatocellular carcinoma; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; TCGA, The Cancer Genome Atlas; LIHC, Liver hepatocellular carcinoma; IF, Intermediate filament; PTM, Post-translational modification; KD, Knockdown; siRNA, Small interfering RNA; EV, Empty vector.

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References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[2] El-Serag HB and Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007; 132: 2557-2576.

[3] Thomas MB, Jaffe D, Choti MM, Belghiti J, Curley S, Fong Y, Gores G, Kerlan R, Merle P, O'Neil B, Poon R, Schwartz L, Tepper J, Yao F, Haller D, Mooney M and Venook A. Hepatocellular carcinoma: consensus recommendations of the National Cancer Institute Clinical Trials

Planning Meeting. J Clin Oncol 2010; 28: 3994-4005.
[4] Haigis MC and Guarente LP. Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction. Genes Dev 2006; 20: 2913-2921.
[5] Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, Kim J, Woo J, Kim JH, Choi BH, He B, Chen W, Zhang S, Cerione RA, Auwerx J, Hao Q and Lin H. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. Science 2011; 334: 806-809.
[6] Nishida Y, Rardin MJ, Carrico C, He W, Sahu AK, Gut P, Najjar R, Fitch M, Hellerstein M, Gibson BW and Verdin E. SIRT5 regulates both cytosolic and mitochondrial protein malonylation with glycolysis as a major target. Mol Cell 2015; 59: 321-332.
[7] Park J, Chen Y, Tishkoff DX, Peng C, Tan M, Dai L, Xie Z, Zhang Y, Zwaans BM, Skinner ME, Lombard DB and Zhao Y. SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. Mol Cell 2013; 50: 919-930.
[8] Nakagawa T, Lomb DJ, Haigis MC and Guarente L. SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. Cell 2009; 137: 560-570.
[9] Wang Y, Zhu Y, Xing S, Ma P and Lin D. SIRT5 prevents cigarette smoke extract-induced apoptosis in lung epithelial cells via deacetylation of FOXO3. Cell Stress Chaperones 2015; 20: 805-810.
[10] Qin K, Han C, Zhang H, Li T, Li N and Cao X. NAD(+) dependent deacetylase Sirtuin 5 rescues the innate inflammatory response of endotoxin tolerant macrophages by promoting acetylation of p65. J Autoimmun 2017; 81: 120-129.
[11] Lu W, Zuo Y, Feng Y and Zhang M. SIRT5 facilitates cancer cell growth and drug resistance in non-small cell lung cancer. Tumour Biol 2014; 35: 10699-10705.
[12] Lai CC, Lin PM, Lin SF, Hsu CH, Lin HC, Hu ML, Hsu CM and Yang MY. Altered expression of SIRT gene family in head and neck squamous cell carcinoma. Tumour Biol 2013; 34: 1847-1854.
[13] Leduc C and Etienne-Manneville S. Intermediate filaments in cell migration and invasion: the unusual suspects. Curr Opin Cell Biol 2015; 32: 102-112.
[14] Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, Tiron C, Lorens JB and Ivaska J. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene 2011; 30: 1436-1448.
[15] Satelli A, Batth I, Brownlee Z, Mitra A, Zhou S, Noh H, Rojas CR, Li H, Meng QH and Li S. EMT circulating tumor cells detected by cell-surface vimentin are associated with prostate cancer progression. Oncotarget 2017; 8: 49329-49337.
[16] Meng J, Chen S, Han JX, Qian BX, Wang XR, Zhong WL, Qin Y, Zhang H, Gao WF, Lei YY, Yang W, Yang L, Zhang C, Liu HJ, Liu YR, Zhou HG, Sun T and Yang C. Twist1 regulates vimentin through Cul2 circular RNA to promote EMT in hepatocellular carcinoma. Cancer Res 2018; 78: 4150-4162.
[17] Song X, Wang J, Zheng T, Song R, Liang Y, Bhatta N, Yin D, Pan S, Liu J, Jiang H and Liu L. LBH589 inhibits proliferation and metastasis of hepatocellular carcinoma via inhibition of gankyrin/STAT3/Akt pathway. Mol Cancer 2013; 12: 114.
[18] Snider NT and Omary MB. Post-translational modifications of intermediate filament proteins: mechanisms and functions. Nat Rev Mol Cell Biol 2014; 15: 163-177.
[19] Zhu QS, Rosenblatt K, Huang KL, Lahat G, Brobey R, Bolshakov S, Nguyen T, Ding Z, Belousov R, Bill K, Luo X, Lazar A, Dicker A, Mills GB, Hung MC and Lev D. Vimentin is a novel AKT1 target mediating motility and invasion. Oncogene 2011; 30: 457-470.
[20] Wang L, Zhang J, Banerjee S, Barnes L, Barnes L, Sajja V, Liu Y, Guo B, Du Y, Agarwal MK, Wald DN, Wang Q and Yang J. Sumoylation of vimentin354 is associated with PIAS3 inhibition of glioma cell migration. Oncotarget 2010; 1: 620-627.
[21] Pullar CE, Chen J and Isseroff RR. PP2A activation by beta2-adrenergic receptor agonists: novel regulatory mechanism of keratinocyte migration. J Biol Chem 2003; 278: 22555-22562.
[22] Kalluri R and Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009; 119: 1420-1428.
[23] Kim SC, Sprung R, Chen Y, Xu Y, Ball H, Pei J, Cheng T, Kho Y, Xiao H, Xiao L, Grishin NV, White M, Yang XJ and Zhao Y. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol Cell 2006; 23: 607-618.
[24] Megee PC, Morgan BA, Mittman BA and Smith MM. Genetic analysis of histone H4: essential role of lysines subject to reversible acetylation. Science 1990; 247: 841-845.
[25] Ren Q and Gorovsky MA. Histone H2A.Z acetylation modulates an essential charge patch. Mol Cell 2001; 7: 1329-1335.
[26] Li LP, Lu CH, Chen ZP, Ge F, Wang T, Wang W, Xiao CL, Yin XF, Liu L, He JX and He QY. Subcellular proteomics revealed the epithelial-mesenchymal transition phenotype in lung cancer. Proteomics 2011; 11: 429-439.

[27] Soltermann A, Tischler V, Arbogast S, Braun J, Probst-Hensch N, Weder W, Moch H and Kristiansen G. Prognostic significance of epithelial-mesenchymal and mesenchymal-epithelial transition protein expression in non-small cell lung cancer. Clin Cancer Res 2008; 14: 7430-7437.

[28] Chen XF, Tian MX, Sun RQ, Zhang ML, Zhou LS, Jin L, Chen LL, Zhou WJ, Duan KL, Chen YJ, Gao C, Cheng ZL, Wang F, Zhang JY, Sun YP, Yu HX, Zhao YZ, Yang Y, Liu WR, Shi YH, Xiong Y, Guan KL and Ye D. SIRT5 inhibits peroxisomal ACOX1 to prevent oxidative damage and is downregulated in liver cancer. EMBO Rep 2018; 19.

[29] Tang Z, Li L, Tang Y, Xie D, Wu K, Wei W and Xiao Q. CDK2 positively regulates aerobic glycolysis by suppressing SIRT5 in gastric cancer. Cancer Sci 2018; 109: 2590-2598.

[30] Du Z, Liu X, Chen T, Gao W, Wu Z, Hu Z, Wei D, Gao C and Li Q. Targeting a Sirt5-positive subpopulation overcomes multidrug resistance in wild-type kras colorectal carcinomas. Cell Rep 2018; 22: 2677-2689.

[31] Wang YQ, Wang HL, Xu J, Tan J, Fu LN, Wang JL, Zou TH, Sun DF, Gao QY, Chen YX and Fang JY. Sirtuin5 contributes to colorectal carcinogenesis by enhancing glutaminolysis in a deglutarylation-dependent manner. Nat Commun 2018; 9: 545.

[32] Yang X, Wang Z, Li X, Liu B, Liu M, Liu L, Chen S, Ren M, Wang Y, Yu M, Wang B, Zou J, Zhu WG, Yin Y, Gu W and Luo J. SHMT2 desuccinylation by sirt5 drives cancer cell proliferation. Cancer Res 2018; 78: 372-386.

[33] Xiangyun Y, Xiaomin N, Linping G, Yunhua X, Ziming L, Yongfeng Y, Zhiwei C and Shun L. Desuccinylation of pyruvate kinase M2 by SIRT5 contributes to antioxidant response and tumor growth. Oncotarget 2017; 8: 6984-6993.

[34] Boggs AE, Vitolo MI, Whipple RA, Charpentier MS, Goloubeva OG, Ioffe OB, Tuttle KC, Slovic J, Lu Y, Mills GB and Martin SS. alpha-Tubulin acetylation elevated in metastatic and basal-like breast cancer cells promotes microtentacle formation, adhesion, and invasive migration. Cancer Res 2015; 75: 203-215.