SEPT2 Protein Crotonylation Promotes Metastasis and Recurrence Through AKT Pathway in Hepatocellular Carcinoma and is Associated with Poor Survival

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Research Article

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Abstract

Background

Hepatocellular carcinoma (HCC) metastasis and recurrence lead to therapy failure. Posttranslational modification (PTM) mechanisms in HCC, especially those of recently discovered PTMs, such as lysine crotonylation (Kcr), are still poorly understood.

Methods

Kcr protein levels in HCC cell lines of MHCC-97H (highly invasive) cells and MHCC-97L (minimally invasive) were detected through stable isotope labeling by amino acids in cell culture (SILAC) and liquid chromatography with tandem mass spectrometry (LC–MS/MS). The differentially crotonylated proteins in these two cell lines were identified through bioinformatics analysis. Protein Kcr was confirmed by immunoprecipitation (IP) and western blotting (WB). The function of Septin2 (SEPT2) crotonylated at K74 (SEPT2-K74cr) in metastasis was examined *in vivo* and *in vitro*. The clinical significance of SEPT2-K74cr in HCC patients was determined by immunohistochemistry (IHC) assay.

Results

The crotonylation rate was higher in highly invasive cells, and higher crotonylation level in HCC cells facilitated cell migration and invasion. The crotonylated protein SEPT2 was significantly hypercrotonylated in highly invasive cells, and the de-crotonylated mutation of K74 in SEPT2 impaired SEPT2 GTPase activity and inhibited HCC metastasis *in vitro* and *in vivo*. Mechanistically, SIRT2 decrotonylated SEPT2, and P85α was found to be the downstream effector of SEPT2. SEPT2-K74R facilitated P85α degradation, suppressing PI3K pathway and inhibiting the epithelial–mesenchymal transition. SEPT2-K74cr was correlated with poor prognosis and recurrence in HCC patients.

Conclusions

We revealed the role of nonhistone protein crotonylation in regulating HCC metastasis and invasion. Crotonylation facilitated cell invasion through the crotonylated SEPT2-K74 (cr)-P85α-AKT pathway. High SEPT2-K74 crotonylation predicted poor prognosis and a high recurrence rate in HCC patients.

Introduction

Hepatocellular carcinoma (HCC), comprising 75%-85% of liver cancer cases, was the sixth most commonly diagnosed cancer and the third leading cause of cancer-related death worldwide in 2020[1]. The five-year survival rate of patients with HCC is less than 20% worldwide[2]. Metastasis and recurrence are major obstacles to improving the long-term survival of HCC patients. Surgery is currently the most

effective treatment[3], but the recurrence rate after surgery remains high, and the five-year recurrence rate is higher than 70% after resection[4]. Therefore, research on HCC metastasis is important.

The complexity of biological functions depends not only on the genome but also on the proteome, and the increase in complexity from the level of the genome to the proteome is compounded by protein posttranslational modifications (PTMs). Although many studies of HCC metastasis and recurrence there have been performed at the genome level, studies on PTMs, especially studies on recently discovered acylation modifications, have been rare. Lysine (K) crotonylation (Kcr), which is similar to acetylation (Kac), is widespread throughout a cell, and both Kac and Kcr sites overlap on histones[5]. However, Kcr involves one more carbon-carbon double bond than Kac. A crotonylated lysine residue is larger and more hydrophobic than an acetylated lysine residue because of the extended crotonyl hydrocarbon chain. Moreover, a C-C π -bond in Kcr results in a rigid and planar protein configuration, which makes it unique among acyl modifications[6]. Functionally, histone Kcr stimulates transcription to a greater degree than Kac[7]. In 2017, Kcr was found in numerous nonhistone proteins and is thought to play roles in multiple cellular functions.[8, 9] Recent studies have shown that crotonylation of nonhistone proteins ensures accurate spindle positioning in mitosis[10] and is related to aging[11] and colorectal cancer progression[12]. However, our understanding of Kcr is far from sufficient.

To explore the role played by this unique modification in HCC, we performed stable isotope labeling by amino acids in cell culture (SILAC) and liquid chromatography tandem mass spectrometry (LC-MS/MS) in highly (MHCC-97H) and minimally (MHCC-97L) invasive HCC cell lines. We found that crotonylation was positively correlated with cell migration and invasion. Septin2 (SEPT2), a member of the septin family of GTPases, was hypercrotonylated in MHCC-97H cells. High levels of SEPT2 crotonylation led to poor prognosis and a high recurrence rate in HCC patients.

Methods And Materials

Patients and tissues

Tissue microarrays (HLivH090Su01, HLivH060CD03) were purchased from Outdo Biotech (China). Thirty-six HCC tissue samples were obtained from the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. The use of these clinical samples was approved by the Institutional Review Board of The First Affiliated Hospital of Sun Yat-sen University. Written informed consent was obtained from each patient.

Cell cultures

The MHCC97L, MHCC97H, Huh7 and HEK293T human cell lines were grown in DMEM (GIBCO, USA). SNU449 and SMMC7721 cell lines were grown in RPMI 1640 medium (GIBCO, USA). All media were supplemented with 10% fetal bovine serum (FBS, GIBCO, USA). The HEK293F cell line was grown in Union 293 medium (Union-biotech, China). The cell lines were free from mycoplasma infection and authenticated by short tandem repeat (STR) assay.

RNA isolation, reverse transcription and qRT-PCR

Total RNA was extracted by TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. Reverse transcription was performed with the PrimeScript RT reagent kit (TaKaRa, Japan). The cDNA samples were diluted 1:5 for gene expression analysis by qRT-PCR and gene amplification by PCR. qRT-PCR was carried out with TB Green™ Premix Ex Taq™ II (TaKaRa, Japan) in a LightCycler 480 real-time PCR system (Roche, USA). Each sample was analyzed three times, and relative mRNA expression was calculated using GAPDH as the internal control.

Plasmids and lentivirus infection

Flag-tagged full-length human wild-type (WT) SEPT2, K74R and K318R SEPT2 mutants and full-length SIRT2 were cloned into a retroviral (pCDH), PcDNA 3.1 + and pGEX-4t-1 vector. SIRT1, SIRT3, and HDAC3 were cloned into a PcDNA 3.1 + vector. Full-length human PIK3R1 (P85α) was cloned into a pCDH vector. Short interfering RNAs (shRNAs) against PIK3R1 were purchased from GENE, China. Transient plasmid transfection was performed using Lipofectamine 3000 according to the manufacturer's instructions (Invitrogen) and DNA transfection reagent (NEOFECT). The medium was changed 24 hours after transfection, and the cells were collected 48 hours after transfection.

To produce lentivirus, HEK293T cells were seeded into 6 cm cell culture plates before transfection. Then, 15 µl of P3000 was diluted in 250 µl of Opti-MEM (GIBCO) with 6 µg of pCDH plasmid, 4 µg of psPAX2 and 2 µg of pMD2.G, and then added to 250 µl of Opti-MEM (with 15 µl of Lipo3000), which was added to the culture plate wells. After 48 hours, the cell supernatant was collected and filtered with a 0.45 µm low protein-binding membrane (Millipore Steriflip). Cells grown to 80% confluence were transfected with the lentivirus in the presence of 8 µg/ml polybrene (Solarbio, China). Puromycin (Solarbio, China) was used to select stably transfected cells.

Western blot analysis and immunoprecipitation (IP)

Total protein was extracted with RIPA lysis buffer (CST, USA) containing protease inhibitors and phosphatase inhibitors (Sigma, Germany). The protein concentration was measured with a Pierce™ BCA Protein Assay Kit (Thermo, USA). Protein samples (15 µg) were separated on a Bis/Tris-polyacrylamide gel (Epizyme, China) by electrophoresis and then blotted onto PVDF membranes (Merck Millipore, Germany). The blots were incubated with the primary antibodies overnight at 4°C and with secondary antibodies (CST, USA) for 1 hour at room temperature. An Omni-ECL™ Femto Light chemiluminescence Kit (Epizyme, China) was used to visualize the proteins. For IP, IP lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% Nonidet P40 [CWBIO, China], 5 mM EDTA, proteinase inhibitor cocktail [CWBIO, China] and PMSF [CWBIO, China], pH = 7.4) was used to extract total protein. The cell lysate was incubated with appropriate antibodies and protein-A/G beads (CWBIO, China) for 12 hours. The immunoprecipitates were washed five times with cold IP lysis buffer and eluted with SDS loading buffer after boiling for 10 min subjected to WB.

Eukaryotic expression and purification of recombinant SEPT2

PcDNA3.1-SEPT2 WT and PcDNA3.1-SEPT2 K74R vectors were transferred into the HEK293F cell line by PEI (Proteintech, USA). An anti-FLAG-tag affinity purification kit (DIA-AN, China) was used to purify recombinant Flag-SEPT2. In brief, anti-Flag affinity gel was added to HEK293F cell lysate (lysed with 0.3% Nonidet P40 IP lysis buffer), mixed at 4°C overnight, and then, the protein was harvested through centrifugation and washed five times with 0.5% Nonidet P40 IP lysis buffer. Recombinant SEPT2 was eluted with arginine-HCl (pH = 3.0) buffer, and the pH was adjusted with 1 M Tris HCl buffer (pH = 8.0). Recombinant SEPT2 was concentrated in an Amicon® Ultra4 tube (Merck Millipore, Germany).

Phosphate determination with malachite green

Phosphate level was determined performed as previously reported[28]. In brief, 5 µl (50 µg) of recombinant protein and 10 µl of 4 mM GTP were added in 25 µl of assay buffer and incubated at room temperature for 15 min. Then, 80 µl of color reagent was added to this mixture, which incubated for 2 min, and finally, 10 µl of citrate solution was added to each tube, which was incubated for 5 min. Absorbance was read at A650 nm. Solutions: assay buffer (40 mM Tris, 80 mM NaCl, 8 mM MgAC₂, 1 mM EDTA, pH = 7.5); A. Malachite green solution (0.05%); B. Ammonium molybdate (4.2%) in HCl; C. Citrate solution (34% of Na₃Citrate•2H₂O in di-H₂O); D. 2% Nonidet P40 in di-H₂O; E. Color reagent: 1 volume of B mixed with 3 volumes of A to which 0.1 ml of solution "D" in 5 ml was added.

GST pull-down assay

The pGEX-4t-1, pGEX-4t-1-SEPT2 WT and PGEX-4T-1-SEPT2K74R, GST-SIRT2 plasmids were transformed into *E. coli* BL21. Protein prokaryotic expression was induced by 1 mM isopropyl-β-D-thiogalactoside (CW BIO, China) at 37°C for 4 hours. Protein was then purified using a GST-tagged protein purification kit (Beyotime, China). Purified proteins were mixed with anti-GST antibody-conjugated beads and cell lysate (lysed with 0.3% Nonidet P40 IP lysis buffer) or recombinant Flag-SEPT2 at 4°C for 12 hours. The beads were subsequently harvested through centrifugation and washed five times with 0.5% Nonidet P40 IP lysis buffer before boiling with SDS-loading buffer and subjected to WB.

In vitro migration and invasion assays

A 24-well plate containing chamber inserts with 8 mm pores (Corning, USA) was utilized to evaluate the migration and invasion of HCC cells. A total of 1×10^5 cells were positioned in the upper chamber precoated with 10% Matrigel (BD Biosciences, USA) (invasion assay) or without with Matrigel (migration assay). In each lower chamber, 750 µL of culture medium supplemented with 10% FBS was added. Cells were incubated at 37°C and allowed to migrate for 8 hours or invade for 24 hours. After incubation, the cells that had migrated or invaded through the pores were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Then, the cells were photographed and counted.

Animal experiments

Male BALB/c-nu mice aged 4 or 5 weeks (19–21 g) were purchased from GemPharmatech Co., Ltd. (Jiangshu, China). To establish the tail vein injection model, Huh7 cells (3×10^6) in 200 μ l PBS were injected into the mouse tail vein. To establish the intrasplenic injection model, 2×10^6 Huh7 cells were injected into the spleen, and splenectomy was performed. Eight weeks after the injection, tumor-bearing mice were sacrificed, and the main organs were removed from the animals. For the orthotopic liver tumor implantation mouse model, 8×10^5 Huh7 cells were injected into the left lobe of the liver, and 10 weeks later, the mice were sacrificed. Tissues were fixed in 10% neutral-buffered formaldehyde for 24 hours at room temperature and embedded in paraffin. Took 4 sections in every 200 μ m. The sections were sliced (5 μ m thick) following the standard histological method, and the slices were stained with hematoxylin and eosin (H&E). All experimental procedures were approved by the Animal Ethics Committee of Sun Yat-sen University, Guangzhou, China (no. 2020000768).

H&E and IHC staining

For H&E staining, paraffin sections were dewaxed in xylene, rehydrated using sequentially decreasing concentrations of ethanol and subsequently washed in PBS and stained with H&E. After staining, the sections were dehydrated in increasing concentrations of ethanol and xylene. For IHC staining, slices were subjected to antigen retrieval by heat treatment for 2.5 min after rehydration. Then, the slides were blocked in 20% goat serum for 30 min and incubated with primary antibodies overnight at 4°C in a dark room. Peroxidase bound to the antibody complex was labeled with a 3,3'-diaminobenzidine chromogen substrate solution provided with a Real™ Envision™ Detection System (DAKO).

Statistical analysis

The results are expressed as the means $\pm$ standard deviation (SD). Statistical analysis and plotting were performed using the SPSS statistical software package (standard version 18.0, IBM, USA) and GraphPad Prism 7.0 software (GraphPad Software, USA). In vitro experiments were repeated at least three times to achieve adequate power for detecting significant changes. One-way ANOVA, Student's t test and two-way ANOVA were performed to compare the variations in groups in the functional assay. A value of $p < 0.05$ was regarded as statistically significant.

Results

The crotonylome was highly expressed in highly invasive HCC cell lines

To determine the role of crotonylation in tumor metastasis, the MHCC-97L (minimally invasive) and MHCC-97H (highly invasive) HCC cell lines, which were isolated from one patient and showed differential invasive potential (Fig. 1A)[13], were studied with the SILAC technique. Differentially crotonylated proteins (**Table S1**) were identified by LC–MS/MS (Fig. 1B), including 1085 crotonylated proteins and 3540 crotonylation sites. An analysis of the subcellular distribution of crotonylated proteins showed that 34.2% of the crotonylated proteins were located in the cytoplasm, 24.2% of the crotonylated proteins in the nucleus, 8.7% of the crotonylated proteins in the cytoskeleton and 7.6% of the crotonylated proteins in

mitochondria (Fig. 1C). Crotonylated proteins were enriched in several metabolic pathways, such as adipogenesis and fatty acid metabolism, oxidative phosphorylation, peroxisome and xenobiotic metabolism pathways (Fig. 1D).

We next analyzed all protein sequences in modified 21-mers constituted with amino acids in specific positions (10 amino acids upstream and downstream of one site) (**Figure S1A**). A motif-X analysis showed that EKE, KE, and EKxE were overrepresented Kcr hotspot sites (**Figure S1B**). Further analysis of differences in the MHCC-97L and MHCC-97H cell proteomes showed that the degree of protein expression and crotonylation was generally increased in MHCC-97H cells, and the increase in crotonylation was more obvious than that in protein expression (Fig. 1E). When compared with MHCC-97L cells, we identified 334 upregulated and 205 downregulated proteins (fold change [FC] > 1.5, $p < 0.05$) (Fig. 1F) and 199 hypercrotonylated and 46 hypocrotonylated sites in the MHCC-97H cells ($FC > 1.5$, $p < 0.05$) (Fig. 1G). In addition, the total crotonylation level was higher in the MHCC-97H cells than in MHCC-97L cells ($p = 0.03$) (**Figure S1C**). The findings were further confirmed by Western blot (Fig. 1H).

Crotonylation was positively correlated with HCC cell migration and invasion

To further investigate the association between crotonylation and metastasis, we analyzed differentially crotonylated proteins in these two HCC cell lines. As shown in Fig. 2A, highly invasive cells had more hypercrotonylated proteins. Gene set enrichment analysis of the differentially crotonylated proteins showed that, more crotonylated proteins were enriched in biological oxidation and metabolism of xenobiotics by cytochrome P450 processes and fewer were enriched in cell junction organization and focal adhesion processes in MHCC-97H cells compared with those in MHCC-97L cells (**Figure S2A and S2B**). Gene Ontology (GO) annotation of the hypercrotonylated proteins in the highly invasive cells revealed that proteins were involved in diverse biological processes, such as the extracellular matrix and cell adhesion molecule binding (Fig. 2B). In addition, we found higher degrees of crotonylation on known metastasis-related proteins in the MHCC-97H cells (**Figure S2C**).

For verification, we increased the overall level of crotonylation in SUN449, Huh7 and SMCC7721 cell lines by adding crotonylation substrate (NaCr, sodium crotonate). An anti-pan-crotonyl-lysine (pan-Kcr) antibody was used to detect the total crotonylation level in cells after NaCr treatment. As shown in **Figure S3A**, the total crotonylation reached a maximum after NaCr (25 mM) treatment. We observed that SUN449, Huh7 and SMCC7721 cells treated with NaCr exhibited an elongated spindle shape (Fig. 2C, **Figure S4A**). In addition, increasing the crotonylation level had a positive effect on the migration and invasion of SUN449, Huh7 and SMCC7721 cells (Fig. 2D and 2E; **Figure S4B, C**). Furthermore, increasing the concentration of NaCr decreased the expression of epithelial markers and enhanced the expression of mesenchymal markers in HCC cells (Fig. 2F, **Figure S4D**). These results indicated that increasing crotonylation exerted a promoting effect on HCC cell migration and invasion.

SEPT2 was identified in the crotonylome

To explore the potential mechanism by which crotonylation facilitates cell migration and invasion in HCC, we further analyzed the proteome. After normalization of protein levels, we selected the 3 most significantly differentially crotonylated protein candidates obtained by comparing MHCC-97H with MHCC-97L Kcr levels, SEPT2, RAB35, and GRB2, and used them in further experiments (Fig. 3A, **Figure S5A**). The results showed that these three candidates were hypercrotonylated in MHCC-97L cells after NaCr treatment (Fig. 3B). Furthermore, analysis of the endogenous crotonylation of these three proteins in MHCC-97H and MHCC-97L cells revealed that the crotonylation level was higher in MHCC-97H cells; these results were similar to those of the SILAC analysis (Fig. 3C).

We chose SEPT2, which was crotonylated to the highest level in MHCC-97H cells, as our main study object. Two crotonylation sites, K318 (MHCC-97H/MHCC-97L = 1.3, $p < 0.05$) and K74 (MHCC-97H/MHCC-97L = 2.19, $p < 0.05$), in SEPT2 were significantly differentially crotonylated, as determined by LS-MS/MS. SEPT2 is crucial in spindle formation and sister chromatid separation, thereby regulating the G2/M phase transition and cell mitosis[14]. Several studies have shown that SEPT2 can facilitate tumor growth and metastasis[15, 16]. We analyzed the protein domains in SEPT2, and the results showed that K74 is in the GTPase domain and that the K318 site is in the variable domain (Fig. 3D). An analysis of protein sequence alignment of SEPT2 homologs in different species revealed that K74 was more evolutionarily conserved than K318 (Fig. 3E, **Figure S5B**). In addition, when lysine residue at the 74 site was replaced with an alanine, SEPT2 crotonylation was significantly reduced, largely abrogating SEPT2 crotonylation, whereas crotonylation of the K318R mutant was slightly reduced (Fig. 3F). These results indicated that K74cr may play a functional role in SEPT2. Then, we purified ectopically expressed SEPT2 WT and SEPT2-K74R in HEK293FT cells (**Figure S5C**) and performed a GTPase assay. The results showed that SEPT2-74R impaired the GTP degradation activity of SEPT2 (Fig. 3G).

The SEPT2-K74R mutant inhibited HCC cell migration and invasion in vitro and in vivo

To investigate the functional role of SEPT2-K74 crotonylation, we stably overexpressed SEPT2 WT and SEPT2-K74R in SUN449, Huh7 and SMCC7721 cells. Forced expression of SEPT2 WT and SEPT2-K74R and whole-cell crotonylation and crotonylation of SEPT2 with or without NaCr treatment were verified by WB (Fig. 3H; **Figure S5D**). We observed increased metastatic potential of the HCC SEPT2 WT cells treated with NaCr compared with that of the control cells. However, K74R-overexpressing cells with or without NaCr inhibited metastasis compared with the effect of the SEPT2 WT and control cells (Fig. 4A and 4B; **Figure S6A**). A wound healing assay showed similar results (Fig. 4C; **Figure S6B**).

To validate the inhibitory effect of SEPT2-K74R on tumor metastasis *in vivo*, we performed tail vein injection, orthotopic liver tumor implantation and splenic vein injection with mouse models. For the tail vein injection mouse model, three groups of Huh7 cells carrying a stable overexpression vector, SEPT2 WT or SEPT2-K74R, were injected into the tail vein of NCG mice (N = 8 each group). The mice were sacrificed 8 weeks later, and the HCC metastasis in the lungs was analyzed by H&E staining (**Figure S7A**). The results showed that mice in the SEPT2-K74R group obtained better nutrition (**Figure S7B**) and weighed more than those in other groups (Fig. 5A), and fewer mice in the SEPT2-K74R (4/8) group than in

the SEPT2-WT (8/8) and vector (8/8) groups exhibited lung metastases (Fig. 5B). Mice injected with SEPT2-K74R cells exhibited fewer lung metastases than mice in the SEPT2 WT or vector group (Fig. 5C and 5D). In addition, we observed liver metastasis in the vector (2/8) and SEPT2 WT (3/8) groups but not in the SEPT2-K74R group (Fig. 5E, **Figure S7C**).

For the orthotopic liver tumor implantation mouse model, the groups were established as described above. Huh7 cells were injected into the left lobe of the liver, and the mice were sacrificed 10 weeks later (Fig. 5F). The all the liver lobes of were separately embedded in paraffin and stained with H&E. The results showed that the relative liver weight was higher in the vector and SEPT2 WT groups (**Figure S8A**). We observed fewer metastases in the left lobe of the liver (**Figure S8B**) and the total liver (Fig. 5G-5I) in the K74R group than in the other groups. In addition, fewer mice exhibited metastases in the other liver lobes liver (1/8) or lung (0/8) in the K74R group than in the vector group (6/8 with liver and 2/8 with lung metastasis) and SEPT2 WT group (5/8 with liver and 3/8 with lung metastasis) (Fig. 5J and 5K, **Figure S8C**). For the intrasplenic vein injection mouse model, Huh7 cells were injected into the spleen, and mice were sacrificed 8 weeks later (**Figure S9A**). More intrahepatic metastases incidents were macroscopically and microscopically observed in the vector and SEPT2 WT groups than in the K74R group (**Figure S9B-S9E**).

SIRT2 decrotonylated SEPT2

To identify SEPT2 decrotonylases, we coexpressed SEPT2 WT and four decrotonylases, SIRT1, SIRT2, SIRT3 and HDAC3, that had been verified in a previous study[6] in 293T cells (**Figure S10A**). Notably, only SIRT2 showed SEPT2 decrotonylation ability, and the other decrotonylases exerted no effect on SEPT2 Kcr. To verify the interaction between SIRT2 and SEPT2, we performed a co-IP assay and found that SEPT2 interacted with SIRT2 (**Figure S10B**). GST pull-down assay led to a similar result (**Figure S10C**). Furthermore, SIRT2 decronylated SEPT2 in a dose-dependent manner (**Figure S10D**), and knockdown of SIRT2 expression led to a significant increase in SEPT2 crotonylation (**Figure S10E**). Therefore, our results indicated that SIRT2 decrotonylated SEPT2.

Crotonylation promoted cell invasive capability mediated through the SEPT2-K74cr-P85α-Akt pathway

SEPT2 is crucial in spindle formation and sister chromatid separation and plays an important role in mitosis. A previous study showed that P85α, a subunit of PI3K that is important in the epithelial-mesenchymal transition (EMT)[17, 18], interacted with SEPT2 to regulate mitosis and that SEPT2 GTPase activity was crucial to mitosis[19, 20]. Therefore, we hypothesized that P85α may be related to SEPT2-K74cr.

To determine the interaction of SEPT2 and P85α, we performed co-IP and GST pull-down assays and found that the K74R mutation impaired the interaction of SEPT2 with P85α (Fig. 6A and 6B). We observed a reduction in P85α expression in SEPT2-74R-overexpressing cells (Fig. 6C), while the mRNA expression of P85α was not changed in SNU449 cells and even increased in Huh7 cells (**Figure S11A**),

indicating that SEPT2-K74R regulated P85 α protein level mainly at the posttranscriptional level. For verification, SEPT2-74R was transiently overexpressed in SNU449 cells, and these cells were treated with the protein synthetase inhibitor CHX in FBS-free medium. The results showed that the P85 α protein level was decreased in SEPT2-K74R-overexpressing cells treated with CHX (Fig. 6D). In addition, we observed a decrease in the stability of P85 α in SIRT2-overexpressing cells (**Figure S11B**). To identify the protein degradation pathway through which the K74R mutant regulated P85 α level, SNU449 cells transiently overexpressing SEPT2-74R were treated with the proteasome inhibitor MG132 and the autophagy inhibitor Baf1A. The results showed that MG132 exerted no effect on P85 α degradation, but Baf1A slowed P85 α degradation in CHX-treated cells, indicating that SEPT2-K74R enhanced the P85 α stability by influencing the autophagic degradation of P85 α (**Figure S11C**). Since P85 α is a key protein in the PI3K-Akt pathway, we identified its downstream targets in this pathway. The results showed that PI3K-Akt pathway activity in SEPT2-K74R cells and SEPT2-K74R mouse tumor tissues was significantly decreased, N-cadherin expression was decreased, and E-cadherin expression was increased in the SEPT2-74R group (Fig. 6C, **Figure S11D**).

Furthermore, knocking down P85 α expression in conjunction with NaCr treatment inhibited the invasive capability of SEPT2 WT-overexpressing cells, and this trend was reversed by overexpressing P85 α in SEPT2-K74R cells (Fig. 6E and 6F; **Figure S12A and S12B**). We also observed that P85 α was overexpressed in SEPT2-WT cells after NaCr treatment but not in P85 α -knockdown or SEPT2-K74R-overexpressing cells (Fig. 6G). These results suggested that NaCr facilitated the EMT through the SEPT2-K74Cr-P85 α -AKT pathway.

Crotonylation of SEPT2 led to a poor prognosis and HCC recurrence in patients

To examine the potential clinical importance of SEPT2-K74cr in HCC patients, we generated a site specific anti-SEPT2-K74 antibody specifically for targeting K74cr in SEPT2 for IHC staining. A dot blot and Western blot assays were performed to characterize the specificity of this antibody, and the results showed that K74cr preferentially detected SEPT-K74cr but not unmodified SEPT2 (**Figure S13A**). A total of 126 HCC samples were stained for IHC analysis. Based on the percentage of stained cells and the intensity of the stain, the HCC samples were given a score of 1 to 4 for SEPT2-K74cr level (Fig. 7A). We found that HCC patients with a low SEPT2-K74cr level had a better prognosis ($p < 0.05$) (Fig. 7B), and a lower recurrence rate ($p < 0.05$) (Fig. 7C). In addition, SEPT2-K74cr protein level was higher in the early stage of (stage I/II) than in the advanced stage (stage II/III) ($p < 0.05$) (Fig. 7D and 7E) of HCC patients. Moreover, SEPT2-K74cr level was higher in bone metastasis tissue compared with that in its corresponding primary HCC tumor (Fig. 7F). There were no significant associations between age, sex, SEPT2-K74cr level, BCLC stage and recurrence status (**Table S3**). In the univariate Cox regression analysis (**Table S4**), BCLC stage and SEPT2-K74cr level were found to be significant prognostic factors ($p < 0.05$), and a low SEPT2-K74cr level was associated with a lower risk of cancer-related death (relative risk [RR], 0.536; 95% confidence interval [CI]; 0.293–0.982; $p < 0.05$). In the multivariate Cox regression analysis, the

SEPT2-K74cr level was an independent predictor for low survival (RR, 0.521; 95% CI, 0.285–0.955; $p < 0.05$) and HCC recurrence in HCC patients (RR, 0.575; 95% CI, 0.330–0.999; $p = 0.05$) (**Tables S5 and S6**).

Discussion

In this study, we used the SILAC method to characterize the crotonylome in two HCC cell lines with differential invasive potential, and found that the highly invasive cell line exhibited a higher crotonylation level. In addition to the level of specific enzymes, the overall degree of crotonylation is largely dependent on the crotonylation substrate and is a very dynamic process. Previous studies have shown that crotonylation and acetylation share a family of writers and erasers[7, 8]; therefore, discerning the specific PTM that is critical for an effect on enzyme activity is difficult. NaCr can be synthesized into crotonyl-CoA, the direct substrate in crotonylation, by acyl-CoA synthetase short chain family member 2 (ACSS2) [21] and can specifically enhance protein crotonylation but not acetylation[9]. Studies have found that NaCr exhibits potentially beneficial effects on acute kidney injury[22], can affect the cell cycle[9] and promotes endoderm differentiation of human embryonic stem cells (hESCs)[23]. To investigate the role played by crotonylation in HCC, we used NaCr to specifically increase overall crotonylation in cells and found that a higher overall crotonylation level facilitated the EMT and increased migration and invasion of HCC cells. Our results expanded the understanding of the effects of overall crotonylation. Crotonylation is derived from crotonyl-CoA. In addition to its precursor NaCr, crotonyl-CoA is an intermediate metabolite in fatty acid oxidation and lysine/tryptophan degradation. Acyl-CoA dehydrogenase short chain (ACADS) and acyl-CoA oxidase (ACOX) can metabolize butyryl-CoA to crotonyl-CoA[23]. Metabolic abnormalities, such as butyryl-CoA accumulation or ACADS and ACOX dysfunction, can result in intracellular crotonylation fluctuation. Exploring the reason that the degree of crotonylation is changed in HCC and whether this change is related to metabolic pathways is of great interest in future studies.

We further demonstrated that SEPT2 was hypercrotonylated in highly invasive cells. SEPT2 is crucial in cytokinesis, and the activity of SEPT2 GTP hydrolysis is indispensable for fiber distribution[19, 25]. We identified that of SEPT2-K74 crotonylation affects SEPT2-K74 GTPase activity, and a crotonylation-abrogating mutation of K74 could inhibited HCC metastasis *in vitro* and *in vivo*. Moreover, we observed an increase in the invasiveness of SEPT2 WT-overexpressing cells after NaCr treatment compared with that of the NC-Cr group. This inferred that SEPT2 GTPase activity not only regulates mitosis but is also associated with tumor invasion and migration, and the crotonylation of SEPT2-K74 may shift the role played by SEPT2 between tumor promoter and inhibitor. Thus modulating crotonylation of SEPT2-K74 may be a potential therapeutic strategy for the prevention and treatment of HCC.

A recent study showed that dynamic crotonylation of EB1 regulated accurate microtubule dynamics in mitosis[10], thus ensuring that vertebrate cells divide in the correct orientation. EB1 has been found to compete with SEPT2 for binding to guanosine' 5'-O-[γ -thio] triphosphate (GTP γ S)-stabilized microtubules[26]. As shown in Fig. 1C, we observed that 8.7% of crotonylated proteins were located in the cytoskeleton, which is a higher percentage than the 1% reported in previous studies[9]. Combined with our

results obtained from a GO analysis, findings showing that crotonylated proteins are enriched in intermediate filaments of the cytoskeleton, and a previous report indicating that histone crotonylation regulates meiosis[27], indicating that crotonylation may play an important role in mitosis.

Postoperative recurrence and high malignancy confound HCC treatment; therefore, finding biomarkers of recurrence and prognosis in HCC is an urgent undertaking. PTMs have been widely studied as predictors of tumor prognosis; for example, glycosylation in tumors is a target for liquid biopsy sampling [24], but crotonylation on this area is still unknown. In our study, by using a site-specific antibody, we found that crotonylation of SEPT2-K74 was higher in patients with HCC recurrence and indicates a poor prognosis; therefore, SEPT2-K74cr can be utilized as a potential recurrence and prognostic predictor in HCC. Our study is the first to elucidate the role played by crotonylation as a tumor biomarker.

Conclusion

In conclusion, our study revealed a novel role played by crotonylation in regulating HCC metastasis and invasion. Crotonylation-modulated SEPT2 promoted cell invasive capability by elevating the activity of the P85 α -Akt pathway. High crotonylation of SEPT2-K74 predicted poor prognosis and a high recurrence rate in HCC patients.

Declarations

Ethics approval and consent to participate

The use of all clinical samples was approved by the Institutional Review Board of The First Affiliated Hospital of Sun Yat-sen University. Written informed consent was obtained from each patient. All experimental procedures involving animals were approved by the Animal Ethics Committee of Sun Yat-sen University, Guangzhou, China (no. 2020000768).

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Author Contributions:

ZN, LXX and YJ designed and supervised the study; ZXY and ZYF performed the *in vitro* assays; ZXY, ZYF, CMK contributed to the *in vivo* studies. LZK, ZXY and XLX performed data analysis; ZXY, LXX, ZN wrote the draft, and all authors revised and approved the final manuscript.

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Figures

Figure 1

The crotonylome in differentially invasive HCC cells

(A) Migration and invasion assays showing that MHCC-97H cells had higher invasive potential than MHCC-97L cells. The data are presented as the means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ (Student's t test).

- (B) Flowchart showing the experimental procedure followed for identifying crotonylated proteins through stable isotope labeling by amino acids in cell culture (SILAC) and liquid chromatography with tandem mass spectrometry (LC-MS/MS).
- (C) Subcellular localization of the identified crotonylated proteins.
- (D) Gene set enrichment analysis (GSEA) showing the five most enriched pathways ($p < 0.05$).
- (E) The difference in protein expression and crotonylation between MHCC-97H and MHCC-97L cells. The level of protein expression and degree of crotonylation were increased in MHCC-97H cells ($-\lg \text{false discovery rate [FDR]} > 0$).
- (F) Scatter diagram showing differentially expressed proteins in the MHCC-97H and MHCC-97L cell lines ($p < 0.05$).
- (G) Scatter diagram showing the differentially crotonylated sites in the MHCC-97H and MHCC-97L cell lines ($p < 0.05$).
- (H) Western blotting (WB) verified that the total crotonylation level was higher in the MHCC-97H cell line.

Figure 2

Crotonylation was positively correlated with HCC cell migration and invasion

(A) Heatmap showing differentially crotonylated proteins in the MHCC-97H and MHCC-97L cell lines.

(B) Gene ontology (GO)-based enrichment analysis of differentially crotonylated proteins.

(C) Morphological changes in SNU449 and Huh7 cells after 25 mM sodium crotonate (NaCr) treatment.

(D) Migration and invasion assays showing that SNU449 and Huh7 cells had higher invasive potential after 25 mM NaCr treatment. The data are presented as the means $\pm$ SD. **p < 0.001, ****p<0.0001 (Student's t test).

(E) Wound healing assays showing that SNU449 and Huh7 cells exhibited greater migration capacity after 25 mM NaCr treatment. The data are presented as the means $\pm$ SD. **p < 0.01, ***p<0.001 (Student's t test).

(F) Western blot analysis showing the changes in the expression of epithelial-mesenchymal transition (EMT)-related proteins after 25 mM NaCr treatment.

Figure 3

Lysine (K) 74 crotonylation of SEPT2 was identified

(A) Scatter diagram based on the fold change (FC) of protein expression ($FC < 2$) and crotonylation ($FC > 1.5$).

- (B) Validation of endogenous crotonylation of SEPT2, GRB2 and RAB35. MHCC-97L cells were treated with 25 mM sodium crotonate (NaCr) for 24 hours. Cell lysates were immunoprecipitated with corresponding primary antibodies, followed by western blotting (WB) with an anti-pan-Kcr antibody.
- (C) Validation of endogenous differences in SEPT2, GRB2 and RAB35 crotonylation in MHCC-97H and MHCC-97L cell lines. SEPT2 crotonylation was highest crotonylation in the MHCC-97H cells.
- (D) The location of differentially crotonylated sites in the SEPT2 domain.
- (E) SEPT2-K74 is evolutionarily conserved in seven species. K74 in SEPT2 is highlighted in red.
- (F) The SEPT2-K74 and SEPT2-K318 mutants were crotonylated to a lesser extent than wild-type (WT) SEPT2. The degree of crotonylation in cells with ectopically expressed Flag-tagged SEPT2 WT, K74R and K318R was analyzed.
- (G) GTPase activity was impaired after SEPT2-K74R mutation. Flag-tagged SEPT2 WT and K74R proteins were purified, and an in vitro GTP hydrolysis assay was performed. Panels 1, 2, 4, and 5 show the negative controls, and panel 3 shows the positive control.
- (H) Western blot analysis of overexpressed Flag-tagged SEPT2 WT and K74R and pan-crotonylation in cells and crotonylation on SEPT2 with or without NaCr treatment.

Figure 4

SEPT2-K74R inhibited cell migration and invasion in vitro

(A) Overexpression of SEPT2-K74R inhibited migration of Huh7 cells (left panel) and SNU449 cells (right panel) with and without 25 mM sodium crotonate (NaCr) treatment.

(B) Overexpression of SEPT2-K74R inhibited the invasion of Huh7 cells (left panel) and SNU449 cells (right panel) with and without 25 mM NaCr treatment.

(C) Overexpression of SEPT2-K74R inhibited the migration of Huh7 cells (upper panel) and SNU449 cells (lower panel) with and without 25 mM NaCr treatment.

The data are presented as the means ± SD. Ns p>0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001 (one-way ANOVA). NC, negative control; WT, wild type; 74R, SEPT2-K74R; NC-Cr, SEPT2-WT-Cr and K74R-Cr indicate NC, SEPT2-WT and SEPT2-K74R cells treated with 25 mM NaCr, respectively.

Figure 5

SEPT2-K74R inhibited cell migration and invasion in vivo

(A) Mice in the SEPT2-K74R group gained the most weight. Huh7 stable cells were injected into the tail vein of NCG mice (8 mice each group). The mice were weighed every week (W) and were sacrificed 8 weeks after the injection.

(B) Quantitative comparison of mice with lung metastases in each group. Fewer mice had lung metastasis in the SEPT2-K74R group. The red column indicates the mice had metastases.

(C) Representative images of lung metastases in the tail vein injection model. After the mice were sacrificed, the lungs were dissected and photographed (upper panel). Blue arrows indicate lung metastases. Lung tissues were stained by H&E (bottom panel).

(D) Quantitative comparison of the number of lung metastases in each group.

(E) Quantitative comparison of mice with liver metastases in each group. The red column indicates the transferred mice.

(F) Flowchart showing the orthotopic liver tumor implantation model. Huh7 stable cells were injected into the left lobe of the liver in NCG mice (8 mice per group).

(G) Images of liver metastases in the orthotopic liver tumor implantation model. After the mice were sacrificed, the livers were dissected, photographed and weighed. Blue arrows indicate liver metastases.

(H) Quantitative comparison of the number of liver metastases in each group. Mice in the SEPT2-K74R group had fewer liver metastases.

(I) Representative images showing liver metastases in the orthotopic liver tumor implantation model. Tissues were stained by H&E.

(J-K) Quantitative comparison of the number of mice with metastases in other lobes of the liver (J) and the number of mice with lung metastases (K). The red column indicates the transferred mice.

The data are presented as the means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA, two-way ANOVA). NC, negative control; WT, wild-type SEPT2; K74R, SEPT2-K74R mutant.

Figure 6

Crotonylation enhanced cell invasive capability by mediating SEPT2-K74Cr-P85α-Akt pathway activity

(A-B) P85α interacts with SEPT2. Whole-cell lysates were immunoprecipitated with an anti-SEPT2 antibody (A) and GST-tagged SEPT2-WT or GST-tagged SEPT2-K74R were purified with anti-GST antibody-conjugated beads (B).

(C) SEPT2-K74R overexpression decreased the expression of downstream P85α.

(D) SEPT2-K74R overexpression decreased P85α stability. Transient overexpression vectors carrying SEPT2 WT and SEPT2-K74R in SNU449 cells were treated with cycloheximide (CHX), and the P85α protein level was determined by WB (upper panel). The lower panel shows the relative protein levels in the different groups. Error bars represent $\pm$ SD on the basis of triplicate experiments.

(E-F) P85α rescued SEPT2 function after sodium crotonate (NaCr) treatment. Knocking down P85α expression inhibited cell migration (E) and invasion (F) after NaCr treatment; overexpression of P85α in SEPT2-K74R-expressing cells rescued the cell migration (E) and invasion (F) abilities after NaCr treatment.

(A) P85α rescued SEPT2 function after NaCr treatment. Quantitative comparison of wound-healing assays performed with Huh7 cells (upper panel) and SNU449 cells (lower panel).

(B) Western blot analysis of proteins expressed in the P85α rescue assays.

The data are presented as the means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA, two-way ANOVA). NC, negative control; WT, wild-type SEPT2; K74R, SEPT2-K74R mutant; Cr, NaCr treatment at 25 mM; sh, short interfering RNA downregulation of p85α expression; oe, p85α overexpression.

Figure 7

SEPT2-K74cr may be a potential biomarker for predicting prognosis and HCC recurrence in patients

(A) Scores indicate crotonylation of SEPT2-K74cr (K74-Cr) in representative tumor tissues as determined by immunohistochemistry (IHC) staining. Scores were calculated on the basis of the staining intensity and percentage of stained cells.

- (B) Patients with high K74-Cr levels had lower overall survival than those with low K74-Cr levels.
- (C) Patients with high K74-Cr levels had a higher recurrence rate than those with low K74-Cr levels.
- (D) Representative images of tissues with low and high stage pathology as determined by hematoxylin and eosin (H&E) and IHC staining.
- (E) Quantitative comparison of the IHC score in the tissues with low and high stage pathology. The data are presented as the means $\pm$ SD. * $p < 0.05$.
- (F) Images showing bone metastasis tissue and the corresponding primary HCC tumor after H&E and IHC staining.
- (G) Graphic model showing the effect of SEPT2-K74 crotonylation in HCC. K74 crotonylation was negatively regulated by SIRT2, and K74 decrotonylation decreased P85 α stability and PI3K pathway activity, which inhibited HCC metastasis. SEPT2-K74 crotonylation can serve as an independent predictor of low patient survival and HCC recurrence.

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