Am J Transl Res 2015;7(1):100-110
www.ajtr.org /ISSN:1943-8141/AJTR0003223

Original Article

Cathepsin S silencing induces apoptosis of human hepatocellular carcinoma cells

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Received October 17, 2014; Accepted November 25, 2014; Epub January 19, 2015; Published January 30, 2015

Abstract: This study was to investigate the potential molecular mechanisms underlying the Cathepsin S (CTSS) silencing induced apoptosis of Hepatocellular Carcinoma (HCC) cells with lentivirus-mediated RNA interference. Real-time quantitative PCR and western blot assay were performed to detect the mRNA and protein expression of CTSS, respectively, in 13 HCC cell lines with different metastatic potentials. Results showed MHCC97-H cells had the highest CTSS expression. Therefore, MHCC97-H cells were used in following experiments. Then, lentivirus-mediated RNAi was employed to silence CTSS expression (shCTSS). Annexin V/FITC staining showed NF-κB was activated in shCTSS cells treated with conditioned medium from shCTSS-PAR2 cells. This implies a probable positive correlation between PAR2 and CTSS. In addition, results demonstrated CTSS induced apoptosis of HCC cells and increased their chemosensitivity via regulating NF-κB and activating cleaved caspase-3. Our results indicate that CTSS silencing by lentivirus mediated RNAi can significantly induce apoptosis and chemosensitivity of MHCC97-H cells. This provides an attractive anti-cancer strategy and a novel strategy for the treatment of human HCC.

Keywords: Hepatocellular carcinoma, melittin, cathepsin S, apoptosis, chemosensitivity, NF-κB

Introduction

Hepatocellular carcinoma (HCC) is the sixth common malignancy and the third leading cause of cancer related death world wide [1]. It has a high mortality in short time, even after chemotherapy and surgical intervention. Thus, it is imperative to develop effective strategies for the therapy of HCC to improve the survival of HCC patients. In recent years, cancer proteomics have been employed to identify diagnostic markers and therapeutic targets for HCC [2]. Cathepsin S (CTSS) has been a focus in studies on proteases and cancer [3]. CTSS expression is at a low level in a majority of tissues and it is predominantly expressed in antigen-presenting cells, such as lymphocytes, macrophage/monocyte and lineage cells [4, 5]. CTSS expression is also found in non-professional antigen-presenting cells such as epithelial cells and various malignant cells [6-8]. Apart from its role in inflammation, CTSS has been reported to have increased activity and expression in various malignant tumors such as colon cancer [9], pancreatic cancer [10], prostate cancer [11, 12], HCC [13], melanoma [14] and brain tumors [8, 15, 16]. CTSS's role in the development and progression of tumors has been confirmed in a variety of studies, and recent studies also reveal that it plays critical roles in invasion and apoptosis of cancer cells. This suggests that to inactivate CTSS may provide a new strategy for the therapy of HCC with high CTSS expression [17-21]. In our previous study, results demonstrated both mRNA and protein expressions of CTSS were at high levels in human HCC cells with high metastatic potential [13]. In addition, our results also showed inhibiting CTSS expression by small interfering RNA (siRNA) (shCTSS) was able to suppress the proliferation, invasion and angiogenesis of human MHCC97-H cells, as well as induce their apoptosis [17]. Currently, little is known about the molecular mechanisms underlying the apoptosis of HCC cells after CTSS silencing. Thus, the present study aimed to confirm CTSS

Table 1. The Origin and Characteristics of 13 Liver Cell Lines

Cell line	Origin	Characteristics
MHCC97-H	Human HCC	High metastatic potential
MHCC97-L	Human HCC	Low metastatic potential
SMMC-7721	Human HCC	Low metastatic potential
Hep G2	Human HCC	
Hep 3B	Human HCC	No metastatic potential
HuH-7	Human HCC	
HCCLM3	Human HCC	High metastatic potential
Bel-7402	Human HCC	
Bel-7403	Human HCC	
Bel-7404	Human HCC	
SK-HEP-1	Human HCC	
Chang Liver	Human hepatic cells	
HL-7702 [L-02]	Human hepatic cells	

silencing induced apoptosis of human HCC cells and to explore the potential molecular mechanisms underlying the CTSS silencing induced apoptosis and chemosensitivity of HCC cells *in vitro* and *in vivo*.

Materials and methods

Cell lines and cell culture

A total of 13 different cell lines were employed into present study (**Table 1**). MHCC97-L cells and MHCC97-H cells [22] were obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China). Other human HCC cell lines (SMMC-7721, HuH-7, HCCLM3, Bel-7402, Bel-7403, Bel-7404, SK-HEP-1) and human liver cell lines (Chang Liver, HL-7702 [L-02]) were from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and the Hep G2, Hep 3B and human embryonic kidney cell line 293T from the American Type Culture Collection (Manassas, VA). Melittin (≥90%) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Doxorubicin (Biotopped, CHINA) and Puromycin from SIGMA-ALDRICH (Sigma, USA). Melittin solution (1 μg/μl) was prepared with sterilized water, stored at -20°C and diluted before experiment.

Construction of plasmids and vector, and transfection

To produce lentivirus expressing CTSS siRNA (GenBank ID: NM_004079) and protease activated receptor-2 (PAR2) (GenBank ID: NM_005242), siRNA sequence for human CTSS (5'-CACAGTTGCATAAAGATCCTA-3') was designed using the siRNA Designer program. The control siRNA, a negative control siRNA without homology to human genome, was created on the basis of a scrambled sequence (5'-TTCTCCGAACGTGTCACGTTT-3'), and cloned into lentiviral plasmid containing RFP reporter gene (pMAgic 8.1, sbo-bio, Shanghai, CHINA). Following digestion with restriction endonuclease, sequencing was done to confirm whether the sequence was correct. As previously described [23], MHCC97-H cells were traced with luciferase using RNAi technique. The genomic library clone IRATp970H0715D (http://www.imagenes-bio.com), contains CTSS and PAR-2 open read frame (ORF) cDNA was used to expand coding sequences using following primers: CTSS: forward: 5'-CGCAAATGGGCGGTAGGCGTG-3' and reverse: 5'-CAGCGGGGCTGCTAAAGCGCATGC-3'; RAR2: forward 5'-ATGCGGAGCCCCAGCGCG-3' and reverse: 5'-TCAATAGGAGGTCTTAAC-3'. The resultant PCR products were cloned into lentiviral plasmid containing the reporter gene of RFP (pLVX-RFP-3FLAG-Puro, sbo-bio, Shanghai, China).

RNA extraction and real-time quantitative PCR

Real-time quantitative reverse transcription-PCR (RT-PCR) was done with Light Cycler (Roche Applied Science) and a SYBR reverse transcription-PCR kit (Takara, Dalian, China). The expression of target genes was normalized to that of β-actin. RT-PCR was performed as described previously [17]. The primers used for real-time PCR were as follows: CTSS: 5'-GCCTGATTCTGTGGACTGG-3'(forward),5'-GATGTACTGGAAAGCCGTTGT-3' (reverse); PAR2: 5'-CTGTGGGTCTTTCTTTTCCGAA-3' (forward), 5'-CAAGGGGAACCAGATGACAGA-3' (reverse); β-actin: 5'-TGACGTGGACATCCGCAAAG-3' (forward), 5'-CTGGAAGGTGGACAGCGAGG-3' (reverse). The mRNA expression was calculated with $2^{-\Delta\Delta Ct}$ method.

Western blot assay

Western blot assay was performed as described previously [17]. Following primary antibodies were used: anti-CTSS antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti-NF-κB antibody (p65/RelA), anti-P-NF-κB antibody (p65/RelA), anti-IKb-α antibody, anti-P-IKb-α,

Table 2. Comparison of the Changes in the Volumes of Implanted Tumors and the Weights of Nude Mice with Implanted Tumors among Different Groups Treated with melittin ($\bar{x}$ ± S)

Group	N	Cells	Melittin-Treated (80 μg/kg per day)	DAY 7 of Volume (mm^3)
A	5	PLVT8	-	101.29 ± 11.5
B	5	PLVT8	+	100.01 ± 20.85
C	5	PLVT1150	-	80 ± 16.62
D	5	PLVT1150	+	79.4 ± 18.17

antibody anti-IKKα antibody, anti-IKKβ antibody, anti-P-IKK-α/β antibody, anti-PAR2 antibody, anti-cleaved caspase-3 antibody (Cell Signaling Technology; CST, USA). Anti-caspase-3 was from Abcam (USA), anti-GAPDH and anti-β-Actin from Kangchen (Shanghai, China).

Flow cytometry

Annexin V-FITC/PI Apoptosis Kit (BD Biosciences, Franklin Lakes, NJ, USA) was use to detect the apoptosis of MHCC97-H cells quantitatively according to manufacturer's instructions. Cell lines with CTSS silencing and PAR2 over-expression were collected and exposed to melittin (4 μg/mL and 8 μg/mL) for 30 min and then to doxorubicin (5 mg/mL) for 24 h [17].

Detection of in vivo tumorigenicity

PLVT8 or PLVT1150 cells (1×10^7 cells/animal) were injected subcutaneous into BALB/c nude mice (Experimental Animal Centre of Guangxi Medical University, Nanning, Guangxi, China) to produce xenografted tumors. A total of 20 nude mice were divided into four groups randomly (**Table 2**). Drug treatment was started on the 7th day after tumorigenesis. Mice in Groups 1 and 2 were treated with saline and those in Groups 3 and 4 with melittin (80 μg/kg/d) via the tail vein once daily. A total of 25 treatments were performed, and then the tumor diameters were measured with a caliper, followed by calculation of tumor volume as follow: a (largest diameter) × b (smallest diameter)^2/2.

Statistical analysis

Statistical analysis was performed with SPSS version 21 (SPSS Inc., Chicago, IL). Experiments were repeated at least three times. Results are expressed as mean ± standard error (S.E.M) or mean ± standard deviation (S.D). Comparisons between two groups were done with Student's t test. A value of $P<0.05$ as considered statistically significant.

Results

CTSS over-expression in human HCC cell line MHCC97-H

CTSS expression was detected in 13 HCC cell lines with different metastatic potentials (**Table 1**). Real-time quantitative PCR and western blot assay were performed. mRNA and protein expressions of CTSS in MHCC97-L cells and MHCC97-H cells were higher than those in other HCC cell lines. Among 13 cell lines, MHCC97-H cells had the highest CTSS expression (**Figure 1A**, **1B**). Therefore, MHCC97-H cells were used in following experiments.

Construction of stable cells with CTSS knock-down and CTSS over-expression

Lentivirus-mediated RNAi technique was introduced to silence or over-express CTSS gene to investigate the influence of CTSS on MHCC97-H cells. Based on lentivirus-mediated RNAi, two difference vector plasmids were used to construct four cell lines to evaluate the knock-down or over-expression of CTSS gene and scrambled gene (negative control group). These cells were labeled with Plvt1150, Plvt8, PSB880 and PSB53, independently. After continuous expansion of cells, cell lines with stable expression were harvested at 21 days (**Figure 1B1**) through puromycin test, and its ultimate concentration was 2 μg/ml.

Knock-down and over-expression of CTSS in MHCC97-H cells by lentivirus-mediated RNAi

In our study, results showed that the mRNA (**Figure 1B2**) and protein (**Figure 1B3**) expressions of CTSS decreased markedly in shCTSS cells in MHCC97-H, but increased significantly in CTSS over-expression cells, when compared with blank group and negative control group. Not only the mRNA and protein expressions of CTSS dramatically reduced in Plvt1105 group (**Figure 1B**), but they increased in CTSS over-expression group. No significant difference was found in CTS expression between blank group and negative control group. Our results demonstrated RNAi technique has high specificity and efficiency to silence CTSS expression and over-express CTSS in MHCC97-H cells.

CTSS silencing induces apoptosis in protease activated receptor-2 (PAR2) and NF-κB dependent manner in MHCC97-H cells

Our results further showed that both mRNA and protein expression of PAR2 decreased significantly in shCTSS cells, but those increased markedly in CTSS over-expressing cells (**Figure 2A**). Furthermore, an increased apoptosis was observed in shCTSS cells as compared to HCC cells when compared with control cells (30.07% vs 18.58%) (**Figure 2B**). To determine the relationship between CTSS and PAR2 and to investigate whether CTSS induces anti-apoptosis of cancer cells through activating PAR2 expression, shCTSS cells with PAR2 over-expression were prepared by transfecting MHCC97-H cells using RNAi technique and to survey whether the outcomes of CTSS knock-down could be eliminate on the basis of transfection with PAR2. When PAR2 was transfected successfully into CTSS knock-down MHCC97-H cells, the apoptosis of CTSS knock-down cells decreased significantly (30.07% vs 19.72%) (**Figure 2B**).

In conclusion, Annexin V/FITC staining showed that NF-κB was activated in shCTSS cells after incubation with conditioned medium from shCTSS-PAR2 cells (**Figure 2B**). This implies a probable positive correlation between PAR2 and CTSS.

CTSS silencing induces tumor apoptosis through Regulating NF-κB activation

Above results implies the importance of NF-κB activation in CTSS signaling pathway. To verify it, the expressions of total and nuclear NF-κB were detected in shCTSS from MHCC97-H cells. Results showed nuclear NF-κB expression reduced significantly in CTSS knock-down cells, but total NF-κB protein expression remained unchanged (**Figure 2C**), implying the importance of NF-κB activation in CTSS signaling pathway. To verify this hypothesis, the expressions of total and nuclear NF-κB were detected in shCTSS-PAR2 cells from MHCC97-H cells. Results showed the expression of nuclear NF-κB reduced significantly in CTSS knock-down cells, but that of total protein remained unchanged. On the basis of above findings, NF-κB is notably activated in PAR2 over-expressing cells with CTSS knocked-down. This suggests that there is a probable positive correlation between PAR2 and CTSS and that CTSS silencing induces apoptosis of cancer cells through PAR2-mediated NF-κB signaling. To confirm this, stable cells with CTSS knock down and PAR2 over-expression were prepared, and labeled with Plvt200 (shCTSS-PAR2). The role of PAR2 in the apoptosis signaling pathway was further investigated in MHCC97-H cells: As compared to non-targeted control cells, CTSS knockdown cells showed decreased expressions of PAR2, nuclear NF-κB, nuclear IKB-α and IKK-α/β; however, the expressions of total NF-κB, IKB-α, IKK-α and IKK-β remained unchanged. Of interest, these were reversed in shCTSS-PAR2 cells (**Figure 2C**). In addition, our results showed CTSS silencing decreased NF-κB activity, but NF-κB activity was revealed in shCTSS-PAR2 cells which was ascribed to the activation of IKK-β and phosphorylation of IKB-α (**Figure 2C**).

shCTSS induces chemosensitivity

Moreover, the response of cells to chemotherapeutics was investigated. The melittin and doxorubicin in shCTSS and scramble shRNA cells were detected by flow cytometry with PI and annexin V-FITC. MHCC97-H cells were treated with melittin (0, 4 and 8 μg/mL) for 30 min and then with doxorubicin (0 and 5 μg/mL) for 24 h and the apoptosis of shCTSS and scramble shRNA cells was determined (**Figure 3A**, **3B**). Cells were harvested at 30 min and 24 h after treatment and subjected to flow cytometry. The percentage of apoptotic cells was calculated. Our results indicated that the apoptotic cells increased in a dose-dependent manner after melittin and doxorubicin treatment (**Figure 3A**, **3B**).

In addition, shCTSS cells were more sensitive to melittin and doxorubicin when compared with scramble shRNA cells: shCTSS cells were more chemosensitive to melittin (4 μg/mL: 36.72% vs 15.32%; 8 μg/mL: 52.98% vs 28.86%) after treatment for 30 min (**Figure 3A**), and also more chemosensitive to doxorubicin at 5 μg /mL (71.83% vs 32.66%) (**Figure 3B**).

CTSS silencing changes the expression of cleaved caspase-3

In the present study, CTSS knock-down also had an impact on the expression of cleaved caspase-3, a marker of apoptosis. Cleaved caspase3 markedly increased in shCTSS cells.

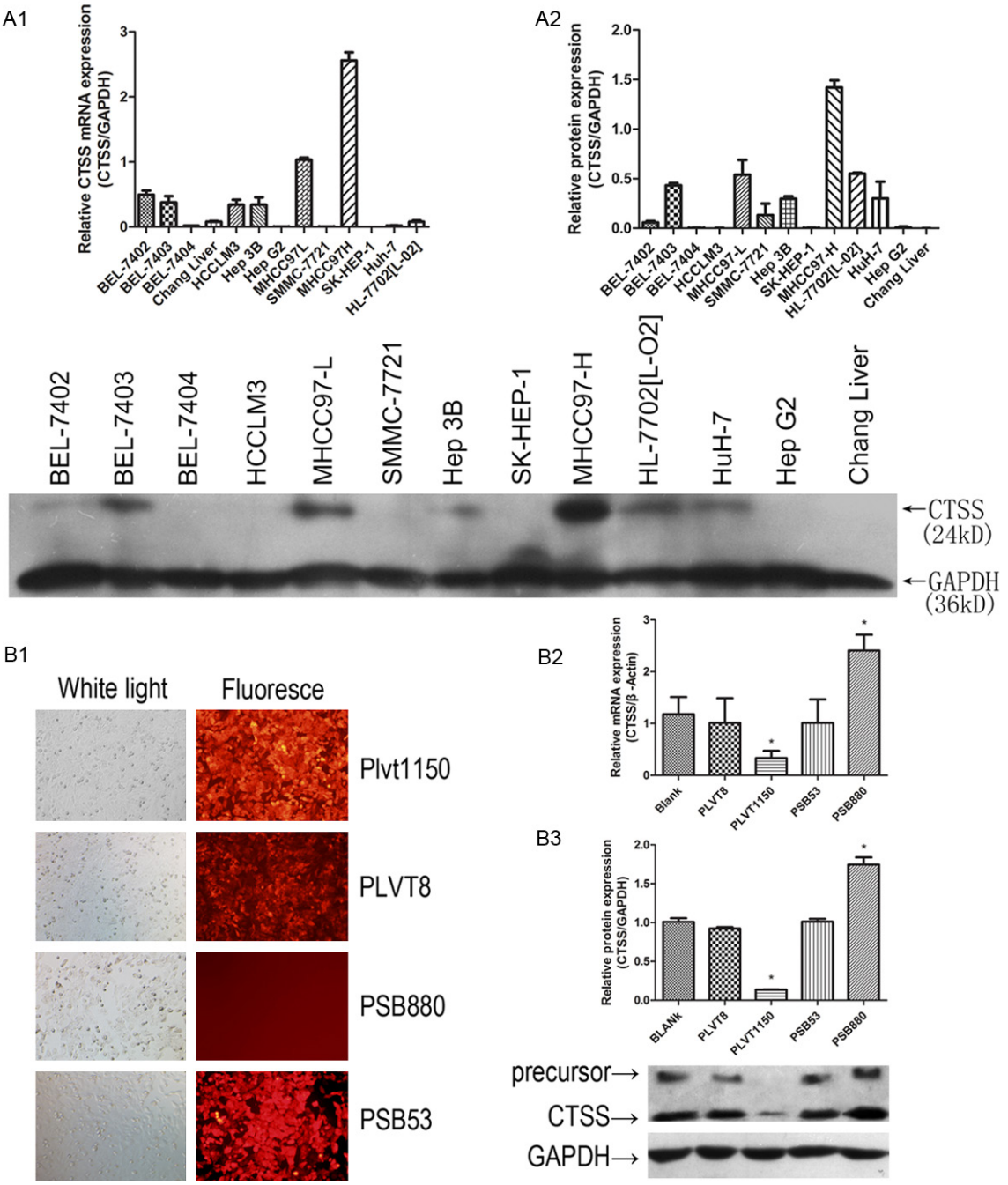

Figure 1. A. CTSS Expression in different HCC cell lines. A1. Real-time quantitative PCR was performed to detect CTSS mRNA expression. A2. Western blot assay was performed to detect CTSS protein expression. B. Construction of stable cell lines with CTSS knock-down and over-expression by lentivirus-mediated RNAi in MHCC97-H cells. B1. mCheery expression under a fluorescent microscope was observed at 96 h after stable infection in 293T cells (200×). B2. Real-time quantitative PCR was performed to evaluate interference efficiency at mRNA level. Plvt1150 significantly inhibited CTSS expression and PSB880 markedly enhanced CTSS expression in MHCC97-H cells. B3. Western blot assay was employed to evaluate interference efficiency at protein level, and results were consistent with those from PCR. (*P<0.05 vs control group).

Aforementioned results indicate that CTSS is associated with the apoptosis of MHCC97-H cells. Since the caspase-3 pathway also is involved in the process of apoptosis, the protein expressions of pro-caspase-3 and cleaved-caspase-3 were detected by western blot assay

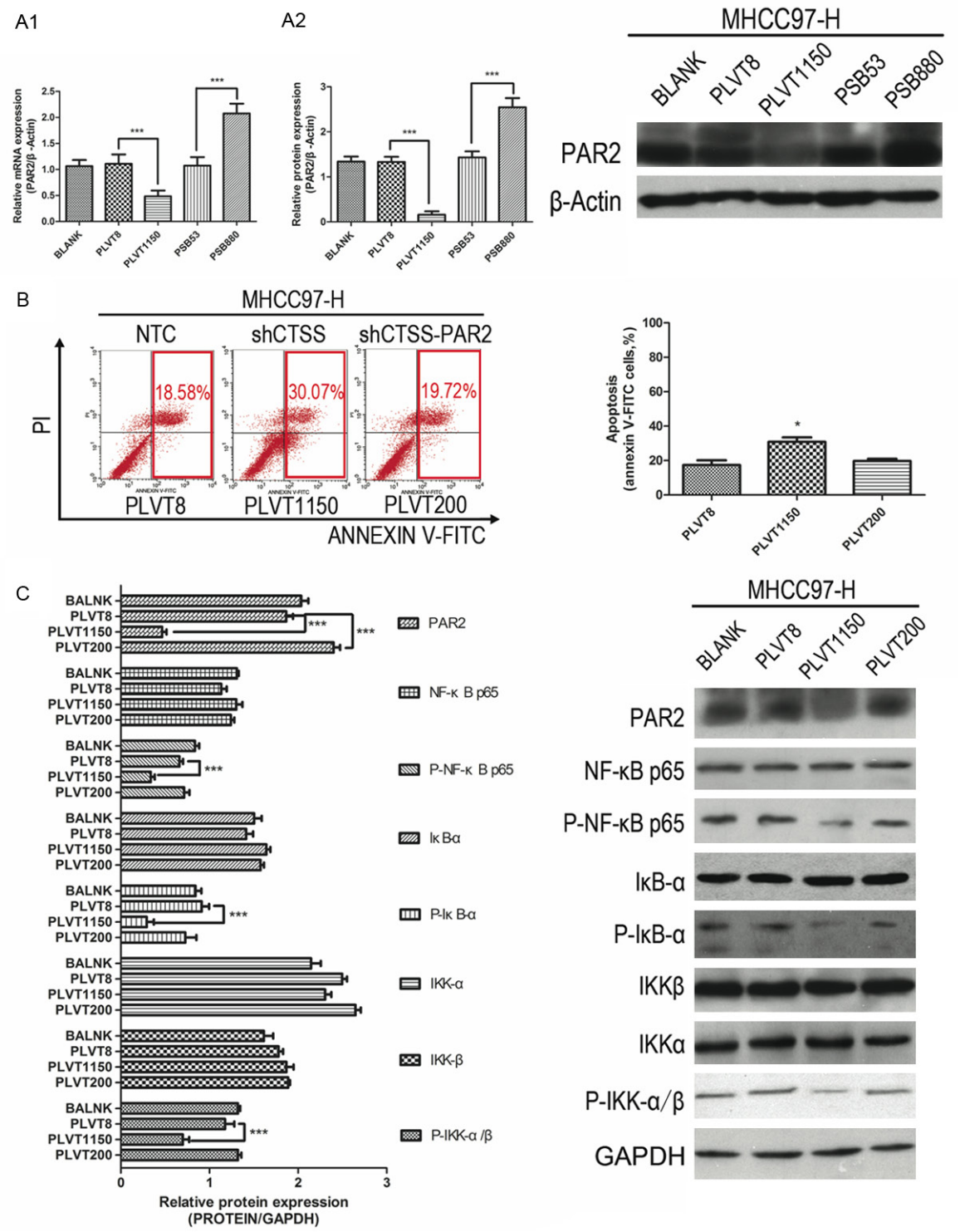

Figure 2. CTSS knock-down induced apoptosis of HCC cells. A. Knock-down and over-expression of CTSS increased and decreased PAR2 expression, respectively, at mRNA and protein levels in MHCC97-H cells. A1. Real-time quantitative PCR was performed to detect PAR2 mRNA expression. A2. Western blot assay was performed to detect PAR2 protein expression. B. CTSS knock-down induced apoptosis of HCC cells: shCTSS cells had higher apoptosis index when compared with control cells (30.07% vs 18.58%), but the apoptosis of CTSS knock-down cells decreased after PAR2 transfection (30.07% vs 19.72%). C. shCTSS induced apoptosis through PAR2 and NF-κB dependent manner. MHCC97-H shCTSS cells were incubated with conditioned medium from shCTSS-PAR2 cells, and results showed an increase in nuclear NF-κB and PAR2 expression, which was comparable to that after incubation with conditioned

medium from NTC cells. PAR2 was efficiently knocked down in MHCC97-H cells by lentiviral-based RNAi. In NTC cells of MHCC97-H cells, up-regulation of both nuclear NF-κB and PAR2 was observed in response to conditioned medium from shCTSS-PAR2 cells. In contrast, nuclear NF-κB and CTSS expressions remained unchanged in PAR2 knock-down cells. shCTSS cells showed inhibited IKBα kinase phosphorylation (***P<0.001).

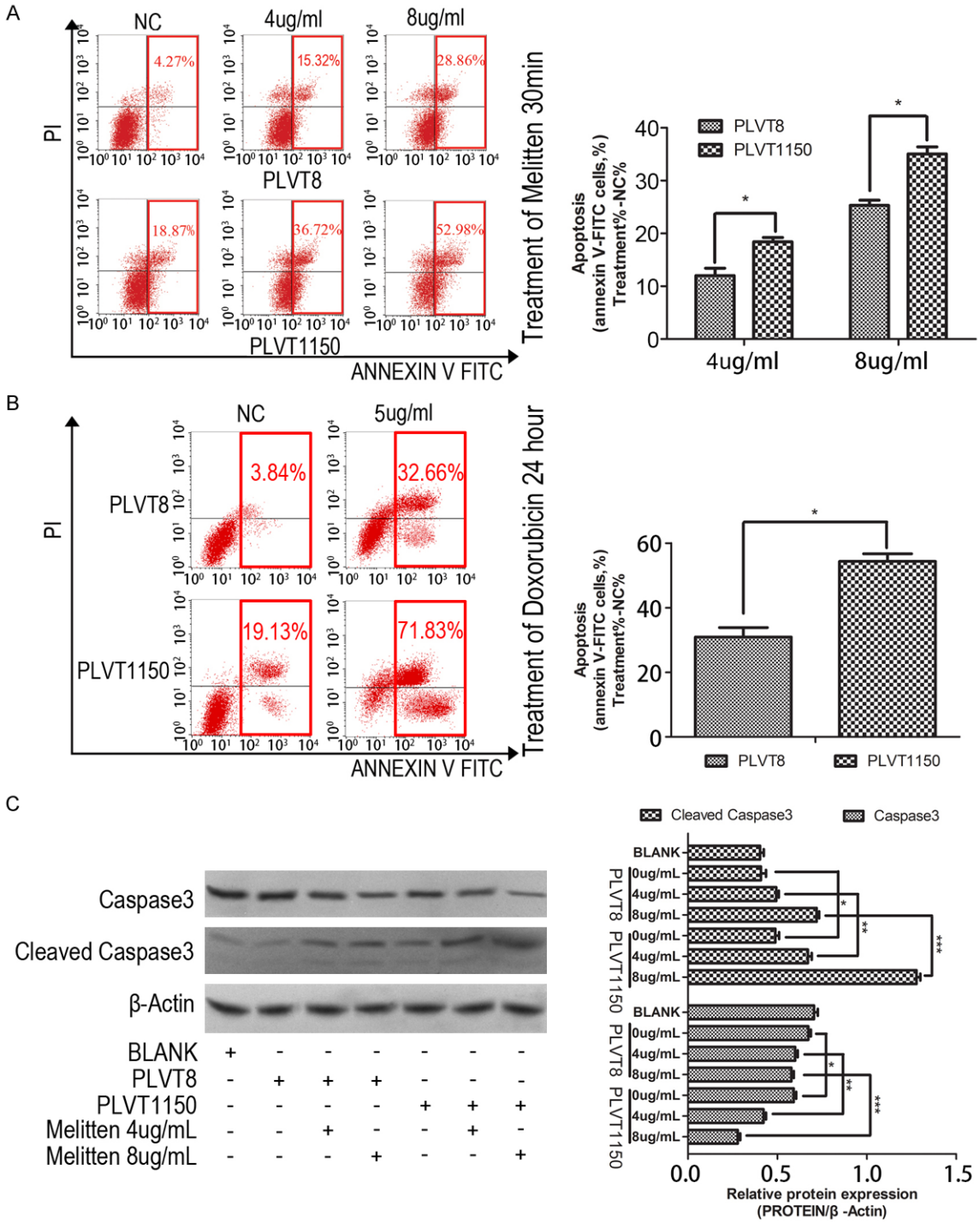

Figure 3. CTSS silencing induced chemosensitivity and apoptosis through activating caspase cascade pathway. A. shCTSS cells were treated with melittin (0, 4 and 8 μg/mL) for 30 min in MHCC97-H cells. When compared with control group, the apoptosis index of shCTSS cells was 18.87% vs 4.27%, 36.72% vs 15.32%, 52.98% vs 28.86%, respectively. B. shCTSS cells were treated with Doxorubicin (0, 5 μg/mL) for 24 h in MHCC97-H cells. When compared

with control group, the apoptosis index of shCTSS cells was 19.13% vs 3.84%, 71.83% vs 32.66%, respectively. C. Detection of chemosensitivity and apoptosis related proteins were performed by western blot assay. Results showed the expressions of caspase-3 and cleaved-caspase-3 changed in MHCC97-H cells treated with melittin and doxorubicin. The lanes contain caspase-3 and cleaved caspase-3 samples from blanks, Plvt1150 and Plvt8. Data are expressed as mean ± SD (*P<0.05; ***P<0.001).

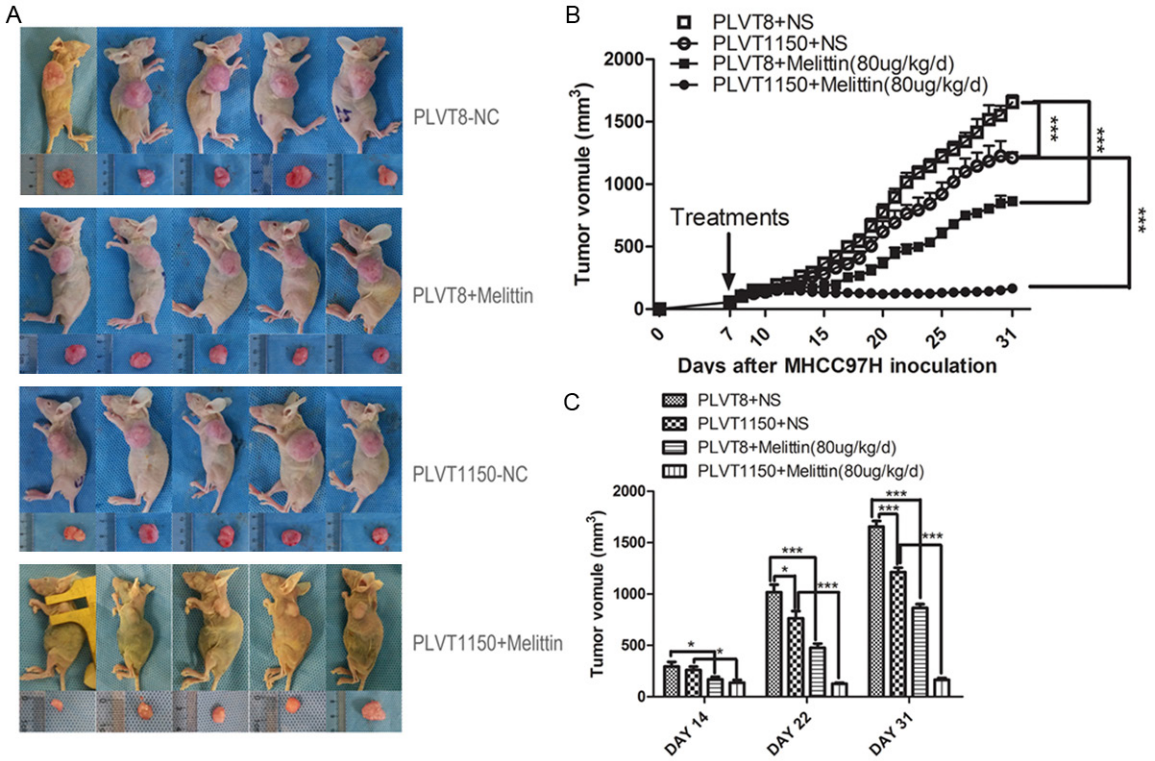

Figure 4. CTSS silencing synergized with melittin in the treatment of HCC *in vivo*. A. Nude mice were randomized into four groups (n=5 per group). Mice in each group were treated for 31 days with either Plvt8-NC (Group A), melittin (80 μg/kg/d) and Plvt8 (Group B), Plvt1150-NC (Group C), or melittin (80 μg/kg/d) and Plvt1150 (Group D). B. 7 days after inoculation of MHCC97-H cells into BALB/c nude mice (n=5 per group), melittin (80 μg/kg/d) was intravenously injected once daily for 7 days. On the indicated days, the tumor volume was determined as follows: $(a \times b^2/2)$. Data are presented as mean ± S.E. (n=5). C. On days 14, 22 and 31, the tumor volume was determined. Data are presented as mean ± S.D. (*P<0.05; ***P<0.001).

after ShCTSS and melittin treatments. As shown in **Figure 3C**, western blot assay showed pro-caspase-3 expression decreased but cleaved-caspase-3 expression increased. These results suggest that CTSS silencing activate caspase-3. CTSS silencing also increased caspase-3 activity (**Figure 3C**).

CTSS silencing and melittin treatment synergistically inhibit human HCC growth

To examine the therapeutic effects of combined CTSS silencing and melittin treatment on HCC *in vivo*, MHCC97-H cells were inoculated into node mice which were then intravenously treated with melittin once daily for 7 days at 7 days after inoculation. Volume of xenografted tumors and body weight of nude mice were determined (described in **Table 2** and **Figure 4C**) to evaluate the therapeutic effects. On day 14, the tumor volume in Group B notably reduced when compared with Group A, and that in Group D significantly reduced when compared with Group C. On day 22, these were confirmed, and that the tumor volume in Group C was also smaller than that in Group A. On day 31, these were further confirmed; (*P<0.05; ***P<0.001). In summary, our results showed that combined melittin and CTSS silencing significantly inhibit the growth of HCC synergistically (**Figure 4B**, **4C**).

Discussion

CTSS is an important gene involved in the invasion, metastasis, and self-renewal and chemotherapy resistance of HCC cells [24]. PAR is a member of G-protein-coupled receptor family and can be self-activated following proteolytic cleavage of their extracellular N-terminal domain. PAR family members consist of PAR1, PAR2, PAR3 and PAR4, that are expressed in epithelial, immune, neural and vascular tissues and have been implicated in inflammation, pain, itch, hemostasis, thrombosis and carcinogenesis [25, 26]. Consistent with wide distribution, PAR2 is involved in numerous physiologic and pathophysiologic processes in response to both endogenous and exogenous proteases. PAR2 has been shown to be activated by several families of proteases, including cysteine protease, CTSS [27].

In this study, our results demonstrated that PAR2 expression decreased in CTSS silencing MHCC-97H cells, whereas PAR2 expression was up-regulated in cells with CTSS over-expression. This indicates a positive correlation between PAR2 and CTSS. In addition, CTSS silencing induced apoptosis of liver cancer cells, and PAR2 over-expression reversed the apoptosis in CTSS knock-down cells. Furthermore, there was a reduction in the phosphorylated NF-κB in CTSS silencing cells accompanied by apoptosis. Based on above findings, we hypothesized that CTSS modulates the apoptosis of HCC cells through activating PAR2 with is dependent on NF-κB pathway. In vitro experiment, CTSS expression was silenced by lentivirus-mediated RNAi, which induced apoptosis and increased chemosensitivity of HCC cells, potentially by activating NF-κB and the caspase-3 signaling pathway in a PAR2 dependent manner.

RNAi has been found to be a tool for the functional analysis of genes and a potential therapeutic strategy for various diseases including cancers [28]. In this study, lentivirus mediated RNAi system was prepared to specifically induce CTSS knock-down in MHCC97-H cells and CTSS over-expression. Our results showed, when compared with negative control group, CTSS silencing cells showed increased apoptosis and were more chemosensitive to melittin and doxorubicin. Furthermore, PAR2 expression was significantly down-regulated in CTSS knock-down cells, but up-regulated in CTSS over-expressing cells.

These results suggest that lentivirus mediated RNAi of CTSS provides an alternative pathway for the therapy of HCC. In our study, HCC cells with the highest CTSS expression were screened from thirteen HCC cells with different metastatic potentials and the CTSS expression was silenced, aiming to clarify the molecular mechanism underlying the influence of CTSS silencing on the apoptosis and chemosensitivity of HCC cells *in vitro* and *in vivo*. Our results demonstrated the CTSS silencing induced apoptosis and chemosensitivity of HCC cells through regulating NF-κB and caspase-3 activation. In addition, silencing of CTSS expression induced HCC cell apoptosis through PAR2 and NF-κB dependent manners.

In summary, our study demonstrates that lentivirus mediated knock-down of CTSS can significantly induce apoptosis and chemosensitivity of MHCC97-H cells. This provides an attractive anti-cancer strategy for the treatment of human HCC. This is supported by the fact that CTSS exerts anti-apoptotic and chemotherapy resistant effects, which makes it a potential molecular target in the therapy of HCC.

Acknowledgements

This work was supported by the Guangxi Natural Science Foundation (No. 2011GXNSFA018284) grant, the Department of Health Care of Guangxi grant (No. GZKZ10-114), and the National Natural Science Foundation of China (No. 81360372) grant.

Disclosure of conflict of interest

None.

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