Original article

Garlic-derived compound *S*-allylmercaptocysteine inhibits hepatocarcinogenesis through targeting LRP6/Wnt pathway

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1. Supplementary materials and methods

S1. Reagents and antibodies

All cell culture consumables and reagents were bought from either Corning Incorporated (Corning, NY, USA) or Gibco (Carlsbad, CA, USA). All antibodies were purchased from Abcam (Cambridge, UK). SAMC and SAC powders (purity > 95%) were supplied by Wakunaga Pharmaceutical Co., Ltd, Japan.

S2. Cell culture, transfection and lentiviral infections

Hep3B, Huh-7 and LO-2 cell lines were provided by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM with 10% (*v*/*v*) FBS at 37 °C with 5% CO_2 using a cell incubator (Shellab, Cornelius, OR, USA). Cells were grown to about 60%–70% confluence before exposing it to treatment. Human *LRP6* gene knockdown was performed as previously described using MISSION® *LRP6*-specific lentiviral shRNA (Sigma–Aldrich, St. Louis, MO, USA)[1]. For stable cell line generation, cells were selected in a growth medium containing 2 μg/mL puromycin[2].

S3. Cell viability assay

The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell viability assay was conducted as described in previous report[3]. For lactate dehydrogenase (LDH) release assay, cell-free supernatant was collected from different groups of cells after centrifugation. Determination of LDH release was conducted by using a lactic dehydrogenase-based *In vitro* toxicology assay kit (Sigma–Aldrich, St Louis, MO, USA). Results were read at 490 nm

in a micro-plate reader (Bio-Rad).

S4. TCF/LEF reporter luciferase assay

The activity of *β*-catenin activity was measured by a luciferase reporter assay of TCF/LEF-dependent transcription with Cignal TCF/LEF Reporter Kit (Qiagen, Valencia, CA, USA). *Renilla* luciferase construct was also co-transfected into each well for normalization. The luciferase activity was measured using the Dual-Luciferase Reporter assay system (Promega, Fitchburg, WI, USA).

S5. RNA extraction and quantitative real-time PCR

RNA extraction, cDNA synthesis, and quantitative PCR were performed as previously described[4]. The primer sequences of human *LRP6* were forward: 5'-TTTATGCAAACAGACGGGACTT-3'; reverse: 5'-GCCTCCAACTACAATCGTAGC-3'. The annealing temperature of this primer pair was 62 °C. Parallel amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control. All real-time PCR procedures including the design of primers, validation of PCR environment and quantification methods were performed according the MIQE guideline[4].

S6. Immunofluorescence of cells

After SAMC incubation, cells were washed thrice with sterile PBS and then immobilized with 4% paraformaldehyde (Sigma–Aldrich) for 10 min on ice and then blocked by PBS containing 1% bovine serum albumin (BSA, Sigma–Aldrich) for 1 h. Antibody of Ki-67 was

then incubated and an appropriate secondary fluorescent antibody was added. Hoechst 33342 was used to visualize the cell nuclei.

S7. Flow cytometry analysis of cell cycle distribution

After different treatments, cells were scraped off and washed with ice-cold PBS for three times and were centrifuged for 5 min (500x*g*) at 4 °C. The cells were then fixed in 70% ethanol for 2 h at 4 °C. Samples were rehydrated with PBS and the cells were incubated for 30 min at room temperature with PBS containing 0.2 mg/mL propidium iodide, 0.2 mg/mL DNAse-free RNAse A (Sigma–Aldrich), and 0.1% Triton X-100 (Fluka, Basel, Switzerland). Four-thousand events were acquired with a FACS SCAN Flow Cytometer (Becton-Dickinson, San Jose, CA, USA) for each sample and the percentage of cells in G0/G1, S and G2/M phases of the cell cycle was determined by the Cell-Quest analytical software (Becton-Dickinson).

S8. Quantification of apoptotic cells and caspase activity

After drug treatment, Hoechst 33342 (5 μg/mL) and propidium iodide (5 μg/mL) were added to each well to stain live cells. Quantifications of apoptotic ratio and caspase-3/7/8 activity were conducted as previously described[3,5].

S9. Cell migration and adhesion assay

The ability of cancer cell migration and adhesion were measured using a Transwell system (8-μm pores, Costar, Cambridge, MA, USA) and a 96-well plate coated with 10 μg/mL

fibronectin (then blocked with 1 μg/mL BSA), respectively. For cell migration assay, cells suspended in serum-free DMEM containing 0.1% bovine serum albumin (BSA) were applied to the upper chamber. DMEM with 20% FBS and 10 μg/mL fibronectin (FN) was added to the lower chamber. After the cells were incubated at 37 °C for 3 h, cells that migrated to the lower side of the upper chamber were stained with hemotoxylin and then counted. For the adhesion assay, cells were washed in serum-free DMEM containing 0.2% trypsin inhibitor and then resuspended in culture medium. An aliquot of 100 μL of suspended cells was added to each well of 96-well plates coated with 10 μg/mL FN and blocked with 1 μg/mL BSA. The plates were incubated for the appropriate periods of time at 37 °C in CO_2 incubator. Non-adherent cells were removed by washing with PBS, and attached cells were analyzed by MTT assay.

S10. Immunohistochemistry of xenografts

The IHC analysis of tumor xenografts was performed on acetone-fixed 5-μm tissue sections. Hematoxylin and eosin (H&E, Sigma–Aldrich) or antibody of LRP6 was used to stain the sections as previously described[6].

S11. SAMC labeling and time-lapse fluorescent microscopy

SAMC (1 mg) and Alexa Fluor 488 TFP ester (100 μg) were dissolved in 2 mL PBS (pH 8.3) and 10 μL of DMSO to prepare 0.5 mg/mL SAMC and 10 μg/μL Alexa Fluor 488 TFP ester stock solutions, respectively. Then 1.6 μL Alexa Fluor 488 TFP ester was dropwise added to 50 μL SAMC solution and the mixtures were stirred for 1 h at room temperature. The

TFP-conjuncted SAMC solution was prepared and stored at 4 °C. Huh-7 cells were incubated in 24-well plates (20,000 cells/well) at 37 °C for 24 h without any additional treatment. The medium in the well was replaced with a fresh medium containing 0.125 μmol/L TFP-conjuncted SAMC and the Huh-7 cells were incubated for 2 h at 37 °C in a CO_2 incubator. The imaging of Huh-7 cells were observed at different times under a Zeiss Observer D1 fluorescent microscope (Carl Zeiss Corp., Oberkochen, Germany). Cell nucleus was stained using Hoechst 33342 (Sigma–Aldrich).

S12. Western blot

Western blot analyses of all proteins were performed as described using *β*-actin as the internal control[7].

References for supplementary materials and methods

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2. Supplementary Tables

Table S1 Clinicopathological characteristics of 48 patients with hepatocellular carcinoma (HCC)

Characteristics		Number of patients (%)
Gender	Male	40 (83.3%)
	Female	8 (16.7%)
Age	<55 years	20 (41.7%)
	≥55 years	28 (58.3%)
Tumor size (diameter)	<5 cm	28 (58.3%)
	≥5 cm	20 (41.7%)
Liver cirrhosis history	Yes	41 (85.4%)
	No	7 (14.6%)
TNM stage	I	24 (50.0%)
	II	10 (20.8%)
	III	9 (18.8%)
	IV	5 (10.4%)
Distant metastasis	M0	45 (93.8%)
	M1	3 (6.2%)

Table S2 Clinicopathologic correlation of *LRP6* transcript in hepatocellular carcinoma (HCC) patients

Parameters		No *LRP6* OE	*LRP6* OE	*P* value
Gender	Male	15	25	0.2395
	Female	1	7	
Age	<55 years	9	11	0.2157
	≥55 years	7	21	
Tumor size (diameter)	<5 cm	8	20	0.5370
	≥5 cm	8	12	
TNM stage	Early (I&II)	13	21	0.3279
	Advanced (III&IV)	3	11	
Distant metastasis	M0	16	29	0.5412
	M1	0	3	
β-catenin OE	Absent	10	6	0.0039
	Present	6	26	

OE, over-expression. Over-expression of *LRP6* was defined as Cases with tumor/non-tumor (T/NT) ratio more than 2 folds. Over-expression of *β*-catenin was measured by nuclear or cytoplasmic immunohistochemical staining.

Table S3 Effects of SAMC on the cell cycle of hepatocarcinoma cell lines

SAMC (μmol/L)	G0/G1	S	G2/M
Hep3B			
0	41.6 ± 1.3	43.5 ± 0.5	14.9 ± 1.1
250	50.8 ± 0.9*	35.3 ± 0.7*	13.9 ± 1.4
1000	65.0 ± 2.2*	21.9 ± 0.6*	13.1 ± 1.5
Huh-7			
0	42.5 ± 1.5	43.1 ± 0.7	14.4 ± 1.4
250	61.0 ± 1.7*	19.2 ± 0.9*	19.8 ± 0.8*
1000	52.5 ± 1.3*	20.2 ± 0.8*	27.3 ± 1.2*

Flow cytometry analysis of Hep3B and Huh-7 hepatocarcinoma cells after 24-h treatment with 0, 250, or 1000 μmol/L SAMC. Values are means ± SEM. *$P < 0.05$, compared with control cells.