Lenalidomide induces apoptosis and inhibits angiogenesis via caspase-3 and VEGF in hepatocellular carcinoma cells

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Abstract. Hepatocellular carcinoma (HCC) remains a disease with a high mortality rate, and the treatment for HCC remains limited. It is necessary to develop efficient and low toxicity drugs for the clinical treatment of HCC. Lenalidomide is a novel analogue of thalidomide and has anti-inflammatory, immunomodulatory and anti-angiogenic effects. The current study investigated the inhibitory effect against HCC cells of lenalidomide and thalidomide. The MTT assay was used to determine the cytotoxicity of lenalidomide and thalidomide, and morphological changes were observed by fluorescence microscopy. Caspase and VEGF protein expression were measured by ELISA analysis and western blotting. It was identified that treatment of cells with lenalidomide and thalidomide led to a dose-dependent inhibition of cell proliferation, and the two drugs were able to induce cells apoptosis and inhibit VEGF expression in HCC cells. In addition, lenalidomide was identified to exhibit greater effects than thalidomide at the same concentration. In conclusion, the results indicated that lenalidomide induces apoptosis and inhibits angiogenesis in HCC cells via caspase-3 and VEGF pathway, and these may provide a potential perspective for lenalidomide's application in clinical.

Introduction

There is a high incidence of hepatocellular carcinoma (HCC) in China, HCC accounts for more than 80% of cases of primary liver cancer (1,2). The majority of patients with either HCC or

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hepatic metastasis carcinoma are not eligible candidates for surgical resection (3). Transarterial chemoembolization and the orally available targeted drug sorafenib have been demonstrated to increase survival in selected candidates (4). It is necessary to investigate and develop efficient and low toxicity drugs for the clinical treatment of HCC.

The use of thalidomide was terminated due to its teratogenicity (5). Lenalidomide is a new analogue of thalidomide and has been demonstrated to be more potent than thalidomide in the stimulation of T-cells, interleukin (IL)-2, and interferon (IFN)-γ production (6,7). Unlike thalidomide, lenalidomide exhibits almost no sedative or constipation-causing properties, and induces only minimal neurotoxicity in the initial clinical application (8). Previous studies have demonstrated that the anti-inflammatory, immunomodulatory and anti-angiogenic of lenalidomide served important roles in its anticancer activity (9,10). Lenalidomide induces apoptosis of myeloma cells and exhibits an immunomodulatory effect on cytokine secretion, enhancing T cell proliferation and IL-2 and IFN-γ production in patients with multiple myeloma (MM), and it additionally increases lysis of autologous MM cells through cytotoxicity mediated by natural killer cells (11,12). However, it remains unclear whether it may be efficacious in solid tumors.

In the current study, SMMC-7721 hepatoma cells were treated with lenalidomide or thalidomide at different concentrations, and it was identified that lenalidomide significantly inhibits proliferation of SMMC-7721 hepatoma cells *in vitro*. The two drugs tested can promote cell apoptosis and inhibit the expression of vascular endothelial growth factor (VEGF). In addition, lenalidomide was identified to be more potent than thalidomide, with observations of cell morphology by microscopy confirming these results. It was suggested that lenalidomide may induce apoptosis through the pathway of caspase-3 activation.

Materials and methods

Cells and reagents. The human HCC cell line SMMC-7721 was purchased from the Soochow University Cell Banks (Suzhou, China). Lenalidomide (Natco Pharma Limited, Hyderabad, India) and thalidomide (Sigma-Aldrich; Merck

Millipore, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck Millipore) to prepare 10 and 40 mM stock solutions. Cells were stained with Annexin V-Fluorescein Isothiocyanate (FITC) following the manufacturer's instructions (Annexin V-FITC Apoptosis kit; Beijing BLKW Biotechnology Co., Ltd., Beijing, China) and analyzed for apoptosis by FACS using CellQuest software version 7.0 (BD Bioscience, Franklin Lakes, NJ, USA). Flag-tagged caspase-3 was purchased Bio-Box Biotech (Beijing, China). VEGF enzyme-linked immunosorbent assay (ELISA) analysis was performed with a commercial VEGF ELISA kit (cat. no. EH010-56; BLKW Biotechnology, Co., Ltd.) following the manufacturer's protocol. The antibodies for caspase-3 (cat. no. 9664P) and VEGF (cat. no. 2478S) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). GAPDH antibody (cat. no. AB22131) was obtained from Bioworld Technology, Inc. (St. Louis Park, MN, USA) The Cell Counting Kit 8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

Cell culture. SMMC-7721 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (10%; Gibco; Thermo Fisher Scientific, Inc.). The SMMC-7721 human HCC cell line was maintained at 37°C in a humidified atmosphere with 5% CO_2.

Cell proliferation assay. CCK-8 assay was used to evaluate the relative cell viability. Briefly, cells were plated in 96-well plates at a density of 5,000 cells/well in the media. The cells were pretreated with compounds at 6.25, 12.5, 25, 50, 100 and 200 μg/ml in a final concentration of 0.25% DMSO in triplicate at 37°C in a humidfied incubator at 5% CO_2 for 48 h. Subsequently, CCK-8 reagent (100 μl/ml medium) was applied and incubated with cells at 37°C, 5% CO_2 for 1 h. Cells were then incubated for an additional 4 h and the optical density (OD) was measured at 450 nm using a VersaMax Microtiter Plate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Relative cell viability was calculated with the following formula: Relative cell viability (%) = OD(treatment group)/OD(control group) x 100%. The experiment was performed in triplicate.

Apoptosis assay. Cells ($5x10^5$) were treated with 100 μg/ml or 200 μg/ml drugs (lenalidomide and thalidomide) for 48 h then washed once with Annexin-V wash buffer (BD Biosciences). Cells were incubated with Annexin-V binding protein (5 μl) and propidium iodide (PI; 10 μl) (BD Biosciences) for 10 min. Cells were diluted with 500 μl wash buffer and analyzed by a FACSCalibur flow cytometer using CellQuest software. Furthermore, cells ($5x10^5$) were treated with the indicated treatments for 48 h, the supernatant was removed, 1 ml 70% cold ethanol was added along the six hole plate edge, then cells were fixed for 15 min. The cells were then washed with cold phosphate-buffered saline and PI (0.5 ml) was added prior to observation under the microscope.

Caspase-3 assay. Cells ($5x10^5$) were treated with 100 μg/ml or 200 μg/ml of the drugs (lenalidomide and thalidomide) for

48 h. Lysates (50 μl/$2x10^6$ cells) were added, then the cells were reprecipitated, placed in an ice bath for 30 min, during which they were oscillated 3-4 times (10 sec each time), then the crude cytosol was obtained as the supernatant as a result of centrifugation at 6,140 x *g* for 20 min at 4°C. Cell lysates (50 μl) were then extracted and mixed with Ac-DEVD-pNA (Sigma-Aldrich; Merck Millipore), incubated for 4 h at 37°C, and then the OD was measured using a microplate reader. Caspase-3 activation was determined by the rate of OD induced and OD control. All experiments were performed in triplicates.

ELISA assay. Cells ($5x10^5$) were treated with lenalidomide and thalidomide at the indicated doses for 48 h. ELISA analysis was performed as previously described (13). VEGF ELISA was performed using 200 μl culture supernatant in duplicates using the Quantikine VEGF ELISA kit (BLKW Biotechnology, Co., Ltd.) according to the manufacturer's instructions. Briefly, 200 μl culture supernatants was added to the wells and they were incubated for 2 h at 37°C. The plate was washed with 400 μl wash buffer twice and 200 μl VEGF conjugate was added followed by incubation for 2 h. Subsequent to the addition of substrate and stop solution, the optical density was determined using a microplate reader (RT-21000; Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China) at 450 nm with wavelength correction of 570 nm.

Western blotting. To determine the level of VEGF proteins, lenalidomide and thalidomide-treated cell lysates were prepared as described. A total of 20 μg proteins was analyzed by western blot analysis. The PVDF membranes with the transferred proteins were incubated with primary antibodies (cleaved caspase-3, 1:500; VEGF, 1:1,000; GAPDH, 1:10,000) at 4°C overnight and horseradish peroxidase-conjugated secondary antibodies (1:10,000; cat. no. 7074P2; Cell Signaling Technology, Inc.) at room temperature for 2 h. The signal was developed by the enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA) and visualized by FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. Data analysis was performed using the SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA). Difference between groups were assessed with Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Lenalidomide inhibits SMMC-7721 cell proliferation. The anti-proliferative rate was detected by CCK-8, results are expressed as the mean ± standard deviation. Treatment of cells with lenalidomide and thalidomide in different concentrations for 48 h led to a dose-dependent induction of the inhibition of cell proliferation. The anti-proliferative effects of lenalidomide were identified to be more potent than that of thalidomide in the 25, 50, 100 and 200 μg/ml groups (P<0.01; Fig. 1A and Table I).

Table I. Cell growth inhibition rates in each drug concentration group.

Drug concentration (μg/ml)	Inhibition rate (%)	
	Thalidomide	Lenalidomide
6.25	1.57±0.291	4.55±0.852
12.5	3.01±0.447	11.05±0.859
25	7.89±0.349	17.35±2.366[a]
50	17.28±1.223	28.44±2.331[a]
100	26.03±1.897	35.51±0.383[a]
200	32.40±1.296	41.87±0.949[a]

[a]P<0.05, lenalidomide vs. thalidomide.

Table II. Expression of activated caspase-3 in each drug concentration group.

Drug	100 μg/ml	200 μg/ml
Thalidomide	13.37±1.95	20.75±1.75
Lenalidomide	16.90±1.54[a]	24.31±2.24[a]

[a]P<0.05, lenalidomide vs. thalidomide.

Table III. Expression of vascular endothelial growth factor in each drug concentration group.

Drug	100 μg/ml	200 μg/ml
Thalidomide	0.4985±0.01097	0.2460±0.01192
Lenalidomide	0.3760±0.01813[a]	0.2255±0.01921

[a]P<0.05, lenalidomide vs. thalidomide.

Lenalidomide promotes apoptosis in SMMC-7721 cells. Cells (5x10^{5}) were treated with lenalidomide and thalidomide at the indicated doses for 48 h. Typical apoptotic morphological alterations were observed using fluorescence microscopy, and included cell nucleus shrinkage, chromatin condensation and the appearance of apoptotic bodies when treated with different doses of the drugs tested (Fig. 2). Lenalidomide was observed to exhibit an increased effect of inducing cell apoptosis than thalidomide at the same concentration (Fig. 2). Caspase-3 activity of samples was detected using a microplate reader, and the OD was analyzed at 405 nm. Activity of caspase-3 was upregulated with increased lenalidomide and thalidomide concentrations. Caspase-3 activity in the lenalidomide groups was greater than in the thalidomide groups at the same concentrations, and this difference was significant (16.69±1.54 vs. 13.37±1.59; 24.31±2.24 vs. 20.75±1.75; P<0.01, P<0.05; Fig. 1B, Table II). Hepatoma SMMC-7721 cell apoptosis was examined using the Annexin-V staining-based FACS assay following lenalidomide and thalidomide treatment for 48 h. The rate of apoptosis is presented in Fig. 3. Lenalidomide has a higher rate
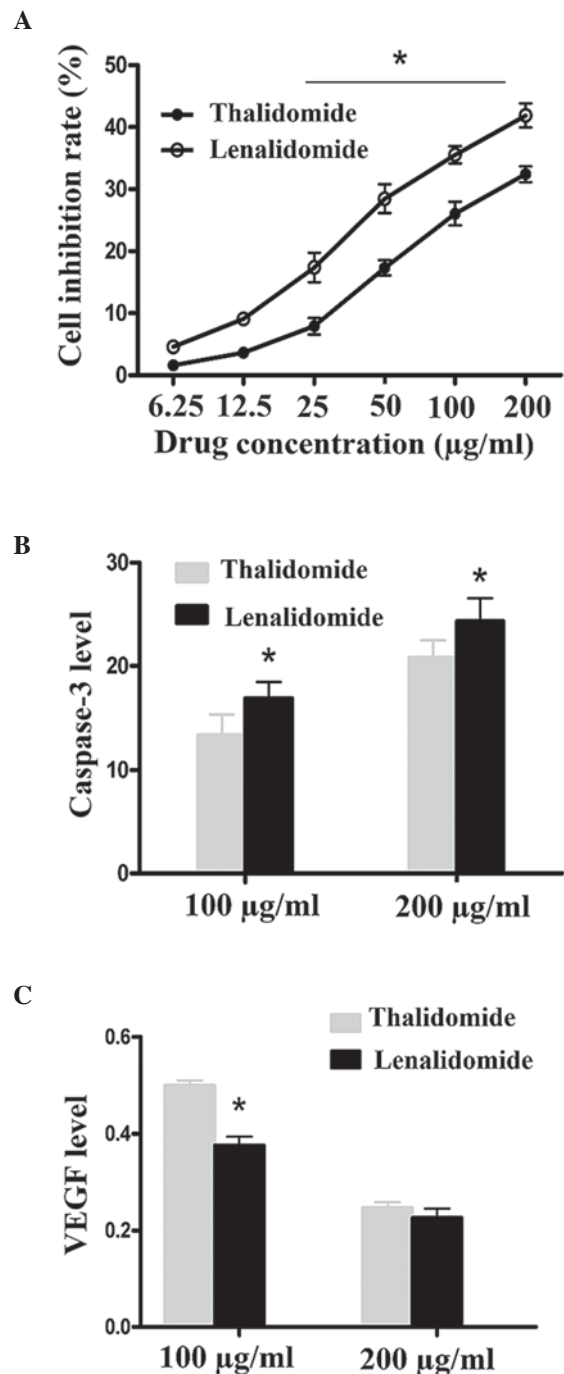
Figure 1. (A) Lenalidomide and thalidomide can significantly inhibit proliferation of SMMC-7721 cells. (B) Caspase-3 activation was indirectly determined by the rate of induced group OD and control group OD. The OD rate in the lenalidomide group was significantly higher than in the thalidomide groups with the same concentration. (C) Lenalidomide significantly inhibits VEGF expression of SMMC-7721 cells *in vitro* and is significantly more efficacious than thalidomide in the 100 μg/ml group. No significant differences were observed between the 200 μg/ml groups. *P<0.05. OD, optical density; VEGF, vascular endothelial growth factor.

of induced cell apoptosis than thalidomide of the same concentration (P<0.01; Fig. 3), and the effect was observed to be greater with the increase of the lenalidomide concentration (Fig. 4).

Lenalidomide inhibits VEGF expression in SMMC-7721 cells. The data were presented as the mean ± standard deviation.
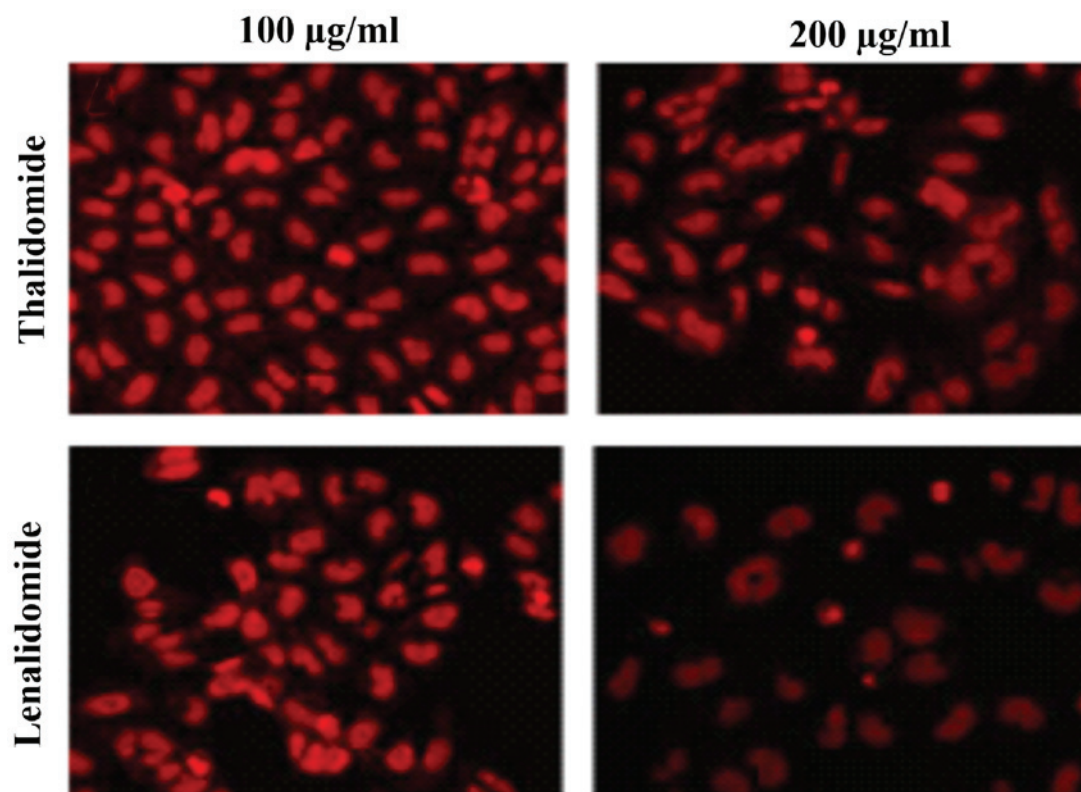
Figure 2. The typical apoptotic morphological alterations were observed by fluorescence microscopy. These included shrinkage of the cell nucleus, chromatin condensation and the appearance of apoptotic bodies with drug treatment.
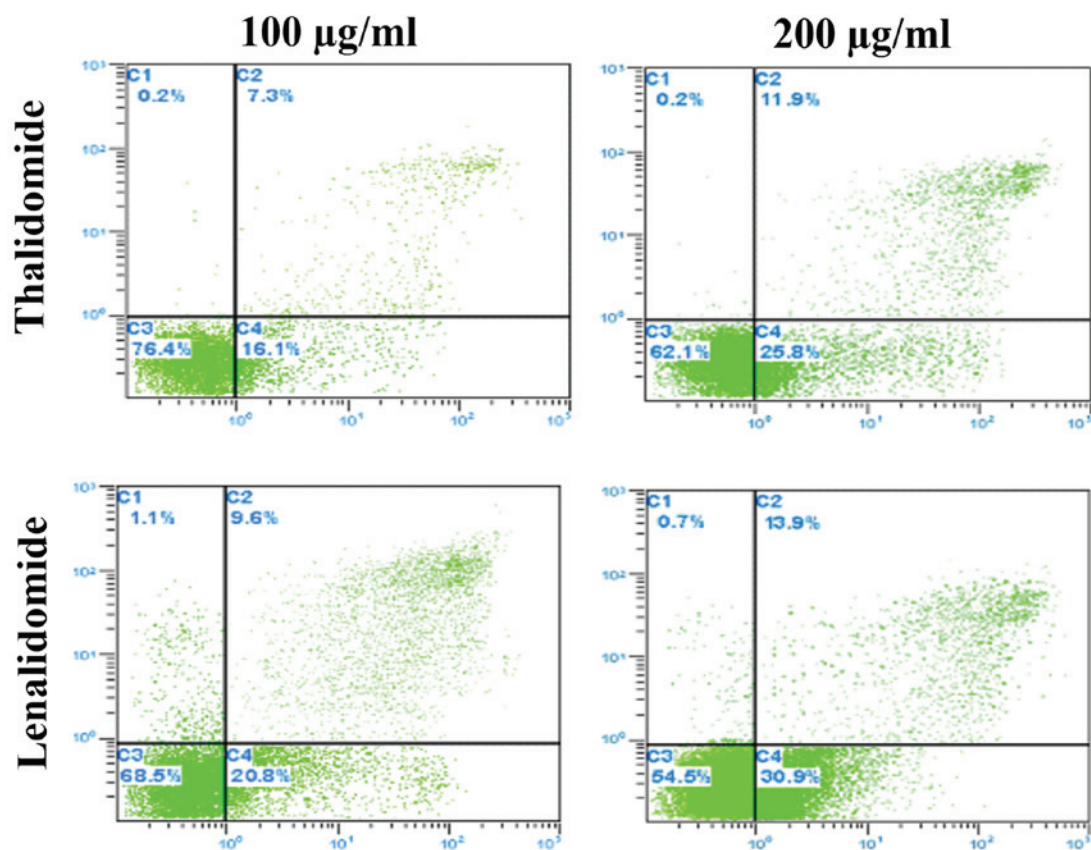

Figure 3. Cells (5×10^5) were treated with 100 or 200 μg/ml drugs (lenalidomide and thalidomide) for 48 h then were detected by Annexin V-fluorescein iso-thiocyanate/propidium iodide. Lenalidomide was observed to have a higher rate of induced cell apoptosis than thalidomide at the same concentration (P<0.01).
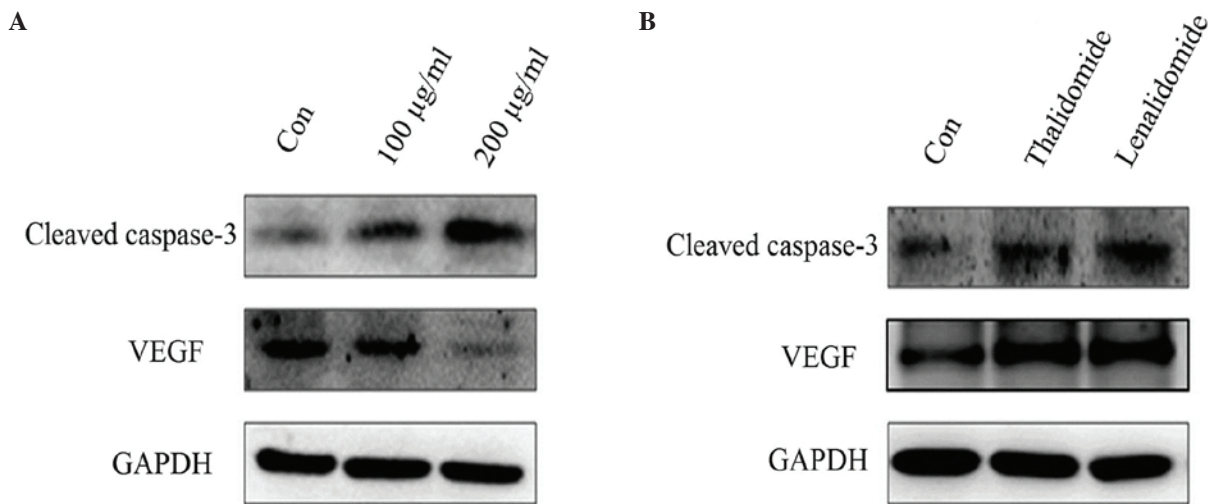
Figure 4. Expression of cleaved caspase-3 and VEGF in SMMC-7721 cells treated with or without lenalidomide; GAPDH was set as internal standard to normalize loadings. (A) Lenalidomide can significantly promote cleaved caspase-3 expression and inhibit VEGF expression of SMMC-7721 cells *in vitro*. (B) Lenalidomide is more potent than thalidomide in 100 ug/m groups and it has not significant difference between 200 ug/m groups. VEGF, vascular endothelial growth factor; Con, control.

In the current study, it was observed that lenalidomide can significantly inhibit VEGF expression of SMMC-7721 cells *in vitro* (Fig. 4) and is more potent than thalidomide in the 100 μg/ml groups (0.3760±0.01813; 0.4985±0.01097; P<0.05). No significant differences were observed between the 200 μg/ml groups (0.2255±0.01921; 0.2460±0.01192; P>0.05; Fig. 1C, Table III).

Discussion

Lenalidomide is a novel analogue of thalidomide and previous studies have demonstrated its anticancer effects (8,9,14). The results of the current study demonstrated that lenalidomide and thalidomide can significantly inhibit the proliferation of the human SMMC-7721 HCC cell line. These suggested that lenalidomide has anti-proliferative activity for HCC cell lines *in vitro*, and the same effect of lenalidomide inhibiting cell proliferation has been observed in multiple myeloma cell lines (15). In addition, in the present study typical apoptotic morphological alterations were identified by fluorescence microscopy, including cell nucleus shrinkage, chromatin condensation and the presence of apoptotic bodies with treatment with different doses of the drugs.

Caspase-3 is an intracellular protease activated early during apoptosis of cells and serves an important role in cell apoptosis. This protease activity can be measured spectrophotometrically by detection of the chromophore (*p*-nitroanilide) subsequent to cleavage from the labeled substrate (DEVD-pNA). In previous studies, Dmoszynska *et al* (11) reported that the mixture of lovastatin and thalidomidemay increases the rate of multiple myeloma cell apoptosis. Ezell *et al* (16) observed that low dose thalidomide treatment of human T leukemic cells exhibited rapid increases in caspase-3 activity, in addition, thalidomide and its immunomodulatory analogs trigger activation of caspase-8, enhancing MM cell sensitivity to Fas-induced apoptosis (17). The current study identified that activity of caspase-3 is upregulated with increases in lenalidomide and thalidomide concentration, and caspase-3 activity in lenalidomide groups is significantly higher than that of the thalidomide groups with the same concentrations (P<0.05; Figs. 1B and 4 and Table II). The present study indicated that lenalidomide inhibits proliferation of SMMC-7721 cells via the induction of cell apoptosis and suggested that caspase-3 may serve an important role in this process.

Angiogenesis serves an important role during tumor growth, invasion and metastasis (18), and VEGF is an endothelial-specific growth factor that stimulates endothelial function and angiogenesis (15). A previous study identified that lenalidomide possesses anti-angiogenic activity, and enhances T cell proliferation in MM patients (8). Tan *et al* (7) demonstrated that thalidomide can suppress VEGF and hypoxia-inducible factor 1α in a dose-dependent manner (P<0.05). In the present study, it was demonstrated that lenalidomide can significantly inhibit VEGF expression of SMMC-7721 cells *in vitro*, and is more potent than that of thalidomide in the 100 μg/ml groups (P<0.05; Figs. 1C and 4 and Table III). No significant difference was observed between the 200 μg/ml groups (P>0.05), These results indicated that the anti-angiogenesis activity of lenalidomide was induced through suppression of VEGF, and this may be a critical factor in the inhibition of cell proliferation.

Taken together, the results suggest that the inhibition of SMMC-7721 cell proliferation by lenalidomide *in vitro* is more potent than that of thalidomide and in addition, induction of apoptosis and inhibition of angiogenesis may be two potential mechanisms for its anti-HCC activity.

Acknowledgements

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