PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2004;10(19):2779-2784
World Journal of Gastroenterology
 ISSN 1007-9327

• ESOPHAGEAL CANCER •

Inhibitory effect of ubiquitin-proteasome pathway on proliferation of esophageal carcinoma cells

Wei-Guo Zhang, Jie-Ping Yu, Qing-Ming Wu, Qiang Tong, Sheng-Bao Li, Xiao-Hu Wang, Guo-Jian Xie

Wei-Guo Zhang, Qing-Ming Wu, Qiang Tong, Sheng-Bao Li, Xiao-Hu Wang, Guo-Jian Xie, Digestive Department, Taihe Hospital, Yunyang Medical College, Shiyan 442000, Hubei Province, China
Jie-Ping Yu, Digestive Department, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
Correspondence to: Wei-Guo Zhang, Digestive Department, Taihe Hospital, Yunyang Medical College, Shiyan 442000, Hubei Province, China. zwg789@sina.com
Telephone: +86-719-8801431
Received: 2004-02-02 **Accepted:** 2004-02-18

Abstract

AIM: To investigate the inhibitory effect of ubiquitin-proteasome pathway (UPP) on proliferation of esophageal carcinoma cells.

METHODS: Esophageal carcinoma cell strain EC9706 was treated with MG-132 to inhibit its UPP specificity. Cell growth suppression was evaluated with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. DNA synthesis was evaluated by ^{3}H-thymidine (^{3}H-TdR) incorporation. Morphologic changes of cells were observed under microscope. Activity of telomerase was examined by telomeric repeat amplification protocol (TRAP) of PCR-ELISA. Cell cycle and apoptosis were detected by flow cytometry (FCM). DNA fragment analysis was used to confirm the presence of apoptosis. Expression of p27^{kip1} was detected by immunocytochemical technique.

RESULTS: After exposed to MG-132, the growth and value of ^{3}H-TdR incorporation of EC9706 cells were obviously inhibited. Cells became round, small and exfoliative under microscope. TRAP PCR-ELISA showed that light absorption of cells gradually decreased after exposed to 5 μmol/L of MG-132 for 24, 48, 72 and 96 h (P<0.01). The percentage of cells at G_0/G_1 phase was increased and that at S and G_2/M was decreased (P<0.01). The rate of apoptotic cells treated with 5 μmol/L of MG-132 for 48 and 96 h was 31.7% and 66.4%, respectively. Agarose electrophoresis showed marked ladders. In addition, the positive signals of p27^{kip1} were located in cytoplasm and nuclei in MG-132 group in contrast to cytoplasm staining in control group.

CONCLUSION: MG-132 can obviously inhibit proliferation of EC9706 cells and induce apoptosis. The mechanisms include upregulation of p27^{kip1} expression, G_1 arrest and depression of telomerase activity. The results indicate that inhibiting UPP is a novel strategy for esophageal carcinoma therapy.

Zhang WG, Yu JP, Wu QM, Tong Q, Li SB, Wang XH, Xie GJ. Inhibitory effect of ubiquitin-proteasome pathway on proliferation of esophageal carcinoma cells. *World J Gastroenterol* 2004; 10 (19): 2779-2784
http://www.wjgnet.com/1007-9327/10/2779.asp

INTRODUCTION

Esophageal carcinoma is common in China. Previous studies have shown that its occurrence and progression are complicated, and are associated with the changes of multi-genes and molecules[1-3]. The ubiquitin-proteasome pathway (UPP) is the major system employed by eukaryotes for the selective degradation of cellular proteins that play key roles in cellular processes such as cell cycle regulation, differentiation, signal transduction, gene transcription, antigen presenting and transmembrance localization of proteins[4,5]. In this study, we investigated the inhibitory effect of UPP on proliferation of esophageal carcinoma cells by using specific ubiquitin proteasome to find a new strategy for esophageal carcinoma therapy.

MATERIALS AND METHODS

Materials

Esophageal carcinoma cell strain EC9706 was presented by professor Ming-Rong Wang, China Academy of Medical Sciences. MG-132 was purchased from Calbiochem Co. Ltd (USA), and dissolved in dimethylsulfoxide (DMSO) as a 40 mmol/L stock solution and stored at -20 ℃. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and DMSO were bought from Sigma Co. Ltd (USA). ^{3}H-thymidine (^{3}H-TdR) was provided by Beijing Atomic Power Research Institute. Telomeric repeat amplification protocol (TRAP) ELISA telomerase detection kit was obtained from Intergen Company (USA). Monoclonal mouse antibody of p27^{kip1}, ultra sensitive S-P kit, and 3,3-diaminobenaidine (DAB) kit were purchased from Fuzhou Maixin Biotechnology Co. Ltd. RPMI 1640 medium was obtained from GIBCOBRL Company (USA). Low melting-temperature agarose was obtained from Promega Company (USA). DNA-PREP™ LPR and DNA-PREP™ stain were obtained from America Beckman Coulter Company.

Cell culture

Human esophageal carcinoma cell strain EC9706 was maintained in RPMI 1640 medium supplemented with 100 mL/L fetal calf serum (FCS), 100 kU/L penicillin, 100 mg/L streptomycin and 2 mmol/L *l*-glutamine in a humidified incubator containing 50 mL/L CO_2 at 37 ℃, the medium was changed every 2-3 d.

MTT assay

EC9706 cells growing exponentially were chosen. Cell density in the suspension was adjusted to 2×10^5 cells/mL. After addition of 200 mL/well of the final cell suspension, 96-well plates were incubated for 24 h. Then the cells were treated with 0.5, 1.0, 2.5, 5.0, 10, 15, 20 μmol/L (DMSO≤0.5 g/L) of MG-132, respectively, as the different observation groups. The cells treated with an equal amount of DMSO instead of MG-132 served as the control. The background was conducted using only cell-free culture medium. After cultivated for 24, 48, 72 and 96 h, 10 μL of stock MTT solution (5 g/L) was added to the cells in each well, followed by a further incubation at 37 ℃ for 4 h. The culture medium was carefully removed, 100 μL of DMSO was added to each well and culture was vibrated for 20 min. The absorbance

of samples was measured three times for each group with three wells at a wavelength of 550 nm with the enzyme linked immunosorbent assay meter (PR 2100, SANOFI company, France). The inhibitory rate (IR) was calculated according to the formula: IR = [1-(absorbance of MG-132 group-absorbance of background group)/(absorbance of control group-absorbance of background group)]×100%.

Incorporation test of ^{3}H-TdR

As described above, experimental group and control group were cultured for 3 h with 100 mL/L FCS RPMI 1640, and then for 12 h with FCS-free RPMI 1640. MG-132 and 1 μ Ci ^{3}H-TdR were added to each group, which was rinsed with PBS after 24, 48, 72 and 96 h and fixed with methyl alcohol and absolute ethyl alcohol for 10 min each. Finally, 200 μL of 0.1mol/L NaOH was added, then 200 μL of each was taken after blowing, and mixed in 5 mL scintillation liquid for overnight. On the following day, the count per minute (CPM) of ^{3}H was tested three times for each group with three wells.

Morphologic changes of cells

Morphologic changes of EC9706 cells were observed under microscope 24, 48, 72 and 96 h after treated with MG-132 (5 μmol/L).

Telomerase assay

The cells (10^5-10^6) treated with MG-132 (5 μmol/L) for 24, 48, 72 and 96 h were collected, respectively. After addition of 300 μL telomerase assay lysis buffer (1×CHAPS), the cells were lysed on ice. The lysate was incubated on ice for 30 min and then centrifuged at 13 000 *g* for 25 min at 4 ℃. The supernatant (2 μL) was added to reaction solution containing 10 μL of TRAP buffer, 2 units of Taq polymerase and 48 μL of DH_2Oqs. PCR was carried out through 33 amplification cycles, each cycle consisting of denaturation at 94 ℃ for 30 s, primer annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 30 s. The amplified product was added to block/dilution buffer (250 μL), and incubated at 37 ℃ for 30 min, and 5 μL of TRAP reactant was then added and mixed. After incubated at 37 ℃ for 60 min, 100 μL working solution of anti-DNP Ab was added and incubated for 30 min, then 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution and 100 μL of stop reagent were added. The absorbance value in each well was read at the wave lengths of 450 nm and 690 nm on an enzyme linked immunosorbent assay meter. Telomerase activity was considered positive when the absorbance value of a sample was at least 0.8 units. When those were lower than 0.2 units, they were regarded as negative.

Flow cytometry detection

After cell cycle was synchronic, the cells of experiment group were treated with MG-132 (5 μmol/L) for 48 h and 96 h. The collected cells were added with DNA-PREP™ LPR and DNA-PREP™ stain, respectively, after they were washed with PBS and centrifuged. Cell cycle and apoptosis were detected by flow cytometry (Epics XL, Beckman Coulter Company, USA) and SYSTEM II™ software was used to dispose the data.

DNA ladder demonstration

As described above, the cells (7×10^6/sample, both attached and detached cells) were lyzed with hypotonic lysis buffer (10 mmol/L edetic acid, 5 g/L Triton X-100, Tris-HCl, pH 7.4) for 15 min on ice and precipitated with 25 g/L polyethylene glycol and 1 mol/L NaCl for 15 min at 4 ℃. After centrifugation at 16 000 *g* for 10 min at room temperature, the supernatant was treated with proteinase K (0.3 g/L) at 37 ℃ for 1 h and precipitated with isopropanol. After centrifugation, each pellet was dissolved in 10 μL of Tris-EDTA (pH 7.6) and electrophoresed on a 17 g/L agarose gel containing ethidium bromide. DNA ladder pattern was identified under ultraviolet light.

Immunocytochemical staining

EC9706 cells cultured with MG-132 (5 μmol/L) for 48 h were fixed with dimethyl ketone at 4 ℃. The cells carrying the detected antigen were stained following SP immunocytochemical staining method using anti-p27*kip1* as primary antibody[6]. PBS was substituted for primary antibody as negative control.

Statistics

The data were expressed as mean±SD. The difference between each group was analyzed by *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Inhibitory effect of MG-132 on EC9706 cell growth

The growth of EC9706 cells treated with 0.5-20 μmol/L of MG-132 was significantly inhibited compared with that of control group. While the cells exposed to MG-132 for 24 h produced a certain inhibitory effect, but only exceeding 48 h did MG-132 show significant effect. When the dose of MG-132 exceeded 5 μmol/L, only slight increases in IR of the cells were observed (Figure 1).

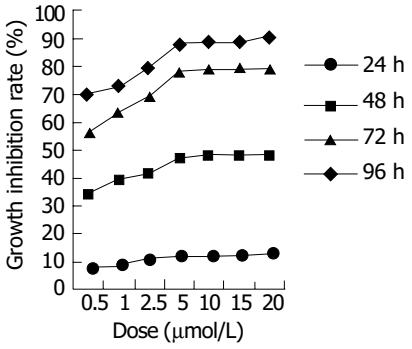

Figure 1 MTT assay of EC9706 cells after exposed to MG-132.

Table 1 Evaluation of DNA synthesis by ^{3}H-TdR incorporation (mean±SD)

Group	24 h	48 h	72 h	96 h
Control MG-132 (μmol/L)	4 295.52±136.32	5 236.17±221.36	5 642.92±105.41	5 863.43±206.58
0.5	3 764.68±97.37[a]	2 879.83±86.25[b]	1 918.73±76.49[b]	1 759.29±89.23[b]
1.0	3 526.14±101.42[a]	2 643.29±79.38[b]	1 547.25±68.94[b]	1 366.18±52.49[b]
2.5	3 402.34±93.44[a]	2 567.76±68.21[b]	1 260.37±51.27[b]	910.25±45.37[b]
5.0	3 324.78±65.43[a]	2 411.56±69.34[b]	840.79±41.17[b]	517.83±41.26[b]
10.0	3 301.29±59.28[a]	2 360.40±49.28[b]	820.56±39.76[b]	498.71±40.14[b]
15.0	3 294.12±67.33[a]	2 324.25±47.30[b]	810.17±45.61[b]	485.26±37.56[b]
20.0	3 280.54±62.46[a]	2 320.60±46.83[b]	804.63±51.34[b]	476.90±38.41[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* control.

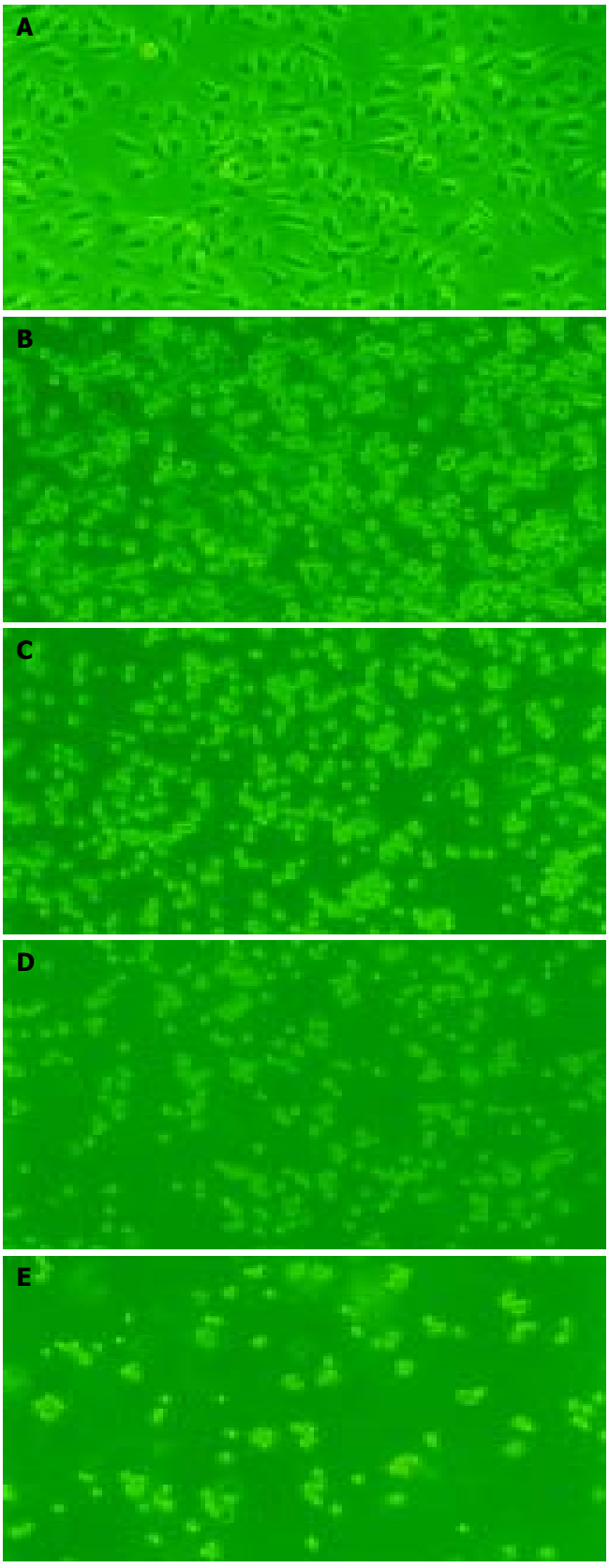

Figure 2 Morphologic changes of EC9706 cells observed under microscope after treated with 5 μmol/L of MG-132. A: control group; B: EC9706 cells treated with MG-132 for 24 h; C: EC9706 cells treated with MG-132 for 48 h; D: EC9706 cells treated with MG-132 for 72 h; E: EC9706 cells treated with MG-132 for 96 h (×200).

Inhibition of DNA synthesis

The values of ^{3}H-TdR incorporation of MG-132 group were decreased compared with the control group (Table 1).

Morphologic changes of cells

EC9706 cells became round, small and exfoliative after exposed to MG-132 under microscope (Figure 2).

Inhibition of telomerase activity

After treated with MG-132 (5 μmol/L) for 24, 48, 72 and 96 h, respectively, EC9706 cells showed a gradual decrease in values of *A* compared with the control group (P<0.01). Furthermore, telomerase activity was negative (Table 2).

Table 2 Effect of MG-132 on telomerase activity of EC9706 cells (mean±SD)

Groups	Light absorption (*A*)			
	24 h	48 h	72 h	96 h
Control	1.871±0.061	2.234±0.092	2.907±0.113	3.025±0.120
MG-132	0.154±0.008[b]	0.085±0.006[b]	0.072±0.004[b]	0.067±0.003[b]

[b]P<0.01 *vs* control group.

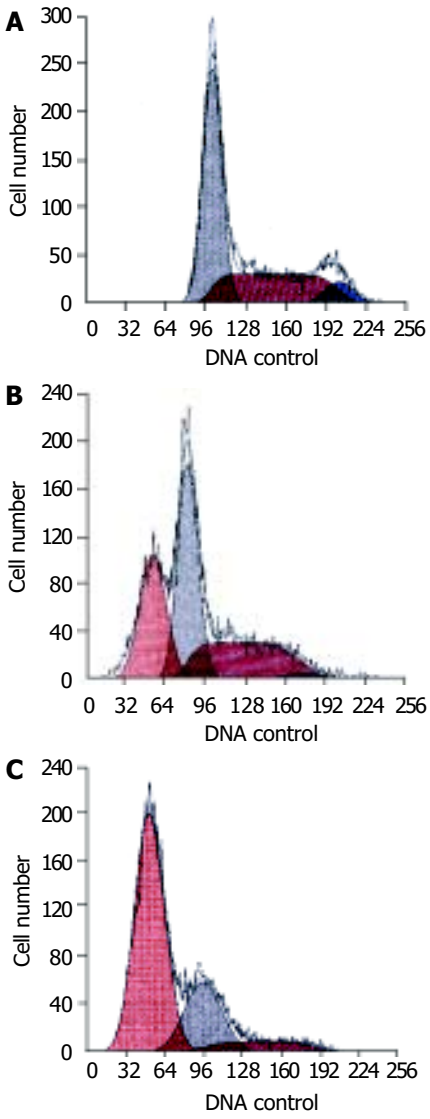

Figure 3 Cell cycle and apoptosis of EC9706 cells exposed to 5 μmol/L of MG-132. A: control group. Apoptotic sub-G_1 peak was not found; B: EC9706 cells exposed to MG-132 for 48 h. The ratio of apoptotic cells was 31.7%; C: EC9706 cells exposed to MG-132 for 96 h. The ratio of apoptotic cells was 66.4%.

Changes of cell cycle and apoptosis

The percentage of cells at G_0/G_1 phase was increased and that

at G_2/M and S was decreased (P<0.01). The rate of apoptotic cells treated with 5 μmol/L of MG-132 for 48 and 96 h was 31.7% and 66.4%, respectively (Table 3, Figure 3).

Table 3 Effect of MG-132 on cell cycle of EC9706 cells (mean±SD, %)

Groups	48 h			96 h		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	40.4±3.9	46.5±4.3	13.1±1.0	44.6±4.1	40.9±2.3	14.5±1.4
MG-132	67.5±5.1[b]	29.3±2.8[b]	3.2±0.2[b]	73.1±5.2[b]	24.6±2.1[b]	2.3±0.1[b]

[b]P<0.01 *vs* control group.

DNA ladder

Agarose electrophoresis showed marked ladders in MG-132 group, while the ladder was not detected in control group. Furthermore, DNA fragmentation was more apparent at 96 h (Figure 4).

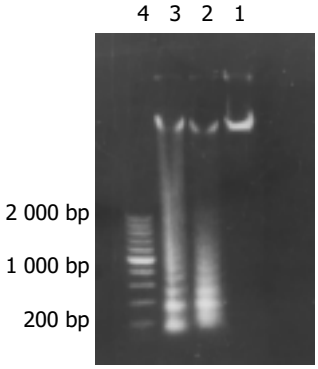

Figure 4 Results of DNA agarose electrophoresis. Lane1: control group; Lane 2: EC9706 cells exposed to 5 μmol/L of MG-132 for 48 h; Lane 3: EC9706 cells exposed to 5 μmol/L of MG-132 for 96 h; Lane 4: 200, 400, 600, 800, 1 000, 1 200, 1 400, 1 600, 1 800, 2 000 bp ladder markers.

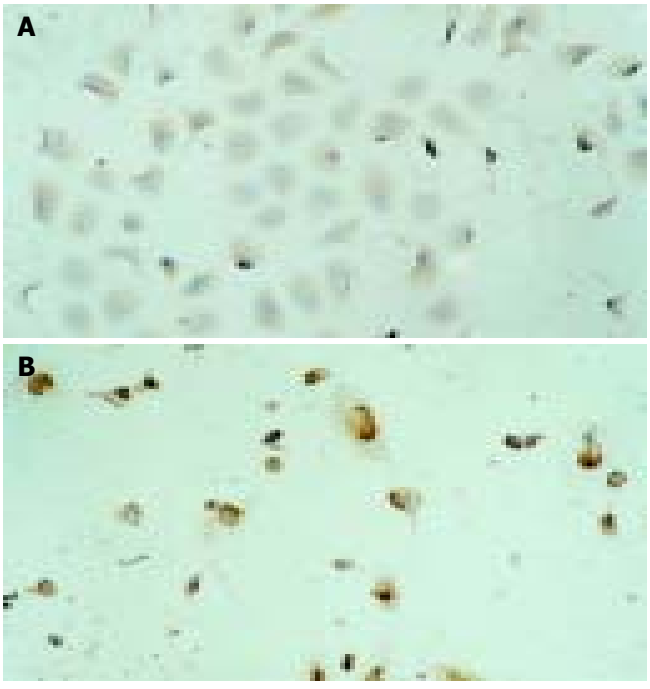

Figure 5 Results of immunohistochemical staining of EC9706 cells. A: control group. Immunohistochemical staining of $p27^{kip1}$ protein located in cytoplasm of EC9706 cells; B: MG-132 group. Immunohistochemical staining of $p27^{kip1}$ protein located in cytoplasm and nuclei (×200).

Expression of p27kip1

In the control group, the cytoplasm was stained in brownish yellow and the nuclei were stained in blue. In the experiment group, both the cytoplasm and nuclei were stained in brownish yellow, indicating that the expression of $p27^{kip1}$ in EC9706 cells was increased after treated with MG-132 (Figure 5).

DISCUSSION

Proteolytic degradation by ubiquitin-proteasome system involves ATP-dependent covalent attachment of a macromolecular chain of ubiquitin (Ub) molecules to the target protein, followed by degradation through the multicatalytic 26s proteasome. The conjugation of Ub, a highly conserved 8.6 kDa protein, to its target protein is mediated by the serial actions of three enzymes. E1, the Ub-activating enzyme, activates Ub in an ATP-dependent manner. E2, the Ub-conjugating enzyme, catalyzes the attachment of Ub to the substrate protein. E3, the Ub-ligase, serves as a scaffold between E2 and the substrate and provides recognition specificity of the substrate[7-10]. A protein tagged with a polyubiquitin chain is recognized and degraded by the 26S proteasome complex. This complex is composed of a 19S regulatory subcomplex and two 20S catalytic subcomplexes. UPP is extensively involved in physiological and biochemical processes. Some experiments showed that the low expression of some anti-oncogene including p53, $p27^{kip1}$ in tumor cells was associated with the increasing activity of ubiquitin proteasome which leads to degradation of expression products of anti-oncogene, and have proved that deubiquitination of p53 is an important pathway for p53 stabilization[11,12]. Moreover, the degradation accommodation of some transcription factors was regulated by UPP, such as *NF-κB, c-fos, c-jun, c-mos, c-myc* and *MATa*[13-17]. So UPP is closely associated with the occurrence and development of malignant tumor.

Ubiquitin proteasome inhibitors include peptide aldehyde, borofax peptide and 3, 4-dichloro isocoumarin. MG-132, also known as carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, a reversible, effective and specific peptide aldehyde inhibitor of ubiquitin-proteasome, could block UPP through inhibiting ubiquitin-mediated proteolysis by binding to and inactivating 20S and 26S proteasomes[18-22].

In our study, esophageal cancer cell line EC9706 was exposed to MG-132 to observe whether UPP could be inhibited. We found that the proliferation of cells was obviously inhibited in a dose- and time-dependent manner. The results also revealed some anti-tumor mechanisms of MG-132. First, MG-132 could up-regulate the expression of $p27^{kip1}$. $p27^{kip1}$ was recently found[23] as a anti-oncogene with function of negative regulation of cell cycle, getting involved in the inhibitive reaction of cytokines, inducing cell differentiation and apoptosis, enhancing cell's adherence and regulating the resistance to medicines for noumenal tumors. $p27^{kip1}$ protein is a cyclin dependent kinase inhibitor (CDKI) that could block G_1/S transition of cell cycle by inhibiting the action of cyclin E-CDK2 complex and cyclin D-CDK4 complex[24-26]. $p27^{kip1}$ expression decreases in esophageal cancer and it may correlate with the histologic differentiation. Reduction of $p27^{kip1}$ has been considered to be an independent prognostic indicator of esophageal cancer[27-30]. The nuclear localization signal of $p27^{kip1}$ contains a protein kinase B (PKB/Akt) consensus site at threonine 157, and phosphorylation of $p27^{kip1}$ by PKB/Akt has been found to impair its nuclear import[31,32], which is a key procedure to play its functional role[33]. We found that $p27^{kip1}$ protein localized in cytoplasm of EC9706 cells showed low expression, but that localized both in cytoplasm

and nuclei of EC9706 cells showed high expression after treated with MG-132. Our previous studies[6,34] demonstrated that the growth of EC9706 cells and tumors implanted in nude mice was obviously inhibited, apoptosis was induced and cell cycle was arrested in G_1 phase by up-regulating $p27^{kip1}$. Second, MG-132 could depress telomerase activity. The activation of telomerase was closely associated with cyclin. It has been reported that inhibition of UPP could not only increase the expression of $p27^{kip1}$, but also increase the expression of p53[35]. Moreover $p27^{kip1}$ and p21 regulated by p53 could inhibit cyclin and result in decreased telomerase activity[36,37]. Third, MG-132 could cause G_1 arrest, which may be involved in changes of cell cycle regulatory factors such as $p27^{kip1}$. Fan *et al*.[38] obtained the same results as ours. But Ling *et al*.[39] tended to consider ubiquitin proteasome inhibitors to cause G_2 arrest. The difference may be involved in the different types of cells. The last, MG-132 could induce apoptosis, which may be closely associated with the functions mentioned above.

In conclusion, MG-132 can obviously inhibit proliferation of EC9706 cells and induce apoptosis. The mechanisms include upregulation of $p27^{kip1}$ expression, G_1 arrest and depression of telomerase activity. The results indicate that inhibiting UPP is a novel strategy for esophageal carcinoma therapy.

REFERENCES

1 **Zhang WG**, Wu QM, Wang XH, Xie GJ, Yu JP. Relationship between expression of survivin gene and biological characteristics in human esophageal carcinoma. *J Chinese Physician* 2003; **5**: 1378-1380

2 **Wu QM**, Li SB, Wang Q, Wang DH, Li XB, Liu CZ. The expression of COX-2 in esophageal carcinoma and its relation to clinicopathologic characteristics. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 11-14

3 **Li SB**, Wu QM, Wang Q, Wang XH, Xie GJ. Effects of adenovirus-mediated human COX-2 antisense RNA on synthesis of DNA and proteins in esophgeal carcinoma cell line. *Shijie Huaren Xiaohua Zazhi* 2003; **11**: 517-521

4 **King RW**, Deshaies RJ, Peters JM, Kirschner MW. How proteolysis drives the cell cycle. *Science* 1996; **274**: 1652-1659

5 **Fuchs SY**. The role of ubiquitin-proteasome pathway in oncogenic signaling. *Cancer Biol Ther* 2002; **1**: 337-341

6 **Wu QM**, Yu JP, Tong Q, Wang XH, Xie GJ. Inhibition of adenovirus-mediated p27*kip1* gene on growth of esophageal carcinoma cell strain. *World J Gastroenterol* 2003; **9**: 2404-2408

7 **Hellmann H**, Estelle M. Plant development: regulation by protein degradation. *Science* 2002; **297**: 793-797

8 **Whitehouse AS**, Tisdale MJ. Increased expression of the ubiquitin-proteasome pathway in murine myotubes by proteolysis-inducing factor (PIF) is associated with activation of the transcription factor NF-kappaB. *Br J Cancer* 2003; **89**: 1116-1122

9 **von der Lehr N**, Johansson S, Larsson LG. Implication of the ubiquitin/proteasome system in Myc-regulated transcription. *Cell Cycle* 2003; **2**: 403-407

10 **Yun J**, Lee WH. Degradation of transcription repressor ZBRK1 through the ubiquitin-proteasome pathway relieves repression of Gadd45a upon DNA damage. *Mol Cell Biol* 2003; **23**: 7305-7314

11 **Lim MS**, Adamson A, Lin Z, Perez-Ordonez B, Jordan RC, Tripp S, Perkins SL, Elenitoba-Johnson KS. Expression of Skp2, a p27*kip1* ubiquitin ligase, in malignant lymphoma: correlation with p27*kip1* and proliferation index. *Blood* 2002; **100**: 2950-2956

12 **Li M**, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* 2002; **416**: 648-653

13 **McDade TP**, Perugini RA, Vittimberga FJ Jr, Callery MP. Ubiquitin-proteasome inhibition enhances apoptosis of human pancreatic cancer cells. *Surgery* 1999; **126**: 371-377

14 **Salvat C**, Aquaviva C, Jariel-Encontre I, Ferrara P, Pariat M, Steff AM, Carillo S, Piechaczyk M. Are there multiple proteolytic pathways contributing to c-Fos, c-Jun and p53 protein degradation *in vivo*? *Mol Biol Rep* 1999; **26**: 45-51

15 **Lafarga M**, Berciano MT, Pena E, Mayo I, Castano JG, Bohmann D, Rodrigues JP, Tavanez JP, Carmo-Fonseca M. Clastosome: a subtype of nuclear body enriched in 19S and 20S proteasomes, ubiquitin, and protein substrates of proteasome. *Mol Biol Cell* 2002; **13**: 2771-2782

16 **Gregory MA**, Hann SR. c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. *Mol Cell Biol* 2000; **20**: 2423-2435

17 **von der Lehr N**, Johansson S, Wu S, Bahram F, Castell A, Cetinkaya C, Hydbring P, Weidung I, Nakayama K, Nakayama KI, Soderberg O, Kerppola TK, Larsson LG. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. *Mol Cell* 2003; **11**: 1189-1200

18 **Mailhes JB**, Hilliard C, Lowery M, London SN. MG-132, an inhibitor of proteasomes and calpains, induced inhibition of oocyte maturation and aneuploidy in mouse oocytes. *Cell Chromosome* 2002; **1**: 2

19 **Steinhilb ML**, Turner RS, Gaut JR. The protease inhibitor, MG132, blocks maturation of the amyloid precursor protein Swedish mutant preventing cleavage by beta-Secretase. *J Biol Chem* 2001; **276**: 4476-4484

20 **Bonuccelli G**, Sotgia F, Schubert W, Park DS, Frank PG, Woodman SE, Insabato L, Cammer M, Minetti C, Lisanti MP. Proteasome inhibitor (MG-132) treatment of mdx mice rescues the expression and membrane localization of dystrophin and dystrophin-associated proteins. *Am J Pathol* 2003; **163**: 1663-1675

21 **Shibata T**, Imaizumi T, Tamo W, Matsumiya T, Kumagai M, Cui XF, Yoshida H, Takaya S, Fukuda I, Satoh K. Proteasome inhibitor MG-132 enhances the expression of interleukin-6 in human umbilical vein endothelial cells: Involvement of MAP/ERK kinase. *Immunol Cell Biol* 2002; **80**: 226-230

22 **Banerjee D**, Liefshitz A. Potential of the proteasomal inhibitor MG-132 as an anticancer agent, alone and in combination. *Anticancer Res* 2001; **21**: 3941-3947

23 **Polyak K**, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. p27*kip1*, a cyclin-CdK inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* 1994; **8**: 9-22

24 **Manne U**, Jhala NC, Jones J, Weiss HL, Chatla C, Meleth S, Suarez-Cuervo C, Grizzle WE. Prognostic significance of p27*kip-1* expression in colorectal adenocarcinomas is associated with tumor stage. *Clin Cancer Res* 2004; **10**: 1743-1752

25 **Center DM**, Cruikshank WW, Zhang Y. Nuclear pro-IL-16 regulation of T cell proliferation: p27(*kip1*)-dependent G0/G1 arrest mediated by inhibition of Skp2 transcription. *J Immunol* 2004; **172**: 1654-1660

26 **Wang G**, Reisdorph R, Clark RE Jr, Miskimins R, Lindahl R, Miskimins WK. Cyclin dependent kinase inhibitor p27(*kip1*) is upregulated by hypoxia via an ARNT dependent pathway. *J Cell Biochem* 2003; **90**: 548-560

27 **Yasunaga M**, Tabira Y, Nakano K, Iida S, Ichimaru N, Nagamoto N, Sakaguchi T. Accelerated growth signals and low tumor-infiltrating lymphocyte levels predict poor outcome in T4 esophageal squamous cell carcinoma. *Ann Thorac Surg* 2000; **70**: 1634-1640

28 **Shamma A**, Doki Y, Tsujinaka T, Shiozaki H, Inoue M, Yano M, Kawanishi K, Monden M. Loss of p27(*kip1*) expression predicts poor prognosis in patients with esophageal squamous cell carcinoma. *Oncology* 2000; **58**: 152-158

29 **Shibata H**, Matsubara O, Wakiyama H, Tanaka S. The role of cyclin-dependent kinase inhibitor p27 in squamous cell carcinoma of the esophagus. *Pathol Res Pract* 2001; **197**: 157-164

30 **Taniere P**, Martel-Planche G, Saurin JC, Lombard-Bohas C, Berger F, Scoazec JY, Hainaut P. TP53 mutations, amplification of P63 and expression of cell cycle proteins in squamous cell carcinoma of the oesophagus from a low incidence area in Western Europe. *Br J Cancer* 2001; **85**: 721-726

31 **Liang J**, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med* 2002; **8**: 1153-1160

32 **Viglietto G**, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A, Santoro M. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27*kip1* by PKB/Akt-mediated phosphorylation in breast cancer. *Nat Med* 2002; **8**: 1136-1144

33 **Singh SP**, Lipman J, Goldman H, Ellis FH Jr, Aizenman L, Cangi MG, Signoretti S, Chiaur DS, Pagano M, Loda M. Loss or altered subcellular localization of p27 in Barrett's associated adenocarcinoma. *Cancer Res* 1998; **58**: 1730-1735

34 **Zhang WG**, Wu QM, Tong Q, Yu JP. Inhibitory effect of p27*kip1* mediated by adenovirus on model of esophageal carcinoma in nude mice. *Shijie Huaren Xiaohua Zazhi* 2003; **11**: 512-516

35 **Fan XM**, Wong BC, Wang WP, Zhou XM, Cho CH, Yuen ST, Leung SY, Lin MC, Kung HF, Lam SK. Inhibition of proteasome function induced apoptosis in gastric cancer. *Int J Cancer* 2001; **93**: 481-488

36 **Liu XJ**, Wu QM, Tong Q, Zhang WG, Liu CZ, Yu JP. The effect of p27*kip1* cDNA on the telomerase activity and cell cycle of SGC7901 cells. *Shiyong Aizheng Zazhi* 2003; **18**: 253-256

37 **Harada K**, Kurisu K, Sadatomo T, Tahara H, Tahara E, Ide T, Tahara E. Growth inhibition of human glioma cells by transfection-induced P21 and its effects on telomerase activity. *J Neurooncol* 2000; **47**: 39-46

38 **Fan XM**, Wong BC, Wang WP, Zhou XM, Cho CH, Yuen ST, Leung SY, Lin MC, Kung HF, Lam SK. Inhibition of proteasome function induced apoptosis in gastric cancer. *Int J Cancer* 2001; **93**: 481-488

39 **Ling YH**, Liebes L, Ng B, Buckley M, Elliott PJ, Adams J, Jiang JD, Muggia FM, Perez-Soler R. PS-341, a novel proteasome inhibitor, induces Bcl-2 phosphorylation and cleavage in association with G2-M phase arrest and apoptosis. *Mol Cancer Ther* 2002; **1**: 841-849

Edited by Kumar M and Wang XL **Proofread by** Xu FM