

Akt associates with nuclear factor κ B and plays an important role in chemoresistance of gastric cancer cells

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Abstract. The ubiquitously expressed serine-threonine kinase Akt and the transcription factor NF- κ B both are involved in cell proliferation and apoptosis. Furthermore, the activation of Akt or NF- κ B has been suggested to associate with chemoresistance of human tumors. The exact mechanism and interreaction of Akt and NF- κ B pathway on chemoresistance in gastric cancer is still unknown. We explored the function of Akt and NF- κ B pathway on chemoresistance in human gastric cancer cells. MTT method was used to analyze the influence of chemotherapeutics and the combined use of wortmannin or MG-132 on the growth of SGC-7901 cells. Apoptosis of SGC-7901 was detected by TUNEL and Annexin V/PI methods. The protein level of NF- κ B was analyzed by immunocytochemical staining. EMSA was used to confirm the increased nuclear translocation of RelA. The protein level of p-Akt and p-I κ B α were analyzed by Western blotting. Etoposide and doxorubicin suppressed the growth of SGC-7901 time and dose-dependently. Combined use of wortmannin or MG-132 can suppress growth further. Chemotherapeutics induced apoptosis of SGC-7901 and activated Akt and NF- κ B, combined use of wortmannin or MG-132 induced apoptosis further and attenuated the activation of NF- κ B. The combined use of wortmannin attenuated the activation of Akt, but combined use of MG-132 did not attenuate the activation of Akt. The activation of NF- κ B is a branch mechanism of Akt anti-apoptosis effects. The chemotherapeutics induced apoptosis and induced the activation of Akt and NF- κ B in SGC-7901 cell, suppression the activation of Akt or NF- κ B can increase the effects of chemotherapeutics. NF- κ B is a downstream target of Akt.

Introduction

Gastric cancer remains one of the major health problems worldwide, and it is one of the most common cancers and the leading cause of cancer-related deaths in China. Chemotherapy is an important therapeutic modality for gastric cancer besides surgical resection, although the success rate of this treatment is limited because of chemoresistance. The rationale of anticancer chemotherapy relies mainly on DNA damaging insults in rapidly dividing tumor cells, imposing a strong apoptotic trigger. However, some tumor cells can obtain chemoresistance through adjusting some survival signal transduction pathway. Therefore, the efficacy of chemotherapeutics is severely limited because of chemoresistance. Several studies have suggested that the serine-threonine kinase Akt and the transcription factor NF- κ B, are the key molecules for protecting cells from undergoing apoptosis, and that the Akt and NF- κ B mediated survival signaling pathway is associated with chemoresistance of human tumors (1-3).

Akt is a serine-threonine protein kinase also known as protein kinase B. It was characterized initially as the human homologue of the viral oncogene v-Akt from the transforming retrovirus Akt8. The induction of Akt activity is primarily under the control of the phosphoinositide products of PI3K. PIP2 and PIP3 bind to the PH domain of Akt, resulting in translocation of Akt to the plasma membrane area (4,5). Akt has been reported to be a signal transduction protein that figures prominently in the mechanisms of carcinogenesis and chemoresistance (3,6,7).

The NF- κ B transcription factor is a pleiotropic activator that participates in the induction of a wide variety of cellular genes. In addition to its role in inflammation and immune response, NF- κ B has also been implicated in the suppression of apoptosis, cellular survival, transformation, and oncogenesis (8-10).

Several reports have shown that Akt and NF- κ B play a critical role in promoting cell proliferation and inhibiting cell death, and the increased activity of Akt and NF- κ B could be directly or indirectly related to the resistance to chemotherapy (11-13). It has been reported that Akt was upstream of NF- κ B in several signaling events, but some reports indicated that this may not necessarily be the case and that Akt is a downstream target of NF- κ B (14,15). The exact mechanism and the interreaction of Akt and NF- κ B

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pathway on chemoresistance in gastric cancer is not clear. Thus, the present study was under-taken to elucidate the role of Akt and NF- κ B pathway, and the correlation between Akt activation and NF- κ B activation in the development of chemoresistance in gastric cancer.

Materials and methods

Materials and reagents. Etoposide, doxorubicin, wortmannin and MG-132 were purchased from ALEXIS Biochemicals. The mouse monoclonal anti-NF- κ B/p65 antibody was purchased from Santa Cruz Biotechnology. The mouse monoclonal anti-phospho-Akt (ser473), anti-phospho-I κ B α antibodies and Western blot assay kit were purchased from Cell Signal Tech. The EMSA assay kit was purchased from Promega Tech. The TUNEL and Annexin V/PI assay kit were purchased from Roche Tech.

Cell culture. The human gastric cancer cell line SGC7901 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and maintained at 37°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO₂ and 95% air. For experimental purposes, cells were plated in 35-mm dishes at 1x10⁶ cells/dish and grown in RPMI-1640 medium for 5-7 days.

MTT assay. Cultured cells were seeded at a density of 4x10³ cells/well on a 96-well plate. At 24 h after seeding, various concentrations of chemotherapeutics (doxorubicin, 0, 0.03, 0.3, 3 μ mol/l; etoposide, 0, 2, 20, 200 μ mol/l) were added to the culture medium. Combined use of chemotherapeutics and Akt inhibitor (wortmannin) or NF- κ B inhibitor (MG-132), requires pretreatment by wortmannin (40 nmol/l) or MG-132 (10 μ mol/l) for 2 h. Then various concentrations of chemotherapeutics (as above) were added to the culture medium. Viability of cells at 0, 3, 6, 12, 24 h after drug treatment was evaluated by the MTT assay. Controls were treated with DMSO vehicle at a concentration equal to that in drug-treated cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well at a concentration of 500 μ g/ml, and plates were incubated for 4 h at 37°C. After 4 h, media were aspirated, cells were lysed with 400 μ l DMSO. Cells were incubated for a further 10 min at 37°C with gentle shaking. Absorbance readings at 570 nm were determined using a computer-controlled microplate analyzer. The inhibitory rate (%) is calculated using the following equation: inhibitory rate (%) = (1-OD_A/OD_C) x 100, where OD_A, the OD value of group with treatment of drugs and OD_C the OD value of group without treatment of drugs.

Immunocytochemistry. Cultured cells were seeded on slides. At 24 h after seeding, various concentrations of chemotherapeutics (as above) were added to the culture medium. For combined use of chemotherapeutics and Akt inhibitor or NF- κ B inhibitor, cells were pretreated by wortmannin (40 nmol/l) or MG-132 (10 μ mol/l) for 2 h. Then various concentrations of chemotherapeutics (doxorubicin, 0.3 μ mol/l; etoposide, 20 μ mol/l) were added to the culture medium. Immunostaining

was performed as previously described (16). In specimens containing positive cells, the positive cells were counted in ten randomly selected fields x200 or x400 magnification for each sample, and the average was expressed as the density of positive cells.

Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL). The drug intervention was the same as above. Apoptosis of SGC-7901 were detected by TUNEL, and TUNEL staining was carried out according to TUNEL assay kit manual. Positive signals were defined as presence of a distinct brown color nuclear staining of the cancer cells or morphologically defined apoptotic bodies. The apoptotic index (AI) was determined by counting a total of at least 1,000 cells nuclei in 10 randomly chosen fields at x400 magnification.

Annexin V/PI staining. The drug intervention was the same as above. Cells suspended at each chosen time point after various concentrations of anticancer treatment were fixed with ice-cold 75% ethanol, washed twice with PBS, and incubated in 20 μ g/ml RNase A for 30 min at 37°C. Subsequently, cells were stained with Annexin-V-Fluorescein and propidium iodide (PI), and measured for DNA content by flow cytometry (Becton-Dickinson, Helmetta, NJ).

Electrophoretic mobility shift assay. The drug intervention was the same as above. Nuclear extracts were harvested according to protocols described previously (17). The protein concentrations of nuclear extracts were determined by Bio-Rad protein assay. Nuclear protein extracts were analyzed by EMSA for NF- κ B nuclear translocation as previously described (18,19). Nuclear extracts were incubated with a γ -³²P-labeled oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') containing a consensus NF- κ B binding site for 30 min at room temperature. For super-shift experiments, 2 μ g of mouse monoclonal antibodies against the p65 subunit (Santa Cruz Biotechnology) of NF- κ B were incubated with the nuclear extracts 10 min before the addition of the γ -³²P-labeled probe and then analyzed as described. For specific competitor experiments, superfluous non-labeled probe was incubated with the nuclear extracts. Samples were electrophoresed at 100 V and 4°C, and gels were dried and exposed to X-ray film (Kodak).

Immunoblot analysis. The drug intervention was the same as above. Cells were lysed in ice-cold lysis buffer (1X PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, 60 μ g/ml aprotinin, 10 μ g/ml trypsin inhibitor and 10 μ g/ml leupeptin). After 10-min centrifugation at 10,000 g at 4°C, the supernatants were transferred into new microcentrifuge tubes and the protein concentration of the supernatant was measured using BCA protein assay. Then stored under at -20°C. Cell lysates (50 μ g) were separated on SDS-PAGE gels. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes. For detection of proteins, membranes were blocked using 5% non-fat dried milk in Tris buffer containing 0.1% Tween (TBS-T). Then incubated at 4°C overnight with anti-phos-

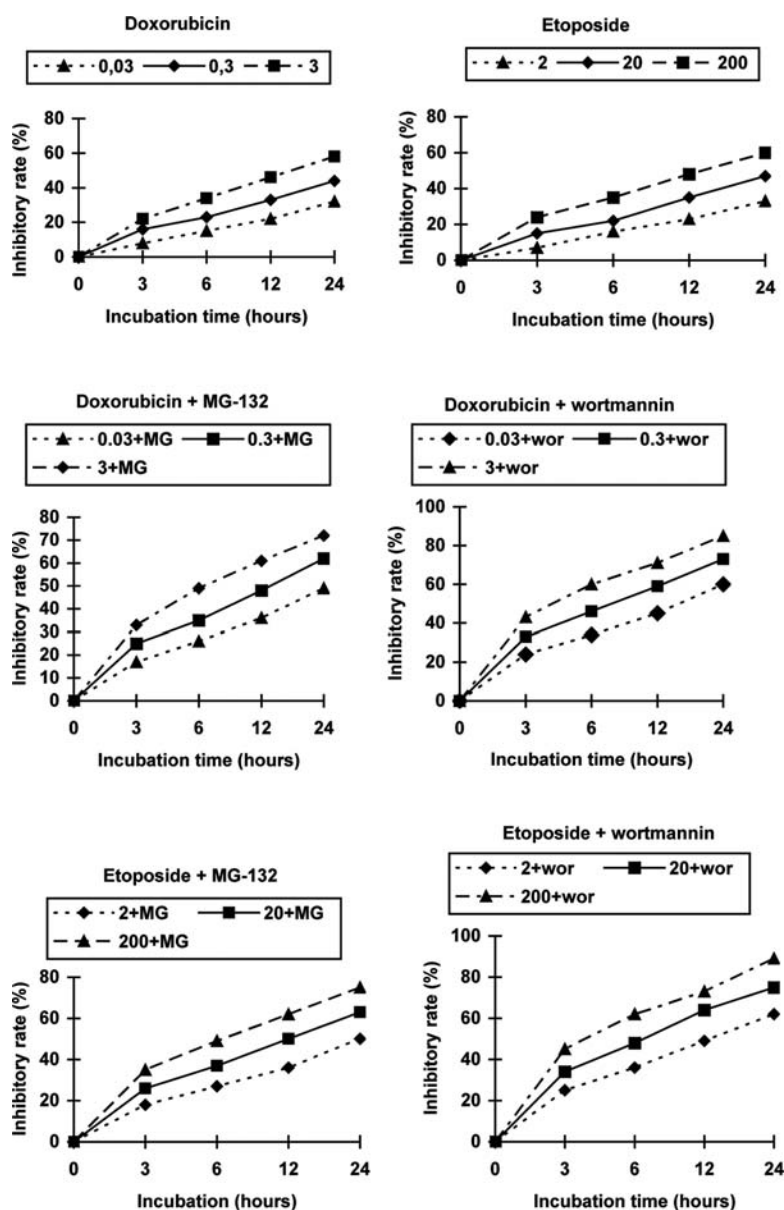


Figure 1. Effect of chemotherapeutics and combined use of inhibitor on growth of gastric cancer cells. An MTT assay was used to detect the growth of gastric cancer cells. Inhibitory rate was significantly increased time and dose-dependently ($p < 0.05$, Student's t-test). Combined use of wortmannin suppresses cell growth further than combined use of MG-132 ($p < 0.05$, Student's t-test).

phor-Akt (ser473) antibody (1:1000) and anti-phospho-I κ B α antibody (1:1000) diluted in TBS-T containing 5% non-fat milk, then subsequently with horseradish peroxidase conjugated anti-mouse antibodies (1:2000). Peroxidase activity was visualized by enhanced chemiluminescence detection.

Statistical analysis. All experiments were repeated at least 3 times. Results are expressed as mean \pm standard deviation (SD). All statistical analyses were performed with SPSS12.0 statistical package for Microsoft Windows. Student's t-test was used to compare continuous variables among groups. A p-value of < 0.05 was considered significant.

Results

Chemotherapeutics inhibit cell growth, combined use of wortmannin or MG-132 inhibits cell growth further. The

effect of chemotherapeutics or jointly using inhibitor on cell growth was measured using MTT assay (Fig. 1). Etoposide or doxorubicin can suppress the growth of SGC-7901, combined use of wortmannin or MG-132 can suppress its growth further, time and dose-dependently. Furthermore, combined use of wortmannin can suppress cell growth further than combined use of MG-132. However, the differences on the inhibitory rate between etoposide and doxorubicin were not statistically significant.

Chemotherapeutics induce NF- κ B/p65 expression, combined use of wortmannin or MG-132 attenuates its expression. The MTT assay indicated growth of cells was inhibited strongly after chemotherapeutics or the combined use of inhibitor for 12-24 h. For better accuracy, we chose 12 h for this treatment time, and the drug concentration (doxorubicin, 0.3 μ mol/l; etoposide, 20 μ mol/l) in later experiments. NF- κ B/p65 protein

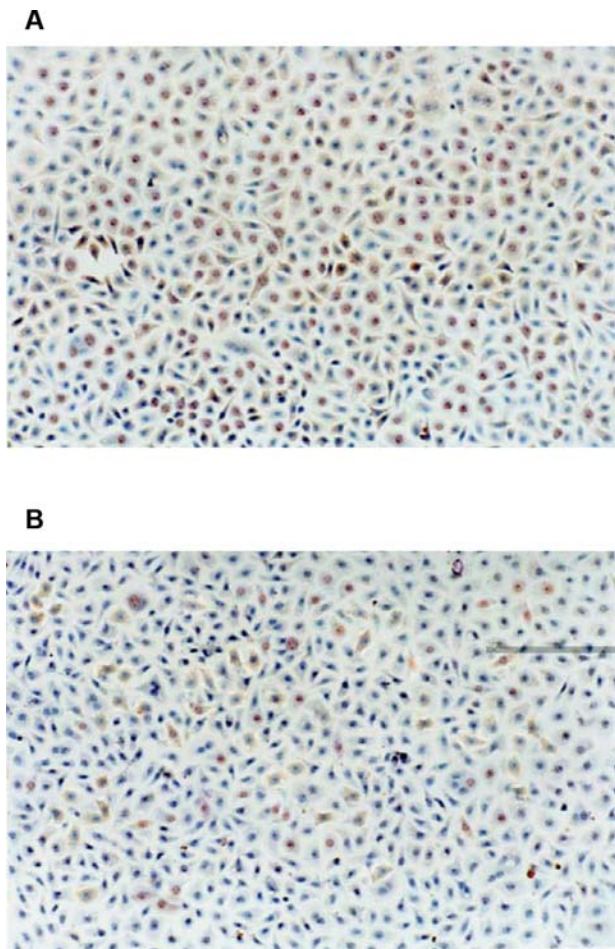


Figure 2. Immunocytochemical staining of NF- κ B/p65. Positive staining of NF- κ B/p65 was observed in the cytoplasm and nuclear staining. (A) The NF- κ B/p65 expression in cells treated by doxorubicin (0.3 μ mol/l) (x200). (B) The NF- κ B/p65 expression in cells treated by doxorubicin (0.3 μ mol/l) + MG-132 (10 μ mol/l pretreatment) (x200).

expression was measured by immunocytochemical staining. Positive staining of NF- κ B/p65 was mainly observed in the cytoplasm, and nuclear staining was also detected. Etoposide or doxorubicin induce the NF- κ B/p65 expression, and its expression was attenuated by the combined use of wortmannin or MG-132. However, the differences on the inhibitory rate between combined use of wortmannin and MG-132 were not statistically significant (Fig. 2).

The effect of chemotherapeutics and combined use of inhibitor on NF- κ B-DNA binding activity. We found that etoposide or doxorubicin induced a dose-dependent increase in NF- κ B-DNA binding activity. Combined use of wortmannin or MG-132 can attenuate NF- κ B-DNA binding activity (Fig. 3). The differences on the inhibiting effect between combined use of wortmannin and MG-132 were not statistically significant. To confirm the specificity of NF- κ B-DNA binding, we performed super-shift analysis with antibodies specific for p65 and a competitive study with a 50-fold excess of unlabeled oligonucleotide. An antibody specific for p65 which recognizes NF- κ B/p65 heterodimer, unlabeled oligonucleotides diminished the intensity of NF-

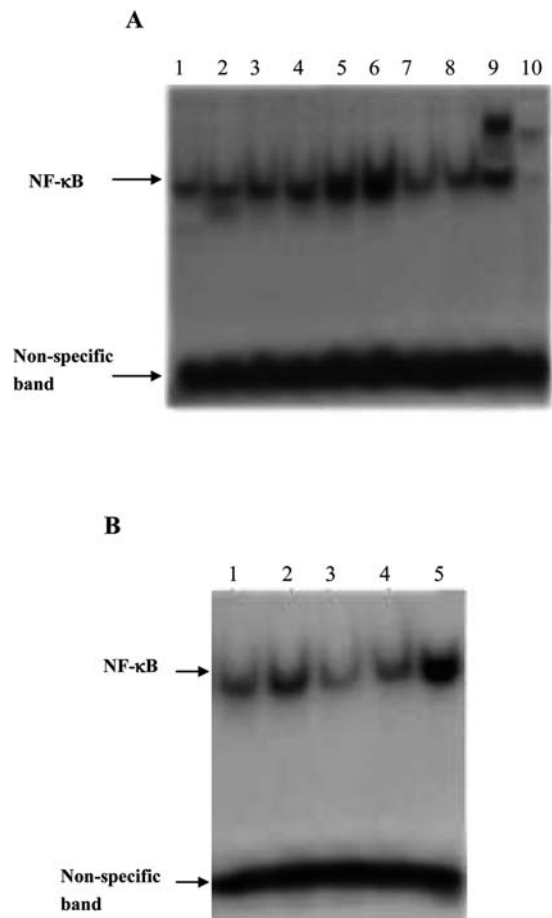


Figure 3. EMSA was used to detect NF- κ B-DNA binding activity. (A) The effect of etoposide, doxorubicin and combined use of MG-132 (10 μ mol/l pretreatment) on NF- κ B activity. Lane 1, etoposide 2 μ mol/l; lane 2, doxorubicin 0.03 μ mol/l; lane 3, etoposide 20 μ mol/l; lane 4, doxorubicin 0.3 μ mol/l; lane 5, etoposide 200 μ mol/l; lane 6, doxorubicin 3 μ mol/l; lane 7, etoposide 20 μ mol/l + MG-132; lane 8, doxorubicin 0.3 μ mol/l + MG-132; lane 9, super-shift experiments; lane 10, specific competitor experiments. (B) The effect of etoposide, doxorubicin and combined use of wortmannin (40 nmol/l pretreatment) on NF- κ B activity. Lane 1, doxorubicin 0.3 μ mol/l + wortmannin; lane 2, doxorubicin 0.3 μ mol/l; lane 3, etoposide 20 μ mol/l + wortmannin; lane 4, etoposide 20 μ mol/l; lane 5, positive control.

κ B/p65 complexes, indicating that the complex was the NF- κ B binding-specific band.

The effect of chemotherapeutics and combined use of inhibitor on phosphorylation of Akt and I κ B α . The etoposide or doxorubicin increased the phosphorylation of Akt (ser473) and I κ B α dose-dependently. Combined use of wortmannin can decrease phosphorylation of Akt and I κ B α , however, there is no obvious change in the phosphorylation of Akt after jointly using MG-132. Meanwhile, jointly using wortmannin or MG-132 can decrease phosphorylation of I κ B α , and the differences on the inhibiting effect between two inhibitors were not statistically significant (Fig. 4).

Chemotherapeutics induce apoptosis, combined use of inhibitor induces apoptosis further. In our study, Annexin/PI staining and TUNEL staining were both used to detect cell apoptosis. Because TUNEL staining has low sensitivity to

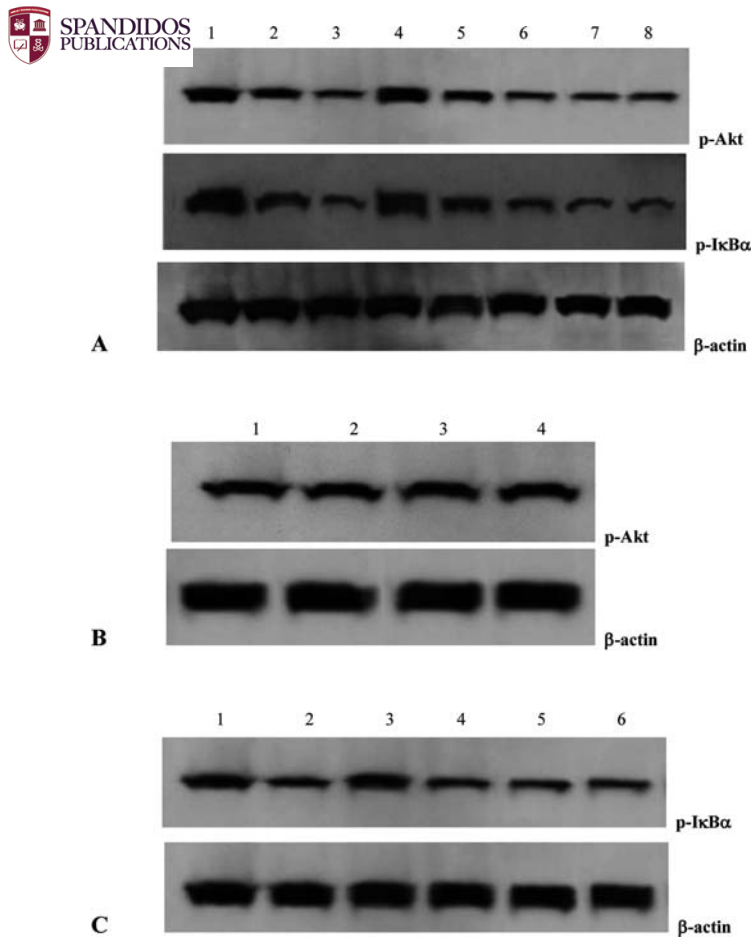


Figure 4. Immunoblot was used to detect p-Akt and p-IκBα. (A) The effect of etoposide, doxorubicin and combined use of wortmannin (40 nmol/l pretreatment) on p-Akt and p-IκBα. Lane 1, doxorubicin 3 μmol/l; lane 2, doxorubicin 0.3 μmol/l; lane 3, doxorubicin 0.03 μmol/l; lane 4, etoposide 200 μmol/l; lane 5, etoposide 20 μmol/l; lane 6, etoposide 2 μmol/l; lane 7, etoposide 20 μmol/l + wortmannin; lane 8, doxorubicin 0.3 μmol/l + wortmannin. (B) The effect of etoposide, doxorubicin and combined use of MG-132 (10 μmol/l pretreatment) on p-Akt. Lane 1, doxorubicin 0.3 μmol/l; lane 2, doxorubicin 0.3 μmol/l + MG-132; lane 3, etoposide 20 μmol/l; lane 4, etoposide 20 μmol/l + MG-132. (C) The effect of etoposide, doxorubicin and jointly using wortmannin (40 nmol/l pretreatment) or MG-132 (10 μmol/l pretreatment) on p-IκBα. Lane 1, doxorubicin 0.3 μmol/l; lane 2, doxorubicin 0.3 μmol/l + MG-132; lane 3, etoposide 20 μmol/l; lane 4, etoposide 20 μmol/l + MG-132; lane 5, doxorubicin 0.3 μmol/l + wortmannin; lane 6, etoposide 20 μmol/l + wortmannin. Immunoblotting result of β-actin is used to show equal loading.

distinguish apoptosis from necrosis, therefore, Annexin/PI staining (Fig. 5) was used to analysis apoptosis. However, we can observe morphological change of a single cell by TUNEL staining (Fig. 6), so the two methods were adopted. Both etoposide and doxorubicin induced apoptosis of SGC-7901 in a concentration dependent manner. Combined use of wortmannin or MG-132 can induce apoptosis further. Furthermore, combined use of wortmannin can induce cell apoptosis further than combined use of MG-132.

Discussion

Chemotherapy is an important therapeutic modality for gastric cancer, however, the therapeutic efficacy of this

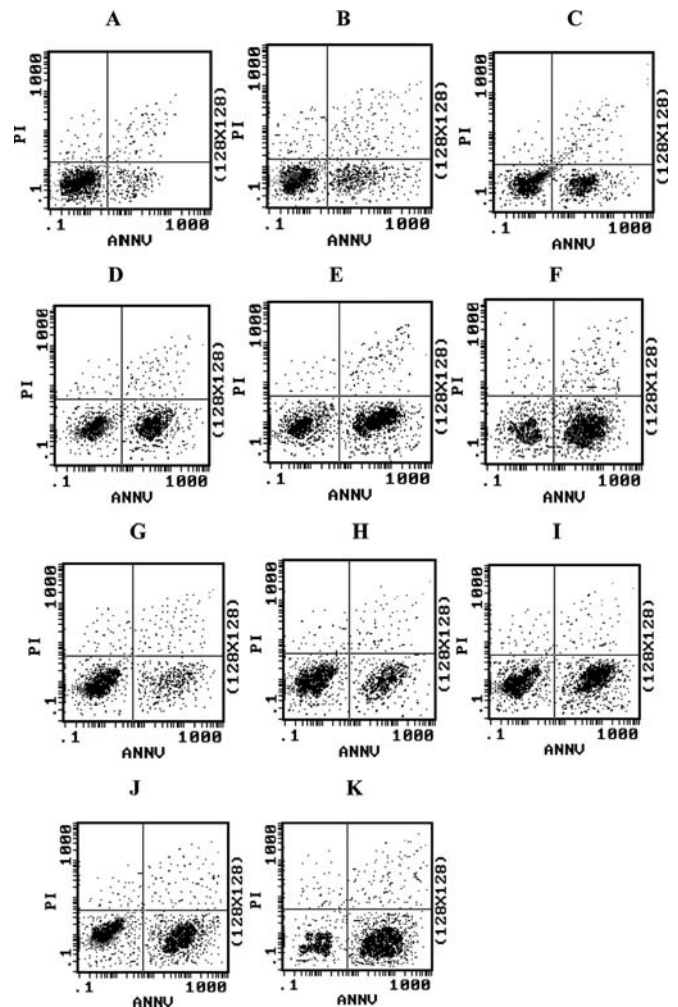


Figure 5. Annexin/PI staining was used to detect apoptosis. Viable, apoptosis and necrotic cell phenotype: Annexin V⁻/PI⁻, Annexin V⁺/PI⁻, Annexin V⁺/PI⁺. (A) Control group; (B) doxorubicin 0.03 μmol/l; (C) doxorubicin 0.3 μmol/l; (D) doxorubicin 3 μmol/l; (E) doxorubicin 0.3 μmol/l + MG-132 (10 μmol/l pretreatment); (F) doxorubicin 0.3 μmol/l + wortmannin (40 nmol/l pretreatment); (G) etoposide 2 μmol/l; (H) etoposide 20 μmol/l; (I) etoposide 200 μmol/l; (J) etoposide 20 μmol/l + MG-132 (10 μmol/l pretreatment); (K) etoposide 20 μmol/l + wortmannin (40 nmol/l pretreatment). Apoptosis increased significantly induced by chemotherapeutics and combined use of inhibitor, time and dose-dependently (p<0.05, Student's t-test). Combined use of wortmannin can induce cell apoptosis further than combined use of MG-132 (p<0.05, Student's t-test).

method decreases when cancer cells develop resistance to chemotherapeutics. Block of cell apoptosis is the main reason of tumor cell resistance to chemotherapeutics. Several studies have suggested that activation of the serine/ threonine kinase Akt and the transcription factor NF-κB are implicated in the protection of cells from apoptosis, and that the Akt or NF-κB-mediated survival signaling pathway play a critical role in cancer chemoresistance (1,2,20,21). This raises the question whether inhibiting the activation of these two signaling pathways, would improve the curative effect of chemotherapy. Would the interaction of Akt and NF-κB be beneficial in chemoresistance?

Akt was characterized initially as the human homologue of the viral oncogene v-Akt from the transforming retrovirus Akt8 (22). Three isoforms of Akt have been identified, the Akt1, Akt2 and Akt3, all of which share a high degree of

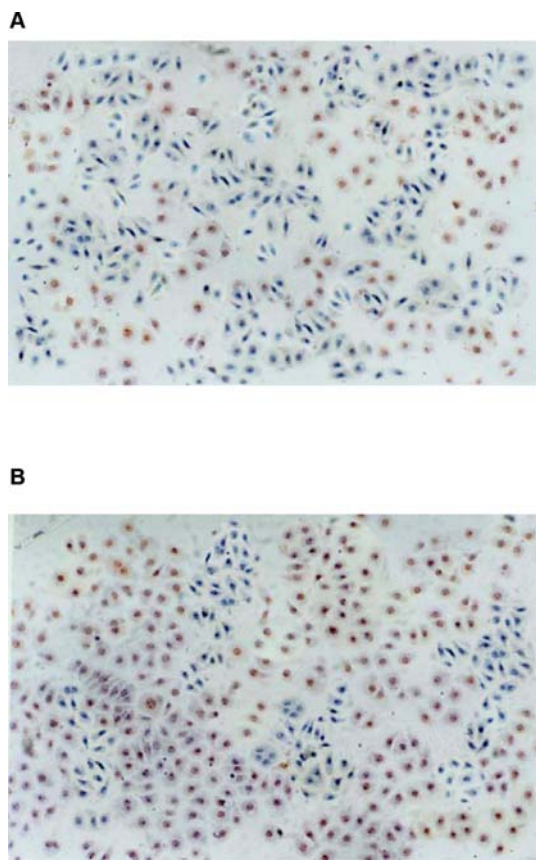


Figure 6. TUNEL was used to detect apoptosis. Positive signals were defined as presence of brown color nuclei. (A) The apoptosis induced in cells treated by doxorubicin ($0.3 \mu\text{mol/l}$) ($\times 200$). (B) Apoptosis induced in cells treated by doxorubicin ($0.3 \mu\text{mol/l}$) + wortmannin (40 nmol/l pretreatment) ($\times 200$).

homology at the amino acid level (23,24). Activation of Akt by extracellular stimuli occurs in a phosphatidylinositol-3-kinase (PI3K)-dependent manner. Phosphorylation of threonine 308 in the activation loop and serine 473 in the COOH-terminal activation domain results in full activation of Akt (25,26). Furthermore, Akt regulates a vast array of fundamental cellular responses and plays a critical role in controlling the balance between cell survival and apoptosis (27,28).

NF- κ B is a family of dimeric transcription factors that control the expression of numerous genes involved in cell growth, regulation of apoptosis, and neoplastic transformation (29,30). The Rel/NF- κ B family comprises NF- κ B1 (p50), NF- κ B2 (p52), and the Rel proteins, RelA (p65), RelB, and c-Rel, which have a high level of sequence homology within their NH₂-terminal 300 amino acids, the Rel homology domain. The most common dimer is the RelA (p65)/NF- κ B1 (p50) heterodimer, i.e., NF- κ B. In most unstimulated cells, NF- κ B proteins are sequestered in the cytoplasm and are complexed with specific inhibitor proteins called I κ B that render the NF- κ B proteins inactive (31). Stimulation of cells leads to phosphorylation and degradation of I κ B and allows translocation of NF- κ B to the nucleus, resulting in expression of target genes. The activation of NF- κ B has been suggested to associate with chemoresistance of human tumors (32).

In our study, etoposide or doxorubicin suppressed the


growth and induced apoptosis of SGC-7901, activated Akt and NF- κ B at the same time. Combined use of wortmannin or MG-132, induced apoptosis further and improved the curative effect of chemotherapy. The results indicated that the activation of Akt and NF- κ B may be one of the mechanisms of gastric cancer cell chemoresistance.

NF- κ B activation requires degradation of I κ B α to free NF- κ B from the heterotrimeric I κ B/NF- κ B complex and to translocate it into the nucleus. I κ B α is the major endogenous inhibitor of NF- κ B activation (32). In our study, chemotherapeutics induced Akt activation, I κ B α phosphorylation and degradation, and then NF- κ B activation. The effect of chemoresistance was attenuated by wortmannin. We speculated whether activation of NF- κ B by chemotherapy may go through the Akt/I κ B α pathway. Therefore, we examined the Akt, NF- κ B and I κ B α levels in our experiments. Our results show that wortmannin can attenuate activation of Akt and NF- κ B. MG-132 can attenuate activation of NF- κ B, however, it can not attenuate activation of Akt. At the same time, wortmannin and MG-132 can inhibit the phosphorylation of I κ B α . Furthermore, the differences on the inhibiting effect on NF- κ B activation and I κ B α phosphorylation between combined use of wortmannin and MG-132 were not statistically significant.

From these results, we consider that Akt and NF- κ B pathway plays an important role in the chemoresistance of gastric cancer cells, NF- κ B is a downstream target of Akt, the activation of NF- κ B is a branch mechanism of Akt resist to apoptosis, and there should exist other regulatory pathway in Akt resistance to apoptosis. For example, Akt phosphorylates the pro-apoptotic Bcl-2 partner Bad (22), which binds to and blocks the activity of Bcl-x, a cell survival factor. Akt can also inactivate initiation caspase-9 and repress the Forkhead transcription factor FKHRL-1, which regulates the apoptosis-inducing Fas ligand expression (33).

However, the correlation between Akt and NF- κ B remains controversial. For example, contradictory findings suggesting NF- κ B activation independent of Akt activation were also reported in HeLa cells and ovarian cancer cells (15). Some other studies have reported that there was a positive reciprocal regulatory loop and crosstalk between NF- κ B and Akt (34). Several reports have described that Akt is a downstream target of NF- κ B (14). Delhase *et al* (15) and Lawrence *et al* (35) point out that I κ B degradation and NF- κ B activation rely on the I κ B kinase (IKK), which consists of two catalytic subunits, IKK α and IKK β . Different cell types and different stimulating signals to determine Akt phosphorylation in IKK sub-units differently, thus determining whether Akt participates in the regulation of NF- κ B, or Akt accepts the regulation of NF- κ B (36). These disparate observations point to deficiencies in our understanding of correlation between Akt and NF- κ B, and also suggest that the Akt/NF- κ B connection is cell-type and stimulus-specific.

In conclusion, our results demonstrate that the chemotherapeutic drugs can induce apoptosis and induce the activation of Akt and NF- κ B in the SGC-7901 cell line, inducible Akt and NF- κ B activities are involved in chemoresistance of gastric cancer cells. NF- κ B is a downstream target of Akt, and the activation of NF- κ B is a branch

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 m of Akt resistance to apoptosis. New strategies for chemotherapy of gastric cancer should be designed to more specifically block Akt/NF- κ B as to decrease the number of resistant cells.

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