Bax is involved in the anticancer activity of Velcade in colorectal cancer

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Received September 19, 2016; Accepted June 8, 2017

DOI: 10.3892/etm.2017.4857

Abstract. Numerous chemotherapeutic agents promote tumor cell death by activating the intrinsic apoptosis signaling pathway. This pathway is regulated by mitochondrial dysfunction, which occurs through an intricate process controlled by complex interactions between B-cell lymphoma 2 (Bcl-2) family members and other cellular proteins. Bcl-2-associated X protein (Bax) is a proapoptotic protein that is an essential component of the intrinsic apoptosis signaling pathway. Patients lacking Bax may be less sensitive to chemotherapy due to an impaired intrinsic apoptosis signaling pathway. The present study demonstrated that Bax expression in colorectal cancer (CRC) tissues was typically increased compared with that in adjacent normal tissues. Furthermore, $Bax^{-/-}$ HCT-116 cells exhibited reduced proliferation and colony formation ability compared with $Bax^{+/+}$ HCT116 cells, although the rate of apoptosis of these cells remained unchanged. However, $Bax^{-/-}$ HCT116 cells became more resistant to apoptosis when treated with Velcade. The results of the present study provide novel insights into the relevance of Bax expression to the prognosis of CRC.

Introduction

Colorectal cancer (CRC) remains one of the leading types of cancer in humans, causing >550,000 mortalities annually in the United States (1). In addition, the incidence of CRC has increased in Eastern and Western countries over the past decades (2). To improve the survival of patients with CRC, personalized therapy targeting reliable biomarkers is required. B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), a proapoptotic protein belonging to the Bcl-2 family is one of the most promising apoptosis-associated biomarkers in several types of human cancers. Bax is an essential component of the intrinsic apoptosis signaling pathway. Patients lacking Bax may be less sensitive to chemotherapy due to an impaired intrinsic apoptosis signaling pathway (3). In CRC, Bax is positively correlated with improved survival outcomes (4,5). However, a previous study reported that the dysregulation of Bax could lead to the resistance of human CRC cells to treatment with sulindac, an effective nonsteroidal anti-inflammatory drug (NSAID) with a chemopreventive effect on CRC (6). Thus far, the use of Bax as a biomarker to aid in clinical decision-making has not been substantiated. Further studies should focus on elucidating the significance of Bax on the apoptosis of CRC cells in response to chemotherapy.

Velcade, also known as bortezomib, is a specific and selective inhibitor of the 26S proteasome (7). Velcade was the first proteasome inhibitor approved by the US Food and Drug Administration for the treatment of relapsed or refractory multiple myeloma and mantle cell lymphoma (8). Similar to other proteasome inhibitors, Velcade can stabilize several proteins by inhibiting their degradation; these proteins include transcriptional factors (proto-oncogenes c-Myc, c-Fos and c-Jun), p21, cyclin-dependent kinase inhibitor p27, cellular tumor antigen p53 (p53), nuclear factor κB inhibitor α, cyclins and Bcl-2 family members (Bcl-2 homologous antagonist/killer and Bax) (9,10). Additionally, Velcade can induce the apoptosis of tumor cells through the intrinsic mitochondrial, extrinsic death receptor and endoplasmic reticulum stress response apoptosis signaling pathways (11-13). Clinical studies have revealed that Velcade exhibits limited efficacy against solid tumors when used as a single agent (14-18). However, reports from phase I/II clinical trials have indicated that when combined with other chemotherapy drugs Velcade can significantly sensitize tumor cells to chemotherapy (19). The combination of Velcade with oxaliplatin, leucovorin and 5-fluorouracil as a treatment for CRC has previously been studied (20,21). One clinical trial revealed that 5 of 13 patients had a partial response to Velcade (21). Further trials are required to verify the effectiveness of Velcade. Furthermore, the underlying molecular mechanisms of this regimen remain unclear.

The present study revealed that Bax expression was increased in CRC specimens compared with adjacent normal

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Key words: colorectal cancer, Velcade, Bax, apoptosis

tissues. $Bax^{-/-}$ HCT116 cells revealed slow growth and modest colony formation ability. When treated with Velcade, the $Bax^{-/-}$ HCT116 cells demonstrated a higher resistance to apoptosis compared with $Bax^{+/+}$ HCT116 cells. These results indicate that Bax is a potential marker for predicting the response of patients with CRC to Velcade therapy. In addition, these results suggest that Bax is associated with the anticancer activity of Velcade in CRC.

Materials and methods

Tissue samples. In total, 13 pairs of CRC and adjacent normal tissue samples (intraoperative tissue was snap-frozen in liquid nitrogen, at -196˚C) were collected from patients with CRC after resection surgery at the Inner Mongolia Medical University Hospital (Hohhot, Inner Mongolia, China) between July and November 2008. The patients selected for the present study were aged between 39 and 76 (8 males and 5 females), did not receive prior radiotherapy or cytotoxic chemotherapy, and were histologically confirmed with colorectal adenocarcinoma. Patients that had received prior radiotherapy or cytotoxic chemotherapy were excluded. All sample collection and handling processes were conducted in accordance with the National Institutes of Health's guidelines and regulations. All participants were verbally informed about the purpose of the present study and provided written informed consent. The present study was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University.

Materials. HCT116 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2. $Bax^{-/-}$ HCT116 cells were established and characterized according to a previously published protocol (6). Antibodies directed against Bax (cat. no. 2772, 1:1,000 dilution), poly ADP-ribose polymerase (PARP; cat. no. 9542; 1:1,000 dilution), Bcl-2 (cat. no. 2872; 1:1,000 dilution), p53 (cat. no. 9282; 1:1,000 dilution) and β-actin (cat. no. 4970; 1:10,000 dilution) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Velcade was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell proliferation assay. The cell proliferation rate was measured using the cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies Inc., Rockville, MD, USA) according to the manufacturer's protocol. $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells were seeded into 96-well plates at a density of 2,000 cells/well. Once the cells had attached to the wells, the appropriate reagents were added and cell proliferation was assessed by determining the absorbance of each well at a wavelength of 450 nm over 6 days using a microplate reader. Data are presented as the mean ± standard deviation (SD) of at least three wells from each group. Each experiment was performed in triplicate.

Clonogenic assay. A clonogenic assay was performed as described previously (22). Cells from each group were seeded into 6-well plates in triplicate at a density of 400 cells/well and cultured for ≤3 weeks until visible colonies had formed. $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells reached 80% confluence were treated with 50 or 100 nM Velcade for >10 days until visible colonies had formed. The cell colonies were fixed by methanol for 15 min at room temperature and stained with a solution containing 0.5% crystal violet and 25% methanol for 30 min at room temperature and washed with tap water to remove excess dye. The number of colonies was counted under a light microscope at room temperature.

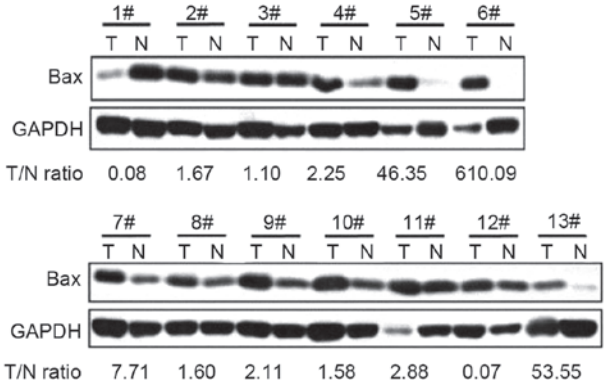

Figure 1. Differential expression of Bax protein in human CRC tissues. The protein level of Bax in 13 pairs of fresh frozen CRC and adjacent normal tissues was examined by western blotting. CRC, colorectal cancer; N, normal tissue; T, tumor tissue; Bax, B-cell lymphoma 2-associated X protein.

Western blot analysis. $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells were treated with different doses of Velcade (0, 0.2, 1 and 2 nM) for 12 h or treated with 1 nM Velcade for 0, 6, 12 and 24 h. Cells were harvested and lysed with 1X SDS lysis buffer [50 mM Tris-HCl (pH 6.8), 10% glycerol and 2% SDS] to extract the total protein. Following the quantification of protein concentration using a Bicinchoninic Acid Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.), 20 μg of protein from each sample were separated by SDS-PAGE on a 10% gel and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked in 5% non-fat milk for 2 h at room temperature and then incubated at 4˚C overnight with primary antibodies. The membranes were washed three times with Tris-buffered saline containing 0.5% Tween-20 (TBST). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. SC-2357; 1:10,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature and washed with TBST again. Finally, target protein bands were visualized using an Enhanced Chemiluminescence Detection system (Engreen Biosystem, Ltd., Beijing, China). GelQuant.NET software version 1.7.8 (biochemlabsolutions.com/GelQuantNET.html) was used for quantification of the intensity of each band. The expression ratio in tumor (T) vs. normal (N) tissue was calculated using the following formula: $(T_{Bax}/T_{GAPDH})/(N_{Bax}/N_{GAPDH})$.

Apoptosis assay. $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells were treated with Velcade (0, 10, 50 and 100 nM) for 12 h at 37˚C. The rate of apoptosis was then quantified using a fluorescein isothiocyanate-labeled Annexin V/propidium iodide apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to

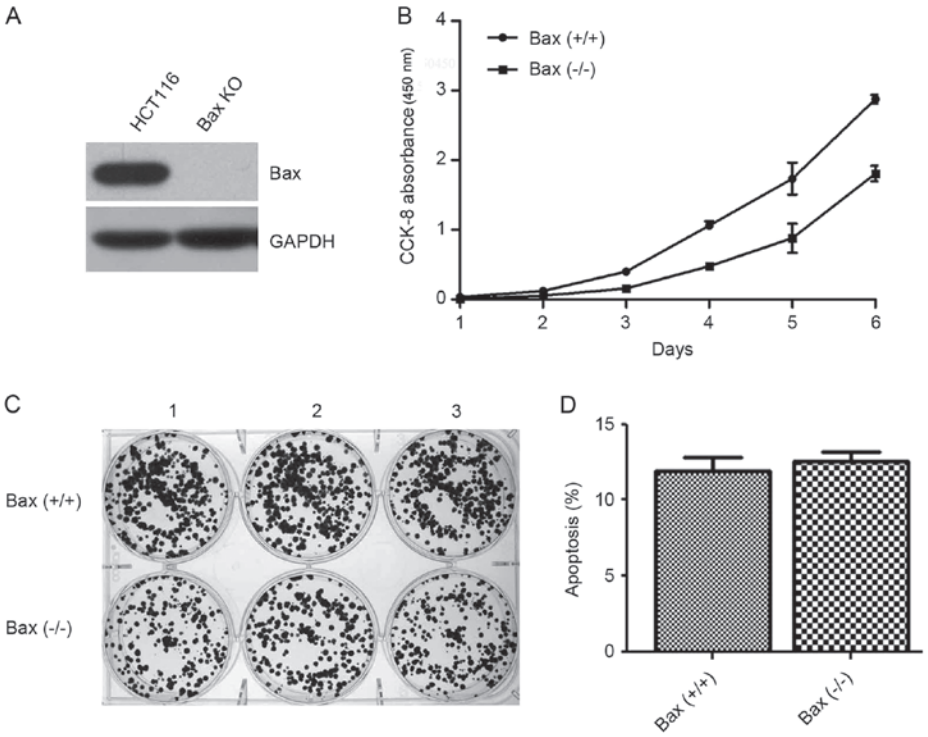

Figure 2. Deletion of Bax in HCT116 cells inhibits cell growth and colony formation. (A) Genetic knockout of Bax in HCT116 cells was verified by western blot analysis. (B) The proliferation of HCT116 cells with or without Bax was examined using the CCK-8 assay. (C) Results of the clonogenic assay in HCT116 cells with or without Bax. (D) Flow cytometry analysis was performed to evaluate the apoptosis of HCT116 cells with or without Bax. Bax, B-cell lymphoma 2-associated X protein; KO, knockout; CCK-8, Cell Counting Kit-8.

the manufacturer's protocol using a FACS Calibur cytometer (BD Biosciences, San Diego, CA, USA).

Statistical analysis. Data are presented as the mean ± SD. The Student's t-test was used to compare the statistical significance of differences between the mean values of two groups. One-way analysis of variance and the Bonferroni method were employed when there were more than three test groups. Each experiment was performed in triplicate. $P<0.05$ was considered to indicate a statistically significant difference. All the statistical analysis was performed using the SPSS software 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Differential expression of Bax protein in human CRC tissues. The expression level of Bax protein was examined in 13 pairs of fresh frozen CRC and adjacent normal tissues by western blotting. Bax was identified to be differentially expressed in CRC tissue compared with normal colorectal tissue (Fig. 1). The expression of the Bax protein was increased in the majority of the CRC tissues compared with that in the corresponding normal tissues (11/13, 85%). Therefore, decreasing the expression of Bax may be beneficial in inhibiting CRC growth.

Deletion of Bax in HCT116 cells inhibits cell growth and colony formation. To investigate the function of Bax in CRC progression, $Bax^{-/-}$ HCT116 cells were established and western blot analysis was performed for verification (Fig. 2A). The effect of Bax on cell proliferation was then evaluated using CCK-8 and colony formation assays. The results demonstrated that the absence of Bax suppressed the proliferation of HCT116 cells (Fig. 2B and C). However, the knockout of Bax did not affect the rate of apoptosis (Fig. 2D).

Deletion of Bax confers HCT116 cells with resistance to Velcade-induced apoptosis. The downregulation of Bax may enhance the resistance of tumor cells to Velcade (6). Thus, the rate of apoptosis in $Bax^{-/-}$ HCT116 cells was examined following exposure to Velcade, a commonly used drug for the treatment of patients with progressive myeloma. The effect of increasing amounts of Velcade on the rate of apoptosis was measured using flow cytometry. The results revealed that the apoptosis rate of $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells markedly increased with increasing doses of Velcade (Fig. 3A). However, the percentage of $Bax^{-/-}$ HCT116 cells that underwent apoptosis was lower compared with that of $Bax^{+/+}$ HCT116 cells at each Velcade dose. Notably, the difference between $Bax^{+/+}$ HCT116 cells at 50 and 100 nm of Velcade was statistically significant ($P<0.05$; Fig. 3A). This indicates that the absence of Bax may increase the resistance of HCT116 cells to Velcade. To validate this phenotype, the expression of an indicator of apoptosis, cleaved PARP, was examined. Cleavage of PARP was reduced in $Bax^{-/-}$ HCT116 cells when exposed to Velcade (Fig. 3B and C), indicating that Bax deletion reduces Velcade-induced HCT116 cell apoptosis.

Deletion of Bax increases the resistance of HCT116 cells to Velcade. Considering that the absence of Bax suppressed the proliferation of HCT116 cells, the effect of Bax on HCT116 cell proliferation was evaluated using a colony formation assay. The results revealed that the deletion of Bax significantly

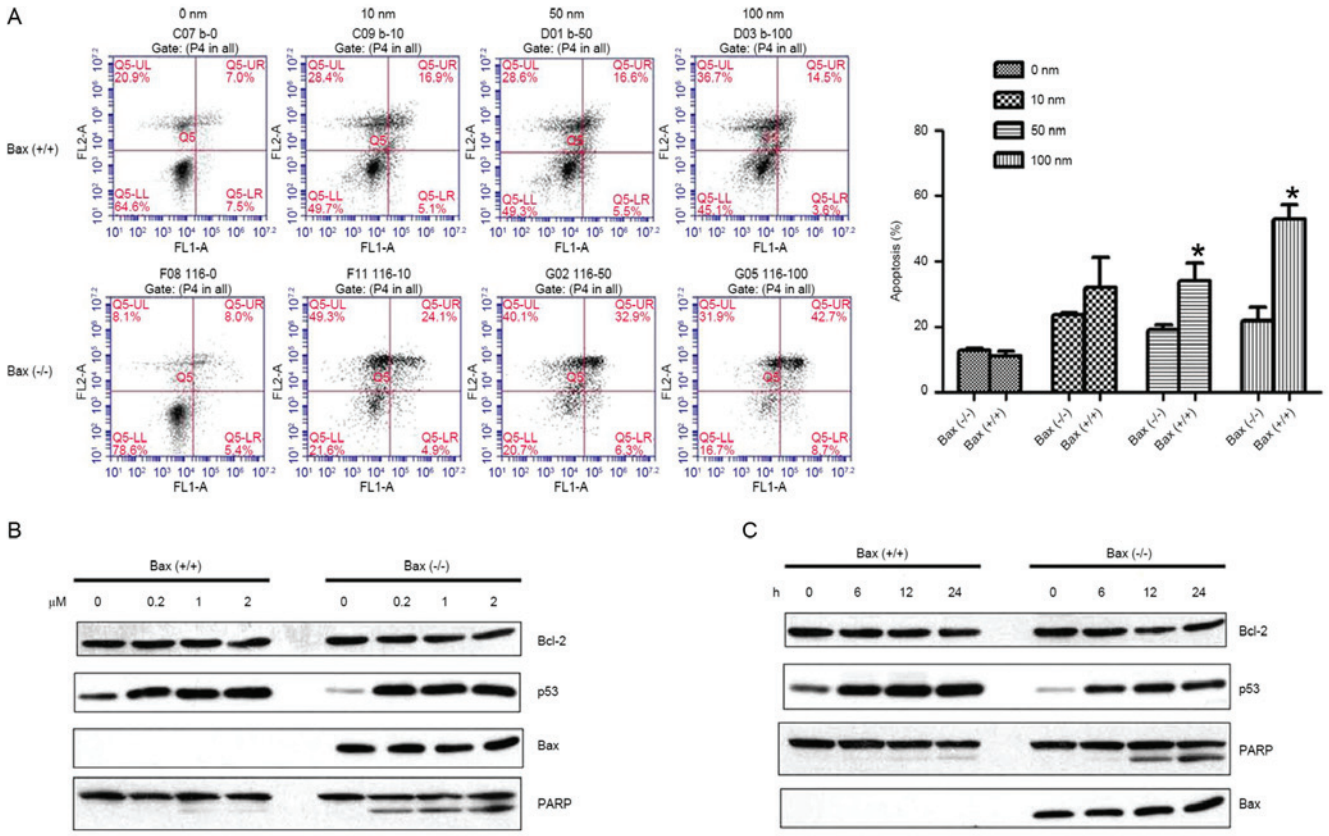

Figure 3. Deletion of Bax confers HCT116 cells with resistance to Velcade-induced apoptosis. (A) $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells were incubated with different doses of Velcade (0, 10, 50 and 100 nM) for 12 h and then flow cytometry analysis was performed to evaluate apoptosis. (B) $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells were treated with different doses of Velcade (0, 0.2, 1 and 2 nM) for 12 h and then the level of Bax protein was examined via western blotting. (C) $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells were treated with 1 nM Velcade for 0-24 h prior to western blot analysis of Bax expression. $^{*}P<0.05$ vs. the $Bax^{-/-}$ group. Bax, B-cell lymphoma 2-associated X protein.

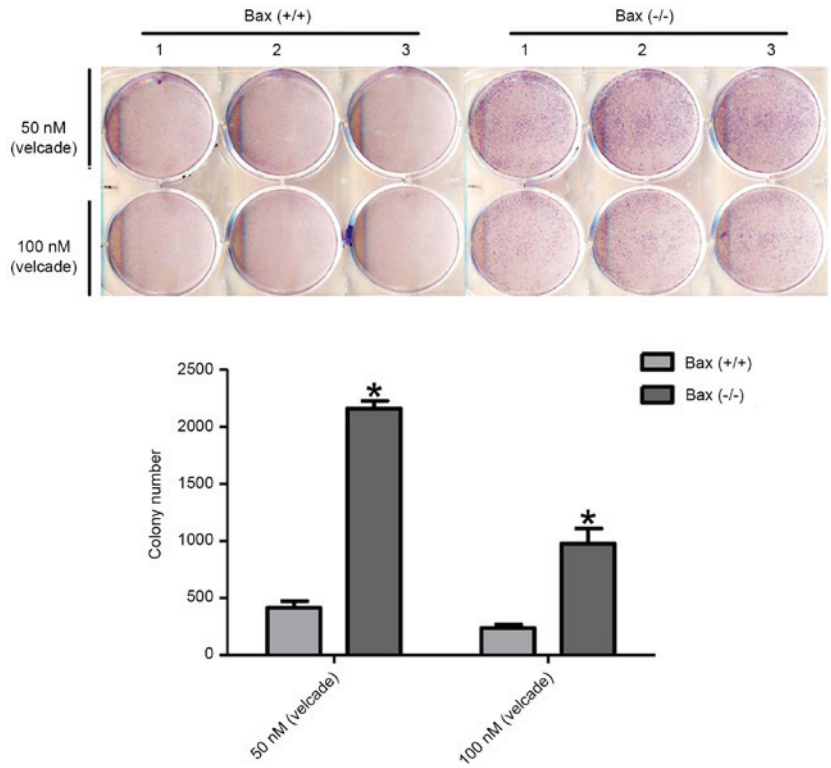

Figure 4. Deletion of Bax increases the resistance of HCT116 cells to Velcade. HCT116 cells with or without Bax were treated with 50 or 100 nM Velcade in triplicate and cultured until visible colonies had formed. The colonies were then stained with crystal violet and images were captured. The colonies were subsequently counted. $^{*}P<0.05$ vs. the $Bax^{+/+}$ group. Bax, B-cell lymphoma 2-associated X protein.

enhanced the resistance of HCT116 cells to Velcade compared with the $Bax^{+/+}$ HCT116 cells ($P<0.05$; Fig. 4).

Discussion

To date, few biomarkers for predicting the response of patients with CRC to chemotherapy have been identified, which has limited the development of personalized medicine for the treatment of CRC. The association between Bax and the prognosis and response of patients with CRC to surgery and chemotherapy has been analyzed in a series of prospective studies (23,24). Bax expression was identified to be correlated with improved survival outcomes in patients with CRC, indicating that Bax is a promising biomarker for CRC prognosis and positively affects the response of patients to chemotherapy (25). Another study demonstrated that in cultured CRC cells the absence of Bax reduced the apoptotic response to chemopreventive agents, including NSAIDs (6).

In the present study, Bax expression was examined in paired CRC and adjacent normal tissues. Bax was identified to be aberrantly expressed CRC compared with normal tissues. In addition, $Bax^{-/-}$ HCT116 cells demonstrated reduced proliferation and colony formation. When treated with Velcade, $Bax^{-/-}$ HCT116 cells exhibited increased resistance to Velcade compared with $Bax^{+/+}$ HCT116 cells. These results indicate that Bax is involved in Velcade-induced apoptosis in CRC cells and is a novel biomarker for the prognosis of patients with CRC. In addition, the role of Bax in the anticancer activity of Velcade highlights the potential use of Velcade as an adjuvant and/or neoadjuvant therapy for patients with CRC.

Acknowledgements

The present study was supported by the Inner Mongolia Natural Science Foundation (grant no. 2011BS1117) and the Inner Mongolia Medical University Science and Technology Million Project (grant no. NY2011BW005).

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