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THE CONSTRUCTION OF THE EUKARYOTIC EXPRESSION PLASMID pcDNA3.1/AZURIN AND THE INCREASED APOPTOSIS OF U2OS CELLS TRANSFECTED WITH IT

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Abstract: In our previous study, we demonstrated that azurin could selectively trigger apoptosis in human osteosarcoma cell line U2OS cells. However, the rate of apoptosis (35.8 ± 3.2%) is not very high, and azurin is too expensive to obtain readily. To solve these problems, we constructed a eukaryotic expression plasmid containing the azurin gene with an influenza virus haemagglutinin 9 peptide HA epitope tag, and transfected the recombinant plasmid pcDNA3.1(+)/azurin into U2OS cells. RT-PCR and Western blot analysis validated the successful transfection and the expression of the azurin-HA protein. Conspicuous apoptosis of the transfected cells was detected by flow cytometry (FCM) and the DNA ladder test. The apoptosis rate reached 64.3 ± 13.1%. The transcriptional levels of the Bax and p53 genes increased significantly in U2OS cells transfected with pcDNA3.1(+)/azurin, but the Bcl-2 mRNA level decreased. There was no difference in the levels of Bcl-xl mRNA and Survivin mRNA. We propose that the transfection of the recombinant plasmid pcDNA3.1(+)/azurin can significantly induce apoptosis in U2OS cells. This is closely associated with the up-regulation of the transcriptional level of the Bax and p53 genes, and the down-regulation of that of the Bcl-2 gene.

Key words: Azurin, Transfection, Osteosarcoma, Apoptosis

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Abbreviations used: FCM – flow cytometry; GFP – green fluorescent protein; HA – haemagglutinin 9 peptide HA epitope; hCMV – human cytomegalovirus; IAP – inhibitor apoptosis protein; PCR – polymerase chain reaction; RT – reverse transcription; UV – ultraviolet

INTRODUCTION

Osteosarcoma is the most common malignant tumor of bone tissue. It mainy occurs in teenagers, with high malignancy, early metastasis and poor prognosis. The current common treatments, such as surgical operation and chemotherapy, is suboptimal, and the prognosis for patients remains dismal. Therefore, novel therapeutic strategies are constantly being studied. The various candidates for new treatments of osteosarcoma include new chemotherapeutic drugs, vaccines, monoclonal antibodies, and approaches using dendritic cells. Recently, the old idea of using live or attenuated pathogenic bacteria or their products in the treatment of cancer has attracted considerable interest.

The infection of tumor-bearing mice with live attenuated cells of *Salmonella typhimurium* has been reported to induce tumor regression [1, 2]. A significant regression of subcutaneous tumors in mice was observed when anaerobic bacteria were combined with various chemotherapeutic agents. The anti-tumor activity of these pathogens has been attributed to the activation of the immune response against the tumor antigen, the preferential growth of certain bacteria within the tumor, and the inhibition of angiogenesis. However, live micro-organisms with or without chemotherapeutic agents can produce significant morbidity and mortality. Therefore, studies are in progress to separate the pure metabolites or any other components of the microbial cell that might have anti-tumor activity. Recently, azurin has become one of the representative bacterial products applied in the treatment of tumors.

Azurin is one of the copper-containing redox proteins called cupredoxins, which are elaborated from the pathogenic bacteria *Pseudomonas aeruginosa.* Different cupredoxins are produced by different aerobic bacteria as agents of electron transfer [3]. Recent evidence confirmed that azurin could selectively trigger cell death in some cancer cells, such as melanoma and human breast cancer [4-6]. *In vitro* studies revealed a direct physical interaction between azurin and p53. After internalization by tumor cells, azurin combines with the tumor suppressor protein p53, enhancing its intracellular level, and inducing apoptosis via caspase-mediated mitochondrial pathways.

In our previous study, we found that azurin showed no toxicity to normal human liver cells, but could selectively trigger cell apoptosis in human osteosarcoma U2OS cells [6]. This suggest considerable potential in the treatment of osteosarcoma. However, the maximal rate of apoptosis of the cells, induced by 200 mg/L azurin for 48 h, is 35.8 ± 3.2%, which is too low. Increasing the concentration of azurin could increase the rate of apoptosis, but it is difficult and expensive to obtain sufficiently large amounts of active, purified azurin protein. Therefore, we considered the construction of a plasmid containing azurin cDNA with which to transfect U2OS cells.

MATERIALS AND METHODS

Materials

The prokaryotic expression plasmid pQE30/azurin was successfully constructed by our research group [7]. The azurin cDNA was amplified from the DNA of *Pseudomonas aeruginosa* PAO1 collected from patients with respiratory disease. The eukaryotic expression vector pcDNA3.1(+) containing the hCMV promoter was obtained from Invitrogen and the plasmid pGEM T Easy was bought from Promega. Hind III, EcoR I enzyme and T4 DNA ligase were purchased from TaKaRa. *E. coli* X-L1-Blue and anti-HA-tag antibody were obtained from the Institute of Cancer Research of Zhejiang University. HRP-conjugated secondary antibody was purchased from Boshide. An endotoxin-free extraction kit was obtained from Qiagen. M-PER® Reagent was bought from Pierce. RPMI-1640 medium and fetal bovine serum were obtained from Gibco.

Cell line and culture conditions

U2OS, a human osteosarcoma cell line, was purchased from the American Type Culture Collection. The cells were cultured in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37ºC in a humidified atmosphere containing 5% CO_2.

Amplification of the azurin-encoding gene containing an influenza virus haemagglutinin 9 peptide HA epitope tag (HA tag)

The azurin-encoding gene was amplified by polymerase chain reaction (PCR) from the expression plasmid pQE30/azurin, which had been successfully constructed by our research group. The PCR was carried out in a 50 μl reaction volume using 1 μl of 2 μg/μl pqe30/azurin, 1 μl of 10 pM target primers, 1 μl of 10 mM dNTP-Mix, 3 μl of 25 mM $MgCl_2$, 5 μl of 10 × PCR buffer and 1 μl of 2.5 U Taq polymerase. PCR was performed under these conditions: 3 min 95ºC preincubation, followed by 30 cycles of denaturation (30 s, 95ºC), annealing (20 s, 56ºC), and extension (1 min, 72ºC), followed by a final extension of 5 min at 72ºC. PCR products were separated on a 2% agarose gel containing 0.2 μg/ml ethidium bromide. The forward primer used was 5'-ATAAGCTTACCA TGGCCGAGTGCTCGGTGGACAT-3', which included the Hind III enzyme restriction site (underlined). The reverse primer used was 5'-ATGAATTCTCA *CGCGTAATCTGGGACGTCGTAAGGGTA*CTTCAGGGTCAGGGTGCCCT-3', which included the EcoR I enzyme restriction site (underlined) and the sequence of the HA tag (in italics) [8]. The extracted DNA was digested by Hind III and EcoR I (TaKaRa). The digested products were evaluated with 1.5% agarose gel electrophoresis.

Construction of the sequencing plasmid

The depurant PCR products and the sequencing vector pGEM T Easy were ligated by 1 μl T4 DNA ligase (TaKaRa) at a ratio of 3:1 at 4ºC overnight. This recombinant plasmid was named pGEM T Easy/azurin. The bacterial *E. coli*

X-L1-Blue transformed with pGEM T Easy/azurin was cultured in LB medium with anti-aminobenzyl penicillin overnight at 37ºC. The recombinant plasmid was extracted with the Qiagen extraction kit according to the manufacturer's instructions. Then it was digested by EcoR I and subjected to DNA sequencing by the Shanghai Yingjun Biology Company.

Construction of the recombined plasmid pcDNA3.1(+)/azurin

The recombinant plasmid pGEM T Easy/azurin was digested by Hind III and EcoR I at 37ºC overnight. The azurin-encoding gene was segregated, reclaimed and depurated. Then the depurant products were ligated with pcDNA3.1(+) by 1 μl T4 DNA ligase (TaKaRa) at a ratio of 3:1 at 4ºC overnight. The pcDNA3.1(+)/azurin plasmid was extracted with the Qiagen extraction kit according to the manufacturer's instructions, and digested by Hind III and EcoR I at 37ºC for 3 h. Finally, the products were separated, extracted and purified.

Transfection

The pcDNA3.1(+)/azurin was extracted with an endotoxin-free extraction kit as per the manufacturer's instructions. The transfection of U2OS cells was carried out in 6-well plate according to the instructions for the Lipofectamine 2000^{TM}. Four μg of pcDNA3.1(+)/azurin, 4 μg of pcDNA3.1(+)/GFP and the control pcDNA3.1(+) were transfected separately into U2OS cells. The efficiency of transfection was observed by fluorescence microscopy and detected by flow cytometry (FCM).

The DNA ladder assay

For the qualitative analysis of DNA fragmentation, the cells were lysed in a lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.1% of Triton X-100. They were incubated with RNase A and proteinase K at 37ºC for 60 min. The procedures for DNA extraction and precipitation were repeated by the addition of hydroxybezene and chloroform. Finally, the soluble DNA fragments were precipitated by the addition of sodioum acetate and ethanol. DNA pellets were dissolved in TE and loaded onto a 2.0% agarose gel and separated at 100 V for 45 min. The DNA fragments were stained with ethidium bromide and visualized by transillumination under UV light.

Quantification of apoptosis and cell-cycle analysis by FCM

Cells were grown in 6-well plates transfected with pcDNA3.1(+)/azurin for 72 h. Adhered cells were then removed by 0.25% trypsin, washed twice with PBS, and fixed in cold alcohol for 12 h. After removal of the fixed solution, the cells were stained with PI staining solution (50 mg/l PI, 0.1% TritonX-100, 100 mg/l RNase) for 30 min at 4ºC in the dark. After washing, the cells were resuspended in PBS and analyzed by flow cytometry. A minimum of 10,000 cells were analyzed for each sample.

Western blot

Cells were lysed by 200 µl M-PER® Reagent (Pierce company). After centrifugation at 14000 × g for 10 min at 0ºC, the supernatants were collected, and the proteins were separated on 12% SDS-PAGE. After electrophoresis, the protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST and incubated overnight with polyclonal anti-HA tag antibody at 37ºC for 1 h. After three washes with TBST, the membrane was incubated at 37ºC for 1 h with horseradish peroxidase-conjugated secondary antibody diluted with TBST (1:500). The detected protein signals were visualized by an enhanced chemilluminescence reaction system (Amersham, Arlington Heights, IL).

RT-PCR quantitation

Total RNA was extracted using the Trizol agent. The reverse transcription (RT) was performed for 1 h at 42ºC with 1 µl of 200 U Mo-AMV reverse transcriptase, 1 µl of 62.5 µmol random oligohexamer primers, 2 µl of 10 mM dNTP-Mix, and 2 µg of total RNA [9]. The polymerase chain reaction (PCR) was carried out in a 20 µl reaction volume using 3 µl of the previous RT reaction product, 1 µl of 10 pM of the target primers, 1 µl of 10 mM dNTP-Mix, 1.2 µl of 25 mM $MgCl_2$ and 0.5 µl of 2.5 U Taq polymerase. PCR was performed under these conditions: 3 min 95ºC preincubation, followed by 30 cycles of denaturation (30 s, 95ºC), annealing (20 s, 55ºC), and extension (1 min, 72ºC), followed by a final extension of 5 min at 72ºC. The PCR products were separated on a 2% agarose gel containing 0.2 µg/ml ethidium bromide. The results were finally analyzed using Kodak Digital Science 1D and expressed as the respective ratios relative to β-actin. The primers for RT-PCR were designed using PCRDESNA Primer Design software and were shown in Tab. 1.

Tab. 1 Description of the designed primers for the RT-PCR.

Gene	Forward primers (5'~3')	Reverse primers (5'~3')	Product size [bp]	Anne-aling tempe-rature [ºC]
p53	CGCTGCCCCCACCATGAGC	CTGGAGTCTTCCAGTGTGATGA	259	58
Bax	GGCCCACCAGCTCTGAGCAGA	GCCACGTGGGCGGTCCCAAAGT	479	62
Bcl-2	TGGAGGAGCTCTTCAGGGAT	AGGCACCCAGGGTGATGCAA	304	58
Bcl-xl	TTGGACAATGGACTGGTTGA	GTAGAGTGGATGGTCAGTG	780	58
Survivin	CAGATTTGAATCGCGGGACCC	CCAAGTCTGGCTCGTTCTCAG	206	60
β-actin	CACCAACTGGGACGACATGG	GTCCAGACGCAGGATGGCAT	310	58

Statistical analysis

The data was expressed as the means ± SD. The statistical analysis was done with Student's t test, and a value of $P < 0.05$ was taken as statistically significant.

RESULTS

Amplification of azurin-HA

The azurin-encoding bacterial expression plamid pQE30/azurin was constructed by our group, and the cDNA fragment encoding azurin was PCR-amplified using pQE30/azurin as a template. It was then digested by Hind III and EcoR I, and evaluated by 1.5% agarose gel electrophoresis. A cDNA fragment of 410 bp (Fig. 1A) was obtained, which we expected to be HA-tagged azurin.

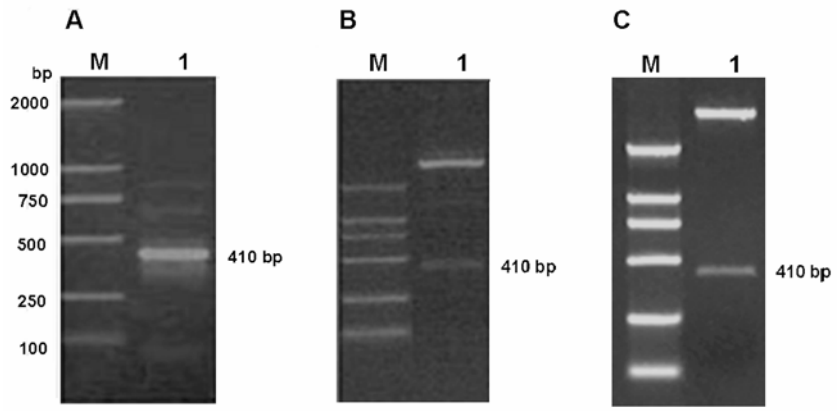

Fig. 1. Insertion of the azurin-containing HA epitope tag cDNA into pcDNA3.1(+). A – The recombinant plasmid pQE30/azurin was amplified, digested and evaluated with 1.5% agarose gel electrophoresis (M - the marker; 1 - the digested product of pQE30/ azurin). B – The plasmid pGEM T Easy/azurin was digested and segregated by 1.5% agarose gel electrophoresis (M - the marker; 1 - the digested product of pGEM T Easy/azurin). C – The plasmid pcDNA 3.1(+)/azurin was digested by Hind III and EcoR I and segregated by 1.5% agarose gel electrophoresis (M - the marker; 1 - the digested product of pcDNA 3.1(+)/azurin). The markers in panel B and panel C are the same with the marker in panel A.

Evaluation of pGEM T Easy/azurin

To confirm the sequence of the 410-bp cDNA fragment, the sequencing plasmid pGEM T Easy/azurin was constructed. The plasmid pGEM T Easy/azurin was digested by EcoR I and segregated by 1.5% agarose gel electrophoresis. Two cDNA fragments of 410 bp and 3.0 Kb (Fig. 1B) were obtained. The DNA sequencing of the 410-bp fragment tested by the Shanghai Yingjun Biology Company concurred with the Genebank sequence (gi:151060). This demonstrates that the recombinant plasmid pQE30/azurin contains the correct sequence of azurin-HA.

Evaluation of the pcDNA3.1(+)/azurin

To transfect human osteosarcoma U2OS cells, 410 bp of HA-tagged azurin cDNA was inserted into the eukaryotic expression vector pcDNA3.1(+). The plasmid pcDNA3.1(+)/azurin was digested by Hind III and EcoR I and segregated by 1.5% agarose gel electrophoresis. Two cDNA fragments of 410 bp and 5.0 Kb were obtained (Fig. 1C). This demonstrates that the azurin-containing HA epitope tag cDNA was successfully inserted into pcDNA3.1(+).

Expression of azurin in the transfected cells

Cells were transfected with pcDNA3.1(+)/azurin for 72 hours. Total RNA was extracted to perform RT-PCR. Cell protein was extracted to perform Western blot analysis. A band was observed at the 410-bp mark in the RT-PCR analysis in cells transfected with pcDNA3.1(+)/azurin. Untreated cells and cells transfected with pcDNA3.1(+) alone had no such band (Fig. 2). The Western blot analysis showed that there was expression of azurin-HA protein in the cells transfected with pcDNA3.1(+)/azurin, but nothing in the control cells (Fig. 3). The molecular weight of the azurin-HA protein is about 14.4 kD. This means that pcDNA3.1(+)/azurin was transfected into the U2OS cells and that HA-tagged azurin can be expressed in U2OS cells.

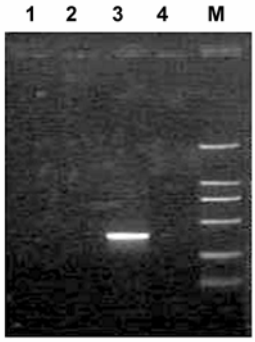

Fig. 2. Transcription of the azurin gene in U2OS cells. Cells were transfected with pcDNA3.1(+)/azurin or pcDNA3.1(+) for 72 hours. Total RNA was extracted and RT-PCR was performed. 1 – untransfected cells; 2 – cells transfected with pcDNA3.1(+); 3 – cells transfected with pcDNA3.1(+)/azurin; 4 – the reverse transcription products of 3 were electrophoresed to exclude contamination by the plasmid; M – marker (the same as the marker in Fig. 1).

Transfection rate

To test the transfection rate of pcDNA3.1(+)/azurin, U2OS cells were transfected with pcDNA3.1(+)/azurin and pcDNA3.1(+)/GFP at the same time. The transfection efficiency of pcDNA3.1(+)/GFP was regarded as the transfection efficiency of pcDNA3.1(+)/azurin. The fusion of azurin and GFP is a good way to obtain the exact transfection rate of pcDNA3.1(+)/azurin. However, it does affect the expression of azurin, so we only chose this method

to get the transfection rate. The transfection rate was detected by FCM according to the fluorescence intensity (Fig. 4). The transfection rate of the cells transfected with pcDNA3.1(+)/GFP and pcDNA3.1(+)/azurin was 38.1 ± 4.9%. We believe that the transfection rate of cells transfected with pcDNA3.1(+)/azurin is approximately 38.1 ± 4.9%.

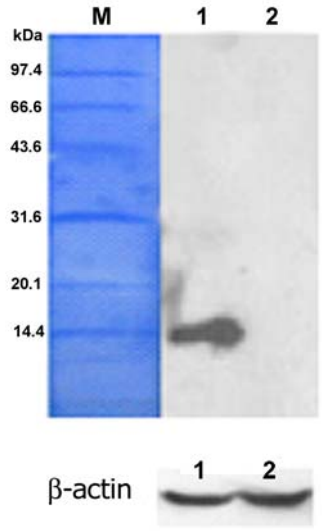

Fig. 3. The expression of azurin in U2OS cells by Western blot analysis. Cells were transfected with pcDNA3.1(+)/azurin or pcDNA3.1(+) for 72 hours. Cell protein was extracted and Western blot analysis was performed using anti-HA antibody. M – marker; 1 – cells transfected with pcDNA3.1(+)/azurin; 2 – cells transfected with pcDNA3.1(+).

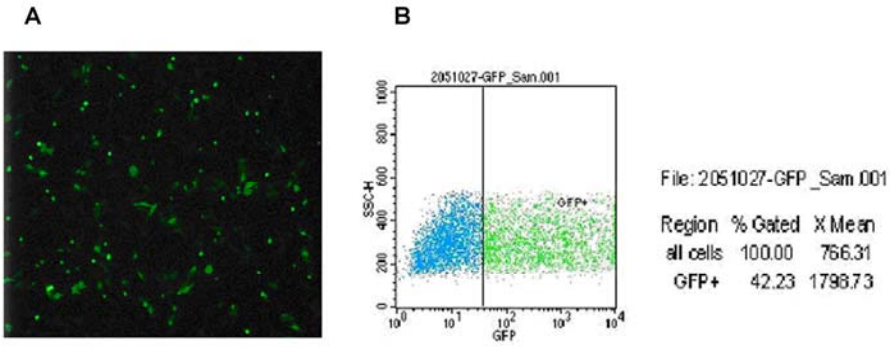

Fig. 4. U2OS cells transfected with pcDNA3.1(+)/GFP and pcDNA3.1(+)/azurin. A – Cells expressing GFP showing green fluorescence under fluorescence microscopy. B – The rate of transfection was 42.2%, as analyzed by FCM.

Flow cytometry (FCM)

The percentages of G_0/G_1 phase, S phase and G_2/M cells phase in the cells transfected with pcDNA3.1(+)/azurin for 72 h were all lower than those for cells

transfected with pcDNA3.1(+) and for untransfected cells. The apoptosis rate of cells transfected with pcDNA3.1(+)/azurin was 64.3 ± 13.1%, obviously higher than that for untransfected cells (10.2 ± 1.9%) and cells transfected with pcDNA3.1(+) (16.4 ± 7.2%) (Fig. 5, Tab. 2). In our previous study, the maximal apoptosis rate that the azurin protein could induce in U2OS cells was 35.8 ± 3.2% when the cells were directly treated with 200 mg/L azurin for 48 h. This demonstrates that the transfection of azurin can induce U2OS cell apoptosis more effectively than direct treatment with the azurin protein.

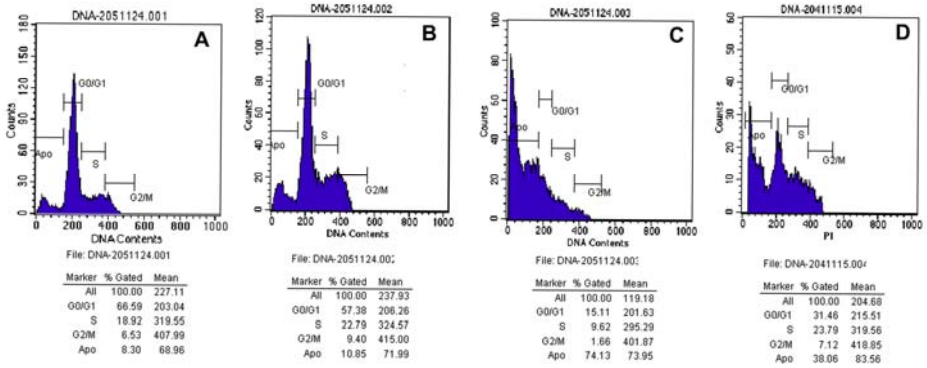

Fig. 5. The apoptosis rate of the studied cells. Cells were transfected with pcDNA3.1(+)/azurin or pcDNA3.1(+) for 72 h. The cell cycle and apoptosis rate was analyzed by FCM. A – untransfected cells; B – cells transfected with pcDNA3.1(+); C – cells transfected with pcDNA3.1(+)/azurin; D – cells treated directly with 200 mg/l azurin protein for 48 h.

Tab. 2. The apoptosis rate and cells in various stages of the cell cycle (%) as detected by FCM after 72 h of transfection (x ± s, n = 3).

	G_0/G_1	S	G_2/M	Apoptosis
Blank group	61.98 ± 5.60	19.57 ± 2.48	8.80 ± 3.40	10.16 ± 1.87
Control group	54.54 ± 6.02	19.32 ± 3.00	10.05 ± 0.56	16.42 ± 7.18
Experimental group	19.03 ± 5.55**▼▼	11.86 ± 3.38	5.26 ± 4.35	64.35 ± 13.12*▼

Blank group – untransfected cells; Control group – cells transfected with pcDNA3.1(+); Experimental group – cells transfected with pcDNA3.1(+)/azurin. *p < 0.05, **p < 0.01 Experimental group vs. Blank group, ▼P < 0.05, ▼▼P < 0.01 Experimental group vs. Control group.

DNA ladder assay

Cells were transfected with pcDNA3.1(+)/azurin for 72 h. Total DNA was extracted and was electrophoresed on 1.8% agarose gel. The cells transfected with pcDNA3.1(+)/azurin showed a typical DNA "ladder" (Fig. 6). The others showed none.

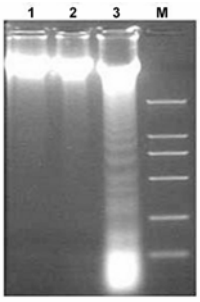

Fig. 6. A DNA ladder assay of cells. Cells were transfected with pcDNA3.1(+)/azurin or pcDNA3.1(+) for 72 hours. All of the DNA was extracted and electrophoresed on 1.8% agarose gel. 1 – untransfected cells; 2 – cells transfected with pcDNA3.1(+); 3 – cells transfcted with pcDNA3.1(+)/azurin; M – marker (the same as the marker in Fig. 1).

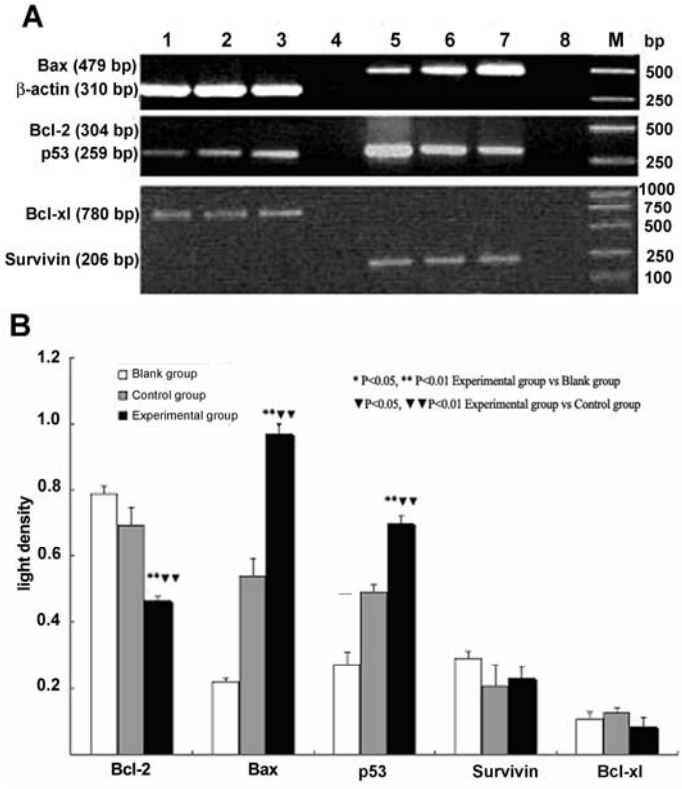

Fig. 7. The transcription of Bax, Bcl-2, Bcl-xl, p53 and Survivin mRNA in U2OS cells. A – Cells were transfected with pcDNA3.1(+)/azurin or pcDNA3.1(+) for 72 h. The RNA was extracted and RT-PCR was performed. The products were electrophoresed on agarose gel. 1, 5 - untransfected cells; 2, 6 - cells transfected with pcDNA3.1(+); 3, 7 - cells transfected with pcDNA3.1(+)/azurin; M - marker. B – The optical density of bands was analyzed by Kodak Digital Science ID software. The transcriptional levels of the five kinds of mRNA were expressed as their respective ratios relative to β-actin. Blank group - untransfected cells; Control group - cells transfected with pcDNA3.1(+); Experimental group - cells transfected with pcDNA3.1(+)/azurin.

The expression of Bax, Bcl-2, Bcl-xl, p53 and Survivin mRNA

When expressed, the azurin protein effectively promotes U2OS cell apoptosis. The pro-apoptotic gene Bax and p53 mRNA levels in the cells transfected with pcDNA3.1(+)/azurin was obviously higher, and the anti-apoptotic gene Bcl-2 mRNA level was obviously lower than the levels in cells transfected with pcDNA3.1(+) or in untransfected cells. There was no significant difference in the levels of Bcl-xl mRNA and Survivin mRNA between those two groups (Fig. 7).

DISCUSSION

We constructed a eukaryotic expression plasmid containing HA-tagged azurin gene with a HA epitope tag. The recombinant plasmid pcDNA3.1(+)/azurin was found to have the correct sequence of azurin-HA by PCR amplification, restriction endonuclease mapping and sequencing (Fig. 1). We transfected the recombinant plasmid pcDNA3.1(+)/azurin into human osteosarcoma cell line U2OS cells and observed the induction of apoptosis and the molecular mechanism of this induction. According to the fluorescence microscopy and FCM analysis, the recombinant plasmid pcDNA3.1(+)/azurin could successfully transfect U2OS cells. The expression of 14.4 kD of azurin-HA protein in cells transfected with pcDNA3.1(+)/azurin was confirmed by RT-PCR and Western blot analysis. The molecular weight of HA is very small, and the molecular weight of azurin is about 14 kD, so this is in agreement with our previous results [7].

After 72 h of transfection, DNA fragmentation typical for apoptosis was detected (Fig. 6). In the cell-cycle analysis, a significant reduction of the cell population in the G_0/G_1, S and G_2/M phases was observed. The rate of apoptosis in pcDNA3.1(+)/azurin-transfected cells was much higher than that in cells transfected with pcDNA3.1(+) and in untransfected cells (Fig. 5, Tab. 2). Moreover, the apoptosis rate induced by transfected azurin (64.3 ± 13.1%) was higher than the maximal apoptosis rate induced directly by azurin protein (35.8±3.2%) [6]. As it is very expensive to obtain purified azurin protein, the construction of pcDNA3.1(+)/azurin saves a lot of research outlays and creates a basis for further study.

We concluded that pcDNA3.1(+)/azurin could induce apoptosis in U2OS cells. We then investigated the mechanism of this induction. Apoptosis is a genetically programmed event that can take place through a variety of internal or external stimuli, and these signals are regulated by two distinct pathways, involving either the death receptor (extrinsic) or the mitochondria [10, 11]. In the mitochondrial pathway, a variety of death signals trigger the release of several pro-apoptotic proteins. A number of apoptosis-related proteins, such as Bcl-2 family members, display both anti- and pro-apoptotic functions by forming homo- or heterodimers [12]. They are important regulators of apoptosis [13]. In these proteins, Bax mediates p53-induced apoptosis and increases sensitivity to chemotherapically induced apoptosis [14, 15]. Meanwhile, Bcl-2 is an anti-

apoptotic protein. When it is activated or prevalent, apoptosis is prohibited. Abnormal overexpression of Bcl-2 has frequently been observed in many types of human cancer. The ratio of Bax to Bcl-2 rather than the level of anti-apoptotic Bcl-2 alone is important in cell survival or apoptosis in response to death stimuli [16]. In our study, we established the mRNA levels of the Bcl-2 family members such as Bax, Bcl-2 and Bcl-xl by RT-PCR after 24 h of the transfection. We found that azurin up-regulated the transcriptional level of Bax and down-regulated the transcriptional level of Bcl-2 in U2OS cells. However, no change in the Bcl-xl transcriptional level was found (Fig. 7). The average ratio of Bax to Bcl-2 of cells transfected with pcDNA3.1(+)/azurin was higher than that of cells transfected with pcDNA3.1(+) and untransfected cells. This suggests that the induction of apoptosis by azurin transfection in U2OS cells is associated with the Bcl-2 family.

Survivin is a newly identified inhibitor apoptosis protein (IAP) that is involved in the control of apoptosis and the mitotic spindle checkpoint at the G_2/M phase boundary [17]. It plays an important role in the suppression of apoptosis by either directly or indirectly inhibiting the activity of caspases. To investigate the role of Survivin in apoptosis induced by pcDNA3.1(+)/azurin transfection in U2OS cell, we assessed the mRNA of Survivin by RT-PCR. No difference was detected between the three studied groups (Fig. 7). This indicates that Survivin did not participate in the negative regulation of apotosis induced by azurin in U2OS cells.

p53 is a well-known inducer of mammalian cell apoptosis. As a transcriptional regulator, p53 is a major player in an intricate networking system that controls cell growth and death, and cellular regulation. Several researchers concluded that functional p53 was important for the apoptotic pathway induced by azurin [18, 19]. In the presence of the azurin protein, wt p53 form a complex at mid-region with the N-terminal of azurin [5, 19-21]. It is stabilized by the protein-protein interaction [22, 23]. The stable p53 is not only able to up-regulate transcriptionally pro-apoptotic genes such as Bax, Apaf-1, caspase-9 and PUMA, but also able to repress some antiapoptotic genes like Bcl-2 [24, 25]. Moreover, the complex can transport the Bax protein to the mitochondria and enhance the release of mitochondrial cytochrome c to the cytosol, which activates the caspase cascade and induces apoptosis [10]. In our previous study, azurin was found to induce apoptosis in U2OS cells but not in MG63 cells, because U2OS cells contain wild-type p53 while MG63 cells are devoid of the p53 gene. In this study, we established the mRNA level of wt p53. We found that the p53 transcriptional level of U2OS cells tansfected with pcDNA3.1(+)/azurin was higher than those of cells transfected with pcDNA3.1(+) and untransfected cells (Fig. 7). It might be concluded that azurin protein is not only able to stabilize the wt p53 protein but also to up-regulate the transcription of the p53 gene. However, the mechanism of this up-regulation was not investigated, meaning further study is needed.

In summary, we successfully constructed the eukaryotic expression plasmid pcDNA3.1(+)/azurin. Our results demonstrate that U2OS cells show a higher rate of apoptosis when transfected with pcDNA3.1(+)/azurin compared to when they are treated directly with azurin protein. The induction of apoptosis by pcDNA3.1(+)/azurin is closely associated with the down-regulation of Bcl-2 and the up-regulation of Bax, which is also consistent with the previous conclusion. The increased expression of p53 was previously estimated by us, and confirmed in this study.

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