

# Preliminary study on XAGE-1b gene and its mechanism for promoting tumor cell growth

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Received January 30, 2013; Accepted May 21, 2013

DOI: 10.3892/br.2013.122

**Abstract.** The XAGE-1b gene has been identified in numerous malignancies in the human body. However, little is known regarding its mechanism for promoting tumorigenesis in adenoid cystic carcinoma. The aim of this study was to explore the correlation between tumor cell growth and the XAGE-1b gene. The constructed PCMV-Myc plasmid vector containing the XAGE-1b gene and transfected adenoid cystic carcinoma (ACC)-2 cells was applied to study cell cycle alterations and anti-apoptotic effects. These were assessed by flow cytometry with PI staining and the measurement of cell content at its Sub-G<sub>1</sub> phase, respectively. The fluorescence intensity representing the regulation of XAGE-1b on the transcription factors located downstream of the signaling pathway using the Mercury pathway profiling system was also detected. XAGE-1b overexpression promoted cell growth by shortening G<sub>0</sub>-G<sub>1</sub> and prolonging the G<sub>2</sub>-M phase. Additionally, XAGE-1b overexpression enhanced the anti-apoptotic effects induced by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and serum deprivation in ACC-2 cells. The results of the present study suggested that XAGE-1b gene is crucial in the tumorigenesis of ACC, and its mechanism should be further investigated.

## Introduction

The function of XAGE-1b, a member of the cancer testis antigen (CTA) family, has been previously investigated, with a focus on its expression profile and immunogenicity (1-3). Overexpression of XAGE-1b in adenoid cystic carcinoma-M (ACC-M) and ACC cell lines was observed in an earlier investigation (data not yet published). Results of that study suggested that XAGE-1b is an important gene that is relevant

to the tumorigenesis and metastasis of ACC. Additionally, XAGE-1b overexpression and RNA interference confirmed that XAGE-1b promoted the cell growth and metastasis of ACC *in vivo* and *in vitro* (unpublished data).

XAGE-1b is mainly expressed within the nucleus and transcription activity domains such as GAL-4 are located at its C terminal. Therefore, XAGE-1b potentially functions as a transcription factor. Findings of a previous study showed that GAGE-7, which belongs to the same family as CTAs, inhibited cell apoptosis mediated by interferon or Fas receptor (4). At present, little is known about the definite correlation between alterations of the cell cycle and apoptosis and XAGE-1b overexpression in ACC.

To study the cell cycle, a eukaryotic vector with transient XAGE-1b overexpression was constructed and transfected into ACC-2 cells, and its anti-apoptotic effects were investigated. The downstream signaling pathway and the interaction chaperone in which XAGE-1b was involved were detected using the Mercury pathway profiling system provided by Clontech (5). The cis-acting element of the molecule associated with the cell signaling pathway highlighted the reporting gene of the plasmid system, and the reporting gene expression detected identified the direct or indirect interaction between XAGE-1b protein and its gene enhancer. Results obtained in the present study provide important evidence to elucidate the mechanism for promoting tumor cell growth with XAGE-1b overexpression.

## Materials and methods

**Plasmids and cell lines.** The Mercury™ pathway profiling vector purchased from Clontech (Mountain View, CA, USA) included the cis-enhancement elements, TAL initiator and Luciferase reporter gene that belong to the transcription factors of the signaling pathway. The vectors included pAPI-Luc, pCRE-Luc, pGRE-Luc, pHSE-Luc, pNF- $\kappa$ B-Luc, pSRE-Luc, pP53-Luc, pRB-Luc, pc-Myc-Luc, pE2F-Luc and pTAL-Luc (control plasmid). The PCMV-Myc and the control pRL-SV40 plasmid were purchased from Clontech and Promega (Madison, WI, USA), respectively. The human salivary ACC-2 and 293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). ACC-2 cells cultured in RPMI-1640 were purchased from Gibco

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**Key words:** XAGE-1b gene, adenoid cystic carcinoma, apoptosis, signaling pathway, tumor necrosis factor- $\alpha$ , cancer testis antigen

(Langley, OK, USA) and supplemented with 10% fetal bovine serum (FBS) from Sigma (St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. 293T cells were cultured in DMEM obtained from Gibco and supplemented with 10% FBS. All chemicals used for cell culture were purchased from Gibco.

**Construction of the eukaryotic vector with transient XAGE-1b overexpression.** cDNA of XAGE-1b from ACC-2 cells was obtained and amplified using XAGE-1b primers: PCMV-1b, F: 5'-CCGGAATTCGGATggAgAgCCCCAAAAgAAgA-3' and R: 5'-CCGGTCGAGTTGCGTTGTTTCAGCTTGTC-3' with the restriction sites *Eco*RI and *Xho*I (underlined base sequences) respectively. The fragments obtained were inserted into the PCMV-Myc plasmid designated as PCMV-Myc-1b as the eukaryotic transient overexpression vector.

**Plasmid extraction.** The *E. coli* DH5α containing PGL3-A1, PGL3-A2, PGL3-A3, PGL3-B1, PGL3-B2, PGL3-B3, PGL3 and PRL plasmid were cultured to its logarithm phase and the precipitations were collected by centrifugation. Extraction was performed according to the manufacturer's instructions (Tiangen, Beijing, China). The extracted expression plasmid comprised PCMV-Myc-1b, the control negative PCMV-Myc, the double fluorescent control pRL-SV40 and the Mercury™ pathway profiling vector plasmid, including pAP1-Luc, pCRE-Luc, pGRE-Luc, pHSE-Luc, pNF-κB-Luc, pSRE-Luc, pP53-Luc, pRB-Luc, pc-Myc-Luc, pE2F-Luc and pTAL-Luc.

**Cell cycle alterations with XAGE-1b overexpression.** ACC-2 cells at a density of 2x10<sup>5</sup>/ml were seeded in 6-well plates and cultured for 18-24 h, and grown until 70-80% confluence at 37°C in an atmosphere of 5% CO<sub>2</sub> prior to transfection. PCMV-Myc-1b (2 μg) sequencing and the control negative PCMV-Myc were transfected with 4 μl of Lipofectamine 2000, respectively, and with 3 wells/transfected cells. The cells were continuously cultured for 36 h at 37°C, and the collected cells (1-5x10<sup>5</sup>) were centrifuged at 55.5-111 x g for 5 min and washed with 3 ml PBS. The precipitations were fixed in cold 70% alcohol at 4°C overnight. Subsequent to centrifugation, the sediment was suspended with 3 ml PBS and then centrifuged at 55.5-111 x g for 5 min. The precipitations were stained with 1 ml PI (10 μg/ml, Sigma) containing Rnase A (20 mg/l) and 1.5% Triton X-100 at 4°C for 30 min in the dark. After washing with PBS, the cell cycle was analyzed with a FACSCalibur flow cytometer according to the manufacturer's instructions (Becton-Dickinson and Company, Franklin Lakes, NJ, USA).

**Anti-apoptotic effects of XAGE-1b.** ACC-2 cells (8x10<sup>4</sup>) were seeded in 24-well plates and cultured for 18-24 h, and grown to 70-80% confluence at 37°C in an atmosphere of 5% CO<sub>2</sub>. PCMV-Myc-1b (1 μg) and the control negative PCMV-Myc were transfected with 2 μl Lipofectamine 2000 and with 24 wells/transfected cells. Cells were continuously cultured for 24 h at 37°C. Apoptosis was subsequently induced by tumor necrosis factor-α (TNF-α) (Xinbainuo, Shanghai, China) and serum deprivation, respectively. The cell content representing the number of necrotic and apoptotic cells at the Sub-G<sub>1</sub> phase was detected and analyzed with a FACSCalibur flow cytometer following 48- and 72-h induction, respectively.

**Regulatory effects on the main signaling pathway transcripts with XAGE-1b overexpression.** 293T cells (1x10<sup>4</sup>) were inoculated in 96-well plates and cultured for 24 h, and grown to 70-80% confluence at 37°C in an atmosphere of 5% CO<sub>2</sub>. The plasmids were simultaneously transfected by Lipofectamine 2000. The amount of PCMV-Myc-1b and PCMV-Myc empty plasmid (control) as well as the amount of the reporter plasmid containing cis-acting elements were 150 ng/well, while the amount of pRL-SV40 as an inner referencing plasmid representing the transfection efficiency was 20 ng/well. During the concentration-gradient experiment, the amount of Mercury Pathway Profiling system plasmid and the pRL-SV40 plasmid remained unchanged. However, the content of PCMV-Myc-1b was arranged according to the gradients 300, 150, 100, 75, 50 and 0 ng/well, while the amount in the controls reached 300 ng by PCMV-Myc empty plasmid. Cells were lysed after 36 h culture, and the luciferase activity was detected using the Dual-Luciferase kit (Promega, Madison, WI, USA). Fluorescence intensity was measured by Lumat LB9507 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The relative value of fluorescence intensity was recorded, and the ratio of M1/M2 was calculated.

**Statistical analysis.** Data were presented as the mean values and standard deviation of the sample. Statistical analysis was performed using the two-tailed Student's t-test. P<0.05 was considered statistically significant.

## Results

**Cell cycle alterations of ACC-2 cells.** XAGE-1b overexpression transfected with PCMV-Myc-1b plasmid in ACC-2 cells was observed. Results of the western blot analysis are shown in Fig. 1A. Transient XAGE-1b overexpression in ACC-2 cells with PCMV-Myc-1b plasmid transfection was identified, whereas no overexpression of the target protein with control plasmid transfection or the blank was observed. Therefore, the plasmid could be applied in the study for its transient overexpression of XAGE-1b gene.

Cell cycle alterations are shown in Fig. 1B. G<sub>0</sub>-G<sub>1</sub> phase exhibited a marked decrease in the PCMV-Myc-1b and PCMV-Myc groups compared with the blank. A statistical difference between PCMV-Myc-1b and PCMV-Myc (P<0.05) was observed. S phase increased more evidently than the blank, although no statistical difference was noted. A decrease was observed in the cell cycle of the G<sub>2</sub>-M phase compared with the blank, while G<sub>2</sub>-M phase of PCMV-Myc-1b was increased compared with PCMV-Myc. There was no statistical difference (P>0.05).

**Anti-apoptotic effects of XAGE-1b overexpression.** Cytokine TNF-α and serum deprivation were applied to induce the apoptosis and necrosis of ACC-2 cells (Table I). Fig. 2A and B shows the peak for Sub-G<sub>1</sub>. The results of overexpression and negative control group were compared with those of the induction-free group, and the ratio was used to evaluate the effects of apoptosis-induction.

After a 48-h induction by TNF-α, the ratio of XAGE-1b (0.85±0.23) was lower than that of the negative control

Table I. Groups of antagonizing apoptosis-induced by TNF- $\alpha$  and serum deprivation with XAGE-1b.

Time point	PCMV-Myc-1b			PCMV-Myc		
	TNF- $\alpha$ (1,250 $\mu$ /ml)	Serum deprivation	Normal culture	TNF- $\alpha$ (1,250 $\mu$ /ml)	Serum deprivation	Normal culture
48 h	2A a	2A b	2A c	2A d	2A e	2A f
72 h	2B a	2B b	2B c	2B d	2B e	2B f

Cytokine TNF- $\alpha$  at the concentration of 1,250  $\mu$ /ml containing 1% blood serum culture medium for apoptotic induction. Serum deprivation refers to apoptotic induction by the incomplete medium. Normal culture is the complete medium as the control. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

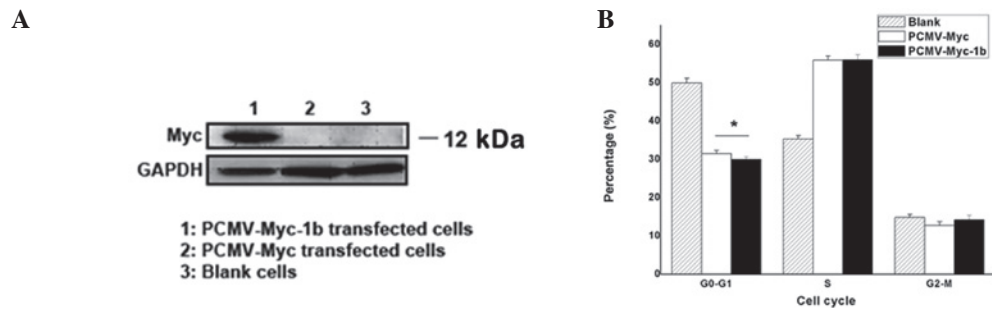


Figure 1. Cell cycle alterations with XAGE-1b gene overexpression in ACC-2 cells. (A) Western blot analysis of PCMV-Myc-1b-transfected cells, PCMV-Myc-transfected and blank cells. (B) Cell cycle alterations in different groups. \*P<0.05.

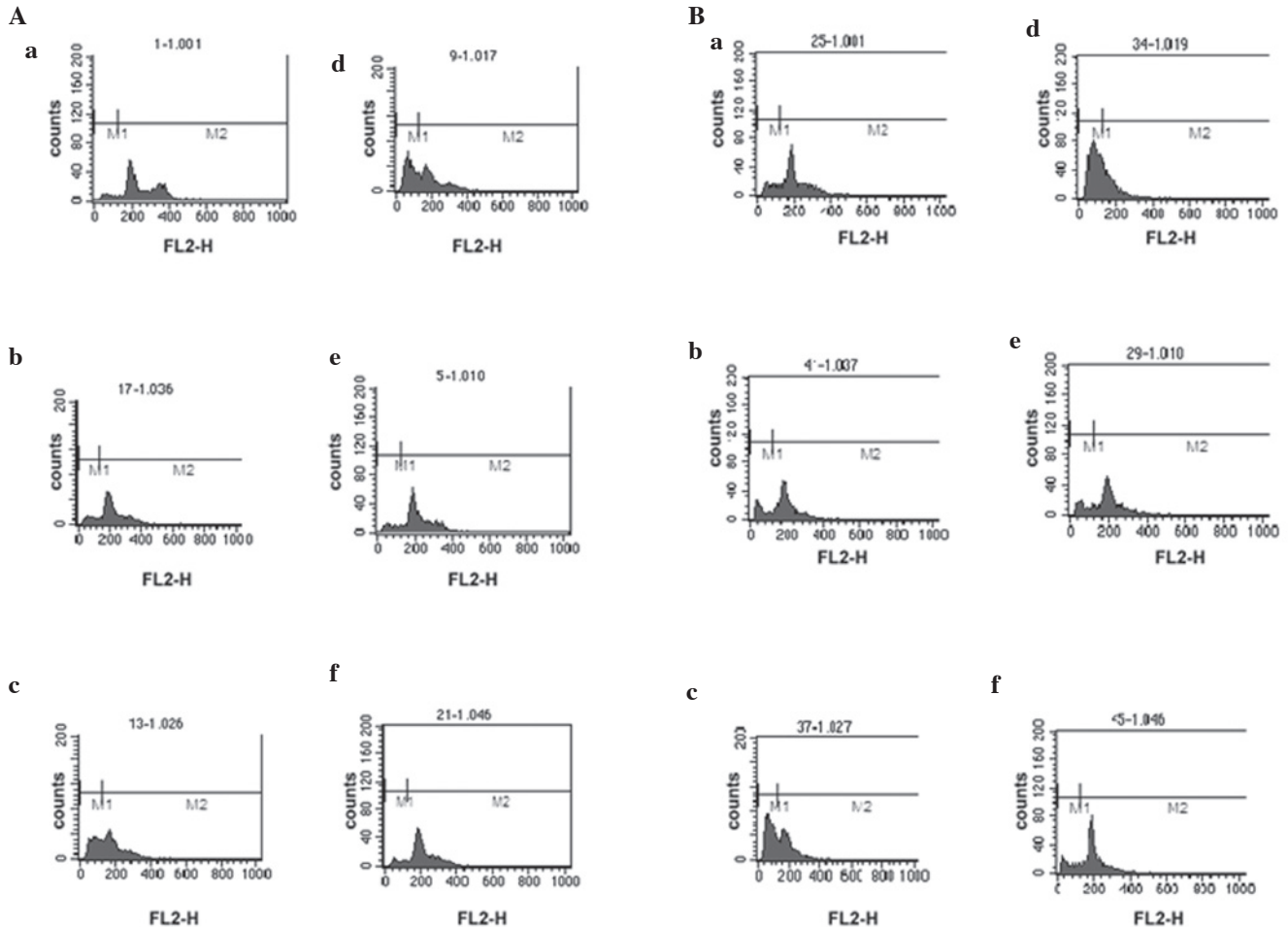


Figure 2. (A and B) The distribution of cell apoptosis induced by TNF- $\alpha$  and serum deprivation with FACS detection in the Sub-G<sub>1</sub> phase. Capital letters represent the different groups (Table I). TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

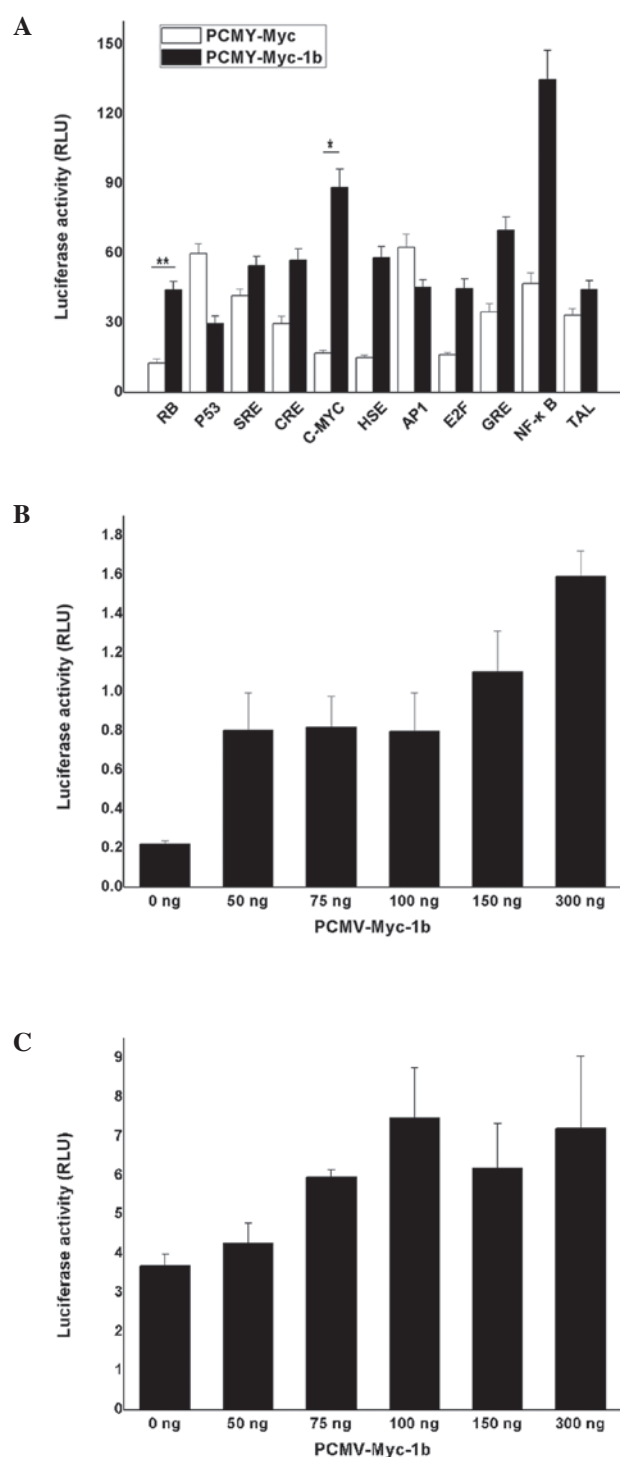


Figure 3. (A) Regulatory effects on the main signaling pathway transcripts with XAGE-1b overexpression (\* $P < 0.05$  and \*\* $P < 0.01$ ). (B) Correlation between the different concentrations of XAGE-1b overexpression and its luciferase activity of c-Myc response element. (C) Correlation between the different concentrations of XAGE-1b overexpression and its luciferase activity of retinoblastoma (RB) response element.

( $0.98 \pm 0.07$ ), although the difference was not significant. However, the ratio of XAGE-1b ( $0.88 \pm 0.08$ ) was significantly lower than that of the negative control ( $1.15 \pm 0.05$ ) ( $P < 0.01$ ) after 72 h of induction. These results suggest an inherent tolerance of antagonizing apoptotic induction by TNF- $\alpha$  in ACC-2 cells. Similarly, after a 48-h induction by serum deprivation, no difference was observed between the ratio of XAGE-1b

( $2.56 \pm 0.60$ ) and the negative control ( $2.36 \pm 0.87$ ). By contrast, the ratio of XAGE-1b ( $2.93 \pm 0.17$ ) was significant lower than that of the negative control ( $3.50 \pm 0.18$ ) ( $P < 0.01$ ) after a 72-h induction. Thus, the results suggest an inherent tolerance in ACC-2 cells. In general, the results showed anti-apoptotic effect after 72-h induction with XAGE-1b overexpression in ACC-2 cells.

**Regulation of the transcripts of the downstream signaling pathway.** The elements of the signaling pathway included RB, E2F, c-Myc, p53, CRE, GRE, HSE, SRE, AP-1 and NF- $\kappa$ B. 293T cells were co-transfected with the vectors containing PCMV-Myc-1b, and the transcription factor containing an element and reporter gene. The ratio of M1/M2 was then calculated. The increased ratio was observed in the groups that included retinoblastoma (RB), CRE, c-Myc, HSE, E2F, GRE and NF- $\kappa$ B when XAGE-1b was overexpressed (Fig. 3A). The t-test showed the evident activity effects on the response element of RB ( $P < 0.01$ ) and c-Myc ( $P < 0.05$ ). However, the ratio of p53 and AP1 decreased slightly, although no statistical difference was observed.

The correlation between the concentration-gradient of c-Myc (Fig. 3B) and RB (Fig. 3C) and XAGE-1b was investigated. The results showed identical activity effects on the transcription factor c-Myc with the XAGE-1b overexpression plasmid exhibiting an increase of 50-100 ng. The final values were ~3 to 4 times higher than those of the PCMV-Myc-1b plasmid at a concentration of zero. The activity of the transcription factor c-Myc was enhanced in accordance with the increased concentration of the expressing plasmid (150-300 ng) and it reached 4 and 7 times higher than that of the expressing plasmid at the concentration of zero. Similarly, the enhancement of the transcription factor RB with XAGE-1b overexpression plasmid increasing from 50 to 100 ng, and the values at a concentration of 100 ng were twice as high as the value at a concentration of zero. This result indicated that the activity of the transcription factor RB is potentially saturated at concentrations of 150 and 300 ng.

## Discussion

CTAs have an expression pattern that is predominantly restricted to testis among normal tissues, but they are expressed in various histological types of cancer. XAGE-1 is a CTA that was demonstrated to be expressed at a significant frequency and to be immunogenic in some solid tumors. Previous findings (6) suggest that the transcription of XAGE-1 gene is initiated from two distinct start sites, resulting in the overlapping transcripts of XAGE-1a and XAGE-1b. Additionally, XAGE-1a contains two in-frame ATG translational start codons, whereas XAGE-1b is initiated downstream of the first ATG start codon. XAGE-1b is potentially the dominant transcript, and its translation is initiated with the second ATG start codon (6). In the present study, XAGE-1b gene overexpression promoted the growth and metastasis of ACC cells *in vivo* and *in vitro*. XAGE-1b-positive expression in the nucleus of ACC cells obtained from the patients was also detected. We hypothesized that this expression affects cell growth and apoptosis by regulating transcription. In the present study, we investigated the manner in which XAGE-1b



overexpression affects cell growth and apoptosis, promotes tumorigenesis.

Apoptosis is regarded as a carefully regulated energy-dependent process, characterized by specific morphological and biochemical features and it elicits a range of non-phlogistic homeostatic mechanisms that regulate the microenvironments of normal and diseased tissues (7,8). Tumorigenesis is thought to be involved in the pathological process with abnormal apoptosis of numerous cells, including the processes of signaling pathway, replication and transcription (9). However, little is known regarding the correlation between the XAGE-1b gene and apoptosis of salivary ACC. In this study, we applied TNF- $\alpha$  and serum deprivation as the inducer of apoptosis to examine the correlation between XAGE-1b overexpression and apoptosis in order to confirm the mechanism of anti-apoptotic effects in the signaling pathway. Apoptosis of salivary ACC induced by TNF- $\alpha$  and the related gene expression of apoptosis has been previously studied with results suggesting that apoptosis induced by TNF- $\alpha$  is capable of increasing the expression of bax and Bcl-2 (10). Neural cell adhesion molecular (NCAM) is involved in the apoptosis of human salivary gland tumor, and its effects mainly depend on NCAM expression through a transcriptional activator of NF- $\kappa$ B (11). Our results suggest that XAGE-1b overexpression reduced G<sub>0</sub>-G<sub>1</sub> phase and increased the G<sub>2</sub>-M phase as compared with the control. The G<sub>0</sub>-G<sub>1</sub> phase was significantly reduced and S phase was increased compared with the blank. Additionally, XAGE-1b overexpression may promote ACC-2 cells to exit the G<sub>0</sub>-G<sub>1</sub> phase immediately, and enter the S or G<sub>2</sub>-M phase rapidly. However, the results demonstrated no significant difference between PCMV-Myc-1b- and PCMV-Myc-transfected cells, which may be associated with the lower transfection efficiency of ACC-2 cells due to lack of selection and comparison of the positive cell lines. Cell cycle alterations suggested this association may affect the cell cycle regulators with XAGE-1b overexpression.

The effects on apoptosis with XAGE-1b overexpression could be regarded as a breakthrough for the exploration and study of the regulatory effects on the cell cycle. Subsequently, serum deprivation and TNF- $\alpha$  were applied as apoptosis inducers in the present study. The mechanisms of apoptosis are extremely complex and sophisticated. The extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway, are connected and have an impact on each other (12). TNF- $\alpha$  is an extrinsic pathway protein that affects a wide range of biological activities, including cell proliferation and apoptosis. In the present study, TNF- $\alpha$  was applied as an apoptosis inducer at concentrations of 2,500  $\mu$ /ml and 1,250  $\mu$ /ml, and after a 48- and 72-h induction, the anti-apoptotic effects with XAGE-1b overexpression were observed. The higher concentration of 2,500  $\mu$ /ml was applied prior to that of 1,250  $\mu$ /ml due to the higher mortality rate observed after 72 h of induction. Anti-apoptosis in ACC-2 cell lines was observed at the concentration of 2,500  $\mu$ /ml as compared to that of 1,250  $\mu$ /ml, and the degree of cell death in the XAGE-1b overexpression group was less than that of the control after 72 h induction. Similarly, when apoptosis induction occurred via serum deprivation, anti-apoptosis was identified, and the degree of cell death in XAGE-1b overexpression group was less than that of the control after 72 h induction. The results demonstrate the

dose-effect relationship between the effects of anti-apoptosis and the amounts of XAGE-1b overexpression.

Tumor necrosis factor- $\alpha$  activated the multi-signaling transcription pathway by recruiting the extracellular ligands and activating the downstream pathway of apoptosis. TNF- $\alpha$  also promoted cell proliferation and differentiation, and contributed to the immune and inflammatory response via transcription factors, such as NF- $\kappa$ B and JHK (13). NF- $\kappa$ B as the key transcription factor is crucial in the anti-apoptotic effects through its involvement in initiating the expression of survival genes, including Bfl-1/A1, Bcl-2 and Bcl-Xl, with the anti-apoptosis induced by TNF (14-16). To determine the exact anti-apoptotic mechanism of XAGE-1b, induction of apoptosis by TNF- $\alpha$  and serum deprivation should initially be conducted. Thus, the mechanism of apoptosis may be crucial for examining the anti-apoptotic mechanism. While examining TNF- $\alpha$ -mediated apoptosis, reactive oxygen species (ROS) produced by TNF- $\alpha$  was found to have an important function in cell death by activating c-Jun N-terminal kinase (17). XAGE-1b overexpression promoted anti-apoptosis induced by TNF- $\alpha$ , the effects of which may involve the increased transcriptional activities of NF- $\kappa$ B and promotion of survival gene expression, or potential interference with other gene expressions associated with the death signaling pathway. The abovementioned hypotheses remain to be confirmed. Serum deprivation-induced cell death, a characteristic of apoptosis, results in a possible increase of death receptor activation and oxygen pressure, DNA breakage, caspase-3 and -9 activation, cytochrome *c* release, bax expression increase, Bcl-2 expression decrease, as well as the reduction of combining activities of NF- $\kappa$ B and lower glutathione content *in vivo* (18-20). XAGE-1b overexpression increased anti-apoptosis induced by serum deprivation, and its mechanism of action and its correlation with the mechanism of anti-apoptosis induced by TNF- $\alpha$  should be investigated.

The regulation of different signaling pathways may explain the results regarding the cell cycle and apoptosis. Results of the present study have shown the different activity of transcriptional factors, including RB, CRE, c-Myc, HSE, E2F, GRE and NF- $\kappa$ B, with XAGE-1b gene overexpression, but inhibitory activity to p53 and AP1. No significant differences were observed among the factors, with the exception of RB and c-Myc. The role of the Myc gene family in the biology of normal and cancer cells has been intensively studied since the early 1980s. Myc gene expression is known to cause tumors and is one of the oncogenes found to be altered in human cancers. Myc is a multifunctional protein that is able to regulate cell cycle, cell growth, differentiation, apoptosis, transformation, genomic instability, and angiogenesis (21,22). The Rb gene is one of the tumor suppressor genes that affect cell proliferation and apoptosis. Additional investigations revealed an improved linear association between activation of the c-Myc response element and XAGE-1b overexpression during the course of the c-Myc concentration-gradient experiment, thereby proving there is a direct or indirect activation of c-Myc element with XAGE-1b overexpression. The c-Myc gene expresses the nucleoprotein-regulating gene function in alteration of the cell cycle, cell growth and metabolism, gene instability, stimulation of angiogenesis, cell malignancy trans-

formation, differentiation and apoptosis. Several target genes including Cdc25A, Cdk4 and cyclin D2 and their expression were promoted, while growth inhibition genes including gas1, p15, p21 and p27 suppressed gene expression (23,24). The regulation of these genes led to cell proliferation and eventually to malignant transformation. However, it was demonstrated that the gradient activity of Rb gene is weak, and the activity only increased twice compared with the control when the saturated concentration was reached, suggesting activation on the RB element is affected by XAGE-1b. The transcription factor NF- $\kappa$ B plays an important role in the process of anti-apoptosis. The value of NF- $\kappa$ B in the PCMV-Myc-1b group with XAGE-1b overexpression greatly increased compared with PCMV-Myc, although no statistical difference was identified. Therefore, we hypothesized that the anti-apoptosis of XAGE-1b is associated with NF- $\kappa$ B.

In conclusion, XAGE-1b is a potential anti-apoptotic agent, with notable anti-apoptotic effects in ACC-2 cells. The mechanism of its anti-apoptotic effects may be associated with XAGE-1b overexpression in ACC-2 cell lines and regulation of the transcription factor of the downstream signaling pathway. To the best of our knowledge, this is the first study to confirm the anti-apoptotic effects associated with the direct or indirect activation of the c-Myc element and the indirect activation of the RB element. The general profile of the XAGE-1b gene, particularly the exact effects associated with the cis-transcription elements in promoting tumor cell growth remain to be investigated.

## Acknowledgements

This study received financial support from the Health Bureau of Zhejiang Province Foundation (no. 2009A055), the Natural Science Foundation of Zhejiang Province (no. 2090053) and was sponsored by the Chinese National Natural Science Foundation (no. 30772591).

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