

Expression levels of the *BAK1* and *BCL2* genes highlight the role of apoptosis in age-related hearing impairment

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Abstract: Age-related hearing impairment (ARHI) is a progressive and a common sensory disorder in the elderly and will become an increasingly important clinical problem given the growing elderly population. Apoptosis of cochlear cells is an important factor in animal models of ARHI. As these cells cannot regenerate, their loss leads to irreversible hearing impairment. Identification of molecular mechanisms can facilitate disease prevention and effective treatment. In this study, we compared the expression of the genes *BAK1* and *BCL2* as two arms of the intrinsic apoptosis pathway between patients with ARHI and healthy subjects. ARHI and healthy subjects were selected after an ear nose throat examination, otoscopic investigation, and pure tone audiometry. RNA was extracted from peripheral blood samples, and relative gene expression levels were measured using quantitative real-time polymerase chain reaction. *BAK1* and the *BAK1/BCL2* ratio were statistically significantly upregulated in the ARHI subjects. The *BAK1/BCL2* ratio was positively correlated with the results of the audiometric tests. Our results indicate that *BAK*-mediated apoptosis may be a core mechanism in the progression of ARHI in humans, similar to finding in animal models. Moreover, the gene expression changes in peripheral blood samples could be used as a rapid and simple biomarker for early detection of ARHI.

Keywords: age-related hearing impairment (ARHI), presbycusis, biomarker, treatment

Introduction

Age-related hearing impairment (ARHI), or presbycusis, is the most common multifactorial sensory disease in the elderly.¹ ARHI has a huge societal impact as a result of its effects on quality of life and is a public health concern.² It is a bilateral, symmetrical, and sensorineural high-frequency impairment. Damage or loss of the inner ear hair cells and spiral ganglion cells is an important cause of ARHI,³ and a study on the auditory organ of aging mice demonstrated that apoptosis is a major form of cell death.⁴ As these cells are not able to regenerate, their loss causes irreversible hearing impairment.

There are two pathways for cell signal transmission in apoptosis: intrinsic and extrinsic. The BCL-2 protein family is responsible for the intrinsic pathway. Genes of this family are divided into two categories that include proapoptotic genes (*BAK1*, *BAX*, *BAD*) and antiapoptotic genes (*BCL2*, *BCLXL*).⁵ An investigation of early-onset hearing loss (HL) in a mouse model demonstrated upregulation of the apoptosis-related genes *Bak1* and *Casp3* during the development of ARHI.⁶ In addition, the MCAT transgenic mice exhibit delayed onset of age-related HL, and investigation of the underlying molecular mechanisms revealed a decreased in the mRNA expression level of *Bak1* in the cochlea and a reduction in cell death.⁷ Moreover, C57BL/6J mice

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with a deletion of the *Bak1* gene exhibited a reduction in age-related apoptotic cell death of hair cells in the cochlea and spiral ganglion neurons, resulting in the postponement of ARHI.⁷

High expression of *Bcl2* and *Bcl-xL* was identified as a key factor in inner ear aging in the mouse cochlea by Tadros et al.⁸ Given the role of apoptosis in ARHI, Yang et al⁶ reported an antiapoptotic treatment that resulted in the preservation of hearing and reduced outer hair cell loss at the cochleae of DBA/J2 mice. Understanding the mechanism of cell apoptosis in ARHI will not only shed light on the intracellular mechanisms underlying aging of the cochlear but also make it possible to manipulate these mechanisms for treatment and prevention of this common human impairment.

The aim of this study was to investigate the role of expression of two genes, representing different arms of the BCL-2 protein family, *BAK1* (proapoptotic) and *BCL2* (antiapoptotic), in peripheral blood samples from ARHI patients in comparison to healthy subjects.

Materials and methods

A total of 52 subjects with ARHI and 29 healthy subjects were selected by otolaryngologist at the ENT and Head & Neck Research Center of Iran University of Medical Science. The participants signed informed consent letters prior to inclusion in the study. This study was approved by the Ethics Committee of Iran University of Medical Sciences.

All subjects completed a questionnaire to determine their medical history and environmental factors that may have affected their ability to hear. Subjects who had experienced factors known to affect hearing sensitivity were excluded from the study.^{9,10} The relevant factors included exposure to ototoxic drugs, early-onset hearing impairment, history of any known cause of sensorineural HL, external or middle ear disease, cognitive dysfunction, neurological or psychiatric disease, brain tumor or vestibular schwannoma, liver cirrhosis, renal failure, cardiovascular disease, history of stroke, and cancer.

Otoscopic examination, tympanometry, pure tone audiometry, and measurement of speech reception thresholds (SRTs) and speech discrimination scores (SDSs) were performed for all subjects at the Audiology Department. Tympanometry was performed using an Amplaid 728 (Amplaid Inc., Milan, Italy) multifrequency admittance meter. Pure tone audiometry was performed using an Amplaid 319 audiometer (Amplaid Inc.). Calibration was performed according to American National Standards Institute standards (ANSI S3.6-2004, ANSI, 2004a). Air conduction thresholds were

measured at 250, 500, 1,000, 2,000, 4,000, and 8,000 Hz, and bone conduction thresholds were measured at 500, 1,000, 2,000, and 4,000 Hz with the same conditions for all subjects. SRT was performed by presenting four spondaic words to each patient, according to the Farsi-language version of the staggered spondaic word test, at different intensities.¹¹ The SRT was defined at the lowest intensity that a patient could repeat 50% of spondees correctly. SDS was performed by presenting 25 unfamiliar one-syllable words at 40 dB above the SRT for the patient. The percentage of words which the patient understood and repeated correctly was defined as the SDS. Audiological exclusion criteria were conductive HL (mean air–bone differences at 500, 1,000, and 2,000 Hz of more than 15 dB); acoustic trauma (differences between air thresholds at 4,000 and 8,000 Hz exceeding 20 dB); or experience of extensive noise exposure (self-report).

Subjects with ARHI had symmetric, bilateral hearing impairment with pure tone average (PTA) values at medium frequencies (1, 2, 4, and 8 kHz) of ≥ 30 dB HL. The healthy subjects had normal ear examinations and PTA values at medium frequencies of < 25 dB HL (Figure 1).

Total RNA samples were extracted from 1 mL of peripheral blood using a QIAamp RNA Blood Mini Kit (Cat No 52304; QIAGEN, Hilden, Germany), according to the manufacturer's instructions. cDNA was synthesized from 1 μ g total RNA using a QuantiTect Reverse Transcription Kit (Cat No 205311; QIAGEN) according to the manufacturer's instructions.

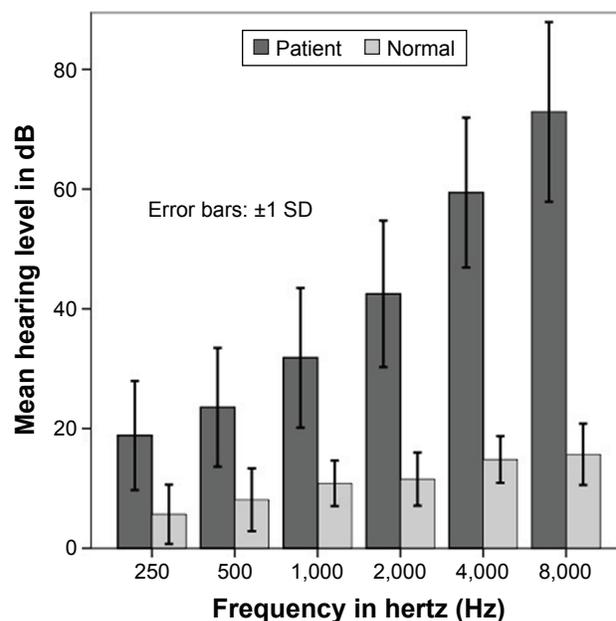


Figure 1 Mean air conduction hearing threshold.
Note: Error bars represent standard deviations (SDs).

Table 1 Sequences of the PCR primers for *BAK1*, *BCL2*, and *ACTB*

Gene	GenBank accession number	Amplicon size (bp)	Reverse primer	Forward primer
<i>BAK1</i>	NM_001188.3	120	CAA ACA GGC TGG TGG CAA TC	TCA TCG GGG ACG ACA TCA AC
<i>BCL2</i>	NM_000633.2	129	CAG CCA GGA GAA ATC AAA CAG AGG	ATC GCC CTG TGG ATG ACT GAG
<i>ACTB</i>	NM_001101.3	131	GTA GTT TCG TGG ATG CCA CA	TCC CTG GAG AAG AGC TAC G

Abbreviation: PCR, polymerase chain reaction.

Quantitative real-time polymerase chain reaction (qPCR) analysis was performed for *BAK1*, *BCL2*, and the housekeeping gene *ACTB*, which was selected as a reference gene. qPCR was performed using specific primers (Table 1) and RealQ-PCR Master Mix with Green DNA dye (Cat No 250507; Ampliqon, Odense, Denmark) in a Rotor-Gene6000 instrument (Corbett life Science, Sydney, Australia). All reactions were performed in duplicate in a total volume of 25 μ L, containing 12.5 μ L of RealQ-PCR 2 \times Master Mix, 0.4 μ L of each primer (10 pmol), 1 μ L cDNA, and 10.7 μ L RNase-free water. A two-step PCR program was used, with an initial activation step for 15 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 20 seconds, and annealing and extension at 60°C for 60 seconds. The threshold cycle (Ct) was determined for *BAK1*, *BCL2*, and *ACTB* in each sample. Gene expression levels were calculated using the following equation:

$$\text{Relative copy number} = 2^{-\Delta\text{Ct}} (\Delta\text{Ct} = \text{Ct}_{\text{GENE}} - \text{Ct}_{\text{ACTB}}).$$

Statistical analysis was performed using SPSS 22 for Windows (IBM Corporation, Armonk, NY, USA). Statistical significance was defined as $P < 0.05$. All numerical variables were checked for normal distribution using the one-sample Kolmogorov–Smirnov test. The Mann–Whitney U -test was used for the comparison of gene expression values between ARHI and healthy subjects. The Spearman’s rank-order correlation was used to evaluate potential correlations between PTA, SRT, and SDS, and gene expression.

Results

The mean ages of ARHI and healthy subjects were 66.7 ± 9 years and 64 ± 6.4 years, respectively. The sex balance was 54.3% male and 45.7% female, with no statistically significant differences in age ($t[79] = -1.5$; $P = 0.13$) and sex ($\chi^2[1, N=81] = 1.6$; $P = 0.2$) between ARHI and healthy subjects.

BAK1 and *BCL2* gene expression levels were determined in ARHI and healthy subjects, relative to those of the *ACTB* gene. The difference between the *BAK1* expression

level in ARHI subjects (median = 1) compared to healthy subjects (median = 0.34) was statistically significant ($U = 484$, $P = 0.008$); hence, the ARHI subjects exhibited elevated levels of expression of the proapoptotic gene, *BAK1*, compared with healthy subjects (Figure 2).

A Mann–Whitney U -test indicated that levels of expression of the antiapoptotic gene, *BCL2*, were also greater for ARHI (median = 1.06) compared with healthy subjects (median = 0.58; $U = 548$, $P = 0.04$) (Figure 3).

Since the balance between pro- and antiapoptotic members of the BCL-2 protein family is a key factor in apoptosis, we evaluated the *BAK1/BCL2* expression ratio, revealing a statistically significant difference between ARHI and healthy subjects ($U = 544$, $P = 0.039$). The *BAK1/BCL2* median ratios for ARHI and healthy subjects were 1.46 and 0.8, respectively (Figure 4).

Next, correlations between the *BAK1/BCL2* ratio and differences in auditory perception were evaluated. Auditory perception measures included PTA, SRT, and SDS. As the data were not normally distributed, we performed Spearman’s rank-order correlation analysis. The results revealed a significant and positive correlation between *BAK1/BCL2* ratio and PTA ($r_s[81] = 0.3$, $P = 0.015$) and SRT

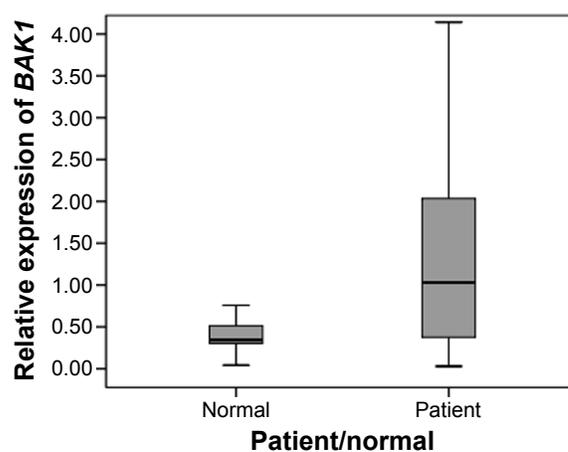


Figure 2 Gene expression of *BAK1* in peripheral blood samples from Iranian patients with ARHI and healthy subjects.

Notes: The difference between the two groups is statistically significant ($U = 484$, $P = 0.008$). The box plots show the median (solid line across the box).

Abbreviation: ARHI, age-related hearing impairment.

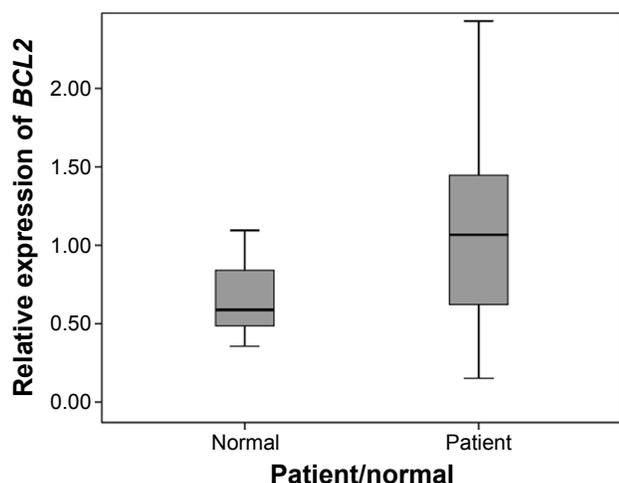


Figure 3 Gene expression of *BCL2* in peripheral blood samples from Iranian patients with ARHI and healthy subjects.

Notes: Difference between the two groups is statistically significant ($U=548$, $P=0.04$).
Abbreviation: ARHI, age-related hearing impairment.

($r_s[81]=0.3$, $P=0.007$). In addition, there was a significant negative correlation between SDS and the *BAK1/BCL2* ratio ($r_s[81]=0.25$, $P=0.03$).

Discussion

BCL-2 protein family members regulate mitochondrial membrane permeabilization during apoptosis, with BAK1 and BAX proteins forming an apoptotic pore within the mitochondrial outer membrane.¹² A recent study demonstrated that the role of the carboxyl terminus of BAK1 in lining the apoptotic pore or aggregating on the membrane surface is key to the destabilization of the mitochondrial outer membrane by BAK1 to generate pores and consequent initiation

of the apoptotic cascade.¹³ Various different tissues in a *BAK1*-deficient mouse model showed a marked reduction in apoptosis,¹⁴ and BAK1 played a central role in apoptosis-mediated murine cerebral cortical neuron degeneration after DNA damage.¹⁵ Furthermore, C57BL/6J mice with a deletion in *Bak1* have reduced apoptotic cell death and exhibit a delay in onset of ARHI.⁷ Oxidative stress, an important factor in age-related diseases, induces *Bak1* expression in primary cochlear cells, leading to apoptosis; however, apoptotic cell death is prevented in *Bak1*-deficient mice, even under conditions of oxidative stress.⁷

This study demonstrated a statistically significant increase in the *BAK1* gene expression level in ARHI compared to healthy subjects (Figure 2). A possible explanation for this could be a general increase in apoptosis among ARHI subjects. This result is in agreement with that reported by Dong et al,¹⁶ demonstrating an association between immune- and apoptosis-related genes and ARHI.

In this study, we found that *BCL2* was significantly upregulated in ARHI subjects (Figure 3). Tadros et al⁸ reported an increase in expression of the apoptosis-related genes, *Bcl2* and *Bcl-xL*, in the cochlea of the mice with age-related HL. They hypothesized that higher expression of *Bcl2* in the age-related HL model, the CBA mouse, may be a protective effect induced during the cochlear aging process.⁸ A previous study of the mechanism of BCL-2 family regulation of the onset of apoptosis in cells demonstrated that BCL2 inhibits the activation of BAK1 by binding to this protein in healthy cells.¹⁷ Neomycin, an aminoglycoside antibiotic, induces cell death of inner hair cells; however, over expression of *Bcl2* prevents neomycin-induced HL in adult mice.¹⁸ The significantly higher expression of *BCL2* in our ARHI subjects provides further support for the role of *BCL2* in a defense response mechanism in age-related HL in humans after the upregulation of *BAK1*.

Experimental evidence indicates that the ratio of anti- and proapoptotic BCL-2 protein family members is a key factor in determining cell death or survival.^{19,20} The *BAK1/BCL2* median ratio was significantly elevated in ARHI compared with healthy subjects, indicating the potential importance of the role of *BAK1* gene expression, which may be indicative of increased apoptosis among ARHI subjects (Figure 4). These results were consistent with data obtained from humans and mouse models, demonstrating a reduced neuron size and a decline in the medial olivocochlear efferent system during ARHI.^{21–23}

This study demonstrated positive correlations between the *BAK1/BCL2* ratio and PTA and SRT values. Hence, our results indicate an association between gene expression levels related to elevated apoptosis and increased PTA and SRT

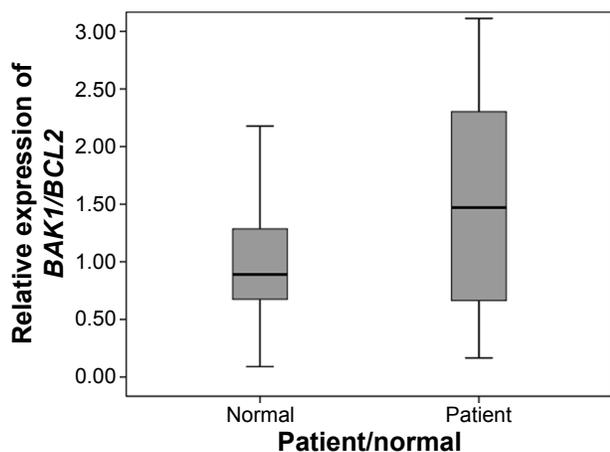


Figure 4 The *BAK1/BCL2* gene expression ratios of Iranian patients with ARHI and healthy subjects. The difference between the two groups is statistically significant ($U=544$, $P=0.039$).

Abbreviation: ARHI, age-related hearing impairment.

scores. SDS values were significantly negatively correlated with the *BAK1/BCL2* ratio. This finding is consistent with the reduced speech understanding exhibited by ARHI patients;³ however, the speech in noise test may be helpful to improving understanding of the relationship between *BAK1/BCL2* ratios and speech understanding in people with ARHI.

Our findings are in agreement with previous studies and support the hypothesis that elevated *BAK1* expression induces the apoptotic pathway in hair cells, initiating the molecular mechanism of impairment. Manipulation of the *BAK1* gene pathway is a treatment target of apoptosis-related diseases.²⁴ Supplementation of mouse food, by increasing coenzyme Q10 and lipoic acid content, reduced *Bak1* expression in the cochlea and prevented age-related HL.⁷

ARHI has profound economic and social health impacts, and the increasing elderly population makes ARHI an important issue for future generations. There is a long delay between the start of ARHI-related bodily changes and signs that are measurable using standard diagnostic tests.²⁵ Therefore, there is a pressing need for a suitable diagnostic biomarker for ARHI, and this is actively being sought by a number of research groups. Observation of a decline in the auditory brainstem gap from middle age in human and animal models was one interesting result in this area.^{23,26} *BAK1* has previously been identified as a predictive and prognostic biomarker in cancer therapy.²⁷ The significantly elevated expression of *BAK1* in peripheral blood samples from subjects with ARHI and the correlation of its expression with increased PTA and SRT values indicate that it may be a suitable and easy-to-use early detection diagnostic biomarker for this impairment. Further studies using a larger study population are required to confirm the present findings and establish their application in clinical practice.

Conclusion

In summary, we demonstrate an upregulation of *BAK1* gene expression and the *BAK1/BCL2* ratio in peripheral blood samples from Iranian ARHI subjects. Both *BAK1* gene expression and the *BAK1/BCL2* ratio were significantly correlated with the result of audiological tests routinely used for the diagnosis of presbycusis. Furthermore, the observation of significant changes in the expression of the apoptosis-related gene, *BAK1*, in peripheral blood samples suggests that it may be a useful biomarker for rapid diagnosis of ARHI.

Acknowledgments

We gratefully appreciate all the volunteers for participating in this scientific research. We thank the department and

audiometry clinic of Rasoul Akram Hospital of Iran University of Medical Science for their kind cooperation during this research. We would like to acknowledge the Iran University of Medical Sciences for supporting this research.

Disclosure

The authors report no conflicts of interest in this work.

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