Original Article

Expression Profile of Apoptotic Genes Bak1 and Bcl2 in Pakistani Presbycusis Patients from Faisalabad and Chichawatni Populations

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ABSTRACT

OBJECTIVE: To analyze the expression profiles of two apoptotic genes, *BAK1* and *BCL2*, in Pakistani ARHI patients' blood samples compared to healthy subjects.

METHODOLOGY: This expression profiles study was conducted at the Sight Centre of Bahawalpur City of Southern Punjab from August 2022 to July 2023. All of the subjects were from Faisalabad or Chichawatni, Pakistan cities. The participants exposed to head injury or trauma, noise exposure, ototoxic medication, chemical exposure, diabetes, liver cirrhosis, neurological disease, psychiatric disease, cognitive dysfunction, middle ear disease, and brain tumour factors were excluded from the study. Individuals with cold or flu were also excluded from the study. RNA was extracted from both the peripheral blood samples of healthy and affected individuals. The quantity of RNA was then determined, and cDNA was synthesized from it. This cDNA was then utilized in qPCR to assess the relative expression of the BAK1, BCL2, and ACTB genes using gene-specific primers.

RESULTS: Our results suggested that ARHI patients had higher levels of *BAK1* expression and *BAK1/ BCL2* ratio than healthy subjects.

CONCLUSION: Thus, apoptosis mediated by *BAK1* may be a key mechanism controlling the development of ARHI. Furthermore, changes in *BAK1* gene expression of blood samples might be utilized as a rapid test for early diagnosis of ARHI.

KEYWORDS: Presbycusis, Age related hearing impairment (ARHI), Apoptosis, *BAK1*, *BCL2*

INTRODUCTION

Age-related hearing impairment (ARHI), commonly known as presbycusis, is a more prevalent multifactorial disorder among aged people. With an increase in the expectancy of human life and the ratio of elderly persons, the incidence of ARHI is also elevating[1]. Individuals aged 65 years or above have this hearing impairment. It is considered an untreatable and unpreventable disorder[2]. ARHI is a crucial health concern with a considerable social impact. Age-related hearing impairment (ARHI) is related to cognition, which has grabbed the attention of many researchers who have studied this disease[3]. This disease has been linked with anxiety, dementia and depression. It is a bilateral, symmetrical, and sensorineural higher frequency impairment in which the hearing threshold tends to damage slowly with age. Gradual degeneration of various sensory cells increases with age and poor diet[4]. This disease is caused by damage and complete loss of stria vascularis cells, spiral ganglion cells, and sensory hair cells. Some critical mechanisms involved in this hearing loss are oxidative stress, apoptosis, mitochondrial dysfunction and inflammation. The primary reason for ARHI is the loss or impairment of spiral ganglion cells and hair cells of the inner ear[5]. ARHI is now widely acknowledged to be associated with ageing-related reductions in the auditory system's capacity for spectral and temporal resolution. Age-related variables that decrease neuronal synchronization are thought to be linked to impaired temporal processing[6].

Apoptosis involves extrinsic and intrinsic signalling pathways. The BCL2 protein family regulates the intrinsic pathway. This family's genes are categorized into two groups: anti-apoptotic and pro-apoptotic. *BCL2* and *BCLXL* are anti-apoptotic genes, while *BAK1*, *BAX*, and *BAD* are pro-apoptotic genes[7].

BAK1 and *Casp3* are apoptosis-related genes whose upregulation has been observed in mice models during the onset of ARHI[8]. The *BAK1* gene accelerates the permeability of the outer membrane of mitochondria, creating big pores that enable cytochrome c release and consequently activate the caspase cascade. Cell death induced by *the BAK1* gene is considered a key mechanism in the development of ARHI disease. Variations in *BAK1* expression can be exploited as a biomarker for the

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early diagnosis of ARHI in peripheral blood samples. Cell destruction of hair cells in the ganglion neurons and cochlea was reduced after the deletion of the *BAK1* gene, delaying the onset of ARHI[9]. Critical factors in the ageing of the inner ear in mice are identified as high expression of mRNA of *BCLX* and *BCL2* genes.

Reactive oxygen species (ROS) are out of equilibrium in ARHI, and the inner ear's metabolism gradually deteriorates. Inflammatory reactions are brought on by mtDNA release into the cytosol, which is brought on by oxidative stress and mtDNA damage. ARHI mice had higher levels of inflammatory cytokines in their inner ears[10]. To identify the vital role of apoptosis in the cochlea, many researchers usually use naturally aged C57BL/6J mice[11]. Exploring the intracellular mechanism of cochlear ageing is crucial to understanding cell death in ARHI, and the methods that can remedy this impairment hold equal weight.

The present study aims to unravel the molecular pathways that underlie ARHI and pinpoint possible biomarkers for early diagnosis and therapeutic intervention. To achieve this, we examine the expression of *BAK1* and *BCL2* in blood samples from Pakistani patients with ARHI compared to healthy individuals, aiming to elucidate the role of apoptosis in the progression of ARHI and identify potential molecular targets for therapeutic intervention.

METHODOLOGY

This case-controlled study was conducted between 2020 and 2021 at the Department of Bioinformatics & Biotechnology, Government College University Faisalabad. Sixty ARHI patients and 40 healthy subjects were selected for this study. The affected individuals were 66±3, and the healthy subjects were 30±5 years old. All the subjects were recruited from two cities in Pakistan, Faisalabad and Chichawatni. Before enrolling for this study, the participants signed the informed consent forms. The Ethical Review Committee of Government College University Faisalabad approved this research. All participants completed a questionnaire to ascertain the environmental factors and medical history that may have influenced their hearing abilities. Participants exposed to factors that can affect hearing were excluded from the study. These factors were any head injury or trauma, noise exposure, ototoxic medication, exposure to chemicals, diabetes, liver cirrhosis, neurological disease, cardiovascular disease, renal failure, psychiatric disease, cognitive dysfunction, middle ear disease, vestibular schwannoma or brain tumour, cancer, early-onset hearing loss, exposure to gunfire, tinnitus, history of stroke, tobacco smoker, and alcohol users. Individuals struggling with cold or flu were also eliminated.

Audiometric Assessment

Pure tone audiometry was performed for all affected and healthy individuals. Air conduction hearing thresholds were measured at 250, 500, 1,000, 2,000, 4,000, and 8,000 Hz. Audiometric profiling revealed that all the affected individuals had moderately severe to severe hearing loss, whereas the healthy subjects had normal hearing (**Figure I**).

RNA Extraction and Quantification

One millilitre of blood was collected from all the individuals under study. After collection, blood was mixed with two millilitres of RBC lysis buffer, vortexed and centrifuged for 4 mins at 10,000 rpm. This step was repeated until a pallet of white blood cells was obtained. Later, 1 ml of Trizol was placed in each tube, vortexed, and kept at room temperature for five minutes. After adding the 50 µl chloroform to this lysate, the tube was kept at room temperature for 15 seconds and centrifugation at 12,000 rpm for 10 minutes. The transparent supernatant was transferred to a fresh 1.5ml tube and centrifuged at 12,000 rpm for 15 mins after adding the 500 µl isopropanol. The supernatant was removed, and the RNA pellet was washed with 500 µl of 70% ethanol and diluted with 20µl of DEPC-treated water. Isolated RNA was quantified by the nanodrop method.

cDNA Synthesis

Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, cat #K1652) synthesized cDNA. 1µg of RNA of each sample and 1µl of oligo (dT) primers were added in PCR tubes separately. RNA-free water was introduced to make the total volume of 12µl in each tube. The tubes were incubated for 5 minutes at 65°C in a thermocycler. After incubation, they were chilled on ice immediately, and RT reaction reagents were added. Later, tubes were incubated for 1 hour at 43°C, and the reaction was completed by heating for 5 minutes at 70°C.

Quantitative Real-Time PCR (qPCR)

Maxima SYBR Green /ROX qPCR Master Mix 2X (Thermo Scientific, Cat number K0222) was applied for qPCR, and the expression of *BAK1*, *BCL2* and *ACTB* genes was determined using the specific primers (**Table I**). For each reaction, 5 µl of SYBR green mix, 0.5µl of each primer (10 µM), 1 µl cDNA, and 2.4µl of H_2O were added. Later, a BioRAD qPCR machine was used to amplify the interested genes. The conditions for the PCR cycle were as follows: 1 cycle of denaturation at 95°C for 15 min, then 40

Table I: PCR primer sequences for *BAK1*, *BCL2* and *ACTB* genes

Accession no.	Gene	Reverse primer	Forward primer	Amplicon size
NM_001101.3	*ACTB*	GTA TCG GTT TGG ATG CA CCA	TCC GAG CTG AAG TACG AGC	131
NM_001188.3	*BAK1*	CAA ACA GGC TGG CAA TC TGG	TCA TCG GGG ACG TCA AC ACA	120
NM_000633.2	*BCL2*	CAG GGA GAA ATC AAA AGG CCA CAG	ATC CTG TGG ATG GAG GCC ACT	129

cycles at 95°C for 20 sec. and 60°C for 60sec before the extension at 72°C for 60sec. then Final extension for 10min at 72°C.

RESULTS

Expression profiling of BAK1 and BCL2 genes

The expression of the apoptotic genes was quantified using the qPCR. *BAK1* and *BCL2* expression profiles were estimated in healthy individuals and ARHI patients. The expression of these genes was relatively compared to the expression of *ACTB*, a housekeeping gene. A higher expression of *BAK1* in the ARHI patients was observed than in healthy subjects (**Figure IIa**). An upregulation of about 4.2-fold was revealed in patients in contrast to normal individuals, indicating a potential impact of the pro-apoptotic gene in hearing impairment. The expression level of the anti -apoptotic gene *BCL2* was also determined. A relative increase in the *BCL2* gene expression was also observed in the patients compared to healthy individuals (**Figure IIb**). A 2.7-fold increase in the expression level of *the BCL2* gene was observed in the patients compared to the healthy subjects.

A relative expression level of anti-apoptotic and pro-apoptotic genes is required in the body to regulate the process of apoptosis. An increase in the pro-apoptotic genes compared to the anti-apoptotic genes will lead to apoptosis. A ratio of *BAK1*/*BCL2* gene expression in both patients and healthy individuals indicated an increased production of *the BAK1* gene (1.6 fold) in ARHI patients compared to the normal individuals (**Figure III**).

Figure I: The mean hearing threshold of affected and healthy individuals

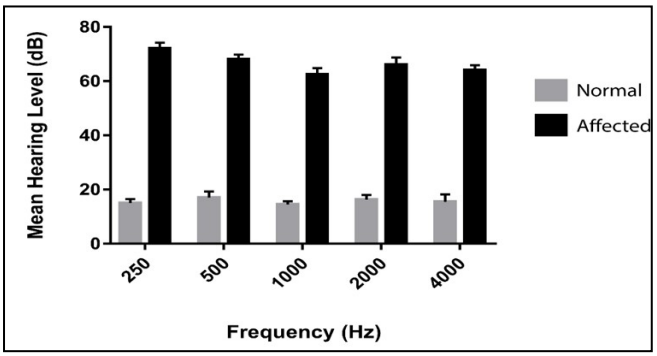


Figure II: Gene expression studies of *BAK1* and *BCL2*: a) *BAK1* gene expression in healthy and ARHI individuals. b) *BCL2* gene expression in both healthy and ARHI individuals

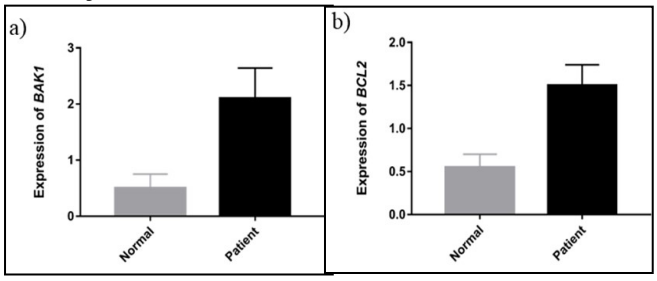
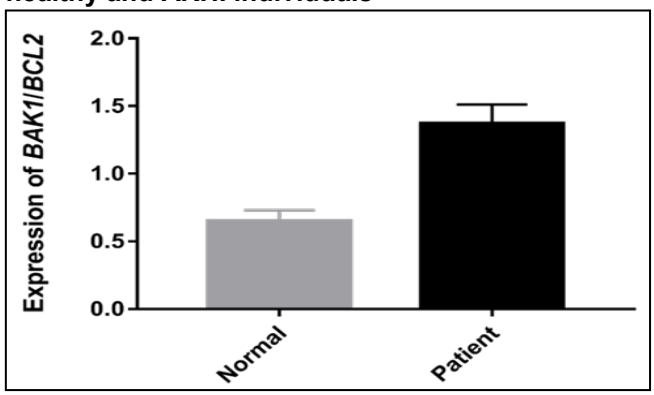


Figure III: Gene expression of *BAK1*/*BCL2* in healthy and ARHI individuals



DISCUSSION

Presbycusis, or ARHI, is considered a progressive disorder of hearing loss in older people. This disease reduces the quality of life and has a significant societal impact[12]. Age and exposure to loud noises are two factors contributing to presbycusis[13]. The burden of hearing loss is remarkably increasing among older people. Approximately two-thirds of people aged 70 and above in the United States suffer hearing loss[14]. The exact cause of ARHL is unknown. However, various studies have shown that oxidative stress, inflammation, apoptosis, and genetic factors are all involved in its pathophysiology. Ohlemiller KK 1999[15] explored the early elevation of cochlear reactive oxygen species (ROS) and the protective effects of antioxidant treatment in acute acoustic trauma, emphasizing the role of oxidative stress in cochlear injury and potential therapeutic interventions. Hwang JH 2012[16] demonstrated a relationship between reactive oxygen species (ROS) with age-related hearing loss (ARHL). Their study revealed a notable correlation between plasma ROS levels and the extent of hearing impairment across both low and high frequencies. Additionally, Kamogashira T 2015[17] further support this notion by suggesting that ROS generation contributes to apoptotic and necrotic cell death pathways in auditory tissues, which are significant factors underlying sensorineural hearing loss, including ARHL.

ARHI is controlled by various genes, which majorly contribute to the apoptosis of the inner ear cells. Upregulation of pro-apoptotic genes *BAK1* and *CASP3* in mice reveals hearing loss at an early age. The *BAK1* gene belonged to the BCL2 protein family, which has been attributed to apoptosis. This gene family includes proapoptotic genes such as *BAD* and *BAX* and antiapoptotic genes such as BCLXL and *BCL2*. Members of the BCL2 protein family[9] control permeabilization of the mitochondrial membrane during apoptosis. Apoptotic signaling in ARHL is triggered by ligands found on cell-surface receptors and takes place by mitochondria-dependent exogenous and endogenous pathways. BCL-2 protein

family members, which localize to the mitochondria, control endogenous apoptosis[13]. *The BAK1* gene has a carboxyl terminus, significantly destabilizing the mitochondrial outer membrane by creating apoptotic pores. Creating the "apoptotic pore" marks the end of the mitochondrial apoptosis[18]. Apoptosis-related mitochondrial injury has been linked to altered morphological dynamics and mitochondrial fragmentation. Mitochondrial fragmentation involving *the BAK1* gene cannot induce maximal cytochrome C release. *BAK1* and *BAX* genes must work together to stimulate a full mitochondrial pathology. The current study demonstrated that BAK and BAX control each other during apoptosis and have unique oligomerization characteristics. During the early stages of mitochondrial outer membrane pore formation, BAK speeds up the recruitment and activation of BAX. In contrast, once inside the membrane, BAX supports the slower accumulation of more BAX molecules to expand pores. As a result, BAX and BAK co-assembly modulate mtDNA release and affect the bystander immune cells[19].

Regarding BAK activation, another study reported that BH3 ligand affinity is less critical than BH3-induced structural changes[20]. *BAK1* gene-mediated mitochondrial fragmentation is decreased in *BAK1* deficient mouse embryonic fibroblasts (MEF) cells[21]. In addition, the MCAT transgenic mice exhibit delayed onset of age-related HL, and investigation of the underlying molecular mechanisms revealed a decrease in the mRNA expression level of Bak1 in the cochlea. Antiapoptotic treatment has been reported to preserve hearing and reduce outer hair cell loss in mice[22]. A direct relationship between a higher expression of BCL2 and BCL-XL and the ageing of the inner ear mouse model has also been identified.

The most well-known model of accelerated ARHI is C57BL/6 mice, in which hearing loss begins at about six months and becomes severe at one year[23]. Caloric restriction (CR), which slows the development of ARHI in C57BL/6J mice, lowers the level of apoptosis in the cochlea by reducing the level of the mitochondrial proapoptotic *BCL2* family member *BAK1*, is known to delay numerous elements of the ageing process in multiple species[24].

Our study showed an elevated expression of *the BAK1* gene in Pakistani ARHI patients compared to healthy subjects. Increased expression of *BAK1* (**Figure IIa**) supports the previous findings, suggesting this gene's crucial role in ARHI patients[22]. Furthermore, the expression of *the BCL2* gene (**Figure IIb**) was also increased in response to the high expression of the *BAK1* gene in ARHI patients when compared to normal subjects. A ratio of *BAK1*/*BCL2* gene expression (**Figure III**) in both patients and healthy subjects indicated a 1.6-fold increase in ARHI patients compared to healthy subjects. In ARHI patients, death of cochlear cells occurs due to increased expression of the *BAK1* gene.

CONCLUSION

This study demonstrates a higher BAK1/BCL2 ratio and BAK1 gene expression in plasma samples of Pakistani ARHI patients. Furthermore, the increase in expression of the *BAK1*/*BCL2* ratio and *BAK1* gene supports the findings of previous studies. It also highlights the significance of targeting apoptotic genes such as *BAK1* for treating apoptosis-related disorders/ diseases like ARHI. This gene's expression profile can be applied as a novel biomarker for the early diagnosis of ARHI disorder.

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Data Sharing Statement: The corresponding author can provide the data proving the findings of this study on request. Privacy or ethical restrictions bound us from sharing the data publically.

AUTHOR CONTRIBUTION

Rauf A: Conceptualization, Writing original Draft
Bhatti R: Methodology, Writing, Review and Editing
Kausar S: Investigation, Methodology
Aslam S: Formating & editing
Nahid N: Conceptualization, Supervision
Qasim M: Project Administration, editing & Review Resources

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