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Association of *SOD2* gene polymorphism with noise induced hearing loss in textile industry employees

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Noise exposure leads to hearing loss in industrial workers due to release of superoxide anions which damage cochlear epithelium in the ear. These free radicals are regulated by superoxide dismutase 2 (SOD2) and polymorphism in this gene may abet in predisposition to noise induced hearing loss (NIHL). The current study describes prevalence of polymorphism at two different loci of *SOD2* gene in NIHL patients and association of smoking habit with NIHL. PCR-SSCP analysis of polymorphic sites revealed mutation in NIHL patients. Results showed that *IVS1+8A/G and IVS3-23T/G* SOD2 polymorphisms are associated with NIHL ($p < 0.05$, CI 95%) irrespective of smoking habit ($p > 0.01$). It is intended that *SOD2* polymorphism plays a significant diagnostic role in determining susceptibility of NIHL in Pakistani industry employees.

Key words: Superoxide dismutase 2, polymorphism, hearing loss, loci, reactive oxygen species.

INTRODUCTION

In industrialized countries, hearing impairment is the most frequent disability and noise exposure appears to be the root cause of hearing impairment (Ries, 1994). Loud noise exposure, vibration and certain drugs like cisplatin and gentamicin can damage small structures involved in hearing (Prasad et al., 2008).

It depends on the level of noise exposure that permanent threshold shift induced by acoustic over stimulation will result into in either complete recovery or loss of sensory hair cells and spiral ganglion neurons, respectively (Endo et al., 2005). It has been demonstrated in experimental systems that noise induces the local release of free radicals which damages the cochlear sensorial epithelium. ROS causes irreplaceable hair cell death involving apoptotic as well as necrotic mechanisms (Henderson, 2006). Consequently, genes involved in the regulation of *ROS*, such as *SOD* gene, may affect the vulnerability of the cochlea to noise-induced hearing loss (NIHL) as well as diseases like cancers, liver damage, Parkinson's disease, Alzheimer's disease and even the process of aging itself (Ries, 1994; Ohlemiller et al., 1999).

Superoxide dismutase (SOD) is one of the primary regulators of ROS. Three members of SOD: copper-zinc containing SOD (Cu/ZnSOD), manganese containing SOD (Mn-SOD, *SOD-2*), extracellular SOD (EcSOD) have been identified in eukaryotes which catalyzes the conversion of superoxide to hydrogen peroxide (H_2O_2) (McCord and Fridovich, 1969).

Three variants of MnSOD are known. In a genetic polymorphic variant of MnSOD, valine is changed to alanine at -9 position in the signal peptide. It was suggested that this change might affect the cellular location and mitochondrial transport of MnSOD (Rosenblum et al., 1996).

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Table 1. PCR conditions for *SOD2* gene screening.

Polymorphic site	Primers, 5´-3´	PCR condition	
		Annealing temperature (°C)	Product size (bp)
IVS1+8A/G	Fwd: 5´ TGTTGTCTAATTTCTTGGGCC 3´ Rev: 5´ AGACTCTGGGTGTTATCTGTTAAG 3´	57	434
IVS3-23T/G	Fwd: 5´ AAAGTTGAAATTGAGAAGATGC 3´ Rev: 5´ GCTTAACATACTCAGCATAACG 3´	53	250

A polymorphic variant of MnSOD at 58 position from isoleucine to threonine decreased thermal stability and reduced enzymatic activity *in vivo* and *in vitro* (Borgstahl et al., 1996; Zhang et al., 1999). Another reported mutation of MnSOD at position 60 from leucine to phenylalanine renders the MnSOD protein sensitive to redox regulation by intracellular thiols (Hernandez and McCord, 2003).

NIHL is one of the most serious issues in Asia as most of the countries are still in developing state and also number of affected laborers is so high. In most of the Asian countries, access and awareness to preventive programs is limited. According to WHO estimates, 278 million people worldwide have a disabling hearing impairment. This could increase to 700 million by 2015 and 900 million by 2025 (Fuente and Hickson, 2011).

The current research aims to determine the prevalence and association of noise induced hearing loss with polymorphism at two different polymorphic sites of *SOD2* gene, that is, *IVS1+8A/G* and *IVS3-23T/G* in industrial workers.

MATERIALS AND METHODS

Sample collection, DNA extraction and PCR

The inclusion criterion for participants was exposure to mean (SD) noise level equivalent to 92.4 dB for 20 years. Age range varied between 30 and 60 years. Peripheral blood samples of 80 NIHL and 20 control individuals were collected in EDTA vacutainers from a production unit of a textile factory at Raiwind road, Lahore, Pakistan. Samples from 40 deaf individuals were also included in the study to detect the genotype of deaf individuals. NIHL cases were defined and identified according to ACOM (2003). The information regarding medical history, age and smoking habit was collected individually. The current study was approved by the local ethical committee and was completed in agreement with second Helsinki Declaration. All the workers participated voluntarily in this study and a written informed consent was obtained from the participants.

Isolation of genomic DNA from blood samples of patients and control individuals was carried out by standard procedures and analyzed qualitatively (Sambrook et al., 1989). The sequence of the oligonucleotide primers used to amplify the polymorphic sites was same as reported by Fortunato et al. (2004) and synthesized by Fermentas. The sequence of oligonucleotide primers used in the study, their PCR product sizes and annealing temperatures are given in Table 1.

PCR was performed in volume of 50 µl containing 5 µl of 10X PCR buffer, 3 µl of 1.5 mM $MgCl_2$, 0.4 µl of 0.2 mM dNTPs, 5 µl of 1 µM/µl forward and reverse primer, 0.2 µl of 1 U/µl Taq DNA polymerase, 26.4 µl of sterile distilled water and 100 ng of DNA sample. Thirty cycles of PCR were performed in thermocycler (Bio-rad, Japan). The PCR profile for amplification was 94°C for 3 min (initial denaturation), followed by 94°C for 30 s (denaturation), 57°C for 30 s (annealing); 72°C for 30 s (extension) and final extension at 72°C for 10 min. Five microliters of PCR product were mixed with ethidium bromide and run on a 1.5% agarose gel in 1x TAE buffer (40 mMTris-acetate and 1 mM EDTA), pH 8.0 at 80 V in an electrophoresis apparatus. PCR products were visualized on Gel documentation system (GDS) (Syngene, USA).

Native polyacrylamide gel electrophoresis

To screen out point mutations in the samples, the restricted samples were resolved on 8% native-PAGE and visualized by staining with ethidium bromide (Sambrook et al., 1989). Non-denaturing polyacrylamide gel was prepared from 30% acrylamide-bisacrylamide (29:1) in 5x TBE buffer (89 mMTris-borate and 2 mM EDTA), 50 µl of 10% ammonium persulphate, 20 µl of TEMED and made final volume up to 7 ml. 10µl of digested sample mixed with 6x loading dye was loaded in the wells and electrophoresed at a constant voltage of 75 V for 2 h. The gel was stained with 50 µg/ml ethidium bromide solution in TBE buffer and visualized under GDS.

Single stranded conformation polymorphism (SSCP) analysis

To determine the polymorphic site mutations at *IVS1+8A/G* in intron 1 and *IVS3-23T/G* in intron 3, SSCP-native PAGE was performed as described (Ho and Crapo, 1988) on each PCR product obtained by using gene specific primers as shown in Table 1.

Statistical analysis

Data was analyzed by using applied test and independent sample 't' test.

RESULTS

Genotype of 120 (80 NIHL and 40 deaf) samples were analyzed and compared with 20 control individuals. Two polymorphic sites were selected: *IVS1+8A/G* and *IVS3-23T/G* as shown in Figure 1. The NIHL group consisted of 35 to 60 years old patients who has been working for more than 20 years in factory and included significantly higher percentage of smokers while congenitally deaf

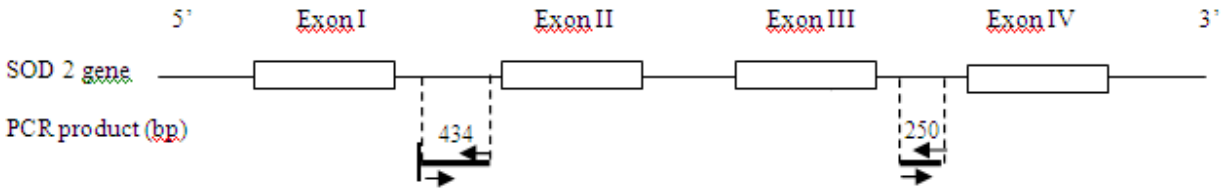

Figure 1. Schematic diagram showing the SOD2 gene and PCR fragments. The expected sizes of each PCR product in bp are shown. Arrows indicate 5′→3′ direction of the primers.

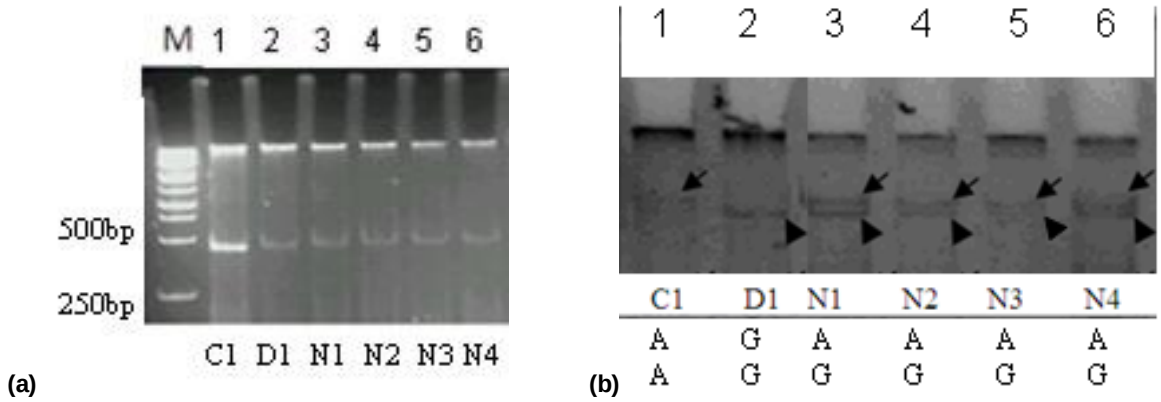

Figure 2. PCR amplification and SSCP analysis of products of *IVS1+8A/G* on intronic region 1 of *SOD2* gene in deaf (D) and NIHL (N1-N4) individuals when compared with the control (C) samples. (a) PCR amplified products of 434 bp. (b) SSCP analysis of PCR products. Difference in mobility shift as indicated by arrows. Lane 1: Homozygous (AA) wild genotype, lane 2: Homozygous (GG) mutant genotype, lane 3-6: heterozygous (AG) mutant genotype.

individuals were selected from deaf rehabilitation center, Lahore, Pakistan.

The amplification of *IVS1+8A/G* and *IVS3-23T/G* produced 434 and 250 bp fragments, respectively, by using specific primer pairs as shown in Figures 2a and 3a.

Analysis of DNA polymorphisms in the SOD2 gene by PCR-SSCP

PCR-SSCP analysis was used to determine the mutation in intron 1 (*IVS1+8 A/G*) and intron 3 (*IVS3-23 T/G*). Three patterns of mobility shifts represented two homozygotes for each allele and one heterozygote. For the intron 1 polymorphism, which is one of the novel polymorphisms as described formerly (Fortunato et al., 2004), control and deaf individuals were homozygous for A/A and G/G, respectively whereas A/G heterozygosity was observed in NIHL individuals (Figure 2b). The most prevalent genotype for deaf and NIHL was G/G (Figure 4). Similarly, *IVS3-23T/G* polymorphism presented two homozygous patterns, that is, T/T and G/G and one heterozygous pattern for both alleles (T/G) as shown in Figure 3 and the most prevalent genotype was G/G in both deaf and NIHL (Figure 5). In both polymorphic sites, it appears that GG genotype is most prevalent and it may be suggested that this genotype is associated with NIHL and deaf patients as confirmed by statistical analysis using applied test and independent sample 't' test ($p < 0.05$) with CI 95%.

DISCUSSION

The present study shows that free radicals produced throughout the body as by products of normal cell metabolism can cause extensive damage to living tissues, including the sensory hair cells in the inner ear, if remain untreated by antioxidant enzymes, which may lead to NIHL

Employees working in the weaving department of textile industry were included in the study because weaving department is one of the areas where noise levels are the highest and workers are more prone to NIHL. ROS are being produced all the time in human body and electron transport chain (ETC) in mitochondria utilizes >90% of body's oxygen whereas 1 to 5% is released as superoxide and H_2O_2 (Boveris et al., 1972). ROS may also be generated from different metabolic activities (Roy et al., 1999).

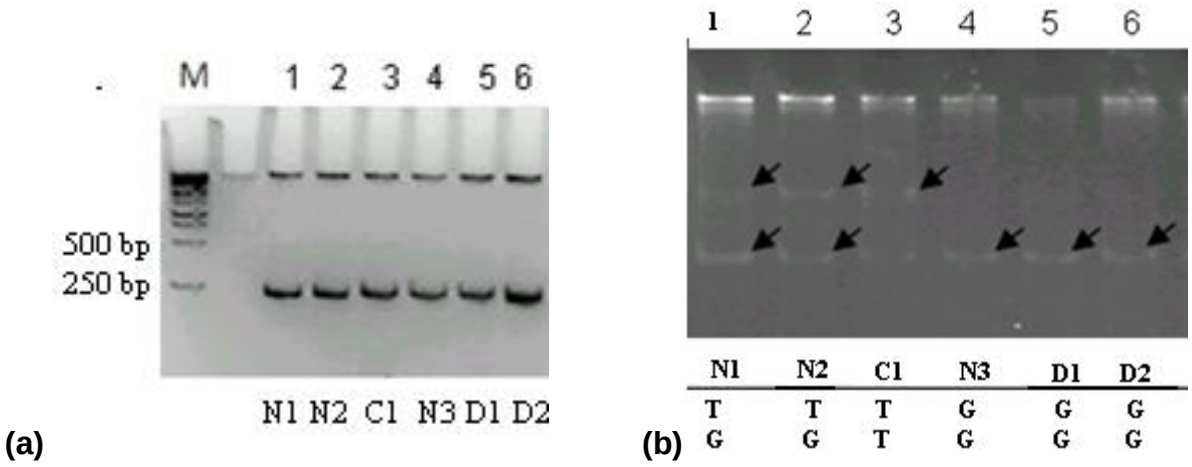

Figure 3. PCR amplification and SSCP analysis of products of *IVS3-23T/G* polymorphic site in intron 3 of *SOD2* gene in deaf (D1 and D2) and NIHL (N1-N4) individuals when compared with the control (C1) healthy individuals. (a) PCR amplified products of 250 bp. (b) SSCP analysis of PCR products. Lane 1: homozygous (TT) wild genotype, Lane 2: heterozygous (TG) mutant genotype, lane 3: homozygous (TT) wild genotype, lanes 4 to 6: homozygous (GG) mutant genotype, lanes 7 to 8: homozygous (GG) mutant genotype.

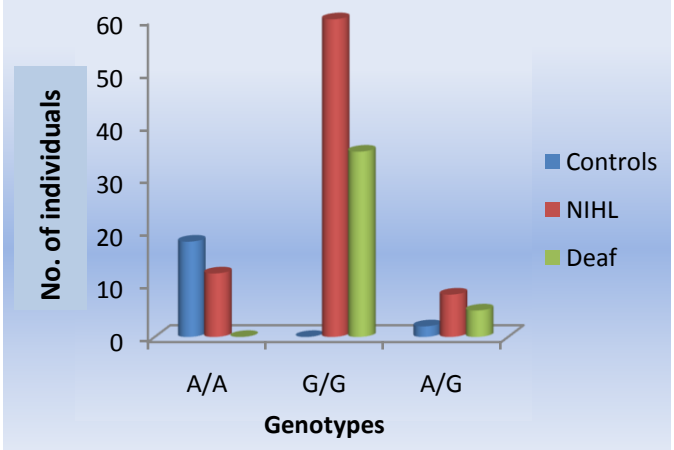

Figure 4. Graph showing most prevalent genotype for IVS1+8A/G region of SOD2 gene in deaf, NIHL and control cases.

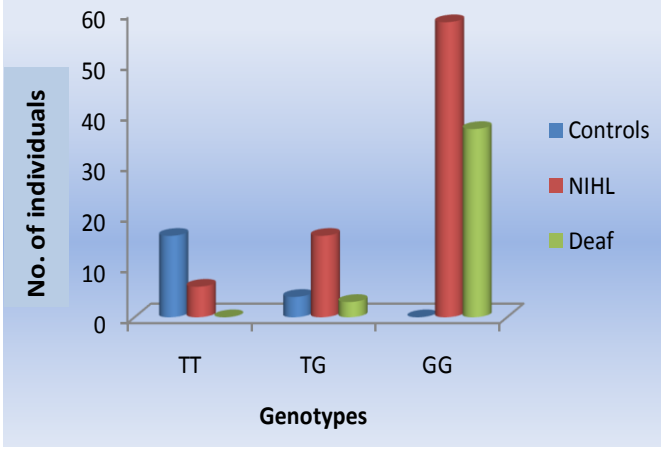

Figure 5. Graph showing most prevalent genotype for IVS3-23T/G region of SOD2 gene in deaf, NIHL and control cases.

As mtDNA has low repair capability and it lacks histone proteins, so it is more exposed to oxidative damage due to ROS, which is continuously generated in mitochondria by ETC (Penta et al., 2001). These ROS are detoxified by two major enzymes: MnSOD and glutathione peroxidase (GPX) 1 (Fridovich and BocaRaton, 1982; Nomura et al., 2001). It is biologically plausible that the *Ile58Thr* polymorphism in *SOD2* play an important role in ROS

Noise-induced cochlear epithelium damage can cause hearing loss in industrial workers. Noise induces the release of free radicals so it has a strong association with damage to cochlear sensorial epithelium. Therefore, gene involved in regulating the ROS could influence cochlear vulnerability to noise. In the present study, an association was established between prolonged expo-sure to loud noise and propensity to NIHL among *SOD2* polymorphic variants.

A significant correlation between noise exposure and hearing loss among polymorphic variants was observed in the the present study as supported by earlier research (Ohlemiller et al., 1999; Fortunato et al., 2004). Reason for this effect of noise is that antioxidant enzyme, SOD2, affect the cochlear membrane vulnerability to damage due to high load of ROS in the body leading to NIHL.

The first study goal was to compare the prevalence of the polymorphic variants of *SOD2* in deaf, NIHL and healthy normal individuals and secondly, to study the

effect of age and smoking on these variants. In reports regarding the effect of smoking on NIHL (Pouryaghoub et al., 2007; Mizoue et al., 2003), it is stated that smoking may accelerate NIHL and smokers are at greater risk of hearing loss as compared to non-smoking industrial workers whereas here we found out that smoking probably do not effect hearing loss ($p > 0.01$). Such a relationship has also been tried to develop in British patients (Palmer et al., 2002) and their findings were similar to our results, however, significant association was observed with frequent headache, tiredness and stress. It is suggested that workers exposed to long-term noise should be discouraged from smoking, the extra risk of smoking on hearing loss, in noisier environment, is small relative to that of noise itself.

In Pakistan, NIHL is a very serious problem and it was observed that hearing loss was significantly associated with noisy environment, working experience of more than 10 years. Random tests in different cities showed that the noise level in most of areas was as high as 70 to 90 dB (A) which is much higher than the acceptable limits. To deal with industrial noise, the organizations are handicapped to take any legal action because of the absence of national standards for noise (Ashraf et al., 2009).

PCR-SSCP method is easy to handle, less time consuming, involve no special equipment or expertise and reagents are simply available. PCR-SSCP technique has been used to genotype β-globin gene in Thai families (Chinchang et al., 2005) but there is no comparative data pertaining to role of genetic variation in SOD2 gene directly related to oxidative stress and noise induced hearing loss progression. It would be of value to compare our findings with those of similar studies in other populations.

Several studies have also emphasized on the use of antioxidants for prevention of NIHL. Antioxidants and magnesium may prevent permanent noise-induced hearing loss when given before and a few days after exposure. Vitamin C, E, α-lipoic acid and n-acetylcysteine (NAC) are observed to reduce noise-induced cochlear damage and hearing loss in guinea pigs (LePrell et al., 2007). Another study supports their role in protecting against noise-induced damage in cyprinid fish (Scholik et al., 2004).

Conclusion

In conclusion, the association of *SOD*2 polymorphisms with neuro-sensorial hearing could represent a marker of susceptibility to NIHL. Free radicals may contribute to the formation of some metabolic changes in workers in noisy environment. PCR-SSCP method is sensitive enough to be used for determination of *SOD2* gene mutations. The pattern of such mutations can be used prospectively for determining these common mutations, at least in our population. Consequently, it is considered that workers exposed to noise are under the risk of free radical mediated ear damage.

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