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Genetic polymorphisms of GSTM1 and GSTT1 and susceptibility to primary DNA damage in steel industry workers

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ABSTRACT

The present study aims to investigate the possible association of detoxifying gene polymorphisms with DNA damage in steel industry workers. 150 steel industry workers and 146 control subjects with no history of occupational exposure to steel dust or any other chemicals were recruited for the study. DNA damage was evaluated using simple and reliable alkaline comet assay in peripheral blood lymphocytes of male steel industry workers. Analysis of GSTM1 and GSTT1 gene polymorphisms was done by multiplex PCR method. The results showed an increase in the frequency of DNA damage in peripheral blood lymphocytes of the steel industry workers compared to the control subjects. The statistical analysis of the results showed that significant DNA damage was observed in both genotypes of GSTM1 and GSTT1 but the frequency was found to be lower in steel industry workers with wild GSTM1 and GSTT1 genes than in those with null genotypes. The findings in this study presented evidence for the DNA damage highlighting an increased genotoxic risk as a result of exposure to dust in steel industry workers. The study also presented evidence for an association of both null genotypes of GSTM1 and GSTT1polymorphisms with DNA damage indicating the influence of GSTM1 and GSTT1 polymorphisms on this biomarker.

Keywords: DNA Damage, Occupational exposure to steel dust, Comet tail length, Gene Polymorphism

INTRODUCTION

The steel and iron industry have been one of the world's most important industries ever since it was first founded. The steel dust that is liberated from the industry contains nickel, chromium, iron, manganese, cobalt,

molybdenum and vanadium which are carcinogenic and mutagenic (Cornelia 2002). Earlier studies have shown an increase in health problems such as lung tumors, diabetes, rheumatoid arthritis, hypertension and cardiovascular diseases in male steel industry workers (Roberto Cappelletti *et al*. 2016, Indira *et al*. 2017).

Hayes (1997) and Sabine Martin (2009) reported that exposure to metals like cadmium, cobalt, nickel, and chromium compounds are carcinogenic in humans. Workers occupationally exposed to heavy metals were considered to be at an elevated risk for developing cancer (Pool *et al*. 1994, Keshava *et al*. 1999, De Flora *et al*. 2000, Gibb *et al*. 2000). Welders are prone to exposure to hexavalent chromium in welding fumes (Langard, 1990; Hartwig *et al*., 2002). Once it passes the cell membranes, in the cell hexavalent chromium is reduced to its trivalent form to react with DNA and leads to damage DNA (De Flora *et al*. 1989, Karahalil *et al*. 1999, Shi *et al*. 1999). In vitro studies have also shown that welding fumes from stainless steel industry are mutagenic in Escherichia coli and Salmonella typhimurium (Hedenstedt *et al*. 1977, Maxild *et al*. 1978). Studies have also indicated an increased frequency of DNA damage, sister chromatid exchanges (SCEs) and chromosome aberrations (CAs) in cultured Chinese hamster cells exposed to welding fume particles of steel (Koshi 1979,Baker *et al*.1986).

Iarmacovai *et al*. (2007) have shown that GSTT1, and GSTM1 polymorphisms modulate chromosomal damage in individuals exposed to genotoxic agents. The present study is aimed to investigate the association of GSTM1 and GSTT1 gene polymorphisms with primary DNA damage in steel industry workers using Comet assay as the bio monitoring tool. The comet assay has been found to be a very sensitive method for measuring DNA damage (Singh, 1988., Tice *et al*., 1995, Kassie., 2000). It is sensitive, quick, reliable and fairly inexpensive way of measuring DNA damage. Due to its sensitivity in detecting genetic damage at the individual cell level and its potential application to different cells, the assay has been adopted as a useful tool in short-term genotoxicity and human biomonitoring studies (Collins *et al*. 1997).
Comet assay is increasingly being used to monitor genotoxic effects in occupationally exposed humans (Kassie *et al*., 2000). Steel industry workers were investigated for genotoxic effects in the current study using the Comet assay. The present study analysed the genotoxic effects of steel dust among steel industry workers.

As we already know that cell consists of proteins and enzymes and their function is to maintain the DNA. One of the cytotoxic damages is the insertion of DNA strand breaks (DSBs) to the DNA helix which triggers apoptosis. Cancer is caused by damage of the DNA and also causes alterations in cells. From environment heavy metals enter in to the body through inhalation, ingestion, and dermal contact which might damage DNA due to the formation of reactive oxygen species thereby increasing the risk of cancer (Tchounwou *et al*., 2012).
Although studies have been carried out on health problems in steel industry workers, no studies have been carried out on the association of GSTT1 and GSTM1 gene polymorphisms with primary DNA damage in steel industry workers. The aim of our study is to evaluate the DNA damage using comet assay as biomarker and to assess the association of GSTT1 and GSTM1 gene polymorphisms as effect modifiers with DNA damage in steel industry workers occupationally exposed to the steel dust.

MATERIALS AND METHODS

Study subjects

The study was carried out on 150 workers of steel industry who were occupationally exposed to steel dust and 146 subjects who were not exposed occupationally to industrial chemicals to serve as control group. Subjects for the present study were selected among the workers of the steel industry situated at Patancheru, Hyderabad, India. All the subjects of steel industry and controls were clinically examined and information on age, literacy, occupation, years of service, socio economic status, habits, hours of work per day, life style, income, living conditions, marital status, health problems, family history, medical history, personal safety measures taken were recorded using a standard questionnaire. The study was approved by the Institutional Ethics Committee of the Centre and written informed consent was obtained from all the participants of the study.

DNA damage analysis by single cell gel electrophoresis (comet assay)

Slides were prepared in duplicate according to the technique of Singh *et al* (1988)[20]. Fully frosted microscopic slides were covered with 140 µl of 0.75% regular melting point agarose (40±42°C). After application of a coverslip the slides were allowed to gel at 4°C for 10 min. Meanwhile, 20µl of whole blood was

then added to 110µl of 0.5% low melting point agarose (37°C). After carefully removing the coverslips a second layer of 110 µl of sample mixture was pipetted out on the pre-coated slides and allowed to solidify at 4°C for 10 min. The coverslips were removed and a third layer of 110 µl of low melting point agarose was pipetted out on the slides and allowed to gel at 4°C for 10 min. The slides (without coverslips) were immersed in freshly prepared, cold lysing solution and refrigerated overnight. Slides were then placed in alkaline electrophoresis solution for 20 min to allow unwinding of the DNA to occur. Electrophoresis was conducted for 25 min at 25 V adjusted to 300 mA by raising or lowering the buffer level in the tank. Slides were then drained, placed on a tray and washed slowly with three changes of 5 min each of neutralization buffer. DNA was precipitated and slides were dehydrated in absolute methanol for 10 min and were left at room temperature to dry. All the slides were then stained with 10% ethidium bromide and for visualization of DNA damage, using a bright field, transmission light microscope (Olympus) at 400X magnification. Comet tail length was measured, using an ocular micrometer fitted in the eyepiece, in 50 cells per subject. The length of the DNA migrated in the comet tail, is an estimate of DNA damage.

Analysis of GSTM1 and GSTT1 gene polymorphisms by multiplex PCR:

GSTM1 and GSTT1 genotyping: Genomic DNA was isolated from 200 µL of whole blood by Spin column kit (Bangalore Genei, India). Multiplex PCR assay was used for analyzing the GSTM1 and GSTT1 gene deletions. To detect the GSTM1 deletion, the primers used were GSTM1 F (5′ GAA CTC CCT GAA AAG CTA AAGC 3′) and GSTM1 R (5′ GTT GGG CTC AAA TAT ACG GTG G-3′). For GSTT1, the primers used were GSTT1 F (5′-TTC CTT ACT GGT CCT CAC ATCTC- 3′) and GSTT1 R (5′-TCACCGGATCATGGCCAGCA-3′). The PCR amplicons were electrophoresed on a 4% agarose gel, stained with ethidium bromide, and the results were documented using a gel documentation system. The presence of GSTM1 and that of GSTT1 genes were indicated by the resulting 215 and 480 bp PCR amplicons, respectively. A DNA sample with GSTM1 and GSTT1 alleles present was run as a positive control in each run. As an internal control, human albumin gene (HAB) was amplified (350bp) using the primers HAB F (5′-CAACTTCATCCACGTTCACC-3′) And HAB R (5′-GAAGAGCCAAGGACAGGTAC-3′) for the authentication of multiplex PCR. Individuals with null (–) genotype of GSTM1 did not have 215 bp band while they did have the 350bp band. Similarly, the individuals with null (–) genotype of GSTT1 did not have 480 bp band.

The PCR protocol included an initial denaturation temperature of 94 °C (5 min) followed by 35 cycles of amplification denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 1 min. A final 10 min extension step (72 °C) terminated the process. The final PCR products were visualized in ethidium bromide stained gel. The size of the GSTM1 was visualized as 215bps, GSTT1 as 480 bps and the HAB internal control as 350 bps fragment.

Statistical analysis: The results were analyzed statistically using Student's t-test to find the significance for the differences between the two groups for the association of detoxifying gene polymorphisms with DNA damage. Mean values and standard deviations were computed for the statistical significance ($P < 0.05$). The differences in the distribution of genotype frequencies were calculated using the $\chi 2$-test. Genotype frequencies were checked for deviation from Hardy–Weinberg equilibrium and were not significantly different from those predicted. Odds ratios and 95% confidence interval (95% CI) were calculated to assess the relationship between GSTM1 and GSTT1 gene polymorphisms.

RESULTS

The results on the demographic characteristics of the study subjects and controls and the frequency of DNA damage in peripheral blood lymphocytes of steel industry workers are presented in Tables 1-2.

Table 1 shows the demographic characteristics of the study subjects and controls. Mean (±SD) age of steel industry workers were 54.21 ± 6.56 years as against the 52.49 ± 9.23 years in the control group. Mean (±SD) duration of exposure of steel industry workers was 13.74 ± 6.05 years.

Comet Mean Tail Length (µm) was studied in a total of 150 subjects using the Comet assay. The results on DNA damage are given in Table 2. The steel industrial workers showed statistically significant increase in DNA mean tail length (11.7 ± 3.67) when compared with controls (9.31 ± 3.12).

Table-1: Demographic characteristics of the study subjects and controls.

Demographic characteristics	Steel industry Workers (%) N=150	Control subjects (%) N=146
Age/ (mean ± SD)years	54.21 ± 6.56	52.49 ± 9.23
<45 years	99 (66.0%)	92 (63.01%)
≥ 45 years	51 (34.0%)	54(36.9%)
Duration of exposure (in yrs):	13.74 ± 6.05	NA
< 20years	92 (61.33%)	NA
≥ 20 years	58 (58.66%)	NA
Smoking habits: Smokers	100 (66.0%)	46 (31.5%)
Non Smokers	50 (33.0%)	100 (68.4%)

Values are mean ± standard deviation.

Table-2: Comet Mean Tail Length (µm) in Steel Industry workers and Controls.

GROUP	**No. of blood Samples collected**	**No. of metaphases successfully screened**	**Comet Mean Tail Length (µm)**
STEEL INDUSTRY WORKERS	150	7500	11.7 ± 3.67*
CONTROL GROUP	146	7300	9.31 ± 3.12

Values are represented as mean ± SD, *P values (<0.05) as compared to controls

TABLE-3: Genotype distributions of GSTM1 & GSTT1 polymorphisms in steel industry workers and Controls.

Genotype	Steel industry workers (n=150)	Controls (n=146)	X^2	OR(95% CI)	*p* value
GSTM1 (wild/wild)	94(62.7)	95(65.1)	1	Reference	
GSTM1 (null /null)	56(37.3)	51(34.9)	0.18	1.11(0.67-1.83)	0.66
GSTT1 (wild/wild)	92(61.3)	91(62.3)	1	Reference	
GSTT1 (null/null)	58(38.7)	55(37.7)	0.03	1.04(0.63-1.71)	0.86

Table 4: Mean DNA damage in steel industry workers and control subjects with null and wild genotypes of GSTM1 and GSTT1.

Genotype	Workers with Basal DNA damage (µm)		Controls with Basal DNA damage (µm)		
	(*n* = 150)	**(Mean ± SD)**	**(*n* = 146)**	**(Mean ± SD)**	***P* Value**
GSTM1 (wild/wild)	94	10.4 ± 1.52	95	9.8 ± 1.99	*p <0.05
GSTM1 (null /null)	56	11.0 ± 1.38	51	10.1 ± 1.90	*p <0.05
GSTT1 (wild/wild)	92	10.2 ± 1.07	91	9.91 ± 1.08	p >0.05
GSTT1 (null/null)	58	11.3 ± 1.59	55	10.2 ± 1.19	*p <0.05

***p*<0.05 significant. Values are Mean ± SD data**

Genotype distributions of GSTM1 & GSTT1 genes:

In our study, we genotyped GSTM1 and GSTT1 polymorphisms in 150 steel industry workers and in 146 control subjects as shown in Table -3. GSTM1 wild gene was present in 65.1% of the control subjects and in 62.7 % of steel industry workers and GSTM1 null gene was present in 34.9% of the control subjects and in 37.3 % of the steel industry workers. Similarly, GSTT1 wild gene was present in 62.3% of the control subjects and 61.3% of steel industry workers and GSTT1 null gene was present in 37.7% of the control subjects and in 38.7% of steel industry workers. The statistical analysis showed the differences in the frequency of genotypes between controls and steel industry workers were not statistically significant.

Table 4 presents the results on DNA damage in steel industry workers with wild and null genotypes of GSTM1 and GSTT1. A statistically significant increase in the DNA damage was noted in both the genotypes of GSTM1, but the DNA damage was found to be higher only in null genotype of GSTT1 when compared with the wild type.

DISCUSSION

Incorporation of biomarkers into epidemiologic studies is important because biomarkers are useful tools for predicting cancer risk. DNA damage can be used as a biomarker of exposure to genotoxic agents and is also commonly regarded as a marker of cancer risk. Several biomonitoring studies in workers exposed to welding fumes have reported mechanisms of metals-induced genotoxicity, mutagenicity and carcinogenicity including direct DNA damage (Brien *et al*. 2003).

Epidemiological studies in steel industry workers have shown that exposure to steel dust increased the risk of developing health problems such as lung tumors, diabetes, rheumatoid arthritis, hypertension and cardiovascular diseases in male steel industry workers (Roberto Cappelletti *et al*. 2016, Indira *et al*. 2017). The results showed that occupational exposure to steel dust significantly elevated DNA damage in the peripheral blood lymphocytes of the steel industry workers.

Our results are in agreement with that of Sudha Sellappa *et al*. (2010) who reported significantly elevated DNA damage in South Indian welding workers. Danadevi *et al*. (2004) also demonstrated genotoxic effects in welders occupationally exposed to chromium using the Comet and micronucleus assays. Hengstler *et al*. (2003) showed occupational exposure to heavy metals induced DNA damage which is a biochemical hallmark of apoptosis.

The steel dust contains nickel, chromium, iron, manganese, cobalt, vanadium, etc and hence the DNA damage might be attributed to the combined effects of these metals. Induction of apoptosis has been recognized as a possible outcome of DNA damage (Wyllie *et al*. 1980). Maxild (1978) and Cornelia (2002) have demonstrated carcinogenicity and mutagenicity of heavy metals like chromium, nickel cadmium, cobalt in steel industry workers. Hayes (1997) reported carcinogenicity of metals in humans. Quievryn *et al*. (2003) showed genotoxicity and mutagenicity of chromium (VI) generated DNA adducts in human and bacterial cells. Comet tails were observed in cells of humans and animals treated with nickel-refining fumes with different concentrations (Wang *et al*. 2016). Nickel triggers apoptosis, cellular changes such as oxidative stress and DNA damage that contribute to toxicity and carcinogenesis (Kasprzak *et al*. 1997, Wang *et al*. 2016). Zorawar Singh (2015) showed DNA damage as an index of genetic toxicity among iron-based industries. Workers have been exposed to iron among iron-based industries and over intake of iron can increase the risk of these free radicals to cause DNA damage. These free radicals are also responsible for mutations and transformation (Bridges and Zalups, 2005). Exposure to the metals occurs via inhalation, ingestion or skin contact, of which the most common occupational route is inhalation (Lide 1998, Wise *et al*. 2004).

In addition, studies carried out in invitro also showed evidence for an increase in DNA damage in cultured Chinese Hamster cells exposed to chromium metal (Yang *et al*. 1992, Cheng, 1997, Liu, 1996).Tsapakos *et al*. (1983) showed Chromium (VI) - induced DNA lesions in rat kidney, liver, and lung.Similarily, in chicken embryo hepatocytes, the metal chromium induced DNA damage (Cupo *et al*. 1985).

Impact of genetic polymorphisms as biomarkers of susceptibility is play an important role in understanding the DNA damage involved in mutagenesis and carcinogenesis and could help to minimize risks in exposed workers (Shiv *et al*. 2012). GSTM1 is one of the most key subclasses of GSTs, which has potent

protective role against cancer compared to other GST subtypes (Lavender *et al*. 2009).GSTM1and GSTT1 genes are the key enzymes which are involved in the detoxification of genotoxicant chemicals like nickel, chromium, iron, manganese, etc. In our study; we observed that wild and null GSTM1 and GSTT1 genotypes are associated with DNA damage. However, some studies reported that GST genotypes do not have any influence on DNA damage among coke-oven workers and graphide electrode workers (Moretti *et al*. 2007 and Chen *et al*.2006).Studies have also shown that GSTM1 polymorphism is not associated with DNA damage in PAH exposed coal- tar workers (Giri *et al*. 2011). Van delft *et al*. (1998) and Chen *et al*. (2006) observed no effects of genetic variation of GSTM1 on DNA damage among PAH exposed coke-oven workers. However, a number of studies have also reported that genetic polymorphisms in GSTM1 have been associated with susceptibility to DNA damage in populations exposed to PAHs (Leng *et al*. 2004). Massimo Moretti (2007) showed that DNA damage was associated with polymorphisms of GSTM1, EPHX, CYP1A1 genes in workers of graphite electrode manufacturing plant. The GSTT1 null genotype increased DNA damage compared to GSTM1 null genotype in white blood cells among styrene-exposed workers (Buschini *et al*. 2003).

Ours is the first investigation to show the association of the polymorphism of GSTT1 and GSTM1 genes with primary DNA damage in steel industry workers. Our study revealed that both wild and null genotypes of GSTT1 and GSTM1 varients are associated with increased risk of DNA damage in workers of the steel industry.

The steel industry workers are exposed to complex mixtures of heavy metals and their byproducts and thus combined effect maybe greater than the sum of their individual effects on DNA damage. Hence preventive and therapeutic measures may be considered for steel industry workers to control the adverse effects of steel dust.

CONCLUSION

The study reveals that there is a significant increase in the frequency of DNA damage in the peripheral blood lymphocytes of steel industry workers. Our study has also confirmed positive association between increased DNA damage with both null and wild genotypes of GSTM1 and GSTT1 genes in steel industry workers. Hence appropriate precautionary measures have to be taken to prevent or minimize the exposure of the workers to steel dust.

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Conflict of interest:
None of the authors of this paper had any personal or financial conflicts of interest.

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