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ROLE OF *ALAD* ISOFORMS IN ANEMIA AND CORRELATION OF BLOOD LEAD LEVEL WITH HEMOGLOBIN CONCENTRATION IN AUTOMOBILE PAINT WORKERS OF KARACHI, PAKISTAN

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ABSTRACT

Lead induced anemia is a common health issue for occupational lead exposed workers particularly in developing countries. This study was performed on automobile paint workers from different workshops in Karachi, Pakistan to get an estimate of degree of lead poisoning and its effects on them. The blood lead level (BLL), Hemoglobin (Hb) concentration and other hematological parameters were determined and genotyping of delta aminolevulinic acid dehydratase (*ALAD*) gene was performed through PCR-RFLP.

Automobile paint workers showed significantly increased BLL as compared to the control group ($p < 0.0001$). Mean cell volume was found to be significantly decreased in automobile paint workers as compared to control group ($p < 0.001$). Odd ratio shows greater chances of anemia with duration of exposure to the lead environment. Positive correlation of Hb concentration is observed with BLL in anemic automobile paint workers. The correlation is found to be increased for *ALAD 1-2* genotype in anemic automobile paint workers. Simple linear regression models showed positive association of Hb concentration with BLL in non-anemic automobile paint workers with *ALAD 1-2* genotype while the association was negative with *ALAD 1-1*genotype.

Positive correlation between Hb concentration and BLL of anemic automobile paint workers shows the possibility of lead induced anemia even at low BLL due to the continuous and prolonged duration of exposure to lead. The positive association of *ALAD 1-2* shows that the workers having this genotype have increased risk of anemia.

Key-words: Lead poisoning, lead induced anemia, *ALAD* genotypes, occupational lead exposure, Hemoglobin

INTRODUCTION

Exposure to lead is common in automobile industries, auto repair workshops, battery recycling plants, construction, ceramic, paint, chemicals, and plastic industries etc. High blood lead level (BLL) is known to be associated with lead poisoning but study has shown that even low BLL is also toxic for the heath due to continuous exposure (Tong *et al.,* 2000). The severity however depends upon the intake of nutrition, age factor and genetic makeup of the individual.

Occupational lead exposure is a serious health problem for workers where the lead dust enters into the body through respiratory tract and then gets distributed into the interstitial spaces, tissues and bones (Philip and Gerson 1994; Lyn Patrick, 2006; Markowitz, 2000).

Anemia is an associated clinical outcome of lead poisoning that results in decreased production of hemoglobin (IPCS, 1995). Marked inhibition of globulin synthesis and delayed regeneration of blood cells has been observed in lead exposed workers (White, 1975; Grandgean *et al.,* 1989). In earlier studies, hematocrit (HCT) values of lead exposed workers were found to be negatively correlated with BLL whereas decreased concentration of mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) was observed among lead workers at automobile workshops (Fontana *et al.,* 2004; Dongre *et al.,* 2011).

Lead enters in erythrocytes and binds itself to its possible binding sites (Barbosa Jr *et al.,* 2005). Delta-aminolevulinic acid dehydratase (*ALAD*) is an enzyme which is present in erythrocytes and catalyzes the second step in porphyrin and heme biosynthesis pathway (Wetmur *et al.,* 1991). Cofactor zinc is essential for the enzymatic activity of *ALAD* (Jaffe, 2000). The replacement of zinc with lead inhibits the enzymatic activity which prevents the conversion of aminolevulinic acid (*ALA*) into porphobilinogen due to which iron cannot be incorporated into the protoporphyrin ring that further leads to anemia (Jaffe, 2000, Lyn Patrick, 2006; Chatterje, 2005).

Studies have shown that polymorphism of *ALAD* is found to be associated with difference in lead toxicokinatics (Onalaja and Claudio, 2000). The replacement of guanine (G) to cytosine (C) in the coding region of *ALAD* resulted in amino acid substitution from asparginine to lysine at 59th position in the polypeptide chain (Keladaet *al.,* 2001; Wetmur*et al.,* 1991). Asparginine is neutral amino acid whereas lysine is positively charged amino acid so the *ALAD 1-2* genotype is more electronegative as compared to *ALAD 1-1* genotype (Keladaet *al.,* 2001). It is supposed

that the substitution may lead to the conformational change that increases the lead binding affinity in lead exposed individuals with *ALAD 1-2* and *ALAD 2-2* genotypes (Keladaet *al.,* 2001; Wetmur*et al.,* 1991).

To the best of our knowledge no study is available on BLL and its toxic effects on hematological parameters in automobile paint workers in our population. This study explains the correlation of Hb with low BLL in anemic and healthy automobile paint workers and the effect of polymorphism of *ALAD* gene on association of Hb with low BLL in the automobile paint workers.

MATERIALS AND METHODS

Data collection

Data on personal information, period of exposure etc. was collected from one hundred and sixty male automobile paint workers from workshops in different areas of Karachi and forty unexposed individuals as control group. Information was obtained from individuals by using questionnaire and assistance was provided in reading and writing when requested. Written consent was taken from all the participants as per the requirement of the ethical committee of the Department of Genetics.

Blood sample collection and hematological analysis

2mL venous blood was collected in duplicate glass vacutainers (ATLAS-LABOVAC) containing K3 EDTA as an anticoagulant. Hematological analysis was done with one set of the sample on same day of collection whereas second set of samples were placed at -40^{o}C till further use. Hb concentration, HCT, MCV, MCH and MCHC were analyzed in laboratory using automatic analyzer (Sysmex, KX21).

Blood cell morphology of samples was observed by making smear on the glass slide followed by fixation using methanol as described by (Houwen, 2000). Staining was performed with leishman stain and slides were observed under the light microscope and photographed.

Atomic absorption spectrophotometery

The blood lead level of all samples was determined by atomic absorption spectrophotometer with graphite furnace (Perkin-Elmer) according to the method described in Sole *et al.,* (1998).

Genotyping

The genomic DNA was extracted from whole blood by commercially available DNA extraction kit (Promega). Amplification reaction of *ALAD* gene was performed in final volume of 50µL as per the method of Schwartz *et al.,* (1995) with the modifications that instead of whole blood, extracted genomic DNA was used as a template and instead of nested PCR, amplification was performed with simple PCR using reported internal primer by Wetmur *et al.,*(1991) 5'-CAGAGCTGTTC-CAACAGTGGA-3' (Sense) and 5'-CCAGCACAATGTGGGAGTGA- 3' (Antisense).

Reaction mixture was prepared by using 2x master mix (Merck), 0.5µg template and 200ng of each primer. The PCR cycles consisted of initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 94^{o}C for 1 min, annealing of primers at 60 °C for 45 sec and extension at 72°C for 1 min with the final extension at 72°C for 10 min. The amplified product was run on a 1% agarose gel for the confirmation of fragment of 839 base pairs as per NCBI Accession No NG_008716. Restriction Fragment Length Polymorphism (RFLP) analysis was performed by using MspI restriction enzyme (Thermo Scientific) as described in Wetmur *et al.,* (1991). All the results were analyzed on 1% agrose gel under UV light in gel doc system (SCEI-PALS).

Statistical analysis

Statistical analysis was performed by SPSS version 20. The normality of data was analyzed before regression analysis and log transformation was performed where needed to normalize the data set.

RESULTS

Hematological parameters and BLL

The BLL of automobile paint workers and control group were found to be µ= 0.462µg/dL and µ= 0.173µg/dL respectively whereas the mean difference was significantly increased BLL at $p< 0.0001$. The mean Hb concentration, HCT and MCHC were found to be in normal ranges of values but the MCV was found to be decreased significantly at $p< 0.001$ in automobile paint workers as compared to the control group (Table 1).

The automobile paint workers having BLL ≥0.50µg/dL showed significantly decreased Hb concentration as compared to the workers having <0.50µg/dL BLL at $p<0.05$.

PCR-RFLP analysis for *ALAD* genotype

RFLP analysis with the *MspI* restriction enzyme of the amplified fragment showed single band of 547 bp and 476 bp for homozygotes *ALAD 1-1* and *ALAD 2-2* genotypes respectively. The heterozygote *ALAD 1-2* genotype showed both the fragments. 85.6% automobile paint workers in our study were found to have *ALAD 1-1* genotype (137 out of 160). 12.5% automobile paint workers showed *ALAD 1-2* genotype (20 out of 160) and 1.8% automobile paint workers showed *ALAD 2-2* genotype (3 out of 160). In the control group 72.5% individuals showed *ALAD 1-1* genotype (29 out of 40), 25.0% showed *ALAD 1-2* genotype (10 out of 40) and 2.5% individuals showed *ALAD 2-2* genotype (1 out of 40).

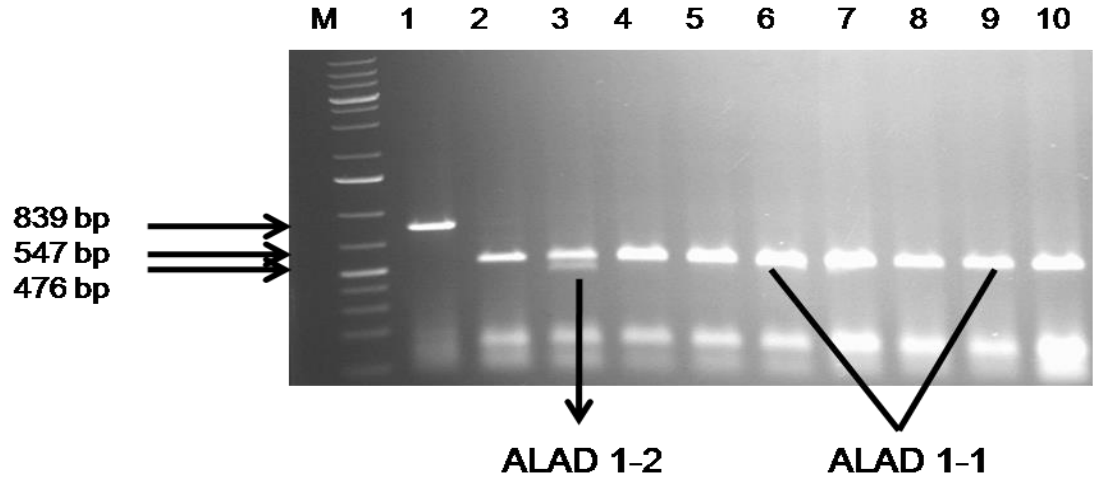

Fig..1. Image of gel electrophoresis of RFLP analysis after PCR showing *ALAD*1-1 and *ALAD* 1-2 genotypes. M = DNA Marker (Fermentas 1kb plus general ruler) lane 1, amplified undigested fragment of *ALAD*, lane 3, *Msp*I digested DNA showing two distinct bands (547 and 476 bp) of heterozygous *ALAD* 1-2 individual, lane 2 and 4-10, *Msp*I digested DNA showing one band (547 bp) of homozygous *ALAD* 1-1 individual.

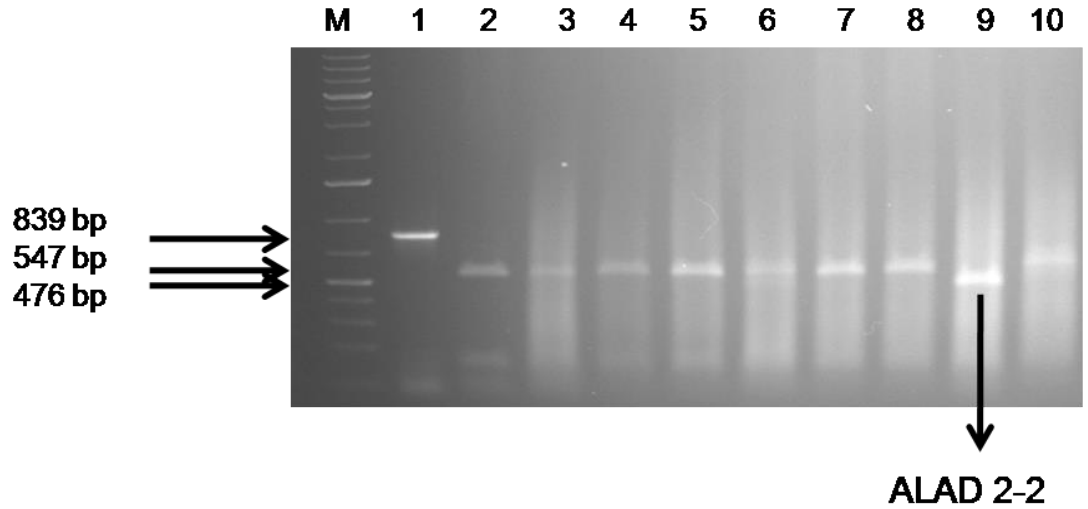

Fig..2. Image of gel electrophoresis of RFLP analysis after PCR showing *ALAD*1-1 and *ALAD* 2-2 genotypes. M = DNA Marker (Fermentas 1kb plus general ruler) lane 1, amplified undigested fragment of *ALAD*, lanes 2-8 and 10, *Msp*I digested DNA showing one distinct band of 547 bp of *ALAD* 1-1 homozygote, whereas lane 9 showing one band of 476 bp of *ALAD* 2-2 homozygote.

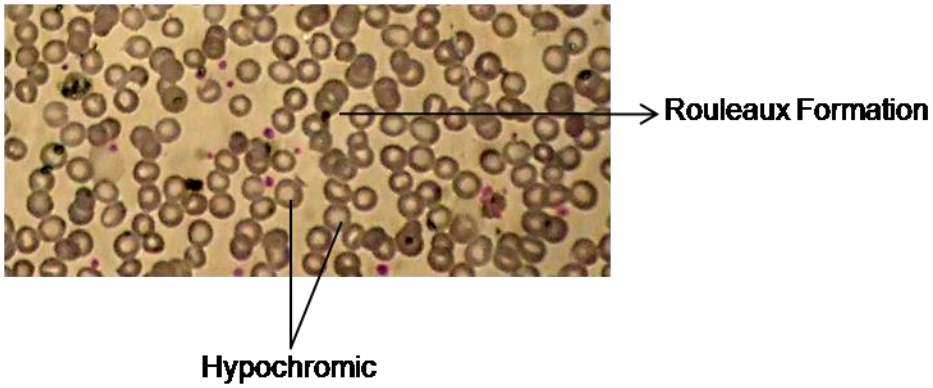

Fig..3. Image of RBC morphology of individual exposed to lead environment. Hypochromic blood cells (completely transparent hollow space in the center) and Rouleaux formation (clumping of the cells) is prominent.

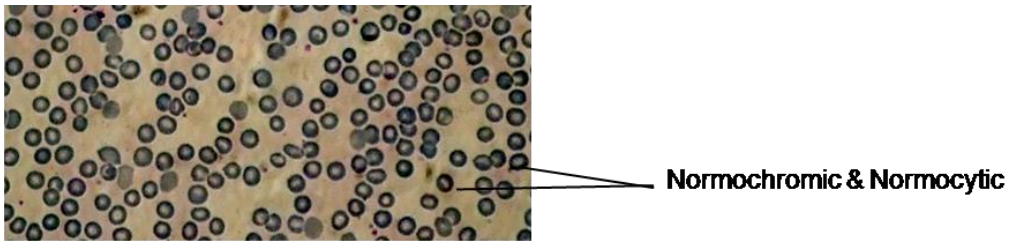

Fig..4. Image of RBC morphology of individuals with no known exposure to lead environment (control group). Normal blood cells with no Rouleaux formation no transparent hollow space in the center) and absence of Rouleaux formation.

Table 1. Comparison of mean blood lead level and different hematological parameters ± SME between automobile paint workers and control group.

BLL and Hematological Parameters	Automobile paint Workers (n=160)	Controls (n=40)	p-Value
Blood Lead Level	0.46 ± 0.43	0.17 ± 0.13	0.0001***
Hb	13.89 ± 1.38	13.56 ± 0.92	0.085^{NS}
HCT (%)	42.59 ± 3.63	41.90 ± 2.63	0.17^{NS}
MCV	86.40 ± 6.33	90.08 ± 6.91	0.001**
MCH	28.34 ± 2.69	29.13 ± 2.73	0.09^{NS}
MCHC	32.42 ± 1.33	32.31 ± 0.97	0.61^{NS}

* Significant difference; ** Highly significant difference; ^{NS} Non significant difference

Microscopy of blood smears through compound microscope

Analysis of blood smear test of automobile paint workers showed RBC with altered morphology including hypochromia, anisocytosis, poikilocytosis, elliptocytosis and rouleaux formation (Figure 3) whereas the blood cells morphology of control group showed normochromic and normocytic cells (Figure 4).

Average BLL, Hb and percentage of anemic automobile paint workers with respect to *ALAD* genotypes

The *ALAD 1-1* genotype showed increased BLL and decreased Hb concentration along with decreased percentage of anemic automobile painters whereas *ALAD 1-2* genotype showed decreased BLL and increased Hb concentration along with increased percentage of anemic automobile painters.

Correlation of Hb with low BLL in anemic and normal automobile paint workers

The correlation analysis performed through person's correlation. The anemic automobile paint workers showed good positive correlation (r = 0.37) of low BLL with Hb concentrations whereas the non anemic automobile paint workers showed negative correlation (r = -0.008) between low BLL and Hb concentration.

Correlation of Hb with low BLL in anemic automobile paint workers with *ALAD* genotypes

The correlation analysis performed through person's correlation. The anemic automobile paint workers with *ALAD 1-1* genotype showed increased correlation (r = 0.21) between Hb concentration as compared to the *ALAD 1-2* genotype which showed correlation (r = 0.48) between Hb concentration and BLL.

Odd ratio for anemia with duration of lead exposure

Logistic regression analysis performed to analyze the chances of individuals becoming anemic with increase in duration of exposure to lead gave the odd ratio OR = 1.02. The odd ratio >1 indicated that there is a greater chance of anemia in individuals with the increase in duration of exposure to lead.

Table 2. Linear regression model evaluating effect of *ALAD* genotypes on association of hemoglobin and low BLL in non anemic automobile paint workers.

Regression Models Of Hemoglobin		B	Std. Error	Beta	Sig.	Empirical Results < 0.05	R^2
*ALAD*1-1	(Constant)	14.526	0.126	--	0.0001	Sig	0.002
	Blood Lead Level	-0.085	0.190	-0.044	0.654	NS	
*ALAD*1-2	(Constant)	13.942	0.226	--	0.0001	Sig	0.123
	Blood Lead Level	0.464	0.357	0.351	0.219	NS	

^{Sig} Significant difference; ^{NS} Non significant difference

Association of hemoglobin and blood lead level with *ALAD* genotypes

Simple linear regression models (Table 2) showed the association of BLL with Hb concentration in automobile paint workers with ALAD *1-1* and *ALAD 1-2* genotypes. The β coefficient (β = -0.044) showed negative association of BLL with Hb concentration with *ALAD 1-1* genotype. Whereas the β coefficient for *ALAD 1-2* (β = 0.351) showed positive association of BLL with Hb concentration. Though the p-values showed insignificant level but the effect of *ALAD 1-1* genotype is found to be negative on association of BLL with Hb concentration in automobile paint workers even at low BLL.

DISCUSSION

Occupational lead exposure is still a serious health problem for lead exposed workers in developing countries as the environmental condition of workshops is generally not suitable for the workers. There is lack of awareness, education and training programs for workers with no regular monitoring of workshops. Safety measures are also not generally adapted by the workers themselves.

Accumulation of lead in the body causes lead poisoning which is an environmental disease that occurs due to the lead exposure by lead based occupations which include storage batteries, paints and pigments etc. (Katzung, 2004; Markowitz, 2000; Patnaik, 2002). Although it is claimed that lead is no longer used in the paints still we analyzed some randomly collected paint samples from the local markets. The results of atomic absorption spectrophotometery showed average 110.82 ppm lead was present in the paint samples. This study was designed to determine the association of BLL with Hb concentration and the effects of *ALAD* genotypes on this association in lead exposed workers of automobile workshops in Karachi, Pakistan.

The atomic absorption spectrophotometery of our automobile paint workers showed mean BLL value μ=0.462μg/dL whereas the highest acceptable BLL for lead exposed workers by Occupational Safety and Health Administration (OSHA) is 40μg/dL (https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=10031).

Our automobile painters showed low BLL similar to the earlier study on Turkish lead workers by Tozun *et al.*, (2009). The statistical analysis showed significantly increased BLL in automobile painters at p<0.0001 as compared to the control group. It has been reported that intoxication could also occur at low lead level with continuous exposure (Tong *et al.*, 2000). Lead toxicity is a major cause of anemia among lead exposed workers. Lead enters into blood stream and attacks on erythrocytes where it binds with its possible binding sites which become a cause of decrease in Hb concentration and in this way it induces anemia (Markowitz, 2000; Jang *et al.*, 2011). Moreover,

presence of lead in erythrocytes increases its chances for spleenic sequestration thereby cause of anemia (Jang *et al.,* 2011).

Study by Kalahasthi *et al.,* (2012) showed insignificant difference in the hematological parameters between lead exposed group with an average of 47µg/dL of BLL. Interestingly, our study shows significantly decreased MCV at $p< 0.01$ in automobile paint workers whereas not in the study of Kalahasthi *et al.,* (2012). Studies by Dongre *et al.,* (2011) and Fonte *et al.,* (2007), reported that the alteration of hematological parameters at 47 and 148 µg/dL of blood lead level respectively.

To analyze the correlation of Hb with BLL we dichotomozed the workers into anemic and non anemic automobile paint workers because we have found low BLL in our population. Our result showed positive correlation of BLL with Hb concentration in anemic automobile paint workers however, the correlation was found to be negative among non anemic automobile paint workers. Studies by Kalahasthi *et al.,* (2012), Lilis *et al.,* (1978) and Kim *et al.,* (2004) showed positive correlation of BLL with Hb concentration in lead exposed workers at high BLL as compared to our study. It shows that the lead exposed workers may have lead indued anemia even at the low BLL and at continuous exposure. Therefore it is suggested that the low blood lead level is also associated with Hb concentration in lead exposed workers.

For the correlation anlysis of Hb and BLL with *ALAD* genotypes, we dichotomized anemic automobile paint workers into two group with respect to the *ALAD 1-1* and *ALAD 1-2* genotypes which showed the greater correlation of Hb with BLL in automobile paint workers with *ALAD 1-2* genotype.

The results of linear regression model showed negative association of BLL and Hb concentration in automobile paint workers with both *ALAD 1-1* genotype ($\beta = -0.044$) whereas the association was found to be positive with *ALAD 1-2* genotype ($\beta = 0.351$), thus indicating that chances of development of anemia is associated with *ALAD 1-2* genotype. Studies of Kim *et al.,* (2004), however showed greater chances of having anemia with increased BLL with *ALAD 1-1* genotype whereas with *ALAD 1-2* genotype, Hb was found normal. Interestingly the odd ratios in our study also showed increased chances of anemia with duration of exposure though BLL was found low.

CONCLUSION

Our research shows that there is a serious danger to the health of automobile paint workers even with low blood lead level, if there is a continuous and prolonged exposure to lead environment. Our study showed positive correlation of low BLL with Hb. The positive association and correlation of Hb and low BLL with *ALAD 1-2* genotype in non-anemic and anemic automobile paint workers respectively may shows that *ALAD 1-2* genotype is more prone to lead induced anemia. It is further predicted by correlation analysis that it is quite likely that continuous exposure to lead even at low level can develop conditions like anemia. This emphasizes the need for regular monitoring of working environment and health checkups of workers associated with industries and workshops with potential lead exposure.

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