Association between δ-aminolevulinate dehydratase G177C polymorphism and blood lead levels in brain tumor patients

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Abstract. As the δ-aminolevulinic acid dehydratase (ALAD) G177C polymorphism affects the toxicokinetics of lead in the body, and the corresponding exposure to lead may increase the risk of adult brain tumors, we hypothesize that there is a possible association of the ALAD G177C genotype and the risk of brain tumors in human. Therefore, the aim of the present study was to clarify the role of the ALAD enzyme gene polymorphism at position G177C in the pathogenesis of brain tumors and its correlation to lead exposure. The ALAD gene polymorphism at position G177C was genotyped using the polymerase chain reaction with restriction fragment length polymorphism method and measured the blood lead level by atomic absorption in 81 brain tumor patients and compared the results with 81 controls. The frequency of the GC genotype (ALADI-2) was significantly increased in primary brain tumor patients compared to the control group. The genotype frequency of ALAD2 (ALADI-2 and ALAD2-2) was significantly higher in the meningioma patients but was not significant in glioma patients. There was no significant difference in the number of patients and blood lead level when compared with the control. There was a significant increase when compared to ALADI regarding a mean value of the lead level. The genotyping of the ALAD G177C polymorphism in the present study revealed a significant association between ALAD2 and brain tumors. The ALAD G177C polymorphism may modify the lead kinetics in the blood, is associated with higher blood lead burden and may provide a biomarker of neurotoxic risk.

Introduction

Community and occupational exposures of adults to lead has been recognized for centuries, particularly in Egypt, as unfavorable effects on the hematopoietic, gastrointestinal, urinary, cardiovascular and nervous systems have been well-documented (1). The International Agency Research on Cancer recently reclassified inorganic lead to a ‘probable’ human carcinogen due to evidence from animal carcinogenicity and limited human carcinogenicity (2). Whereas certain epidemiological studies have reported an increased risk of brain tumors with potential lead exposure (3), particularly meningioma (4,5), another study has reported no significant association between lead and brain cancer (6).

Increasing attention has focused on interpreting the genetic factors that elucidate the differences in symptoms between individuals who have had similar lead exposures. Identification of such genes would aid in the explanation of variation in the association between biological markers of lead exposure and measures of organ dysfunction.

The δ-aminolevulinic acid dehydratase (ALAD) gene codes for the enzyme ALAD that catalyzes the second step of heme synthesis in which two molecules of ALA condense to form porphobilinogen. The most commonly known polymorphism in the gene, ALAD G177C (dbSNP ID: rs1800435) contains a G-to-C transversion at position 177 of the coding region, resulting in the substitution of asparagine for lysine. ALAD G177C has two co-dominant alleles: ALADI and ALAD2 (7).

ALAD can be inhibited by various chemicals, including lead, trichloroethylene, bromobenzene and styrene (8), however, the polymorphic differences in enzyme binding or chemical uptake are more comprehensive for lead. Individuals with the ALAD2 allele have been shown to exhibit higher blood lead levels compared with the ALADI homozygotes, possibly due to tighter binding of lead by the ALAD2 enzyme (9).

How the ALAD genotype influences the distribution of lead to target organs remains unknown. However, it has been documented that the binding of lead molecules inactivates δ-ALAD and causes a rise in the levels of its substrate, δ-ALA (δ-ALA) (10). In the brain, excess δ-ALA disrupts the γ-aminobutyric acid/glutamate system in several ways, creating potential for neuroexcitotoxic events and cell death (11).

Keywords: brain tumor, lead, δ-aminolevulinic acid dehydratase gene, polymorphism, meningioma

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Previous studies have indicated that ALAD2 may be protective against the neurotoxic/neurobehavioral effects of lead (12,13). However, it has also been observed that ALAD2 may confer an increased risk of meningioma (14).

The possible genetic susceptibility and the potential association between lead and brain tumor risk have not been previously evaluated in the Egypt population. The aim of the present study was to examine the associations between the ALAD G177C polymorphism and blood lead burden in adult brain tumor Egyptian patients.

Materials and methods

The present study included 81 patients with brain tumors and 81 gender- and age-matched healthy control subjects. The study was carried out in Departments of Neurosurgery and Medical Biochemistry, Faculty of Medicine, Zagazig University (Zagazig, Egypt).

Participants. The patient group included 81 patients with brain tumors with surgery performed in the Department of Neurosurgery and confirmed by histopathology. The ages of the patients were >18 years old. Only patients with primary brain tumors were included. Patients were excluded if they had secondary brain tumors or they were <18 years old.

The control group included 81 gender- and age matched subjects to the patients. They were selected randomly from the same population as the patients, and were collected from cases admitted in the Department of Neurosurgery for a variety of non-neoplastic conditions.

All the participants were recruited from Zagazig University Hospital and Department of Neurosurgery. All the subjects were of Egyptian nationality. Written informed consent was signed by all the participants. The study design was approved by the Ethics Committee of Faculty of Medicine, Zagazig University.

All the participants were subjected to: i) History; demographic data were collected from the patients in the form of age, gender and any systemic disorder, past history of systemic treatment. ii) Examination; all subjects underwent complete neurological examination. iii) Radiological investigations by computed tomography and magnetic resonance imaging (MRI); post-operative histopathology to detect the type of the brain tumor for the patients.

DNA extraction. DNA was isolated and purified from whole blood (EDTA) using the manufacturer’s instructions (Qiagen GmbH, Hilden, Germany). The purified DNA was stored at -20°C for later use.

Genotyping of ALAD G177C gene polymorphism. A polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) assay was used for detection of the ALAD G177C (rs 1800435) polymorphism, as described by Lee et al (15), using the following primers: Forward, 5’-AGA CAG ACA TTA GCT CAG TA-3’ and reverse primers, 5’-GGC AAA GAC CAC GTC CAT TC-3’.

PCR was carried out in a final volume of 50 µl containing 100 ng of template DNA (5 µl), 1.0 µM of each primer (1 µl) (MBI Fermentas GMBH, St. Leon-Rot, Germany), 25 µl of 2X I-Taq™ PCR Master mix (Intron Biotechnology, Gyeonggi-do, South Korea) and 18 µl deionized water.

Cycling conditions were initial denaturation of 94°C for 10 min, followed by 32 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and finally 72°C for 7 min using DNA Thermal Cycler 480 (serial no, P 16462; Perkin-Elmer, Norwalk, CT, USA).

Digestion by restriction enzyme. The digestion of the 916-base pair (bp) ALAD genomic sequence containing the ALADI/ALAD2 polymorphic site was performed using restriction endonuclease MspI (MBI Fermentas GmbH). It was performed in a 25-µl volume (2.5 µl 10X Buffer, 1 µl 10 U/µl MspI, 15µl PCR product and 6.5 µl deionized water).

The samples were incubated at 37°C for 1 h. Digestion of the PCR fragments produced one fragment, 582-bp, represented ALADI-1 genotype. Two fragments, 582 and 511-bp, representing the ALADI-2 genotype, while one fragment, 511-bp, represented the ALAD2-2 genotype.

Gel electrophoresis detection of ALAD genotype. The digestion products were separated in 2% agarose using Submarines Gel Electrophoresis System (Pharmaica Biotech by InterTek Semko AB, Kista, Sweden) and submarine chamber (Maxicell, EC360; M-E-C Apparatus Corp., St Petersburg, FL, USA), subsequently visualized with ethidium bromide staining under ultraviolet trans-illumination (Herolab GmbH Laborgerate, Wiesloch, Germany) with the 100-bp SiZer™ DNA marker (Intron Biotechnology) and photographed.

Atomic absorption spectroscopic measurement of blood lead. Lead was determined directly in whole blood using graphite furnace atomic absorption spectrometry, according to Miller et al (16). Measurement was achieved by atomic absorption using the Buck model 210 VGP atomic absorption spectrophotometer (Buck Scientific Instrument Manufacturing Company AA, East Norwalk, CT, USA) with a graphite furnace option with background correction; the measurement was read at wavelength 283.3 nm following electro-thermal atomization. The absorption is proportional to lead content and is used to yield quantitative lead content on the basis of comparison with standard series.

Statistical analysis. The results for continuous variables are expressed as mean ± standard deviation (SD). The means of the groups were compared by the analysis of variance (ANOVA) test. The statistical significances of differences in the frequencies of variants between the groups were tested using the χ² test. In addition, odds ratios (OR) and 95% confidence interval (CI) were used. Genotype frequencies in cases and controls were tested for Hardy–Weinberg equilibrium and any deviation from the expected frequencies was tested for significance using the χ² test. P<0.05 was considered to indicate a statistically significant difference. Data were analyzed using SPSS software version 11 (SPSS, Inc., Chicago, IL, USA).

Results

Demographic data of patients and controls. There was no significant difference with regards to age (P=0.54) and
gender (P=0.89) in the control when compared to the primary brain tumor patients. The demographic data for the study groups are shown in Table I.

Types of primary adult brain tumor. Of the 81 patients with primary brain tumor, there were 31 (38.3%) patients with meningioma, 41 (50.6%) with glioma and 9 (11.1%) with other tumors. ANOVA test revealed no statistically significant difference between the three types (F=0.184, P=0.83) (Table II).

Genotypes and allele frequencies of the ALAD G177C gene in the studied groups. The genotype frequencies of ALAD G177C conformed to the Hardy-Weinberg equilibrium in the cases and controls. In patients with primary brain tumor, the frequencies of GG (ALAD1-1), GC (ALAD1-2) and CC (ALAD2-2) genotypes were 56.8, 28.4 and 14.8%, respectively; and in controls, the frequencies were 79.0, 14.8 and 6.2%, respectively. The frequencies of G and C alleles in primary brain tumor patients were 71 and 29%; and in controls were 86.4 and 13.6%, respectively. With regards to the risk of primary brain tumor development, using the GG genotype and G-wild allele as references, there was an increased risk of primary brain tumor with the GC (ALAD1-2) genotype (OR, 2.28, 95% CI, 1.04-4.97 and P=0.03) and the C allele (OR, 2.60, 95% CI, 1.48-4.57 and P=0.0006). There was a significant association between the brain tumor and the ALAD2 (GC and CC) genotypes (OR, 2.86, 95% CI, 1.43-5.72, and P=0.002) (Table III).

### Table I. Demographic data of primary brain tumor patients versus controls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group, n=81</th>
<th>Primary brain tumor patients, n=81</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years ± SD (range)</td>
<td>43.88±11.68 (19-65)</td>
<td>45.09±13.69 (18-67)</td>
<td>0.54a</td>
</tr>
<tr>
<td>Gender, no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36 (44.4)</td>
<td>34 (42.0)</td>
<td>0.89b</td>
</tr>
<tr>
<td>Female</td>
<td>45 (55.6)</td>
<td>47 (58.0)</td>
<td></td>
</tr>
</tbody>
</table>

*aTwo tailed t-test; *bχ² test. SD, standard deviation.

### Table II. Demographic characteristics of primary brain tumor patients following stratification by the tumor type versus controls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Glioma, n=41 (50.6%)</th>
<th>Meningioma, n=31 (38.3%)</th>
<th>Other tumors, n=9 (11.1%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years ± SD</td>
<td>45.73±11.59</td>
<td>44.87±12.00</td>
<td>42.92±19.98</td>
<td>0.83a</td>
</tr>
<tr>
<td>Gender, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16 (39.0)</td>
<td>14 (45.2)</td>
<td>4 (44.4)</td>
<td>0.86b</td>
</tr>
<tr>
<td>Female</td>
<td>25 (61.0)</td>
<td>17 (54.8)</td>
<td>5 (55.6)</td>
<td></td>
</tr>
</tbody>
</table>

*aAnalysis of variance test between three tumor types; *bχ² test. Controls, n=81. SD, standard deviation.

### Table III. Genotypes and allele frequencies of the ALAD G177C gene in the studied groups.

<table>
<thead>
<tr>
<th>ALAD G177C polymorphism</th>
<th>Controls, no. (%)</th>
<th>Primary brain tumor patients, no. (%)</th>
<th>OR (95% CI)</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (ALAD1-1)</td>
<td>64 (79.0)</td>
<td>46 (56.8)</td>
<td>1 (Reference)</td>
<td></td>
</tr>
<tr>
<td>GC (ALAD1-2)</td>
<td>12 (14.8)</td>
<td>23 (28.4)</td>
<td>2.28 (1.04-4.97)</td>
<td>0.03b</td>
</tr>
<tr>
<td>CC (ALAD2-2)</td>
<td>5 (6.2)</td>
<td>12 (14.8)</td>
<td>2.64 (0.88-7.88)</td>
<td>0.07</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>140 (86.4)</td>
<td>115 (71.0)</td>
<td>1 (Reference)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>22 (13.6)</td>
<td>47 (29.0)</td>
<td>2.60 (1.48-4.57)</td>
<td>0.0006b</td>
</tr>
<tr>
<td>ALAD1 (GG)</td>
<td>64 (79.0)</td>
<td>46 (56.8)</td>
<td>1 (Reference)</td>
<td></td>
</tr>
<tr>
<td>ALAD2 (GC,CC)</td>
<td>17 (21.0)</td>
<td>35 (43.2)</td>
<td>2.86 (1.43-5.72)</td>
<td>0.002b</td>
</tr>
</tbody>
</table>

*aχ² test; *bP<0.05. OR, odds ratio; CI, confidence interval.
however, there was no significance with regards to the glioma patients (P=0.08; OR, 2.17; 95% CI, 0.95-4.98) and the other tumor patients (P=0.41; OR, 1.88; 95% CI, 0.43-8.31). The comparison of glioma and meningioma revealed no significant difference between them (P=0.12; OR, 0.47; 95% CI, 0.18-1.23) (Table IV).

Blood lead levels and primary brain tumor risk. In the control group, blood lead levels ranged from 4.5 to 39.0 µg/dl with a mean ± SD of 25.02±12.65 µg/dl and in the brain tumor group, blood lead levels ranged from 4.4 to 62.0 µg/dl with a mean ± SD of 28.61±13.23 µg/dl, however, there was no significant difference (P=0.08) (Table V).

The blood lead levels of primary brain tumor patients following stratification by the tumor types revealed that the mean lead level of glioma patients was 28.86±20.12 µg/dl, meningioma was 29.37±14.35 µg/dl and in other tumors was 24.44±15.97 µg/dl with no statistically significant difference between the three types (F=0.28, P=0.76; ANOVA) (Table VI). When blood lead was categorized by the levels <10, 10-30 and >30 µg/dl, the number of subjects with blood lead level <10, 10-30, >30 µg/dl exhibiting the GG (ALAD1-1) genotype was 37 (5.9%), 3 (17.6%) and 3 (17.6%) respectively, with a significant increase in ALAD2 when compared with ALAD1 (P=0.0001). ALAD1 participants had a lead level with a mean value ± SD of 25.93±12.73 and ALAD2 34.39±17.87 µg/dl, which indicates a significant increase in ALAD2 when compared with ALAD1 (P=0.0007) (Tables VII and VIII).

Discussion
Aside from a small percentage of brain tumors that can be explained by familial syndromes or exposure to ionizing radiation, extremely little is known regarding the etiology of brain tumors (17). Exposure to lead can result in significant adverse health effects to multiple organ systems. As the ALAD G177C polymorphism affects the toxicokinetics of lead in the body and as exposure to lead may increase the risk of adult brain tumors, we hypothesized a possible association of the ALAD G177C genotype and the risk of brain tumors in human (3).
Table VII. Blood lead level in different genotype of studied groups.

<table>
<thead>
<tr>
<th>ALAD genotype</th>
<th>&lt;10, no. (%)</th>
<th>10-30, no. (%)</th>
<th>&gt;30, no. (%)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (ALAD1-1)</td>
<td>37 (33.6)</td>
<td>36 (32.8)</td>
<td>37 (33.6)</td>
<td></td>
</tr>
<tr>
<td>GC (ALAD1-2)</td>
<td>7 (20.1)</td>
<td>6 (17.1)</td>
<td>22 (62.9)</td>
<td></td>
</tr>
<tr>
<td>CC (ALAD2-2)</td>
<td>1 (5.9)</td>
<td>3 (17.6)</td>
<td>13 (76.5)</td>
<td>0.001b</td>
</tr>
<tr>
<td>ALAD1 (GG)</td>
<td>37 (33.6)</td>
<td>36 (32.8)</td>
<td>37 (33.6)</td>
<td></td>
</tr>
<tr>
<td>ALAD2 (GC,CC)</td>
<td>8 (15.4)</td>
<td>9 (17.3)</td>
<td>35 (67.3)</td>
<td>0.0002b</td>
</tr>
</tbody>
</table>

*a2 test; bP<0.05. Percentages were calculated within the genotype.

Table VIII. Mean blood lead level in the ALAD1 and ALAD2 genotypes.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ALAD1 (GG)</th>
<th>ALAD2 (GC,CC)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood lead level, mean µg/dl ± SD</td>
<td>25.93±12.73</td>
<td>34.39±17.87</td>
<td>0.0007b</td>
</tr>
</tbody>
</table>

*a Two-tailed t-test; bP<0.05. SD, standard deviation.

In the present study, the frequencies of ALAD1 and ALAD2 were 79 and 21%, respectively, this was similar to other studies which identified that the ALAD2 allele frequency ranges from 6 to 20% in Caucasian populations, with ~18% of the Caucasian population being ALAD1-2 heterozygotes and 2% being 2-2 homozygotes (18). In comparison, the ALAD2 frequency was 3 to 11% in Asian populations and ~3% in African-American populations (19-21).

The genotyping of the ALAD G177C polymorphism in the present study declared a significant association between ALAD2 and brain tumors. Following stratification of the patients with tumor type, there was a significant association between ALAD2 and meningioma but not with glioma or other brain tumors; a result that corresponds with the results of Rajaraman et al 2006 (14,22).

The increased risk of meningioma in individuals with the ALAD2 allele and the effect being dependent upon exogenous chemical exposures acting on the heme synthesis pathway or being independent of such exposures remains unknown. A direct effect of the ALAD2 polymorphism may be indicated when the ALAD2 allele has lower enzyme activity compared with the ALAD1 allele, as the precursor ALA is believed to be neurotoxic and genotoxic (23). It is also possible that the increased risk of meningioma in ALAD2 individuals occurs in the presence of chemicals that influence the heme synthesis pathway.

Several chemicals inhibit ALAD enzyme activity, including lead, trichloroethylene, bromobenzene and styrene (8). Additionally, Montenegro et al (24) and Fujihara et al (25) suggested that the significant inter-ethnic differences in the distribution of ALAD G177C variants may help us to understand the inter-ethnic disparities in susceptibility to lead toxicity and brain tumors.

The meta-analysis conducted by Scinicariello et al (26) concluded that ALAD2 carriers generally exhibit higher blood lead levels in adults at increased levels of lead exposure compared to the ALAD1 carriers, and the strength of this analysis is based on the aggregation of published studies.

The present study observation of the increased risk with the ALAD2 variant for meningioma, but not for glioma or other types, parallels with the observation of Lacourt et al (27) who confirmed the lack of association between glioma tumors and occupational exposure to selected combustion products, dusts and other chemical agents. The only strongly established risk factors for glioma are rare (ionizing radiation and rare medical conditions), possibly rarer compared to the agents that were examined in the present study.

The results of the present study showed that participants with ALAD2 have a high blood lead level when compared with ALAD1 in the case and control groups. This was in agreement with the results of Ziemsen et al (28), Steenland and Boffetta (6), Fleming et al (29) and Wetmur et al (30). All these studies examined populations with exposure levels that were higher compared to the normal level, with blood lead levels often >30 µg/dl, which was previously designated as a cut-off used as evidence of lead poisoning. These findings led to the suggestion that ALAD2 may be a determinant of increased susceptibility to lead toxicity.

Hypotheses generated to support these results were based on the fact that ALAD2 codes for a more electronegative enzyme, and the ALAD2 protein is believed to be able to bind positively charged lead ion more tightly compared to the ALAD1 protein. Exposure to lead in carriers of the ALAD2 allele may be retained in their blood and tissues longer, increasing the chance of an adverse effect due to the presence of lead, which may initiate oxidative damage and cellular component structural changes, or due to inhibition of ALAD and the consequent accumulation of ALA (31).

From these initial studies, we conclude that the kinetics of lead in blood are modified by the ALAD genotype, although perhaps only at relatively high levels of exposure. Shaik and Jamil (32) reported that blood lead levels did not differ significantly among ALAD1-I, I-2 and 2-2 genotypes. Additionally, Sobin et al (33) reported that there was no association between ALAD2 and sub-clinical blood lead burden.

One explanation for this may be the relatively low number of children with homozygous versus heterozygous for the
polymorphism. A second explanation may be that the impact of the ALAD2 polymorphism is highly variable in cases of lowest level lead exposure.

Sützen et al (34) and Yang et al (35) concluded that ALADI-1 individuals may have a higher blood lead level compared to those with the ALADI-2 genotype.

The present study has a number of strengths. It proved that the ALAD G177C polymorphism may modify the lead kinetics in the blood, is associated with higher blood lead burden and may provide a biomarker of neurotoxic risk. However, the potential bias could be minimized by restriction of inclusion criteria to patients, as confirmed by MRI and histopathology. The homogenous ethnic cohort limited the confounding genetic factors caused by ethnic heterogeneity.

References

34. Sützen et al (34) and Yang et al (35) concluded that ALADI-1 individuals may have a higher blood lead level compared to those with the ALADI-2 genotype.