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ORIGINAL ARTICLE

Founder effect confirmation of c.241A > G mutation in the *L2HGDH* gene and characterization of oxidative stress parameters in six Tunisian families with L-2-hydroxyglutaric aciduria

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L-2-hydroxyglutaric aciduria (L2HGA) is an autosomal recessive neurometabolic disorder characterized essentially by the presence of elevated levels of L-2-hydroxyglutaric acid (LGA) in plasma, cerebrospinal fluid and urine. L2HGA is caused by a deficiency in the L2-Hydroxyglutaric dehydrogenase (L2HGDH) enzyme involved in the oxidation of LGA to the alpha 2-ketoglutarate. LGA has been proposed as an endo- and exogenous cytotoxic organic acid that induces free radical formation and generation of reactive oxygen species (ROS). In this report, we analyzed 14 L2HGA patients belonging to six unrelated consanguineous families the south of Tunisia. The patients were diagnosed with L2HGA disease confirmed on the presence of high level of LGA in urine. We analyzed the *L2HGDH* gene in all probands and identified the same c.241A > G homozygous mutation, which was previously reported in Tunisia. We also used intragenic single nucleotide length polymorphisms (SNPs) and two extragenic microsatellites flanking the *L2HGDH* gene to confirm the founder effect of c.241A > G mutation in the 14 studied cases. In addition, we carried out the measurement of the oxidative stress parameters in the plasma of L2HGA patients which revealed a significant increase in the malondialdehyde levels (MDA), a biomarker of lipid peroxydation, and the reduced glutathione (GSH). A diminution of the antioxidant enzyme activities including superoxide dismutase (SOD), glutathione peroxidase (GPx), was also observed.

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INTRODUCTION

L-2-hydroxyglutaric aciduria (L2HGA-MIM236792) is an autosomal recessive neurometabolic disorder characterized by the presence of elevated levels of L-2-hydroxyglutaric acid (LGA) in plasma, cerebrospinal fluid and urine.[1,2]

Clinically, the affection is characterized by global developmental delay or mental deterioration in the first years of life, cerebellar ataxia, and pyramidal and extra pyramidal signs. Epilepsy and macrocephaly may be additional findings.[3,4] Cerebral MRI characteristically shows subcortical leukodystrophy with the involvement of basal ganglia and dentate nuclei. The centripetal extension of the white matter involvement constitutes a distinct feature that differentiates this disorder from other leukodystrophies.

L2HGA is caused by a deficiency in the L-2-hydroxyglutaric dehydrogenase (L2HGDH), an enzyme involved in the oxidation of LGA to α 2-ketoglutarate. This deficiency leads to the accumulation of LGA in body fluids. Influence of LGA accumulation was studied on various parameters of oxidative stress such as level of lipid peroxidation in the cerebellum and cerebral cortex of young rats via measurement of the malondialdehyde levels (MDA).[5] In fact, LGA has been proposed as an endo- and exogenous cytotoxic organic acid, and its presence in the neuronal cultures induces free radical formation and generation of reactive oxygen species (ROS).[6] The antioxidant enzymes reduce oxidative stress through the inactivation of highly toxic free oxygen radicals and peroxides. In the central nervous system, antioxidant mechanisms include a number of

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cytosolic and lypophilic low molecular weight and enzymatic antioxidants. The key enzymatic and nonenzymatic scavengers are superoxide dismutase (SOD) [EC 1.15.1.1], glutathione peroxidase (GPx) [EC 1.6.4.2] and reduced glutathione (GSH) [EC 1.11.1.9]. The decreased activity of enzymatic scavengers can result from the nonenzymatic glycation along with glycoxidation products followed by inactivation of the modified enzyme.[7,8]

Topçu *et al.*[9] reported the involvement of the *L2HGDH* gene in L2HGA. This gene encodes the L2HGDH enzyme, which is expressed in multiple tissues including brain, testis and muscles with the highest expression in the brain.

Up to now, several pathogenic mutations within the 10 exons of the *L2HGDH* gene have been reported in the literature associated with L2HGA phenotypic spectrum in patients from diverse ethnic backgrounds.[9–15] L2HGDH mutations include missense, nonsense, frameshift and splice site mutations. The majorities are single-base changes resulting in an altered amino acid; rarely large deletions are found. In Tunisia, two missense mutations c.185C>A and c.241A>G are reported.[10,15]

In this report, we described a molecular analysis of 14 patients belonging to six families from southern Tunisia with L2HGA and presenting the same mutation in the *L2HGDH* gene. We suggested also the presence of founder effect of this mutation using intragenic SNPs in the *L2HGDH* gene and two extragenic microsatellite markers. Besides, we evaluated MDA and GSH levels, and SOD and GPx activities in plasma of L2HGA patients.

PATIENTS AND METHODS

Patients

Our study is about 16 patients, two of whom (P1's sister and P6's brother) died and their DNA were not available; therefore, we reported here clinical and molecular analyses of 14 patients from six unrelated consanguineous families from the same region in south Tunisia, including six affected girls and eight affected boys (Figure 1a). For all patients, history and clinical examination were evaluated by pediatric neurologists. All patients underwent urinary organic acid analysis and at least one cerebral MRI with transverse and sagital T1-weighted images and transverse T2-weighted images. Magnetic resonance spectroscopy was performed only for five patients.

Clinical and biological data are shown in Table 1. Biochemical analysis showed that all patients for whom the chromatography of organic amino acids was performed and presented increased urinary concentration of LGA ranging between 432 and 1944 $\mu mol\, mmol^{-1}$ creatine, indicating functional deficiency of the L2HGDH enzyme.

Cerebral MRI showed subcortical leukodystrophy with dentate nucleus involvement in all patients. Basal ganglia involvement was noted in six patients (globus pallidus in six patients and thalamus in one patient) and cysts were noted in three patients. Magnetic resonance spectroscopy was normal in three patients and showed nonspecific findings in two patients including high level of lactate in one patient and decrease in *N*-acetyl aspartate level in one patient.

Methods

DNA extraction. Genomic DNA was extracted from blood samples of the patients and their family members using the standard phenol chloroform procedure.[16] Informed consent was obtained from their legal guardians.

DNA sequencing. PCR amplification was performed for the 10 exons of the gene and their exon–intron boundary regions using appropriate primers. PCR reactions were performed under the touchdown conditions as follows: 95 °C for 5 min, 6 cycles of 95 °C for 40 s, 63 °C for 45 s, 72 °C for 45 s and 30 cycles of 94 °C for 40 s, 58 °C for 45 s for exons 1, 3, 4, 5, 6, 7, 8, 9 and 10 and 59 °C for exon 2, and a final extension step of 7 min at 72 °C. Amplification was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) using 1-U Taq polymerase (Promega, Madison, WI, USA).

The PCR products were purified and sequenced on both strands using the Big Dye v1.1 Terminator Kit on automated DNA sequencer ABI PRISM 3100-Avant (Perkin Elmer, Norwalk, CT, USA). Resulting sequences were aligned with reference sequences using the blast homology programs available at the National Center for Biotechnology Information Web site.

Microsatellites and SNP typing. To check for the founder effect of the identified mutation, we analyzed two SNPs localized in the exon 2 (rs2297995) and intron 5 (rs12433038) of the *L2HGDH* gene (Figure 1b). Genotyping of the selected SNP was performed for all patients and their family members using PCR amplification followed by direct sequencing of the PCR product. Two extragenic microsatellite markers were also selected from genomic sequence of the *L2HGDH* gene using http://genome.ucsc.edu/cgi-bin/ web site. They are located at 176.247 kb (L2HGDH 176GA) proximal and at 199.216 kb (L2HGDH199AC) distal to the *L2HGDH* gene, respectively (Figure 1b). All markers were typed in all patients and also in some available family relatives using the appropriate primers. Genotyping was carried out using an ABI 3100 Genetic Analyzer and the results analysis was performed using Genotyper (version 3.5). A haplotype co-segregating with the disease was derived from the segregation of SNPs and markers within all pedigree. The profile of markers as well as the SNP haplotype was compared among the affected members of the six families.

Oxidative stress parameter measurement

Plasma preparation. Plasma was prepared from whole-blood samples obtained from only six patients and 20 controls by venous puncture using tubes with ethylene diamine tetraacetic acid as anticoagulant reagent. Whole blood was centrifuged at $1000 \times g$, and then plasma was removed and frozen at −80 °C until analysis. Protein concentration in plasma was determined according to the Bradford's method using bovine serum albumin as standard.[17]

Determination of MDA plasma level. Plasmatic rate of MDA was estimated by using the method of Thiobarbutaric acid-reactive substances (TBARS), which were expressed in terms of the MDA level according to Draper and Hadley method.[18] The resulting pink-stained TBARS were estimated through the absorbance at 535 nm. Calibration curve was performed using 1,1,3,3-tetraethoxypropane subjected to the same treatment as that of the samples. MDA was expressed in nanomoles TBARS per mg protein.

GPx activity. GPx activity was determined as described by Flohe and Gunzler.[19] GPx catalyzes the oxidation of GSH by cumene hydroperoxide. GPx activity was measured using hydrogen peroxide (H_2O_2) as substrate. Hundred microliters of serum were incubated at 37 °C with the phosphate buffer (0.1 M, pH7.4) in the presence of glutathione (4 mM) and NADPH. H_2O_2 (5 mM) was added and the absorbance was measured at 412 nm. The enzyme activity was expressed as nanomoles of GSH oxidized per min per mg of protein.

SOD activity. SOD activity was estimated according to Beauchamp and Fridovich[20] using the ethylene diamine tetraacetic acid, riboflavin and NitroBlueTetrazolium. The developed blue color in the reaction was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of NitroBlueTetrazolium by 50% and activity was expressed as units per mg protein.

GSH level. GSH, a major intracellular nonenzymatic antioxidant, was also estimated in patients and in the control's plasma according to Ellman[21] modified by Jollow, Mitchell, Zampaglione and Gillete.[22] This method is based on the development of yellow color when 5,5-dithio-bis 2-nitrobenzoic acid was added to samples containing sulphydryl groups and was estimated by absorbance at 412 nm. Total GSH content in plasma was expressed as μg GSH per ml of plasma.

Statistical analysis. Data are expressed as mean ± s.d. The Student's *t*-test for non-paired samples was used to compare the means from controls and patients. A *P*-value less than 0.05 was considered to be significant.

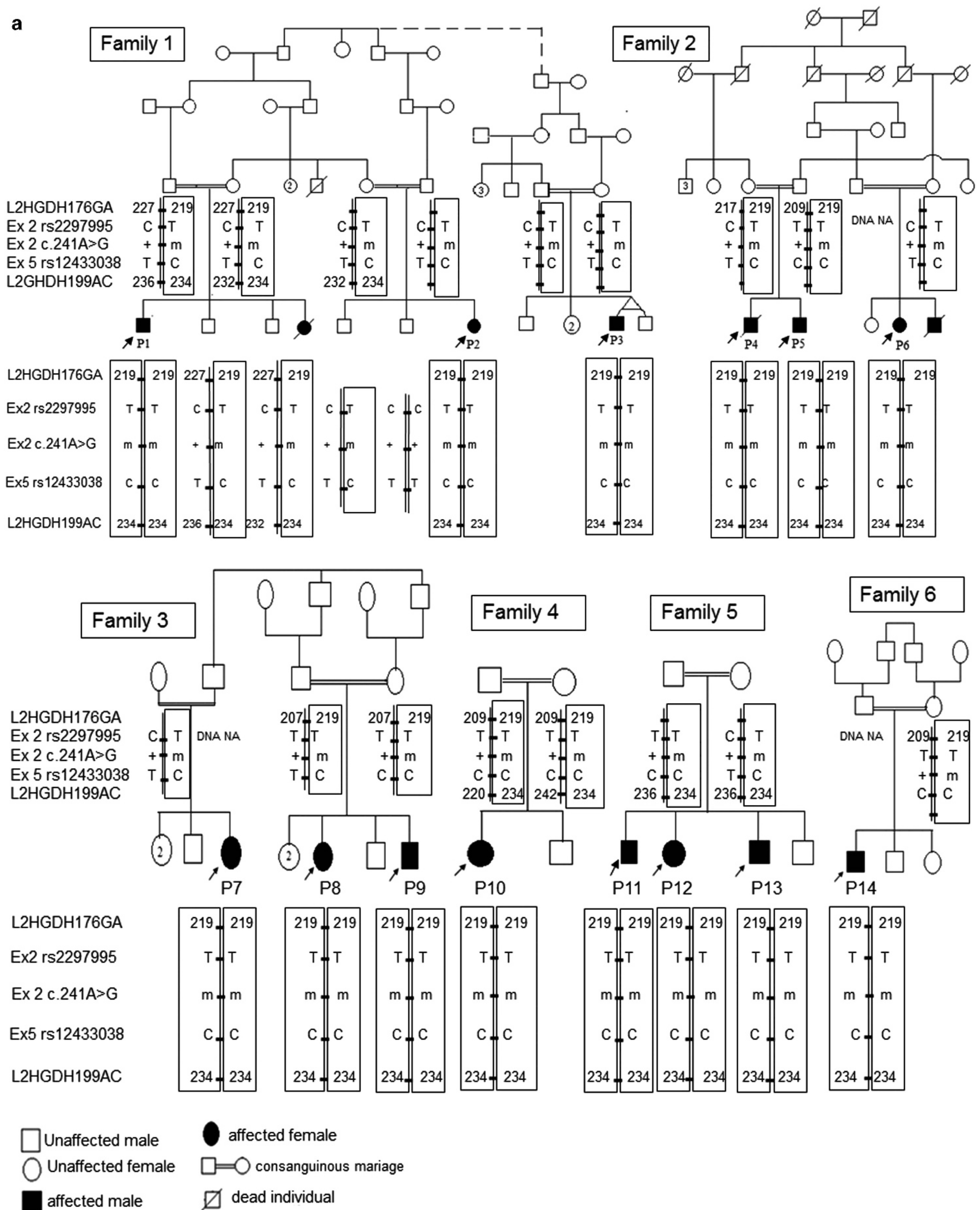

Figure 1 (**a**) Pedigrees of the six Tunisian families showing the inheritance of the c.241A>G mutation. Haplotype study showed a common homozygous haplotype indicated by the framed boxes in the 14 affected individuals. All patients shared the same founder allele: 219 bp for the L2HGDH 176GA marker and the 234 bp for L2HGDH199AC marker transmitted with the c.241A>G mutation. (m) Indicates mutated allele and (+) sign indicates wild-type allele. (**b**) Schematic representation showing the two intragenic SNPs, rs2297995 in the exon 2 and rs12433038 in the exon 5, and the extragenic microsatellite markers L2HGDH 176AG and L2HGDH199AC used in our haplotype analysis and their positions on chromosome 14.

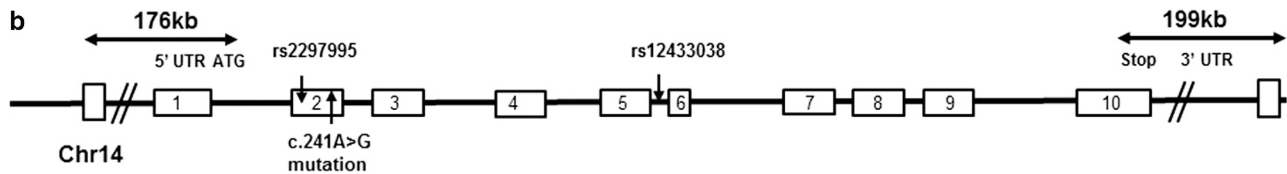

Figure 1 Continued.

Table 1 Clinical findings and urine LGA level expressed in μmol mmol^{-1} creatine of the patients with L2HGA

Patients	*Sex*	*Age (years)*	*Age at onset (months)*	*Presenting symptoms*	*Clinical features*	*Urine LGA level μmol mmol^{-1} creatine*
Family 1						
Patient 1	M	12	4	Global developmental delay	SP, cerebellar ataxia epilepsy	438
Patient 2	F	21	6	Upperlimb tremor	SP, cerebellar ataxia, choreoathetosis epilepsy	1944
Patient 3	M	35	12	Motor difficulties	SP, cerebellar ataxia	ND
Family 2						
Patient 4	M	Died at the age of 12 years		Epilepsy	SP, cerebellar ataxia epilepsy	ND
Patient 5	M	23	12	Global developmental delay	Macrocephaly SP, cerebellar ataxia epilepsy	538
Patient 6	F	28	72	School difficulties	SP, cerebellar ataxia, choreoathetosis	1087
Family 3						
Patient 7	F	12	36	Upperlimb tremor	SP, cerebellar ataxia	ND
Patient 8	F	17	12	Global developmental delay	SP, cerebellar ataxia	ND
Patient 9	M	11	30	Global developmental delay	SP, cerebellar ataxia	ND
Family 4						
Patient 10	F	16	4	Global developmental delay	SP, cerebellar ataxia, extra pyramidal syndrome epilepsy	1560
Family 5						
Patient 11	M	24	24	Upperlimb tremor	Cerebellar ataxia	ND
Patient 12	F	20	8	Global developmental delay	Cerebellar ataxia	1344
Patient 13	M	18	72	Upperlimb tremor	Cerebellar ataxia, ophthalmoplegia epilepsy	825
Family 6						
Patient 14	M	11	78	Headache	SP, cerebellar ataxia	1528

Abbreviations: F, female; LGA, L-2-hydroxyglutaric acid; L2HGA, L-2-hydroxyglutaric aciduria; M, male; ND, not determined; SP, spastic paraplegia.

RESULTS

Molecular analysis

Coding regions and exon–intron boundaries of L2HGDH sequences was performed in the 14 studied patients. Result revealed the same homozygous mutation c.241A>G in the exon 2 of the *L2HGDH* gene in the 14 patients. It was present at the heterozygous state in parents and some siblings and was completely absent in others (Figure 2). The c.241A>G mutation leads to a substitution in lysine (K) to glutamate (E) at the position 81 (p.K81E).

Founder effect study using SNPs and extragenic microsatellite typing

Given that c.241A>G mutation was detected with high frequency in all patients belonging to the six studied families from southern Tunisia, this could be due to a founder effect. A founder effect was expected to result in sharing of allelic sequence polymorphisms in the vicinity of the mutation or close to the gene (linkage disequilibrium due to a common ancestor). We tested at first two intragenic SNPs (rs2297995 and rs12433038) localized, respectively, in exons 2 and 5 of the *L2HGDH* gene. We found that these two SNPs were present at homozygous state in all patients and at heterozygous state in some parents and siblings, suggesting a common haplotype in all patients (Figure 1a). To further strengthen this founder effect, two microsatellite markers localized at 176 kb (L2HGDH 176AG) proximal and 199 kb (L2HGDH199AC) distal to the *L2HGDH* gene were additionally tested. We performed haplotype analysis in patients and in all family members. For both markers L2HGDH 176AG and L2HGDH199AC, homozygous alleles of 219 and 234 bp containing 18 GA and 19 AC repeats, respectively, were shared by the 14 patients harboring the same c.241A>G mutation (Figure 1a). Taken together, these results strongly suggest that a founder effect is responsible for the increased frequency of c.241A>G mutation in Tunisian patients.

Oxidative stress parameter determination

Result measurement analysis showed that MDA, a biomarker of lipid peroxydation, is significantly increased in the plasma of patients

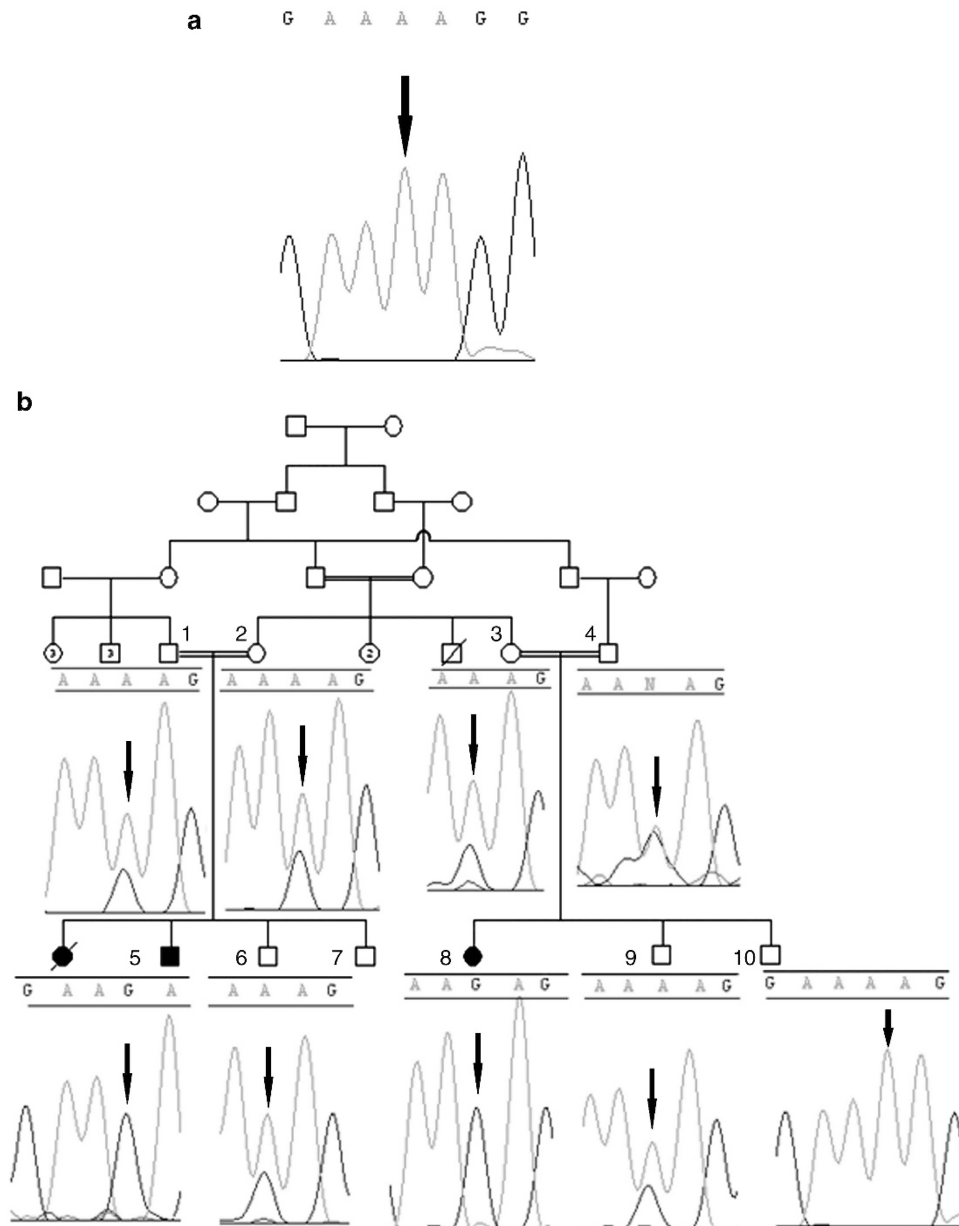

Figure 2 Identification of c.241A>G mutation in the *L2HGDH* gene. Direct genomic DNA sequence of the *L2HGDH* gene from a healthy control individual (**a**). Chromatograms showing an affected individual with c.241A>G pathogenic variants in the *L2HGDH* gene (**b**-5 and **b**-8) and heterozygous individuals (**b**-1, **b**-2, **b**-3, **b**-4, **b**-6, **b**-7, **b**-9). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

compared with controls with a *P*-value of 0.03 ($P<0.05$) (Figure 3a). In addition, GSH levels were significantly low in the plasma of L2HGA patients compared with the controls ($P=0.0122$, Figure 3b). Results showed also a significant decrease in the activities of SOD and GPx with *P*-values of 0.003 and 1.05×10^{-5}, respectively (Figures 3c and d).

DISCUSSION

In the present study, we reported clinical and molecular analysis of the *L2HGDH* gene performed in 14 Tunisian patients with L2HGA, and we evaluated various parameters of oxidative stress in the plasma of patients. Direct sequencing of all exons of the *L2HGDH* gene revealed the presence of the same c.241A>G mutation in exon 2, which was present at homozygous state in all tested patients and at heterozygous state in parents. This mutation was also previously described in three Tunisian patients.[10,15] Clinical and radiologic data of our patients were consistent with previously reported findings except for ophthalmoplegia. Despite the fact that all the patients shared the same mutation c.241A>G on the *L2HGDH* gene, there are different clinical features in the same family or from one family to another. These inter- and intra-familial heterogeneities were also mentioned in the previous study that described this mutation.[15] In fact, and as described previously, c.241A>G mutation is associated mainly with cerebral ataxia and spastic paraplegia in the 14 studied patients, whereas presence of other features was variable such as epilepsy, macrocephaly or ophthalmoplegia, which is firstly described here.

As the 14 patients are from southern Tunisia and carry the same mutation c.241A>G, we emphasized that this variation could be a

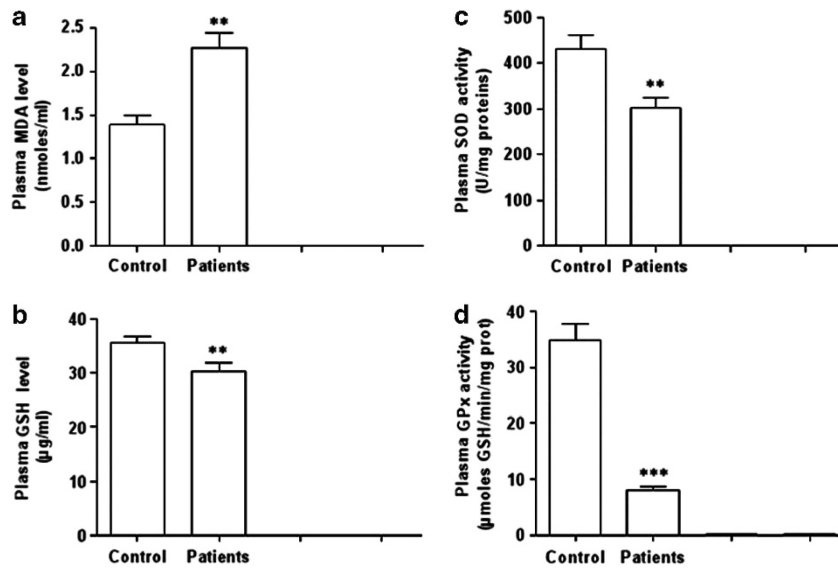

Figure 3 (**a**) TBARS expressed in MDA level in plasma from six patients with L2HGA and 20 controls. Data represent mean ± s.d. (**b**) Nonenzymatic antioxidant level in patient's plasma and control's. Each value represents mean ± s.d. of individual rats per group. Student's *t*-test for unpaired samples: controls vs patients. $^{**}P<0.01$. (**c**, **d**) Antioxidant enzyme activities (SOD and GPx) in patients' sera compared with the controls. $^{***}P<0.001$.

founder mutation. In fact, the six families are from southern Tunisia where rural communities are characterized by high consanguinity and endogamy rates. Studies by Ben Arab *et al.*[23] revealed that geographic isolation, social traditions and parental involvement in mode selection contribute to increase consanguinity in rural Tunisian regions.[23] According to such findings and in agreement with the prevalence of this mutation reported in our patients and the previous studies describing the same mutation in three Tunisian patients from the same southern area,[10,15] we suggested that a clustering of c.241A>G mutation in our six Tunisian families is due to a common founder ancestor. This founder effect has been confirmed in our study by genotyping two intragenic SNPs, rs2297995 and rs12433038, belonging to the exon 2 and exon 5 of the *L2HGDH* gene, respectively. Analysis revealed a common haplotype carried by the 14 patients harboring the same mutation. Moreover, these patients showed also a common haplotype for the two tested extragenic microsatellite markers 176GA and 199CA. Indeed, there was a complete linkage disequilibrium between the allele carrying c.241A>G mutation and a shared two homozygous SNPs and both 219AG and 234AC alleles. Consequently, our haplotype study constitutes further evidence showing that the c.241A>G mutation occurred in a specific founder haplotype in Tunisian population. Nevertheless, evidence for a founder effect had also been reported in other Tunisian patients with different diseases such as nonsyndromic hearing loss,[24,25] congenital factor XIII deficiency,[26] mucopolysaccharidosis type I[27] and for the Pelizaeus Merzbacher Like Disease.[28]

Oxidative stress was shown to be involved in many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and epilepsy.[29] In the present study, we investigated the effect of LGA accumulation on various parameters of oxidative stress. TBARS reflects especially the amount of MDA formation, which is an end product of membrane fatty acid peroxidation.[30] In our results, significant raise of plasma MDA level was noted in tested patients compared with the controls. This could be due to the excessive free radical formation and hence leads to lipid peroxidation and increased cell membrane permeability. These results match with other previous investigations performed *in vitro* on rat brain treated with increased concentration of LGA, which showed that lipid peroxidation is stimulated by LGA in brain tissue and the TAR (a measure of the tissue capacity to react with free radicals) was markedly reduced.[5] In addition, in female carriers of X-linked adrenoleukodystrophy a significant increase in MDA and a decrease in TAR measurements were also observed, reflecting a deficient capacity to rapidly handle an increase in reactive species.[31]

Our data on measurement of antioxidant activities of SOD and GPx showed a significant decrease in SOD and GPx activities in L2HGA patients' plasma when compared with controls. The reduction in the activities of the antioxidant enzymes (SOD and GPx) may be due to the increased generation of ROS such as superoxide and hydrogen peroxide, which in turn lead to the decrease in the activities of these enzymes.[32] However, Latini *et al.*[5] showed that LGA did not affect the activities of the antioxidant enzymatic defenses in the rat brain.

GSH, a major intracellular nonenzymatic antioxidant and a chief constituent of cellular thiol, was significantly reduced in L2HGDH plasma patients. This decrease in the GSH level could result from enhanced oxidative stress. Decreased GSH may be explained on the basis of (a) its utilization in scavenging the free radicals, (b) its involvement in maintaining non-GSH critical protein sulfhydryls in reduced state, (c) acting as cofactor for GST during detoxification of xenobiotics, (d) oxidation of glutathione to its oxidized form by glutathione peroxides in detoxification of hydrogen peroxide and/or lipid peroxides.[33,34]

CONCLUSION

In the present study, we reported a same mutation c.241A>G in 14 Tunisian patients from southern Tunisia and presenting the L2HGA disorder. We provided also a molecular confirmation of a founder ancestor for the c.241A>G mutation in the *L2HGDH* gene using two SNPs, rs2297995 and rs12433038, and two extragenic microsatellite markers. The identification of this founder mutation has important implications on genetic counseling in relatives of these families and the antenatal diagnosis. Furthermore, our data demonstrated a significant increase in the MDA levels and a diminution of the antioxidant enzyme activities. These results indicate that LGA

accumulation in plasma of L2HGA patients may cause toxicity leading to excessive release of ROS, which consequently result in a decrease in the activities of antioxidant enzymes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX

Appendix The Tunisian Network on Mental Retardation study

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