High frequency of the TARDBP p.Ala382Thr mutation in Sardinian patients with amyotrophic lateral sclerosis


Recently, rare mutations in the TARDBP gene have been identified in familial and sporadic amyotrophic lateral sclerosis (ALS) patients. The purpose of this study was to characterize the genetic variability of the TARDBP gene in a cohort of Sardinian ALS patients. The coding region of the gene was analyzed in 97 unrelated patients previously tested negative for superoxide dismutase (SOD1) mutations. The p.Ala382Thr (c.1144G>A) mutation was found in 30 patients (30.9%). The mutation was predominant in familial ALS patients (FALS) as it was represented in 24 of 30 FALS cases (80%) (p < 0.0003). Six cases were apparently sporadic (9% of sporadic ALS patients). No further mutation of TARDBP was found in our cohort of ALS patients. Patients carrying the mutation showed spinal site of onset in 24 cases (80%), an average age at onset of 54.7 ± 11.1 years, not significantly different from patients not harboring TARDBP mutations (56.7 ± 9.6) and a female:male gender ratio of 1:1.1. The haplotype analysis carried out using eight microsatellite markers flanking the gene showed a founder effect for this mutation. Finally, we estimated the age-specific penetrance of the TARDBP p.Ala382Thr mutation in an additional sample of 47 carriers (20 affected and 27 unaffected). The average penetrance to 70 years was 60% (95% confidence interval 41–79%). A trend toward a higher penetrance in males was observed. Even in the presence of a causal mutation, most of the ALS clinical heterogeneity, however, draws upon from a multifactorial context.

Conflict of interest

All authors declare that there is no conflict of interest.

Amyotrophic lateral sclerosis (ALS) is the commonest adult-onset disorder of motor neurons, in which the loss of motor neurons from the brain and spinal cord leads to fatal paralysis and death, generally within 1–5 years (1). Approximately 5% of patients with ALS have a positive family history of the disease. Several genes have been described to be involved in familial ALS (FALS) pathogenesis. Mutations in the Cu/Zn superoxide dismutase gene (SOD1) account for 10–20% of FALS cases (2). Mutations in other genes, including alsin (ALSIN), senataxin (SETX), vesicle-associated membrane protein B (VAPB), dynactin (DCTN1), angiogenin (ANG), d-amino acid oxidase (DAO), optineurin (OPTN), and valocin-containing protein (VCP), have been described as rare causes of FALS (3–6). A characteristic feature of degenerating neurons in FALS patients without SOD1 mutations is...
the presence of cytoplasmic inclusions containing abnormal aggregates of TAR DNA-binding protein (TDP-43), encoded by the TARDBP gene (7, 8). TDP-43 is involved in transcription and splicing regulation, and roles have been suggested in other cellular processes, such as microRNA processing, apoptosis, cell division, and stabilization of the messenger RNA (9–11). Overexpression of mutant, but less so of wild type, human TARDBP caused a motor phenotype in zebrafish (Danio rerio) embryos consisting of shorter motor neuronal axons, premature and excessive branching as well as swimming deficits. Interestingly, knockdown of zebrafish tardbp led to a similar phenotype, which was rescued by coexpressing wild type but not mutant human TARDBP. Together, these approaches showed that TARDBP mutations cause motor neuron defects and toxicity, suggesting that both a toxic gain of function as well as a novel loss of function may be involved in the molecular mechanism by which mutant TDP-43 contributes to disease pathogenesis (12).

The TARDBP gene on chromosome 1p36.22 consists of five coding and one non-coding exon (13). Recently, several TARDBP mutations have been identified among FALS and sporadic ALS (SALS) patients from different populations (13–21). Mutations in the FUS/TLS gene have been reported in FALS patients with a pathology similar to that of the TARDBP gene characterized by neuronal cytoplasmic protein aggregation and defective RNA metabolism (22, 23).

Sardinia has long been of interest for human geneticists. The demographic and genetic features of this population, related to its insularity, offered the opportunity to clarify relevant aspects of the molecular basis of monogenic diseases and promised to be a resource for fine mapping of susceptibility genes for complex diseases. With the aim to characterize the genetic variability of the TARDBP gene in a selected cohort of ALS patients of Sardinian origin, we performed a mutation analysis of the TARDBP gene. During the performance of this study, another study of 135 Sardinian ALS patients found the p.Ala382Thr TARDBR mutation in 39 patients (28.7%), 15 of which were FALS and 24 SALS (24).

Materials and methods

Subjects

Ninety-seven unrelated patients having Sardinian ancestors of two generations were included in the study. Patients were consecutively recruited from May 1996 to March 2004 between the patients who attended the Department of Neurology of the University of Cagliari and from October 2005 to October 2009 the Genetic Counseling Service of the Medical Genetic Department of the University of Cagliari. All patients were diagnosed with definite or probable adult-onset ALS according to the El Escorial revised criteria (25). One hundred ten healthy individuals from Sardinia were also recruited as control subjects. This study was approved by the Institutional Review Board of theBinaghi Hospital, University of Cagliari. All participants provided a signed informed consent.

Mutation screening of the TARDBP gene

Genomic DNA was extracted from peripheral blood leukocytes or cell cultures of fibroblasts using standard procedures (QIAamp, Qiagen GmbH, Hilden, Germany). The entire coding region of TARDBP and an average of 100 nucleotides of flanking intronic regions of each exon were fully sequenced in both the directions in 97 ALS patients. The primer sequences were as previously described (20). Six pairs of primers were used to amplify all six exons of the TARDBP gene, including exon/intron boundaries. Briefly for each exon, 50 ng of genomic DNA were amplified by polymerase chain reaction (PCR) using 20 pmol of specific primers in a final volume of 25 μl containing 1× PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, and 1 U of Taq DNA polymerase (AmpliTaq Gold, Applera, Norwalk, CT). The PCR cycles consisted of an initial denaturation step of 95°C for 5 min, followed by 35 amplification cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s). PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, OH) and sequenced according to the Sanger method on a MegaBACE1000™ automated sequencer, following the instructions included in the DYEnamic™ ET Dye Terminator Cycle Sequencing kit (MegaBACE) (GE Healthcare UK Limited, Chalfont, UK).

In 65 patients, the coding parts of the SOD1 gene have been previously analyzed (26). The analysis of the remaining 32 patients was performed as previously described (26).

Microsatellite marker analysis

Eight microsatellite markers (D1S450, D1S244, D1S2736, D1S2667, D1S1151, D1S434, D1S489, and D1S2697), flanking the TARDBP gene, were genotyped in all the affected patients and in 20 healthy subjects. Genotyping was performed by PCR using dye-labeled primers. Sequence primers were those reported in the uniSTS database.
(http://www.ncbi.nlm.nih.gov/unists/). PCR products were further mixed with deionized formamide and the GeneScan™ 500 ROX™ Size Standard (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. The DNA fragments were electrophoretically separated on an ABI 310 sequencer (Applied Biosystems). The alleles were defined by comparisons with known molecular markers and direct inspection of the electropherograms and were numbered according to the CEPH genotype database version 10 (http://www.cephb.fr/en/cephdb/). The allele composition (haplotype structure) of each haplotype and their frequencies were obtained separately in patients and controls by using the expectation–maximization (EM) algorithm, as implemented in arlequin software (27). In ALS families, the haplotypes were confirmed when possible by allele segregation analysis. Less frequent haplotypes with a variant attributable to the addition or loss of a single repeat were added to the corresponding more frequent haplotype.

Penetration and statistical analysis

For the assessment of the penetration, we have identified 11 patients positive for the TARDBP mutation whose DNAs of relatives were available. Overall, the DNA of 9 ALS patients and 61 relatives not affected were typed at exon 6 of the gene. Thirty-six of them were found positive for the mutation and were subsequently admitted to the analysis of penetration together with the 11 index cases. In total, the analysis was carried out on 47 individuals who carried the mutation (20 affected and 27 unaffected). Age-related penetrance was assessed in a cumulative risk curve representing the probability of a TARDBP mutation carrier to develop the disease at different ages (observation time). Observation time was defined as the age at onset for the affected; otherwise the observation time was censored to the age at interview or the age at death for non-affected relatives. Difference in age-related risk between males and females was assessed by logrank test on Kaplan–Meier inverted survival function (100,000 permutation was applied).

Differences in the age at onset, between the group of patients without TARDBP mutations and the group of patients harboring the p.Ala382Thr were analyzed using the Student’s t-test. The results of all continuous data are presented in this report as mean ± standard deviation. Statistical comparisons of dichotomous variables such as gender distribution, site of onset (spinal or bulbar), and familial or sporadic inheritance were conducted using the chi-squared test. p-Values were corrected using the Bonferroni method for multiple testing. A p value of <0.05 was considered statistically significant. The statistical analysis was carried out considering exclusively unrelated samples (97 ALS patients). Data were analyzed using the software spss version 13 (SPSS Inc., Chicago, IL) or ncss version 7 (Kaysville, UT) as appropriate.

Results

Among the 97 unrelated patients diagnosed with ALS, 63 were males and 34 were females. The mean age of ALS onset was 55.9 ± 10.1 years. There were 30 patients with FALS (30.9%) and 67 with SALS (69.1%). Eighty-two patients (84.5%) showed initial symptoms involving the spinal cord, whereas 15 patients (15.5%) presented with a bulbar-onset ALS.

TARDBP sequence analysis revealed that 30 patients (30.9%) were heterozygous for the missense mutation p.Ala382Thr in exon 6. This mutation was not observed in 110 ethnically matched healthy controls through direct sequencing of exon 6. No further mutation of TARDBP was found in our cohort of ALS patients. No mutations were found in the SOD1 gene. The distribution of different disease-related variables in patients with a wild-type TARDBP and in patients carrying the p.Ala382Thr mutation is shown in Table 1.

In our Sardinian samples, the p.Ala382Thr was predominant in FALS patients as it was represented in 80.0% of FALS cases (24 of 30 cases) (p < 0.0003). The mean age of disease onset was lower in patients carrying the TARDBP mutation p.Ala382Thr mutation (54.7 ± 11.1), compared to the mean age of onset of the patients not harboring TARDBP mutations (56.7 ± 9.6), but this difference did not reach statistical significance.

Table 1. Distribution of disease-related variables in patients with a WT TARDBP and in patients carrying the p.Ala382Thr mutation

<table>
<thead>
<tr>
<th>ALS patients’ clinical feature</th>
<th>TARDBP</th>
<th>p-Value</th>
</tr>
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<tbody>
<tr>
<td>(total sample = 97)</td>
<td>p.Ala382Thr</td>
<td></td>
</tr>
<tr>
<td>WT (n = 67)</td>
<td>p.Ala382Thr (n = 30)</td>
<td></td>
</tr>
<tr>
<td>Mean age at onset (years ± SD)</td>
<td>56.5 ± 9.6</td>
<td>54.7 ± 11.1</td>
</tr>
<tr>
<td>Males</td>
<td>47</td>
<td>16</td>
</tr>
<tr>
<td>Females</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>FALS</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>SALS</td>
<td>61</td>
<td>6</td>
</tr>
<tr>
<td>Spinal onset</td>
<td>58</td>
<td>24</td>
</tr>
<tr>
<td>Bulbar onset</td>
<td>9</td>
<td>6</td>
</tr>
</tbody>
</table>

ALS, amyotrophic lateral sclerosis; FALS, familial ALS; SALS, sporadic ALS; WT, wild type.
(p = 0.41). In agreement with the Mendelian model of autosomal dominant trait transmission, we observed a female:male gender ratio of 1:1.1 in the group of ALS patients with the TARDBP mutated gene, whereas an excess of males (1:2.4) was observed among the ALS patients tested negative for TARDBP mutations. The haplotype analysis carried out using eight microsatellite markers showed a founder effect for this mutation. The distribution of haplotypes found in 30 patients carrying the p.Ala382Thr mutation is shown in Table 2. A large haplotype that spans about 7 Mb (haplotype A) was found to be associated with the p.Ala382Thr mutation in 12 ALS patients. Shorter haplotypes spanning 5.8 (haplotypes B and C), 2.5, and 0.8 Mb (haplotypes D–E) were present in 10, 1, 3, and 2 patients, respectively. In two patients, we were unable to determine haplotypes in an unambiguous way. Finally, we estimated the age-specific penetrance of the TARDBP p.Ala382Thr mutation in a sample of 47 carriers (20 affected and 27 unaffected) whose DNA was available at the moment of the analysis. The average penetrance to 70 years was 60% [95% confidence interval (CI) 41–79%] (Fig. 1). A trend toward a higher penetrance in males was observed. By comparing the gender-specific curves at 70 years, the penetrance at 70 years was 74% (95% CI 49–93%) in males and 42.5% (95% CI 22–70%) in females (Fig. 2). However, probably because of the size of our sample, this difference did not reach statistical significance (logrank chi-square 1.9; p = 0.17).

Discussion

Here, we report on the screening results of the TARDBP gene in a significant group of Sardinian ALS patients. We found a very high impact produced by a single mutation p.Ala382Thr of the TARDBP gene in terms of incidence and prevalence of the disease in the Sardinian island.

Overall, our study confirms the results of Chiò et al. (24). They recently reported a mutational screening of 135 Sardinian ALS patients (15 FALS and 24 SALS) had the TARDBP p.Ala382Thr mutation) and by genotyping they found that 5 cases shared a 94 single nucleotide polymorphism risk haplotype. In our study, the haplotype analysis performed with eight microsatellite markers located in an interval of 7 Mb flanking the gene strongly suggests a founder effect for this mutation. Concerning the possibility of overlapping between the

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>D1S450</th>
<th>D1S244</th>
<th>D1S2736</th>
<th>TARDBP</th>
<th>D1S1151</th>
<th>D1S2667</th>
<th>D1S434</th>
<th>D1S489</th>
<th>D1S2697</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12</td>
<td>0.20</td>
<td>333 (2)</td>
<td>283*</td>
<td>116*</td>
<td>A*</td>
<td>267 (16)</td>
<td>142 (2)</td>
<td>244 (3)</td>
<td>149 (1)</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>0.17</td>
<td></td>
<td>283*</td>
<td>116*</td>
<td>A*</td>
<td>267 (16)</td>
<td>142 (2)</td>
<td>244 (3)</td>
<td>149 (1)</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>0.02</td>
<td>116*</td>
<td>A*</td>
<td>267 (16)</td>
<td>142 (2)</td>
<td>244 (3)</td>
<td>149 (1)</td>
<td>290 (1)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>0.03</td>
<td>333 (2)</td>
<td>283*</td>
<td>116*</td>
<td>A*</td>
<td>267 (16)</td>
<td>142 (2)</td>
<td>244 (3)</td>
<td>149 (1)</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>0.05</td>
<td>333 (2)</td>
<td>283*</td>
<td>116*</td>
<td>A*</td>
<td>267 (16)</td>
<td>142 (2)</td>
<td>244 (3)</td>
<td>149 (1)</td>
</tr>
</tbody>
</table>

| Allele numbers indicate base pairs. Numbers in brackets are according to the CEPH genotype database v10. The * indicates that the allele is not present in the CEPH genotype database version 10. An empty space was left when one allele different from that found in haplotype A was present. In two patients, it was not possible to determine unambiguous haplotypes. |
| Sequence map position expressed in kb are indicated in brackets (map position from human genome build 37.2). |
| Number of haplotypes. |
| Frequencies calculated using the expectation–maximization algorithm. Total number of haplotypes = 60. |
| Nucleotide at position c.1144 of coding sequence of the TARDBP gene (NCBI Reference Sequence: NM_007375.3). |

Fig. 1. Age-specific cumulative risk (penetrance) estimated by Kaplan–Meier inverted survival curve on 47 carriers of the p.Ala382Thr TARDBP mutation. Censored unaffected carriers are represented by a ‘+’ symbol. |
two cohorts, it cannot be ruled out; however, this risk is limited. Most of the patients we describe have previously been studied for polymorphisms of monoamine oxidase A and B (28) and in a search for \textit{SOD1} mutations in the context of an Italian multicenter study (26). Only 12 of these patients could have a potential overlap between the two studies, as they were alive and residing in Sardinia in May 2009, the date of beginning of the recruitment of the patients in Chiò et al. (24). Therefore, data on the frequency of this mutation in Sardinian ALS patients is strengthened by observations from two independent cohorts of patients.

One of the major differences between the two studies concerns the FALS/SALS ratio among patients with the mutation as we found a preponderance of familial cases compared to Chiò et al. (24), who instead found most of the mutations in sporadic patients. These differences might arise from a different way and time of samples recruitment. In our study, the samples were recruited in a period of 13 years (1996–2009) and thus it was not uncommon for patients initially classified as sporadic to be reassessed as familial over the time. Also, the reconstruction of the pedigree up to the grandparents of the patients allowed us to correlate patients who at the moment of anamnesis appeared independent.

The uniqueness of the Sardinian population, with regard to the identification of founder effects, has been previously discussed (29). A strong founder effect has been observed for numerous recessive (30, 31) and dominant monogenic traits (32), suggesting that some disease variants could be unique to the Sardinians. On the other hand, at this moment we are unable to explain the reasons for such a high incidence of this mutation.

By analogy to what has been observed for the MHC loci (33), we may speculate that this mutation has been under the effect of a strong genetic drift. However, we cannot exclude that genetic selection may have played a role which has led to increase of the incidence of this mutation. The discovery of a mutation at a very high frequency in ALS patients may instead contribute to the explanation of recent observations and epidemiological findings. Preliminary unpublished data indicate that ALS incidence in Sardinia may be higher than in other European populations, with a possible cluster in some areas of the island.

The p.Ala382Thr mutation has initially been described in two FALS patients of French origin (13). Subsequently, this mutation was identified in large studies carried out on patients of Italian and French origin (16, 19, 34–36). Although this mutation has been reported in eight sporadic and seven familial Italian cases, there has been an absence of data that these patients may share the same founding event and therefore originate from the same population. However, it is interesting to note that the polymorphic markers D1S2667 and D1S489 shared common alleles (alleles 2 and 1, respectively) in 2 patients of French origin (13), 6 of 8 patients from Italy (16) and 26 of 30 patients from Sardinia.

In this as well as in previous studies, mutations of \textit{TARDBP} have been identified in SALS patients, suggesting an incomplete penetrance of the disease in carriers. On the other hand, the real estimate of the penetrance is of particular importance especially when a single mutation causes a large number of cases. Therefore, we attempted to provide a true estimation of the penetrance intended as the age-specific cumulative risk for carriers of the p.Ala382Thr mutation. Our data indicate that about one carrier of two develop the disease within 70 years. This dimension of high penetrance fits well with the FALS/SALS rate that we observed in our total sample of patients concerning this mutation. To our knowledge, this is the first time that data concerning the penetrance of mutations in \textit{TARDBP} are reported.

Despite the genetic homogeneity, we did not observe any reduction in the clinical heterogeneity traditionally described in ALS patients. Regarding the correlation of this mutation with the clinical phenotype, we did not observe a significant difference between the age or the site of onset among patients carrying the mutation and those of the rest of the sample suggesting that the etiology for
most of the clinical variability in ALS should be searched in the context of a multifactorial model. The major observation among patients who carried the mutation compared to those who did not is that in the first group the female:male gender ratio was not significantly different (1:1:1) from that expected for an autosomal dominant trait, whereas within the second group there was a significant deviation (1:2.4, p = 0.001).

In conclusion, our study confirms that in Sardinia the p.Ala382Thr mutation of the TARDBP gene accounts for a large part of ALS cases. The obtained data contribute to the characterization of the phenotype associated with this mutation. The low genetic heterogeneity of the Sardinian population, as highlighted in this work, is an encouraging basis for further studies aiming to identify genes involved in the pathogenesis of ALS.

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