Environmental and Genetic Factors in ALS: Positive Correlation of Snps in Flavin-Containing Monooxygenase 5 Gene

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Abstract
Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease of unknown etiology characterized by death of upper and lower motor neurons. ALS is defined as a multifactorial disease caused by both environmental and genetic factors. Several data propose an association between toxic environmental factors and sporadic ALS. Flavin-Containing Monooxygenase (FMOs) is a gene cluster encoding for detoxification enzymes. Previous studies have showed an increased expression level of FMO isoform 5 (FMO5) gene (Entrez Gene ID 2330) in brain stem and spinal cord of ALS patients. FMO5, situated on chromosome 1q21.1, is furthermore the unique isoform of FMO genes to be regulated by human progesterone. Here in, we have performed a genetic screening of FMO5 gene in a cohort of 199 SALS patients and 191 control to correlate FMO5 genotype to ALS. Molecular analysis identified a SNP (ID: rs. 894469), not linked with other diseases, in exon seven that was found in 4.5% and 3.1% of patients and controls respectively. A correlation between genotype-sex showed that this SNP is associated with the 80% of females and 20% of males patients. Conversely, in control population, it was found only in males.

Moreover, FMO5 analysis showed a prevalence of a SNP (rs: 894469) in female ALS patients. This study underlines the visible association between FMO SNPs and female ALS patients although their role in the disease onset has yet to be defined.

ABBREVIATIONS
ALS: Amyotrophic Lateral Sclerosis; FMO5: Flavin-Containing Monooxygenase; SNP: Single Nucleotide Polymorphism

INTRODUCTION
Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease with unknown etiology characterized by death of upper and lower motor neurons. ALS is classified as a multifactorial disease caused by both environmental and genetic factors. Most considerable hypothesis about the premature neurodegeneration in ALS is about oxidative stress, protein aggregation, cytoskeletal alterations, glutamate excitotoxicity and chemical exposure [1]. Few recent studies propose an association between toxic environmental factors and sALS [2]. Toxic hypothesis started to be considered after observation of a complex phenotype of ALS with Parkinsonism and Dementia, spread among Chamorro population of Guam island and associated with a toxic amino acid situated in to the seeds of Cycas Micronesia plant. Chamorros indeed used to eat two kinds of bats, Pteropusmariannus and Pteropustokudae, which used to feed with the seeds and leafs of the Cycas Micronesia [3]. Therefore other studies focused attention on populations, as farmers and soccer players, with a direct contact of toxic factors.
as organophosphates and other pesticides [4-7]. These studies showed a high incidence of sALS in both populations, confirming the role of toxic factors in the susceptibility of sALS [5,8].

From these observations, during recent past, arose some studies about Flavin-Containing Monoxygenase (FMOs) and Paraoxonase (PONs) genes, two genes cluster encoding for enzyme involved in to the oxidative metabolism (FMO) and detoxification enzymes (PON). Studies conducted on yeast showed how yFMO should be implicated in oxidative stress and in to the misfolding protein toxicity [9]. In human there are various isoforms of PON and FMO as well. PONs gene cluster is largely studied, about FMO gene cluster the isof orm most studied is FMO3, considering its role in disorder as Trimethylaminuria [10]. Because FMOs are involved in detoxification processes and their functional activity to metabolize chemicals to toxic agents have been documented [11], a possible role of detoxification pathways in ALS may help to understand the involvement of FMO enzymes in disease. Our previous study has showed the tPON and FMO isoforms are differently expressed in brain tissues of sALS patients compared to healthy subjects [12]. Moreover also an ALS animal model study demonstrated the involvement of FMO in ALS [13]. In sALS patients, a significant difference was found in the expression level of FMO isoform 5 (FMO5) gene (Entrez Gene ID 2330) situated on chromosome 1q21.1. In the spinal cord, the mRNA level of this isoform has been detected 100-fold greater than control samples [12]. FMO5 is furthermore the unique isoform of FMOs to be regulated by human progesterone, a typical female hormone [14], interest features considering the different ratio of sALS between male and female. In a previous study has been demonstrated a connection between ALS female patients and a Single Nucleotide Polymorphism (SNP) situated on 3’ Untraslated Region (3’UTR) region of FM01 [15,16]. Epidemiological studies have shown that approximately 10% of ALS cases are familial (fALS), whereas the remaining 90% of cases are considered to be sporadic (sALS) [17]. About gender-data, JALS has an equal male:female sex ratio, sALSis characterized by 1.5:1 male:female sex ratio [17,18].

In the present study we made a molecular screening of FMO5 in sALS patients and controls, to have a complete overview of FMO cluster and shedding a light on its possible implication in to the metabolism dysfunction of xenobiotics.

**MATERIALS AND METHODS**

**Patients and controls**

199 Italian patients with sporadic ALS and 191 Italian healthy subjects were included in this study. We collected peripheral blood samples from 199 SALS and 191 healthy volunteers over 50 years of age after obtaining written informed consent approved by the C. Mondino Institute ethic committee.

ALS patients underwent clinical and neurologic examination at IRCCS National Neurological Institute “C. Mondino” (Pavia, Italy). All patients were diagnosed with ALS as defined by El Escorial criteria.

Moreover all patients have been screened for genetic mutations in ALS candidate genes as SOD1, TARDBP, FUS, C9orf72 and ANG and only negative samples have been included in this study.

The control subjects have been recruited at the Transfusional Service and Centre of Transplantation Immunology, Foundation San Matteo*, IRCCS, Pavia, Italy.

DNA was extracted from white blood cells from peripheral blood using standard procedures by automatic extraction (Maxwell® 16 Blood DNA – Promega).

**High resolution melting (HRM)**

Molecular analysis was performed with post-PCR High Resolution Melting (HRM) method. Both healthy and sALS groups were analyzed by LightCycler® 480 Real-Time PCR System, Roche® for post-PCR HRM analysis. The data were confirmed by Sanger Sequencing method (primers upon request).

**Statistical Analysis**

Graphpad software was used to make χ² test, p value < 0.05 was considered significant. The test was used to detect the statistical significance of the genotype’s frequency between the healthy group vs sALS group, or between Wild Type group vs mutated/SNP group.

**RESULTS**

Two SNPs were found both in controls and patients groups, one on the 7th exon (ref SNP ID: rs894469, Figure 1) and other one on the 9th exon (ref SNP ID: rs72708554, Figure 2). These SNPs are already known in literature but not linked to other diseases or phenotypes. The SNP on 7th exon is a synonymous polymorphism, leading to nucleotide change (CCG to CCA) with no amino acid change, at other hand the SNP on 9th exon is a missense polymorphism (CGG to CAG) with amino acid change from Arginine to Glutamine. The frequencies with which the SNPs were found is higher in sALS than in controls (Table 1).

**Linkage Genotype–Phenotype**

To further characterize rs894469 and rs72708554 phenotypes we made a linkage between genotype and phenotype’s features as onset, age and gender.

District Onset analysis. Linkage analysis has been made between the onset of the disease and the frequency of the SNPs. Four groups were created on the site of onset: Spinal, Bulbar, Spinal/Bulbar and Pseudopolyneuritic. The frequency of rs894469 and rs72708554 has been calculated in each onset group. The data showed that rs894469 was spread rather equally without any statistic difference (data not shown). On the other hand rs72708554 had significant different frequencies related to the disease onset (p < 0.0001) (Table 2). Spinal onset had a lower frequency in sALS with SNP than Wild Type sALS as well Bulbar onset was more frequent in SNP sALS than Wild Type sALS (Table 2).

Moreover, 2 among 9 patients with rs894469 showed another neurological disease, Frontotemporal Dementia (FTD), clinical aspect that often occurs associated with ALS disease.

**Age analysis**

Analysis of the time, age when the disease was diagnosed, SNP sALS and Wild Type sALS patients did not show any significant difference (Data not shown).
Figure 1 Elettroferogram of rs 894469.

Figure 2 Frequency of male and female with SNP RS 894469 in Control group (B - panel) and sALS patients (A – panel).

Table 1: Frequencies of SNPs rs894469 and rs72708554 in sALS patients and in the control group, both not statistically significant.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>sALS</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP</td>
<td>n: 199</td>
<td>n: 191</td>
</tr>
<tr>
<td>rs894469</td>
<td>9 (4.5%)</td>
<td>6 (3.1%)</td>
</tr>
<tr>
<td>rs72708554</td>
<td>6 (5.2%)</td>
<td>2 (1.05%)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.30</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Table 2: Frequencies of rs72708554 SNP correlated with different onset. Wild type column show the frequency of onset in sALS without the SN, while rs72708554 column show the frequency of onset in sALS where SNP has been detected (**p<0.0001).

<table>
<thead>
<tr>
<th>Onset</th>
<th>Wild Type</th>
<th>rs72708554</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal</td>
<td>55.1%</td>
<td>40%</td>
</tr>
<tr>
<td>Bulbar</td>
<td>31%</td>
<td>60%</td>
</tr>
<tr>
<td>Spinal/Bulbar</td>
<td>12.2%</td>
<td>-</td>
</tr>
<tr>
<td>Pseudopolyneuritic</td>
<td>1.7%</td>
<td>-</td>
</tr>
</tbody>
</table>

Gender analysis

A study of the SNPs frequency among the gender of both rs894469 and rs72708554 in sALS and Control has been done. rs894469 showed a different spread in females than males between sALS and Controls groups. In Controls group, the rs894469 was found just in male subjects (100%), whereas female rs894469sALS patients showed a higher frequency. In sALS female, rs894469 had a frequency significant different from female of Controls (20% vs. 80%, sALS male/female; 100% vs. 0%, Controls male/female), p<0.0001 underling a higher frequency in femalesALS than Controls (Figure 2). The correlation between 72708554 and gender resulted negative.

Gene expression analysis

Our previous study (Gagliardi et al, 2013) [12] showed that FM05 mRNA from sporadic ALS lymphocytes was expressed at higher level than in control. No correlation has been found between mRNA level and FM05 genotype. This increased expression was not dependent the presence of SNPs in FM05 gene.

DISCUSSION

A cluster of gene (FMO) encoding for detoxification enzymes are involved in the metabolism of xenobiotic, such as pesticides. This cluster of enzymes is able to catalyze the oxidation of N- as well as S-group present in some compounds [19,20]. Our previous study showed a different expression of FMO genes in spinal cord of ALS patients compared to healthy subjects, moreover a significant different was found for the isoform FM05 [7], suggesting their possible implication in pathogenesis of the disease. The goal of this study is to make a molecular screening of FM05.

None new mutations were found, but two known SNPs: rs894469 (4.2% of patients and 3.1% of healthy subjects) and rs72708554 (5.2% in sALS and 1% in healthy subjects).

Patients with SNP rs894469 showed a premature age onset of the disease, indeed patients with rs72708554 showed a delayed onset. About clinical aspect, the interested data concerned that 2 among 9 patients with rs894469 showed another neurological disease, Frontotemporal Dementia (FTD). This disease is often associated with ALS [21].

A linkage analysis with sex of subjects studied and frequency of SNP was also done. ALS indeed has a different distribution among gender, with a little predominance for males [17,22]. SNP rs894469 showed different distribution among sex. In patients with this SNP 80% were females (p< 0.0001), on the contrary all controls positive for rs were males. rs72708554 was present only in male subjects, ALS and healthy.

Concerning SNPs on FMO cluster and ALS, the only study published so far is our 2006 work [16]. This study demonstrated a correlation among a SNP situated on a regulatory region of FM01 (FM01*1Ng.+27,568) and female sALS Italian patients. The present study shows another SNP (rs894469) placed in a gene, FM05, of the same cluster with a significant frequency only in female patients in Italian population. Future studies with a larger population could be developed to find any significant association among SNPs of FMOs and sALS female patients also out of Italy. Results suggest that the presence of SNPs in FMO cluster are related to the female gender. Even if we still do not know if this SNPs could affect functionality of detoxify enzymes we cannot exclude to consider this SNPs as risk factor in people exposed to pesticides. A possible study object, indeed, could be making a correlation between SNPs of FMO and pesticides exposition in order to clarify a possible link with the disease.
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REFERENCES


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