Original article

Study of Serotonin Transporters Gene in Sporadic Amyotrophic Lateral Sclerosis in French Population

Julien Praline[1,2], Refaat M.Tabagh[1,4*], Maïté Amy[1,2], Patrick Vourc'h[1,2,3], Christian Andres[1,2,3] and Philippe Corcia[1,2]

[1]Inserm U930, CNRS 2448, Tours, France,
[2]Universite´ Francois-Rabelais, Tours, France,
[3]CHRU de Tours, Laboratoire de Biochimie et BiologieMole´culaire, Tours, France,
[4]Zoology Department / Faculty of Science / University of Tripoli-Libya.

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Abstract

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive, invariably fatal neurological disease that attack the nerve cells and responsible for controlling voluntary muscles. The disease belongs to group of disorders known as motor neuron disease which are characterized by gradual degeneration and death of motor neurons. Serotonin is a neurotransmitter existing in both central nervous systems (CNS) and gastrointestinal tracts (GI) and regulates visceral sensation through a paracrine signaling pathway. Our objective was to study if there is a link between the 5-HTTLPR (Linked Polymorphism Region) allele of the serotonin transporter gene and the existence of pyramidal or extra-pyramidal rigidity in different subgroup of ALS patients. This gene has never been studied in ALS. We analyzed the functional polymorphism 5-HTTLPR of the SLC6A4 serotonin transporter gene in 209 patients with ALS and 214 control population of 214 individuals. All subjects were of French origin dating at least three generations. The DNA was extracted from blood cells. The analysis of polymorphism HTTLPR localized in the promoter of the gene of the transporter of the serotonin (SCL6A4) was performed after PCR in 100ng of DNA. The results are analyzed by agaros gel electrophoresis (2 %). We obtained tow bands LL genotype with 512pb or the short allele SS with 469pb. We found no significant differences in allele and genotype frequencies in this 5-HTTLPR polymorphism studied in the SLC6A4 gene of ALS patients versus control patients. However, further studies will be necessary to fully reject the implication of SLC6A4in SLA. Indeed the expression of this gene is regulated by epigenetic processes, and these may still be impaired in SLA. Moreover, effects on mRNA splicing, protein localization, and properties of 5-HTT could be possible mechanisms for influencing SLA risk.

Key words: Amyotrophic lateral sclerosis, 5HTTLPR, serotonin transporter, Genotypes, PCR

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder described worldwide. During the last 10 years, an extensive literature has focused on genetic susceptibility factors in populations from different origins. Currently, there is no universal genetic factor associated with the majority of ALS cases.

Amyotrophic lateral sclerosis (ALS), sometimes called Lou Gehrig's disease, is a rapidly progressive, invariably fatal neurological disease that attacks the nerve cells and responsible for controlling voluntary muscles (muscle action we are able to control, such as those in the arms, legs, and face) (Kelly 2013). The disease belongs to a group of disorders known as motor neuron diseases, which are characterized by the gradual degeneration and death of motor neurons (Ellison 2008).

ALS is characterized by stiff muscles, muscle twitching. This results in difficulty speaking, swallowing and eventually breathing (NINDS 2010). The cause is not known in 90% to 95% of cases; about half of these genetic cases are due to one of two specific genes; it results in the death of the neurons that control voluntary muscles and the diagnosis is based on a person's signs and symptoms with testing done to rule out other potential causes (NINDS 2015). About 5–10% of cases are inherited from a person's parents (Kim and Camilleri 2000).

We suggest two ideas the first that neurotransmitters in particular the serotonin can have a net impact on the phenotype, functional statue and survival. The second we suggests that neurotransmitters transporter probably involved in these modifications both in human and SOD1 transgenic mice. These results showing the involvement of serotonin in the rigidity, we propose to analyses the polymorphism of this gene 5-HTT serotonin transporter carries neurotransmitter to search for a possible association between this gene and clinical parameter ALS patients.

The serotonin is a neurotransmitter existing in both central nervous systems (CNS) and GI tracts, and regulates visceral sensation through a paracrine signaling pathway (Kim and Camilleri 2000). Previous studies have shown that elevated plasma serotonin is associated with IBS-D and decreased plasma serotonin is associated with IBS-C (Atkinson *et al*. 2006). The serotonin secreted by enterochromaffin (EC) cells which not attached to postsynaptic membrane and still

***Corresponding Author:** Refaat Mohamed Tabagh, Department of Zoology, Faculty of science, University of Tripoli. Tripoli, Libya. Tele.+ 218092 404 65 67. E.mail: Refaat_tb@yahoo.com.

in the synaptic cleft, this serotonin will be activated to reuptake the serotonin back mechanism by the transporter (SERT) and allow the retune of the serotonin into enterochromaffin EC cells and activate the effect of serotonin in GI tracts subsequently (Bertrand and Bertrand 2010).

Pharmacological and genetic studies also support a role for serotonin in corticogenesis, and brain development in general, even prior to the formation of synapses (Wellman *et al* 2007). One key regulator of serotonin levels in the cortex and hippocampus is the serotonin transporter (5-HTT), which is involved in the reuptake of extracellular serotonin (Mamounas *et al*. 2000; Salichon *et al*. 2001). 5-HTT is a Na+/Cl-dependant membrane transporter encoded by the SLC6A4 gene (solute carrier family 6 member 4; 17q11.2) (Figure 1) (Heinz 2005). A polymorphism (5HTTLPR) in the promoter region of SLC6A4 determines the neuroanatomical size and functional coupling of the amygdala-frontal cortical circuit in humans (Pezawas *et al*. 2005; Watson et al. 2008).

Figure 1: Schematic structure of the human SLC6A4 gene. Non-coding and coding exons are indicated by white and black boxes, respectively. Locations of the genotyped polymorphisms and alternative splicing are indicated.

In present 70% of subjects of European populations (Heils *et al*. 1995), it is frequency is lower in African populations (37%) but nearly augment in Asian 90% in term of frequency, the second allele (Heils *et al*. 1995). Allele 4, 5 and 6 are very rare in Europe (Meyer-Lindenberg *et al*. 2005).

Multiple genetics studies have shown a significant association between the number of repetitions and neurological disease. In promoter region of SLC6A4 gene the 5HTTLPR polymorphism determines the neuroanatomical size and functional coupling of the amygdala-frontal cortical circuit in humans (Heinz 2005). This circuit has been implicated in several psychiatric disorders including Fragile X and Williams syndrome, this two pathologies characterized by mental deficiency (Pezawas *et al*. 2005).The association between VNTR polymorphism and psychiatric disease has been reported several times (Mazer *et al*. 1997). These results demonstrate the importance of studying the frequency of alleles of the VNTR in ALS to determine if an association exists between the VNTR genotype and presence of rigidity.

The serotonin transporter (5HTT) is a protein of 630 amino acids of the family Na+ dependent transporters, with 12 transmembrane domains. The serotonin transporter gene (SCL6A4) is located 17q11 near NF1gene and bleomycin hydrolase gene. The SCL6A4 gene consists of 16 exons and extends over 38kb (Bradley *et al*. 1997).

Among the described various polymorphisms, some have functional effects, the polymorphism most studied are: The insertion-deletion of 44pb in the promoter region 5` (5-HT Transporter Linked-Polymorphic Region, HTTLPR). This polymorphism is arepetition of a member of 20-23pb. The allele contains 14 repeats (Short S) and 16 repeats (Long L) U 18 repeats (very long) (Heils *et al*. 1995). The allele is associated with increased expression of RNA messanger in vitro (Heils *et al*. 1996), And in vivo (Murphy *et al*. 2004).

A variable number of tandems repeat (Variable Number of Tandem Repeats VNTR) intron 2 (Lesch *et al*. 1994). The allele contains 12 repetitions are more expressed but this expression also depends on sequential variations and the tissues were the alleles are expressed. A version of G to T in the site of polyadenylation in the UTR3` region (5-HTT3`UTR-Variant) (Kim *et al*. 2002), these allelic variant have been studied in numerous pathology neuropsychiatric .A lot of articles have been published on the subject last 10 years in particular depression, bipolar disorder, mood, suicide, autism, and attention hyperactivity deficit disorder.

SS genotype of 5-HTTLPR is associated with a risk of depression or not correlate with treatment anti depression, another neurological disease are studies for example pulmonary hypertension, the genotype SS is associated with late onset of Alzheimer disease and the depression in the Parkinson`s disease (Meyer-Lindenberg et *al*. 2005). This study was designed to research if there is association between polymorphism 5-HTTLPR of the gene encoding serotonin transporter and the ALS phenotype according to weather patients are rigid (extra-pyramidal rigidity, pyramid or not rigid) after genotype study by PCR of ALS patients and control (healthy subjects).

Materials and methods

Currently 209 patients with sporadic defined ALS and 214 probable or possible controls were included in this study, Clinical data and blood samples, collected between 1996 and 2012, were obtained from 209 SALs patients and 214 controls. We have our DNA bank which reserved in -20Callowed us to use the DNA bank for many years and we have a tissue culture of the patients which reserved in -70 permitting us to use DNA extraction and use our DNA bank for a long time (over 100 years). The participants signed consent forms approved by Medical Ethical Review Boards in French that adhere to the principles described in the Declaration of Helsinki. All subjects were of French origin dating at least three generations. To maintain comparability between groups, patients and controls living in France, but of other ethnicity, were excluded from the study.

The DNA was extracted from blood cells in the usual methodology (sambrooke et al. 1989),the analysis of polymorphism HTTLPR localized in the promoter of

the gene of the transporter of the serotonin (SCL6A4) was performed after PCR in 100ng of DNA with 125 µMdNTP, 2% DMSO, 125 µMbuffer 1X, 0,4 U of DNA polymerase DyNAzyme EXT (Finnzymes) and 200 pmol of every primer : forward, 5HTTF2 : 5'-GGCGTTGCCGCTCTGAATGC -3', reverse, 5HTTR2 : 5'-GAGGGACTGAGCTGGACAACCAC-3'. The conditions of PCR are : 94°C5 min, 35 cycles à 94°C 30 s, 63°C 1 min, 72 °C 1 m in, puis à 72°C 10 min. The results are analyzed by Agaros gel electrophoresis (2 %). We obtained tow bands LL genotype with 512pb or the short allele SS with 469pb like show in the gel electrophoresis photo (Figure 2).

The frequencies of polymorphism between patients and controls were compared with a chi2 test. All analysis was performed with JMP statistical software version 7.0.2 (SAS) Institute, Cary, NC, USA).Allele and genotype frequencies in patients and controls were compared with a Chi test x^2. Odds ratios were estimated for alleles and genotype (http://www. hutchon. net/ ConfidOR.htm). Linkage disequilibrium (parameter D') between markers was calculated using the software Haploview v.3.32. LD patterns were analyzed by Spearman rank correlation (Excel) between LD measures.

Results

The Comparing genotypes and polymorphisms of HTTLPR between patients and Controls (Table 1 and 2).The HTTPLR polymorphism is not significantly associated with the Clinic form (bulbar or spinal) ALS (Table 3).No significant difference was observed between two groups of patients in the age of onset (Table 4).

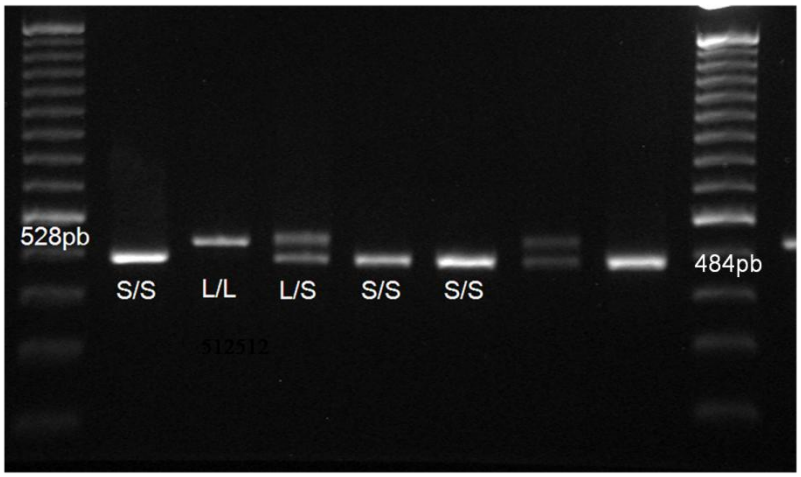

Figure 2: The PCR amplification step allowed differentiating between the short (S,484 bp) Versus the long allele (L, 528 bp) of 5 - HTTLPR. Gel agar electrophoresis (2 %).

Table 1: The number of chromosomes and allele frequencies of L and S HTTPLR for the Patients and controls ($\chi^2 = 0.02$; p = 0.87).

Alleles	Frequencies %	Number	Frequencies %	Number
L	58.4	250	57.9	242
S	41.6	178	42.1	176
Total	100	428	100	418

Table 2: Genotypes of HTTPLR polymorphism for the patients and controls ($\chi^2 = 0.02$; p = 0.99).

Genotype	Frequencies %	Number	Frequencies %	Number
LL	36.5	78	35.9	75
LS	43.9	94	44	92
SS	19.6	42	20.1	42
Total	100	214	100	209

Table 3: Comparison of allele frequencies L and S HTTPLR between two groups of patients according to the clinical form of the disease ($\chi^2 = 2.85$; p = 0.09).

Alleles	Frequencies %	Number	Frequencies %	Number
L	47.5	56	56.6	172
S	52.5	62	43.4	132
Total	100	118	100	304

Table 4: The comparison of allele frequencies L and S HTTPLR between two groups of patients according to age of disease onset (χ^2= 0.02; p = 0.88).

Alleles	Frequencies %	Number	Frequencies %	Number
L	57.4	58	56.5	95
S	42.6	43	43.5	73
Total	100	101	100	168

Discussion and conclusions:

We here report the first genetic study on the serotonin transporter gene in ALS, We have investigated functional polymorphism 5-HTTLPR in these gene in control individuals and ALS patients matched for origin (Central France) and ethnicity (Caucasian). Functional studies have reported that the S allele of 5-HTTLPR was associated with a lower expression of 5-HTT and a lower serotonin reuptake activity (Pezawas *et al*. 2005).

Our results do not support a direct role for the S allele of 5-HTTLPR in SLA. These preliminary results concern the gene of serotonin transporter (SCL6A4). This gene has never been studied in ALS. We did not find any association between a polymorphism in the promoter of this gene and ALS. The numbers of patients are important enough that this conclusion considered is solid. We also tried to search in different disease subgroups (sex, age and ethnical groups) if there were differences in serotonin transporter gene genotype. No significant difference could be detected, noted that the bulbar forms appear to be more S alleles that spinal forms. This would be to check on a larger population; in fact we do not have the sufficient statistical power to conclude with certainty.

Wendland *et al*. (2006) suggested that the effect of 5-HTTLPR on SLC6A4 expression may be due to a nearby (19 bp) rs25531 polymorphism in the promoter. The G allele of this polymorphism is located in a consensus binding site for AP2, a family of transcription factors described as positive or negative regulators of transcription (Eckert *et al*. 2005). Data from Hu *et al*. (2000) has shown that the G allele of rs25531 was associated with a decreased expression of 5-HTT mRNA compared to the A allele.

Serotonergic signaling systems have significant interactions with overlapping functional targets (Pezawas *et al*. 2005). Genetic epistasis between this system was confirmed by studies on SLC6A4 transgenic mice (Daws *et al*. 2007). In the present study on ALS patients, we observed no genetic interactions between 5-HTTLPR polymorphism studied in SLC6A4. However, further studies will be necessary to fully reject the implication of SLC6A4in SLA. Indeed the expression of this gene is regulated by epigenetic processes, and these may still be impaired in SLA. Moreover, effects on mRNA splicing, protein localization, and properties of 5-HTT could be possible mechanisms for influencing SLA risk.

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