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Association of a new polymorphism in ALOX12 gene with bipolar disorder*

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Abstract Bipolar disorder (BPD) is characterised by episodes of excitement interspersed with periods of depression. The role of genetic factors in BPD is indicated by studies in monozygotic twins showing 40–70 % of concordance. Studies using genetic markers showed linkage of genes for affective disorders in different chromosome regions, emphasising the polygenic and multifactorial traits. The main goal of our research is to search non-synonymous SNPs (those that result in modifications in protein sequence) in genes that can be associated with psychiatric diseases as suggested by genomic mapping and/or by physiological function of the protein. Using DNA sequencing we could confirm a new non-synonymous SNP in the conservative domain of the ALOX12 gene (17p13.1), suggested by EST alignment. This SNP is an alteration from G to A that leads to a change of an arginine (A) to a glutamine in one of the most important domains of the protein. This SNP was evaluated by DNA sequencing in 182 patients with BPD and 160 control individuals. An increased presence of allele A among patients (60 % in controls and 73.1 % in cases; $\chi^2 = 6.581$, $P = 0.010$; $OR = 1.8095$, 95 % $CI = 1.1477–2.853$) was found, suggesting an association of this polymorphism with the BPD in this Brazilian sample.

Key words ALOX12 · SNP · bipolar disorder · polymorphism · lithium

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Introduction

The heterogeneity of mood disorders suggests that different combinations of alleles in different families can produce the deleterious phenotype (Avissar et al. 2002). The role of genetic factors in bipolar disorder (BPD) is established, as indicated by studies in which monozygotic twins showing a concordance between 40 and 70 % (Craddock and Jones, 1999) as compared with a prevalence of 1–2 % in the general population.

Lithium salts are the most effective long-term preventive treatment for BPD. It was recently proposed that the neuro-protective propriety of lithium against glutamate excitotoxicity results of its capacity of inhibiting GSK-3β expression, blocking the facilitation of apoptosis produced by this enzyme (Chuang et al. 2002; Kozlovsky et al. 2002; Li et al. 2002). The lipoxygenase pathway also plays an important role in apoptosis (Tang et al. 1996) since the glutathione (GSH) depletion, caused by glutamate toxicity, triggers ALOX12 (arachidonate 12-lipoxygenase) activation, which leads to the production of peroxides and the influx of Ca2+, resulting in cell death (Li et al. 1997) (Fig. 1).

Studies using genetic markers have claimed linkage of genes for affective disorders in different chromosome regions such as 4p (Blackwood et al. 1996), 5q (Coon et al. 1993), 6 (Stancer et al. 1988), 11p (Egeland et al. 1987), 12q (Ewald et al. 1998), 13, 15 (Risch and Botstein, 1996), 18 (Berrettini et al. 1994), 21q (Aita et al. 1999), 22q (Kelsoe et al. 2001) and Xq (Baron et al. 1994), em-

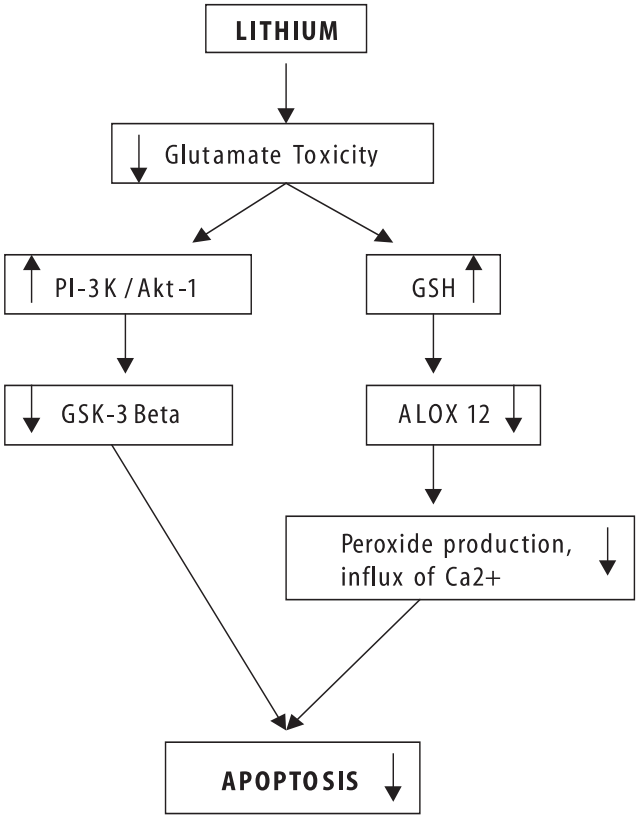

Fig. 1 A hypothetical neuro-protective action of lithium. Lithium intake protects against glutamate toxicity, which activates the PI-3K/Akt-1 expression, leading to an inactivation of GSK-3Beta, and blocking the apoptosis process. Lithium also avoids GSH depletion, which down-regulates ALOX12 expression reducing the levels of reactive oxygen species in the cell and a consequent decrease in apoptosis

phasising the polygenic and multifactorial traits of the disease.

Single nucleotide polymorphisms (SNPs) are the most frequent variations found in the human genome and can be defined as punctual regions of DNA where nucleotides can vary among individuals. SNPs have become very popular in the last few years as one of the most informative genetic markers used in the research of complex diseases. According to theoretical models, if genotypes of patients and controls are compared, a consistent association with affected individuals may be observed for certain alleles.

The main goal of our research is to search non-synonymous SNPs (those that result in modifications in protein sequence) in genes that can be associated with psychiatric diseases by mapping or by physiological function of the protein. While investigating putative polymorphisms in the ALOX12 gene we could confirm a new non-synonymous SNP and associate it with BPD in a sample of Brazilian patients.

Material and methods

Sample

Patients with bipolar disorder selected for this study were recruited at the Institute of Psychiatry, Hospital das Clínicas, FMUSP, Brazil. Diagnosis was made according to DSM-III-R criteria (APA,1987). Control individuals were selected among accompanying persons (not genetically related with patients) and people without psychiatric symptoms attending the same hospital. All patients and controls signed an informed consent form, which was previously approved by the ethics committee of the Faculty of Medicine, University of São Paulo. We deliberately decided to include a higher proportion of elderly people among the controls, in order to be certain of selecting patients that, even at older age, have not developed any psychiatric disorder. A total of 182 BPD patients (60 males and 122 females) and 160 controls (50 males and 110 females) were included in the present investigation. The mean age was 44.33 ± 14.33 (ranging from 18 to 83 years old) for patients and 65.35 ± 18.59 (ranging from 20 to 98 years old) for controls (Table 1).

Polymorphism search

The complete cDNA sequence of the ALOX12 gene was used in the BLAST analysis against a database of human Expressed Sequence Tags (ESTs) available in the GenBank (www.ncbi.nlm.nih.gov/dbEST). Putative polymorphic sites were selected for further analysis only when confirmed by ESTs from different cDNA libraries and when an amino acid alteration resulted (non-synonymous SNP).

DNA extraction, amplification and analysis

DNA was extracted from peripheral blood leukocytes of patients and controls, according standard phenol-chloroform protocols. A fragment of 244 nt of the ALOX12 gene was amplified by PCR in a total volume of 10 µl, containing 5 pmol of each primer, 250 nM dNTPs, 100 ng template genomic DNA with an initial denaturation for 4 min at 95 °C. Thirty-eight cycles were run, with denaturation for 45 s at 95 °C, annealing for 30 s at 60 °C and extension for 30 s at 72 °C. The final extension was for 5 min at 72 °C. The primers used for PCR were ALOX12F (5'-CTGGCAGGATGATGAGTTGTT-3') and ALOX12R (5'-AGTACTGCAGCCTTCCTCTG-3'). The resulting PCR products were inspected in 1% ethidium bromide stained agarose gels and sequenced in an ABI3100 DNA sequencer (Applied Biosystems, Foster City, USA), using standard protocols.

Statistical Analysis

The magnitude of association between the alleles and the occurrence of BPD was measured by the odds ratio (OR) and respective 95% confidence interval (CI), together with the χ^2 test using the level of confidence of $\alpha = 0.05$.

Table 1 Features of the sample

	Males	Females	Mean age	Freq of G/G	Freq of G/A	Freq of A/A
BP Patients N = 182	60	122	44.3 ± 14.3	26.9% (49/182)	46.2% (84/182)	26.9% (49/182)
Controls N = 160	50	110	65.3 ± 18.6	40.0% (64/160)	37.5% (60/160)	22.5% (36/160)

Results

Our database search using the ALOX12 mRNA sequence, against a set of 4.9 million human ESTs, showed four ESTs covering a putative polymorphic nucleotide. These four ESTs were derived from four different cDNA libraries and the alleles A and G were present in the same proportion (2 ESTs for each). After primer design and PCR amplification using a panel of DNA samples, we were able to confirm this SNP by DNA sequencing. The alteration is located in the nucleotide 815 of the 6th exon of ALOX12, and results in a substitution of an arginine to a glutamine in the aminoacid 261 (R261Q).

DNA sequencing of 684 alleles has consistently demonstrated an increased presence of allele A among cases (60 % in controls and 73.1 % in cases; $\chi^2 = 6.581$, $P = 0.010$; OR = 1.8095, 95 % CI = 1.1477–2.853). When comparing the frequency of genotypes G/A and G/G we found the best association between the heterozygous genotype and bipolar patients ($\chi^2 = 5.683$, $P = 0.017$; OR = 1.8286, 95 % CI = 1.1111–3.0094) (Table 1). Nevertheless no significant association was found when the number of G/A to A/A individuals was compared (data not show).

Discussion

The analysis of ESTs obtained by us during the Human Cancer Genome Project (Dias Neto et al. 2000), and other ESTs available in the GenBank (dbEST), suggested the existence of a new non-synonymous SNP in the conserved domain of the ALOX12 gene mapped at 17p13.1 (ALOX12, OMIM *152391). This polymorphism results in the substitution of an arginine to a glutamine at the aminoacid 261 (R261Q) of the protein. The variant aminoacid is part of the lipoxygenase domain, one of the most important and conserved regions of this protein.

Analysis of protein sequences in the GenBank shows that this region is highly conserved among mammals. A comparison of protein sequences of human, mouse, rat, bovine, rabbit and pig shows that few aminoacid positions were altered in this region. The aminoacid change at the position observed in our study (R/Q) can be seen in other species of mammals. In fact, the presence of a glutamine at this position seems to be ancestral, as it is a consensus among the majority of these mammals. However the existence of an arginine in this same position can only be observed in humans and bovines (Fig. 2). Thus, a fixation of the allele coding for an arginine in this position seems to be a recent event in the speciation of mammals.

ALOX12 gene belongs to the lipoxygenase family, is involved in the leukotrienes pathway and is the predominant lipoxygenase expressed in the brain (Bendani et al. 1995; Brash, 1999). This gene is also able to sensitise the nuclear membrane, which is more susceptible to the release of arachidonic acid (a sub-product of phospholipase A2, an important signal transducer in nervous cells). In search of a regulatory molecule that links cPLA2 and sPLA2-IIA, Murakami et al. (2000) found the 12/15-LOX isozyme. cPLA2 activation leads to production of 12/15LOX metabolites, which sensitise the nuclear membrane to be more susceptible to the sPLA2-IIA-mediated arachidonic acid release (Murakami et al. 2000). Lipoxygenase reactions may initiate the synthesis of signalling molecules or be involved in the induction of structural or metabolic changes in the cell (Brash, 1999). Expression analysis of ALOX12 showed higher levels of this gene in the mammary gland, ovary, prostate, brain, lung and heart (www.ncbi.nlm.nih.gov/Unigene).

Rapoport and Bosetti (2002) demonstrated that feeding rats with lithium reduced arachidonic acid turnover within the brain, corresponding to a down-regulation of gene expression and enzyme activity of cytosolic phospholipase A2 (cPLA2), an enzyme that selectively liberates arachidonic acid. Lithium also reduced the brain protein level and activity of cyclooxygenase 2, as well as the brain concentration of prostaglandin E[2], an arachidonate metabolite produced via cyclooxygenase 2. Their results raised the hypothesis that lithium and antimanic anticonvulsants act by targeting parts of the „arachidonic acid cascade," which may be functionally hyperactive in mania. Thus, drugs that target enzymes in this cascade might be candidates for treating mania.

Our results suggest that, in our sample, the presence of the A allele is correlated with BPD. However, we should also consider that this possible association with BPD might in fact be due to a haplotype involving this ALOX12 polymorphism, rather than the R261Q polymorphism itself.

Functional studies are necessary to evaluate the en-

Human - *Homo sapiens* (M58704)	247	LPSRLVLPSGMEELRAQLE 265
Mouse - *Mus musculus* (U04334)	247	LPSRLVLPSGMEELQAQLE 265
Bovine - *Bos taurus* (Y08829)	145	LP**T**RLVLPSGMEELRAQLE 163
Rat - *Rattus norvegicus* (L06040)	248	LP**A**RLV**FPP**GME**K**LQAQL**N** 266
Rabbit - *Oryctolagus cuniculus* (Z97654)	248	LP**A**RLV**FPP**GMEELQAQLE 266
Pig - *Sus scrofa* (D10621)	248	LP**A**RL**KFPP**GMEELQAQLE 266

Fig. 2 Alignment of a region of ALOX12 protein from diverse mammals. Sequences were obtained from the GenBank (accession codes are given in parenthesis) and manually aligned. Aminoacid coordinates are given at the beginning and at the end of the sequences. The amino acids corresponding to the polymorphic site observed in this study are underlined. Other variant positions, with respect to the human sequence, are in bold

zymatic activity of ALOX12 in heterozygous and homozygous cells, confirming a link of this polymorphism and altered enzyme activity.

GenBank/EMBL accession number. ALOX12-arachidonate 12-lipoxygenase, NM_000697

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