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A transcript coding for a partially duplicated form of α7 nicotinic acetylcholine receptor is absent from the CD4+ T-lymphocytes of patients with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE)

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Abstract

Introduction: *It has been suggested that the homomer-forming α7 subunit (CHRNA7) of the neuronal nicotinic acetylcholine receptor (nAChR) is involved in the pathogenesis of common idiopathic generalized epilepsies (IGEs), whereas mutations of the gene coding for the α4 nAChR subunit (CHRNA4) are associated with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). Several genes encoding nAChR subunits, including a partially duplicated isoform of CHRNA7 (CHRFAM7A), are expressed in peripheral blood lymphocytes (PBLs), and are constituents of peripheral receptors corresponding to nAChRs in the brain. Moreover, a 2-bp deletion polymorphism (c.497-498delTG; rs67158670), resulting in a frame shift and truncation of the protein product of the gene, has been found in the CHRFAM7A gene and is associated with some neurological diseases.*

Aim of the study: *CHRFAM7A transcript levels in CD4+ T-lymphocytes were compared between ADNFLE patients harbouring the c.851C>T mutation of the CHRNA4 gene and control healthy individuals in order to determine whether there is any correlation between CHRFAM7A expression in CD4+ T-lymphocytes and the severity of epileptic symptoms. We also tested the hypothesis that the 2-bp deletion polymorphism in the partially duplicated α7 nAChR gene may be related to ADNFLE in these patients.*

Material and methods: *Peripheral venous blood samples were collected from 3 individuals with ADNFLE and from 10 healthy individuals. From the isolated CD4+ T-lymphocytes, RNA was prepared and the CHRFAM7A transcript level was determined by RT-qPCR. In order to compare the CHRNA7 and CHRFAM7A sequences and genotype the -2bp polymorphism, genomic DNA was prepared from PBLs.*

Results: *It has been demonstrated that CHRFAM7A is expressed in CD4+ T-lymphocytes of healthy individuals, the relative abundance of the transcript being nearly equal (about 100 copies per cell), but it is not expressed in ADNFLE patients. Genotype analysis showed that the -2bp polymorphism was found in all patients as well as in seven healthy individuals.*

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Conclusions: *Our observations confirm the hypothesis that the CHRFAM7A gene is expressed in CD4+ T-lymphocytes of healthy individuals and that this expression is legitimate. The observed lack of CHRFAM7A expression in ADNFLE patients might be an important factor in the pathogenesis of ADNFLE.*

Key words: *ADNFLE, nicotinic α7 receptor, CD4+ T cells.*

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels, which belong to a superfamily of homologous receptors. The nAChRs are expressed in the mammalian brain in the form of hetero- or homomeric structures consisting of four transmembrane regions (TM1 through TM4) surrounding a centrally located ion channel [33]. These structures are composed of a number of subunits of multiple subtypes. To date, 9 distinct genes encoding neuronal nAChR subunits (α2 to α10 and β2 to β4) have been found in various species [29]. In the mammalian brain, two types of nAChRs have been distinguished based on their affinity for nicotine or α-bungarotoxin as ligands. Functional receptors as homomers can be obtained by assembling α7, α8 and α9 subunits, whereas other α subunits may co-assemble with at least one type of β subunit to form heteromers [17]. The second type of nAChR, composed exclusively of α7 subunits, exhibits low affinity for nicotine and high affinity for α-bungarotoxin [6]. The nAChRs containing the α7 subunit are expressed throughout the entire central nervous system. These receptors are localised pre- and postsynaptically and modulate both the excitatory and inhibitory pathways. They are predominantly located in cholinergic and non-cholinergic presynaptic terminals, such as GABA-ergic interneurons [12]. The α7 nAChRs are also found in nerve terminals located in peripheral tissues and are involved in neuropeptide release and protection against inflammatory processes [32]. Of particular interest is the function of nAChRs in peripheral blood lymphocytes (PBLs) [21]. These cells produce acetylcholine (ACh), which may exert its effect through autocrine or paracrine transmission. Different nAChR subunits have been detected in blood cells, but their patterns of expression as well as their functions remain largely unknown. It has been reported that α2, α5 and α7 nAChR subunits are expressed in PBLs, raising the possibility that these subunits may serve as a marker of some neurological diseases [44]. However, the expression of genes encoding the α4 and β3 subunits have not been detected in the majority of cell lines derived from peripheral blood tested so far [25].

The genes encoding nAChR subunits have also emerged as candidate genes for inherited idiopathic epilepsies. Mutations of the genes coding for the nAChR α4 (*CHRNA4*), α2 (*CHRNA2*), or β2 (*CHRNB2*) subunits are associated with familial forms of partial epilepsies, classified as autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [22,35,40]. In ADNFLE patients, mutations in the genes encoding these subunits are located within TM1 (*CHRNA2* mutation), within or directly adjacent to TM2 (*CHRNA4* mutations), or are equally distributed between TM2 and TM3 (*CHRNB2* mutations). The function of mutant nAChRs has been extensively studied *in vitro* with the use of electrophysiological methods [39]. *In vivo* PET studies of the distribution of the mutated receptor in the brain of ADNFLE patients have demonstrated decreased density of nAChR in the prefrontal cortex, consistent with focal epilepsy involving the frontal lobe [36].

A number of loci have been reported for juvenile myoclonic epilepsy (JME), including our population-based study testing the hypothesis that the variants of *CHRNA4* confer genetic susceptibility to this form of epilepsy [41]. Linkage studies provide evidence that the gene encoding the α7 subunit of nAChR (*CHRNA7*) is located within the polymorphic locus on chromosome 15q14. This locus, called EJM2 (OMIM ID: 604827), has been shown to contribute to genetic susceptibility to JME in the majority of studied families [50]. Susceptibility loci for the common idiopathic generalized epilepsies (IGEs) – comprising of JME, juvenile absence epilepsy (JAE), childhood absence epilepsy (CAE), and benign epilepsy of childhood with centrotemporal spikes (BECT) – have also been mapped to the 15q13-q14 region [13,24,31,43]. A particularly high expression of *CHRNA7* in the reticular thalamus [4] indicates this gene's role in modulating thalamocortical pathways, which participate in the generation of the primarily generalized seizures seen in IGEs [3].

Out of the nicotinic acetylcholine receptors, the α7 nAChRs are of particular importance in the pathogenesis of Alzheimer's disease (AD). Our investigations

support the involvement of the α4 subunit of nAChR in this process [11]. Increased levels of the *CHRNA7* transcript in the hippocampus, as well as in the lymphocytes, of AD patients have been reported [19,20], suggesting that the elevation in *CHRNA7* gene expression may be associated with AD, and that the receptors localized in PBLs correspond to the same receptors in the brain. Recent observations, however, have revealed that the *CHRNA7* transcripts found in PBLs are products of the gene encoding the duplicated α7 subunit of nAChR (*CHRFAM7A*), but not the "classic" *CHRNA7* [51]. Contradictory results were also obtained in the frontal cortex of AD patients showing no differences in α-bungarotoxin binding [7,49], and a significant decrease of α7 subunit levels [14]. The human gene encoding the α7 subunit is partially duplicated, with both loci mapping to the chromosome 15q13-q14 region and approximately 1.5 Mb apart [38]. Since the two related genes encoding the α7 subunit (*CHRNA7* and *CHRFAM7A*) are present in the human genome, two isoforms of the α7 subunit (α7 and dupα7, respectively) and the corresponding transcripts have been identified. Therefore, the search for mutations of the α7 subunit gene is complicated, since exons 5 through 10 are duplicated and give rise to five new exons (D´-D-C-B-A), which are expressed in the brain and peripheral tissues [7,14,19, 20,38,49,51]. Gault *et al*. [16] demonstrated that the TG deletion polymorphism (c.497-498delTG; rs67158670) causes a shift in the reading frame, introduces a stop codon in exon 6, and results in a non-functional α7 subunit. This -2bp allele is present only in the duplicated exon 6 of the *CHRFAM7A* gene. It has been shown that the presence of the 2bp deletion in the *CHRFAM7A* gene may influence the risk of developing bipolar (BP) and major depressive (MD) disorders [23,27]. According to a recent study, the 2bp deletion polymorphism is a risk factor for auditory sensory gating deficit, which characterizes the majority of patients with chronic schizophrenia [37]. The impact of the α7 AChR partial gene duplication and the -2bp variant on the development of epileptic symptoms remains to be determined.

Aim of the study

Several mutations of the *CHRNA4* gene were associated with ADNFLE and the c.851C>T (S284L) mutation was identified in a Caucasian family with ADNFLE [40], in which PET analysis has demonstrated decreased density of nAChR in the prefrontal cortex of the proband's brain [36]. Since the α7 nAChR subunit is expressed in PBLs, we decided to investigate the CHRFAM7A transcript level in the CD4+ T-lymphocytes from ADNFLE patients and healthy individuals to look for possible involvement of this gene's expression in the pathogenesis of ADNFLE. Since earlier studies have suggested that a deletion in the 15q13-q14 locus might cause epilepsy, we compared the *CHRNA7* and *CHRFAM7A* sequences, and investigated the relation between the c.497-498delTG polymorphism and ADNFLE.

Material and methods

Patients

Peripheral blood samples were taken from three non-smoking ADNFLE individuals of the same family: the male patient (proband), 28 years of age, his father, and the sister of his father (the pedigree is shown in Fig. 1). All patients harbour the c.851C>T mutation of the *CHRNA4* gene (α4-S284L) and suffer from ADNFLE [40].

The proband (III-3 in Fig. 1) was diagnosed and treated in the Department of Developmental Neurology, Poznan University of Medical Sciences in Poznan (Poland). On the basis of neurological examination before treatment with carbamazepine, the patient had 20-30 seizures per night, manifesting as simple motor acts such as head scratching, limb flexion, the sensation of being out of breath, tonic stiffening, and vocalisation. Upon treatment with carbamazepine (600 mg/day), the frequency of seizures decreased to 2 to 3, twice

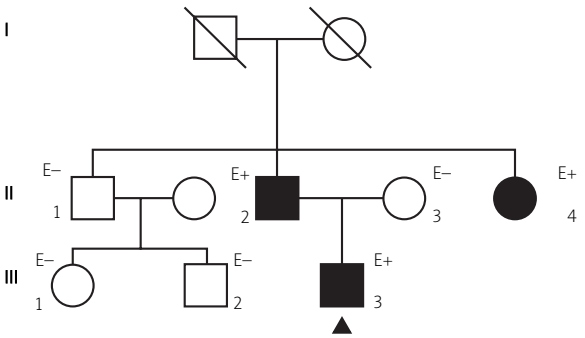

Fig. 1. Pedigree of the family with autosomal dominant nocturnal frontal lobe epilepsy in which the c.851C>T mutation of the *CHRNA4* gene (CHRNA4-S284L) was detected.
Solid symbols – affected; open symbols – unaffected; squares – male; circles – female; E+ – individuals positive for mutation; E– – individuals negative for mutation; solid arrowhead – proband

a month. The attacks (lasting for 10 to 20 s) occurred at the end of the night and throughout naps. During the seizures, the proband exhibited ictal breathing difficulty, but there was no loss of consciousness. After clusters of seizures, postictal phenomena such as motor aphasia (lasting 1 to 2 min) and hyperthermia occur. Interictal surface EEGs are normal, ictal EEGs are not localized, and cerebral MRI shows no abnormality.

Proband's father (II-2 in Fig. 1). At the age of five, nocturnal seizures described as generalized tonic-clonic convulsions were diagnosed. Interictal EEGs and cerebral CT examinations showed no abnormalities and he has never been treated for epilepsy. At present, motor acts with the involvement of multiple body segments such as gross body movements, change in body position and/or rhythmic movements (lasting for 5 to 10 s) can be observed during the night.

Sister of proband's father (II-4 in Fig. 1). Since the age of two she has been treated for epilepsy. The seizures (verbal manifestation and sometimes urinary incontinence) occurred in clusters of about ten a night, and began soon after falling asleep. When she was five, the seizures were observed during the day in the form of general tonic convulsions. Interictal surface EEGs were normal and cerebral CT showed no abnormalities. She was treated with phenobarbital, carbamazepine, valproate and phenytoin, and the frequency and duration of seizures decreased but they were not eliminated. At present, she still has two to three nocturnal seizures lasting for 3 to 5 s.

As controls, 10 healthy male non-smoking blood donors aged 25 to 30, without any neurological disorders, were used.

Written consent of all study participants was obtained, and the study was approved by the local Ethics Committee.

Isolation of genomic DNA and comparison of *CHRNA7* and *CHRFAM7A* sequences

In order to analyse the structure of *CHRNA7* and its partially duplicated form (*CHRFAM7A*), genomic DNA was isolated from PBLs with the use of Blood DNA Prep Plus (A&A Biotechnology, Poland). The sequences of *CHRNA7* and *CHRFAM7A* were compared (http://www.esembl.org), and the fragments that differ between the two genes were identified. These fragments are localized upstream from exon 6, at the 2782 and 3005 positions of *CHRNA7* and *CHRFAM7A*, respectively. In order to amplify these fragments by polymerase chain reaction (PCR), a common pair of the following primers flanking the DNA sequence that is different in the two genes was designed: GCTGGGGTTTTTGATCTTTTAG (forward) and GTGGAGTGGTGAGTGGTGTG (reverse). Following PCR amplification, fragments of the *CHRNA7* and *CHRFAM7A* genes were separated by agarose gel electrophoresis and their molecular size was determined.

Genotyping and sequencing of the -2bp polymorphism

DNA used for genotyping of the -2bp polymorphism was extracted from PBLs. The *CHRFAM7A* fragment harbouring the polymorphism was PCR amplified using the following primers: TCTTCTGTTTCCATCACCCACACA (forward) and GCTTTCTTCCAGGCGGTTAGTCC (reverse). The PCR fragments, 226bp in length, were subjected to polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide gel (500 V, 100 mA, 25 W for 60 min) and were visualized by silver-staining. Templates for direct sequencing were generated using the same primers as those used for amplification. The reaction was conducted using a sequencing kit (*BigDye Terminator v3.1. Cycle Sequencing* system) and an automated DNA sequencer (ABI PRISM310, Applied Biosystems, USA).

Synthesis and sequencing of cDNA from resting and activated $CD4^+$ T-lymphocytes

In order to identify the transcripts encoded by the fragments that are present in *CHRNA4* and *CHRFAM7A*, RNA was isolated from the $CD4^+$ T-lymphocytes according to the original protocol of Chomczynski and Sacchi [5]. Immediately after isolation, one fifth of the poly-A^+ mRNA eluted from oligo$(dT)_{25}$ column was reverse transcribed into cDNA with the use of M-MLV reverse transcriptase (Invitrogen, USA), and the obtained cDNA was used as a template for amplification of the fragment spanning exons 5 and 6 of *CHRNA4* and exons 6 and 7 of *CHRFAM7A*. In order to differentiate the cDNAs amplified, the following primers (all from OLIGO IBB, Poland) were designed for amplification of the different exons of *CHRNA4* and *CHRFAM7A*, CCAGTACATTGCAGACCACCT (forward), TGAACATCCAGAGGAAGATGC (reverse) and CTGAAG-TTTGGGTCCTGGTC (forward), AAGGTGCATCGGGGTAGG (reverse), respectively. The amplified products of *CHRFAM7A* were separated by PAGE in 10% polyacrylamide gel, followed by staining with ethidium bromide. Fragments of the

appropriate length were then excised from the gel, re-amplified, purified, and subjected to sequencing with the use of *BigDye Terminator v3.1. Cycle Sequencing* system and ABI PRISM310 sequencer (Applied Biosystems, USA).

PCR amplification of cDNA from CD4+ T-lymphocytes

In order to detect the *CHRNA4* and *CHRFAM7A* transcripts and estimate the melting temperature (T_m) of the amplified fragments, qualitative analysis was performed. Quantitative analysis (RQ-PCR) was conducted using the *LightCycler* real-time PCR system (Roche Diagnostics, Mannheim, Germany) using SYBR® Green I as the detection dye. The relative abundance of target cDNA in each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal standard and the results were elaborated using the Microsoft Excel computer program.

Results

In order to compare the primary structure of *CHRNA7* and *CHRFAM7A* in patients with ADNFLE and healthy individuals, analysis of genomic DNA fragments, amplified with the use of the same primers, was conducted. Specific amplification products of *CHRNA7* (356bp in size) and *CHRFAM7A* (633bp in size) were obtained in patients and healthy individuals.

Genotyping of the -2bp *CHRFAM7A* polymorphism (rs67158670) was conducted by PAGE analysis of the PCR products in 10% polyacrylamide gel. Among the genotypes found in the control group, there were seven individuals with the c.497-498delTG polymorphism and three with the wild-type/wild-type genotype of *CHRFAM7A*. Among the genotypes found in the patients, the -2bp alleles were present in all individuals. Sequencing analysis (Fig. 2A) of the relevant region of exon 6 in the proband showed a heterozygous c.497-498delTG polymorphism.

In the CD4+ T-lymphocytes of all healthy individuals the fragment of *CHRFAM7A* cDNA of the expected size (170bp) was detected. This fragment was excised from the gel, purified, re-amplified and sequenced. The nucleotide sequence of the purified product was identical to the sequence joining exons 6 and 7 (Fig. 2B).

In an attempt to identify the *CHRFAM7A* transcript in the CD4+ T-lymphocytes of the ten healthy individuals, the T_m of all amplified fragments was measured with the use of *LightCycler*. The T_m was almost identical in all healthy individuals and amounted to 86.0+/–0.1°C (Fig. 3A). In the CD4+ T-lymphocytes of the healthy individuals, the levels of the CHRFAM7A transcript varied from 6.16×10^4 to 1.67×10^5 copies per 10^3 cells (Fig. 3B), the average number being $1.25 \pm 0.36 \times 10^5$ copies per 10^3 cells. This suggests that the basal expression of *CHRFAM7A* in these CD4+ T-lymphocytes was about 100 copies per cell. This result implies a legitimate expression of *CHRFAM7A* in these cells and a possible contribution of the dupα7 subunit to the assembly of the Ca^{2+}-channel in these cells.

Qualitative analysis with the use of *real time* PCR showed lack of this transcript in the lymphocytes of patients with ADNFLE, providing evidence that *CHRFAM7A* is expressed in the CD4+ T-lymphocytes of the healthy individuals tested, but not in the individuals with ADNFLE (Fig. 4). The transcript of *GAPDH* (which is constitutively expressed) was identified in all samples taken from healthy individuals as well as those of the patients with ADNFLE. The T_m of all amplified fragments amounted to 82.0+/–0.1°C (Fig. 4). The possibility that some material was lost during the procedure can be excluded, since GAPDH transcript was detected in the CD4+ T-lymphocytes of the patients as well as in the healthy individuals at similar levels. To ensure the integrity of these results, an additional housekeeping gene, encoding beta-actin (*ACTB*), was used in a qualitative *real time* PCR study (Fig. 4).

Qualitative and quantitative analysis with the use of the *LightCycler* system, excluded the presence of the *CHRNA4* transcript in CD4+ T-lymphocytes of all individuals tested.

Discussion

Expression of nAChR subunits varies across brain regions, with the most abundant α4 subunit being highly expressed in the thalamus and cortex [42] and the α7 subunit in the hippocampus, lateral and medial geniculates, and the reticular thalamic nucleus, a brain region frequently associated with pathophysiology of epilepsy [4]. The presence of *CHRNA7* transcript in various blood cells, including T-lymphocytes, has also been demonstrated [44]. Although the expression pattern of this gene varied in PBLs taken from different donors, Sato *et al.* [44] have demonstrated expression of the α2, α5, and α7 subunits and no expression of the α3, α4, β3, and β4 subunits in these cells. Most of the recent studies have shown that neuron-type

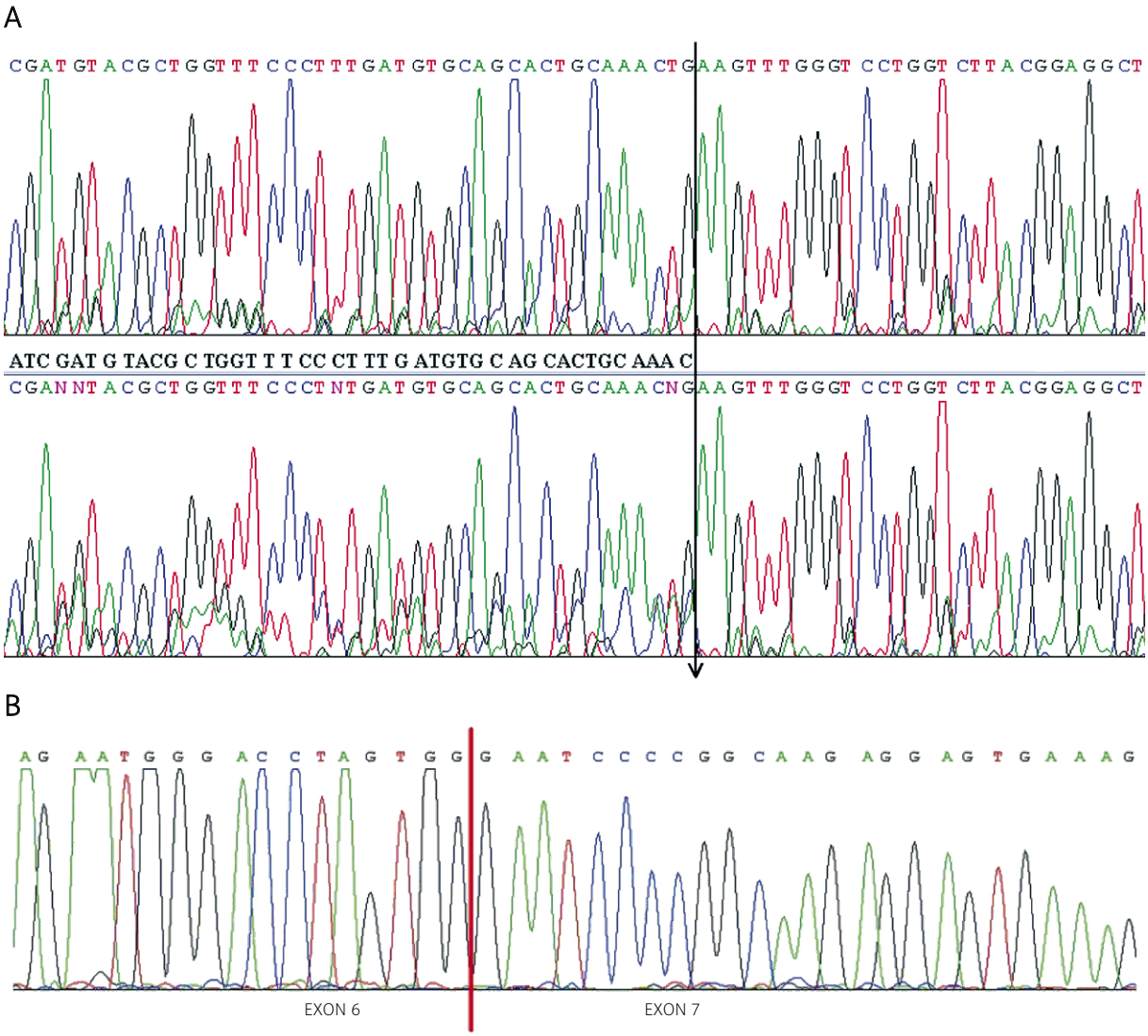

Fig. 2. Sequence analysis of the *CHRFAM7A*. **A**) Nucleotide sequence of the genomic DNA fragment of exon 6 of the *CHRFAM7A* gene. Sequencing of the -2bp polymorphism (only the part of the sequence containing polymorphism is shown). Upper panel, control (healthy individual); lower panel, proband; arrow indicates -2bp deletion (–TG genotype). **B**) Nucleotide sequence of the cDNA fragment corresponding to the region spanning exon 6 and exon 7 of the *CHRFAM7A* transcript. Vertical line indicates the boundary between the two exons.

nAChR subunits are expressed in PBLs, thymocytes, and human leukemic cell lines [25,44]. However, Hiemke *et al.* [21] and Benhammou *et al.* [2] determined, by RT-PCR followed by Southern blot analysis, the expression of the α3 and α4 subunits in the T-lymphocytes. In our study, no *CHRNA4* transcript in the $CD4^+$ T-lymphocytes was detected in all samples taken from 10 healthy individuals and 3 ADNFLE patients, confirming the data published by Sato *et al.* [44] regarding the lack of peripheral expression of this gene.

Consistent with our attempts to identify a mutation of *CHRNA4* responsible for ADNFLE in a Caucasian family [40], we extended our studies and estimated the level of *CHRFAM7A* transcripts in the $CD4^+$ T-lymphocytes of both the patients and the healthy individuals used as controls, since *CHRNA7* is not expressed in PBLs [51]. The cDNA primers used in our study were designed to amplify a region between exon 6 and exon 7 of *CHRNA7* that is conserved between these two genes. In the control group, the basal expression of

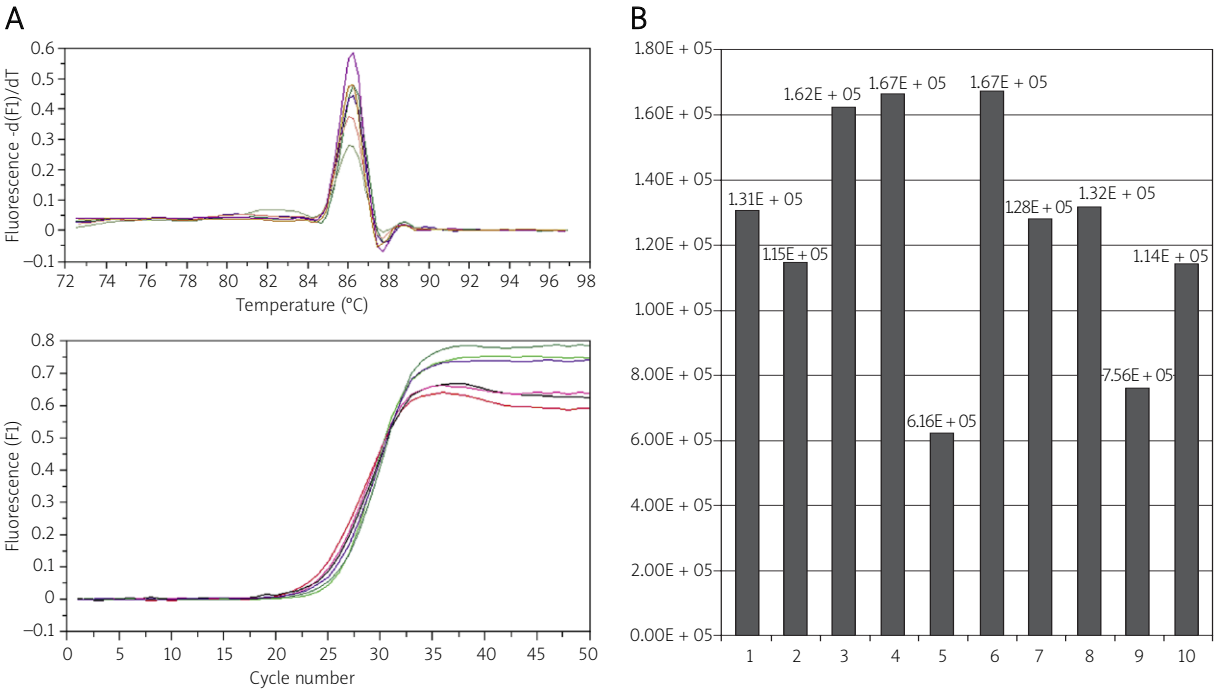

Fig. 3. The *CHRFAM7A* isoform is expressed in CD4+ T-lymphocytes. **A**) Detection of *CHRFAM7A* cDNA by *real-time* PCR. The shapes of the lines represent changes in fluorescence over time, and their level is directly proportional to the amount of double stranded cDNA fragments in the sample. Each colour represents a different amplified sample. The peak shows the melting point of the cDNA fragment. **B**) Quantification of *CHRFAM7A* transcript in controls. The number of copies of *CHRFAM7A* transcript in the samples was normalized to the number of cells (copies/10^3 cells).

CHRFAM7A was about 100 copies per cell. The results of our study showed that, as opposed to the healthy individuals, there was no expression of *CHRFAM7A* in the ADNFLE patients harbouring the S284L substitution in the α4 subunit. This suggests that there might be a link between the expression of *CHRFAM7A* and the occurrence of the symptoms of ADNFLE.

Most recently, de Lucas-Cerrillo *et al.* [9] revealed that basal CHRFAM7A mRNA levels are higher in macrophages. Previously, Benfante *et al.* [1] showed that both the mRNA and protein products of *CHRFAM7A* were reduced in primary monocytes and macrophages after lipopolysaccharide (LPS) treatment. This transcriptional down-regulation was mediated by a direct mechanism dependent on NFκB, suggesting that dupα7 may specifically participate in the inflammatory response of the innate immune system. Current knowledge indicates that functional cross-talk between presynaptic receptors may occur if both receptors are activated by the same neurotransmitter [28]. This interaction will finally produce, through different mechanisms, an integrated response which generates synergistic or antagonistic effects. It has been amply demonstrated that a variable response occurs with different subtypes of heteromeric nAChRs [28]; however, no evidence has so far been produced to support the involvement of the homomeric α7 nAChRs. This could be particularly relevant because, as far as the functional diversity of nAChR subtypes is concerned, recent evidence supports the possibility that α7 and α4β2 nAChR subtypes, which are differently permeable to Ca^{2+} ions, trigger neurotransmitter release via different mechanisms [10]. In our proband with ADNFLE, the PET study of the distribution of the mutated receptor revealed a lower number of α4β2 nAChR in the right prefrontal region of the brain [36]. This confirms that low expression of these subunits might be connected with decreased expression of other nAChR subunits, including those encoded by *CHRNA7*. In the case of ADNFLE, however, no link between clinical symptoms and brain expression of the gene encoding the α7 subunit or the dupα7 isoform has been demonstrated.

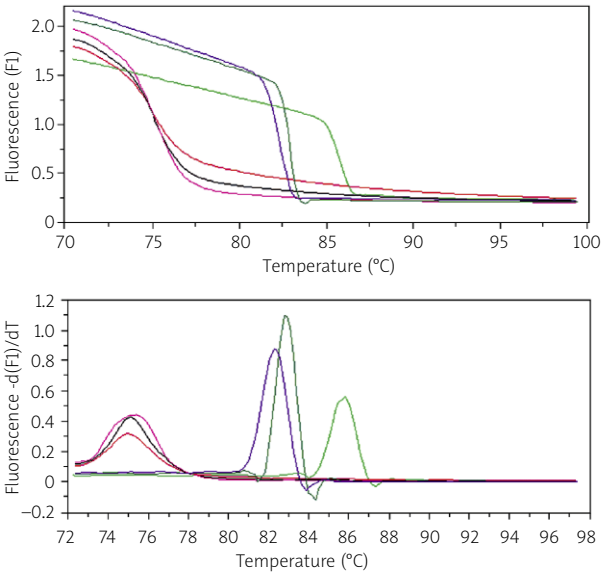

Fig. 4. RQ-PCR analysis of mRNA encoding *CHRFAM7A* fragment in healthy individuals, and *GAPDH* and *ACTB* in the proband with ADNFLE. Light green line, *CHRFAM7A* mRNA of the healthy individual; dark green line, *ACTB* mRNA of the proband; navy blue line, *GAPDH* mRNA of the proband; black, red and pink lines, negative control for RQ-PCR of *CHRFAM7A* in the patients with ADNFLE.

Decreased mRNA levels of the duplicated form of α7 nAChRs in lymphocytes and in the hippocampus of schizophrenic patients have been reported, suggesting that a genetic defect in *CHRFAM7A* expression may be associated with the pathogenesis of schizophrenia [15,34]. A hypothesis was put forward that the level of CHRFAM7A mRNA in PBLs might reflect expression of the α7 nAChRs in the brain and that it might constitute a marker of some psychiatric disorders [34]. Lower expression levels of the gene encoding the α7 nAChR subunit in the frontal lobe of cerebral cortex of patients with schizophrenia seem to support this hypothesis [15]. To date, it is difficult to explain why the *CHRFAM7A* gene is not expressed in $CD4^+$ T-lymphocytes of patients with ADNFLE, while it is expressed in the same cells of normal individuals. The analysis of the clinical variability presented by Steinlein *et al.* [48] suggested that the risk for additional major neurological and psychiatric features might be increased for ADNFLE patients with certain nAChR mutations. However, major neurological features such as schizophrenia-like symptoms, mental retardation or cognitive deficits have been described only in a few families [48]. A relatively high expression of *CHRFAM7A* in T-lymphocytes of the healthy individuals studied, as well as lack of expression of this gene in the same cells of ADNFLE patients, were not a result of contact with nicotine, since all individuals tested were non-smokers and never smoked. We took note of smoking as a factor since the study by Kimura *et al.* [26] demonstrated that nicotine decreased the mRNA level of *CHRFAM7A* in a T-lymphocyte model cell line. We also excluded the effect of antiepileptic drug (AED), carbamazepine, on *CHRFAM7A* expression, since there was no expression of this gene in either the two patients who did not take this drug or in the proband who was treated with carbamazepine. Genetic factors also appear to be significant in disturbances in ratios of the sulphur-containing amino acids, homocysteine (Hcy) and methionine (Met) as well as asymmetric dimethylarginine (ADMA) and arginine (Arg), in epileptic patients treated with variable AEDs. Our previous study [46] demonstrated that AED pharmacotherapy in epileptic patients leads to increase in Hcy and ADMA levels and the feedback control of Hcy over ADMA was disturbed. It is suggested that polymorphisms of genes related to Hcy-to-Met metabolism may have an effect on the regulation of the Hcy and ADMA levels in epileptics treated with AEDs.

Recently, a 15q13.3 microdeletion syndrome (OMIM ID: 612001) has been identified in 0.2-0.3% of individuals with mental retardation and epilepsy, as well as in schizophrenia, autism and some other neuropsychiatric disorders [45,47]. The region on 15q13.3 that contains the 1.5-Mb deletion harbours at least seven genes, including *CHRNA7*. Presently, the 15q13.3 microdeletion has been deemed the main risk factor for IGEs [18]. However, the underlying genetic alterations remain largely unknown in the vast majority of individuals with IGEs [8,30]. In our patients with ADNFLE, as well as in the healthy individuals, analysis of genomic DNA fragments revealed specific amplification products of *CHRNA7* and *CHRFAM7A*, indicating that the absence of the *CHRFAM7A* transcript from T-lymphocytes of the ADNFLE patients was not a result of the 15q13.3 microdeletion. The 2bp deletion in the partially duplicated α7 nAChR gene should also be excluded as the reason of lack of *CHRFAM7A* expression in ADNFLE patients, since *CHRFAM7A* was expressed in CD4+ T-lymphocytes of healthy individuals carrying this polymorphism.

The c.497-498delTG polymorphism is associated with auditory sensory gating deficit characterizing schizophrenic patients [37], and may be implicated in BP

and MD disorders [23,27]. The study described by Hong *et al.* [27] indicated that a genotype variant with greater than two -2bp allele copy number is unlikely. We conducted the c.497-498delTG polymorphism analysis in three patients with ADNFLE and in ten healthy individuals, and -2bp alleles were found in all ADFLE patients as well as in seven members of the control group. This result suggests that the -2bp polymorphism or a nearby polymorphism may only play a secondary role in the pathogenesis of ADNFLE. Determination of the functional impact of the c.497-498delTG *CHRFAM7A* variant on the nervous system needs further exploration.

Conclusions

ADNFLE is a familial partial epilepsy syndrome and the first human idiopathic epilepsy known to be related to specific gene defects. However, there are not many familial cases of ADNFLE with known genetic mutations. Clinically available molecular genetic testing reveals mutations in three genes: *CHRNA4*, *CHRNB2* and *CHRNA2*. Mutations in *CHRNA4* have been found in families from different countries; the S280F in Australian, Spanish, Norwegian and Scottish families, and the S284L in Japanese, Korean, Lebanese and Polish families. Although no link between clinical symptoms of ADNFLE and the genes encoding the α7 or dupα7 subunits has been demonstrated to date, the results of our study showed that, as opposed to healthy individuals, there was no expression of *CHRFAM7A* in the CD4+ T-lymphocytes of the ADNFLE proband and his two family members harbouring the S284L substitution in the α4 nAChR subunit. Since the expression of the α4β2 nAChR in the right prefrontal region of the proband's brain was decreased, our results confirm that low expression of these subunits might be connected with expression of other nAChR subunits, including those encoded by *CHRFAM7A*. This suggests that there might be a link between the expression of *CHRFAM7A* and the occurrence of the symptoms of ADNFLE.

We do recognise that our study was performed in only one family and has a limited scope due to the unavailability of the genetic material of other families with ADNFLE with known genetic mutations in nAChRs. However, we believe that the documentation of our findings is significant as pertains to the genetic and expressive status of the *CHRNAM7A* gene in this particular family with ADNFLE. The results of our study, although made on a small group of ADNFLE patients, seem convincing since the *CHRFAM7A* transcript from the purified CD4+ T-lymphocytes was analysed and the presence of other cell types can be excluded. Moreover, the use of real-time qPCR instead of the routine PCR technique allowed us to precisely quantify the number of transcript copies.

This is the first report showing that the expression pattern of *CHRFAM7A* can be demonstrated in CD4+ T-lymphocytes of the studied healthy individuals, and that there is no expression of this gene in the same cells of the studied patients with ADNFLE, as verified using the RT-qPCR technique. If the level of the CHRFAM7A transcript in the CD4+ T-lymphocytes reflects the expression of nAChR in the brain, it may constitute a biological marker of the disease. However, to substantiate our findings, further studies on a much larger group of ADNFLE patients with diagnosed nAChR mutations are required. Similarly, a large-scale association study is needed to indicate whether there is some evidence supporting the association of the *CHRFAM7A* 2-bp deletion polymorphism with ADNFLE.

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References

1. Benfante R, Antonini RA, De Pizzol M, Gotti C, Clementi F, Locati M, Fornasari D. Expression of the α7 nAChR subunit duplicate form (CHRFAM7A) is down-regulated in the monocytic cell line THP-1 on treatment with LPS. J Neuroimmunol 2011; 230: 74-84.
2. Benhammou K, Lee M, Strook M, Sullivan B, Logel J, Raschen K, Gotti C, Leonard S. [(3)H]Nicotine binding in peripheral blood cells of smokers is correlated with the number of cigarettes smoked per day. Neuropharmacology 2000; 39: 2818-2829.
3. Blumenfeld H. Cellular and network mechanisms of spike-wave seizures. Epilepsia 2005; 46 (Suppl 9): 21-33.
4. Breese CR, Adams C, Logel J, Drebing C, Rollins Y, Barnhart M, Sullivan B, Demasters BK, Freedman R, Leonard S. Comparison of the regional expression of nicotinic acetylcholine receptor alpha7 mRNA and [125I]-alpha-bungarotoxin binding in human postmortem brain. J Comp Neurol 1997; 387: 385-398.
5. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat Protoc 2006; 1: 581-585.
6. Couturier S, Bertrand D, Matter JM. Hernadez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M. A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron 1990; 5: 847-856.

7. Davies P, Feisullin S. Postmortem stability of alpha-bungarotoxin binding sites in mouse and human brain. Brain Res 1981; 216: 449-454.
8. de Kovel CG, Trucks H, Helbig I, Mefford HC, Baker C, Leu C, Kluck C, Muhle H, von Spiczak S, Ostertag P, Obermeier T, Kleefuss-Lie AA, Hallmann K, Steffens M, Gaus V, Klein KM, Hamer HM, Rosenow F, Brilstra EH, Trenité DK, Swinkels ME, Weber YG, Unterberger I, Zimprich F, Urak L, Feucht M, Fuchs K, Møller RS, Hjalgrim H, De Jonghe P, Suls A, Rückert IM, Wichmann HE, Franke A, Schreiber S, Nürnberg P, Elger CE, Lerche H, Stephani U, Koeleman BP, Lindhout D, Eichler EE, Sander T. Recurrent microdeletions at 15q11.2 and 16p13.11 predispose to idiopathic generalized epilepsies. Brain 2010; 133 (Pt 1): 23-32.
9. de Lucas-Cerrillo AM, Maldifassi MC, Arnalich F, Renart J, Atienza G, Serantes R, Cruces J, Sánchez-Pacheco A, Andrés-Mateos E, Montiel C. Function of partially duplicated human α77 nicotinic receptor subunit CHRFAM7A gene: potential implications for the cholinergic anti-inflammatory response. J Biol Chem 2011; 286: 594-606.
10. Dickinson JA, Kew JN, Wonnacott S. Presynaptic alpha 7- and beta 2-containing nicotinic acetylcholine receptors modulate excitatory amino acid release from rat prefrontal cortex nerve terminals via distinct cellular mechanisms. Mol Pharmacol 2008; 74: 348-359.
11. Dorszewska J, Florczak J, Rozycka A, Jaroszewska-Kolecka J, Trzeciak WH, Kozubski W. Polymorphisms of the CHRNA4 gene encoding the α subunit of nicotinic acetylcholine receptor as related to the oxidative DNA damage and the level of apoptotic proteins in lymphocytes of the patients with Alzheimer's disease. DNA Cell Biol 2005; 24: 786-794.
12. Drisdel RC, Green WN. Neuronal alpha-bungarotoxin receptors are alpha7 subunit homomers. J Neurosci 2000; 20: 133-139.
13. Elmslie FV, Rees M, Williamson MP, Kerr M, Kjeldsen MJ, Pang KA, Sundqvist A, Friis ML, Chadwick D, Richens A, Covanis A, Santos M, Arzimanoglou A, Panayiotopoulos CP, Curtis D, Whitehouse WP, Gardiner RM. Genetic mapping of a major susceptibility locus for juvenile myoclonic epilepsy on chromosome 15q. Hum Mol Genet 1997; 6: 1329-1334.
14. Engidawork E, Gulesserian T, Balic N, Cairns N, Lubec G. Changes in nicotinic acetylcholine receptor subunits expression in brain of patients with Down syndrome and Alzheimer's disease. J Neural Transm Suppl 2001; 61: 211-222.
15. Freedman R, Adams CE, Leonard S. The alpha7-nicotinic acetylcholine receptor and the pathology of hippocampal interneurons in schizophrenia. J Chem Neuroanat 2000; 20: 299-306.
16. Gault J, Robinson M, Berger R, Drebing C, Logel J, Hopkins J, Moore T, Jacobs S, Meriwether J, Choi MJ, Kim EJ, Walton K, Buiting K, Davis A, Breese C, Freedman R, Leonard S. Genomic organization and partial duplication of the human α7 neuronal nicotinic acetylcholine receptor gene. Genomics 1998; 52: 173-185.
17. Gotti C, Fornasari D, Clementi F. Human neuronal nicotinic receptors. Prog Neurobiol 1997; 53: 199-237.
18. Helbig I, Mefford HC, Sharp AJ, Guipponi M, Fichera M, Franke A, Muhle H, de Kovel C, Baker C, von Spiczak S, Kron KL, Steinich I, Kleefuss-Lie AA, Leu C, Gaus V, Schmitz B, Klein KM, Reif PS, Rosenow F, Weber Y, Lerche H, Zimprich F, Urak L, Fuchs K, Feucht M, Genton P, Thomas P, Visscher F, de Haan GJ, M?ller RS, Hjalgrim H, Luciano D, Wittig M, Nothnagel M, Elger CE, Nürnberg P, Romano C, Malafosse A, Koeleman BP, Lindhout D, Stephani U, Schreiber S, Eichler EE, Sander T. 15q13.3 microdeletions increase risk of idiopathic generalized epilepsy. Nat Genet 2009; 41: 160-162.
19. Hellstrom-Lindahl E, Mousavi M, Zhang X, Ravid R, Nordberg A. Regional distribution of nicotinic receptor subunit mRNAs in human brain: Comparison between Alzheimer and normal brain. Brain Res Mol Brain Res 1999; 66: 94-103.
20. Hellstrom-Lindahl E, Zhang X, Nordberg A. Expression of nicotinic receptor subunit mRNAs in lymphocytes from normal and patients with Alzheimer disease. Alzheimer Res 1997; 3: 29-36.
21. Hiemke C, Stolp M, Reuss S, Wevers A, Reinhardt S, Maelicke A, Schlegel S, Schroder H. Expression of alpha subunit genes of nicotinic acetylcholine receptors in human lymphocytes. Neurosci Lett 1996; 214: 171-174.
22. Hoda JC, Wanischeck M, Bertrand D, Steinlein OK. Pleiotropic functional effects of the first epilepsy-associated mutation in the human CHRNA2 gene. FEBS Lett 2009; 583: 1599-1604.
23. Hong CJ, Lai IC, Liou LL, Tsai SJ. Association study of the human partially duplicated alpha7 nicotinic acetylcholine receptor genetic variant with bipolar disorder. Neurosci Lett 2004; 355: 69-72.
24. Jallon P, Latour P. Epidemiology of idiopathic generalized epilepsies. Epilepsia 2005; 46 (Suppl 9): 10-14.
25. Kawashima K, Fujii T. The lymphocytic cholinergic system and its contribution to the regulation of immune activity. Life Sci 2003; 74: 675-696.
26. Kimura R, Ushiyama N, Fujii T, Kawashima K. Nicotine-induced Ca^{2+} signaling and down-regulation of nicotinic acetylcholine receptor subunit expression in the CEM human leukemic T-cell line. Life Sci 2003; 72: 2155-2158.
27. Lai IC, Hong CJ, Tsai SJ. Association study of nicotinic-receptor variants and major depressive disorder. J Affect Disord 2001; 66: 79-82.
28. Marchi M, Grilli M. Presynaptic nicotinic receptors modulating neurotransmitter release in the central nervous system: functional interactions with other coexisting receptors. Prog Neurobiol 2010; 92: 105-111.
29. Mc Gehee DS. Molecular diversity of neuronal nicotinic acetylcholine receptors. Ann NY Acad Sci 1999; 868: 565-577.
30. Mulley JC, Scheffer IE, Desai T, Bayly MA, Grinton BE, Vears DF, Berkovic SF, Dibbens LM. Investigation of the 15q13.3 CNV as a genetic modifier for familial epilepsies with variable phenotypes. Epilepsia 2011; 52: 139-142.
31. Neubauer BA, Fiedler B, Himmelein B, Kampfer F, Lassker U, Schwabe G, Spanier I, Tams D, Bretscher C, Moldenhauer K, Kurlemann G, Weise S, Tedroff K, Eeg-Olofsson O, Wadelius C, Stephani U. Centrotemporal spikes in families with rolandic epilepsy: linkage to chromosome 15q14. Neurology 1998; 51: 1608-1612.
32. Norman GJ, Morris JS, Karelina K, Weil ZM, Zhang N, Al-Abed Y, Brothers HM, Wenk GL, Pavlov VA, Tracey KJ, Devries AC. Cardiopulmonary arrest and resuscitation disrupts cholinergic anti-inflammatory processes: a role for cholinergic α7 nicotinic receptors. J Neurosci 2011; 31: 3446-3452.
33. Paterson D, Nordberg A. Neuronal nicotinic receptors in the human brain. Prog Neurobiol 2000; 61: 75-111.

34. Perl O, Strous RD, Dranikov A, Chen R, Fuchs S. Low levels of alpha7-nicotinic acetylcholine receptor mRNA on peripheral blood lymphocytes in schizophrenia and its association with illness severity. Neuropsychobiology 2006; 53: 88-93.
35. Phillips HA, Favre I, Kirkpatrick M, Zuberi SM, Goudie D, Heron SE, Scheffer IE, Sutherland GR, Berkovic SF, Bertrand D, Mulley JC. CHRNB2 is the second acetylcholine receptor subunit associated with autosomal dominant nocturnal frontal lobe epilepsy. Am J Hum Genet 2001; 68: 225-231.
36. Picard F, Bruel D, Servent D, Saba W, Fruchart-Gaillard C, Schollhorn-Peyronneau MA, Roumenov D, Brodtkorb E, Zuberi S, Gambardella A, Steinborn B, Hufnagel A, Valette H, Bottlaender M. Alteration of the in vivo nicotinic receptor density in ADNFLE patients: a PET study. Brain 2006; 129: 2047-2060.
37. Raux G, Bonnet-Brilhault F, Louchart S, Houy E, Gantier R, Levillain D, Allio G, Haouzir S, Petit M, Martinez M, Frebourg T, Thibaut F, Campion D. The 22 bp deletion in exon 6 of the 'alpha 7-like' nicotinic receptor subunit gene is a risk factor for the P50 sensory gating deficit. Mol Psychiatry 2002; 7: 1006-1011.
38. Riley B, Williamson M, Collier D, Wilkie H, Makoff A. A 3-Mb map of a large segmental duplication overlapping the alpha 7-nicotinic acetylcholine receptor gene (CHRNA7) at human 15q13-q14. Genomics 2002; 79: 197-209.
39. Rodrigues-Pinguet N, Jia L, Li M, Figl A, Klassen A, Truong A, Lester HA, Cohen BN. Five ADNFLE mutations reduce the CA^{2+} dependence of the mammalian α4β2 acetylcholine response. J Physiol 2003; 550: 11-26.
40. Rozycka A, Skorupska E, Kostyrko A, Trzeciak WH. Evidence for S284L mutation of the CHRNA4 in a white family with autosomal dominant nocturnal frontal lobe epilepsy. Epilepsia 2003; 44: 1113-1117.
41. Rozycka A, Steinborn B, Trzeciak WH. The $1674^{+11}C>T$ polymorphism of CHRNA4 is associated with juvenile myoclonic epilepsy. Seizure 2009; 18: 601-603.
42. Rubboli F, Court JA, Sala C, Morris C, Chini B, Perry E, Clementi F. Distribution of nicotinic receptors in the human hippocampus and thalamus. Eur J Neurosci 1994; 6: 1596-1604.
43. Sander T, Schulz H, Saar K, Gennaro E, Riggio MC, Bianchi A, Zara F, Luna D, Bulteau C, Kaminska A, Ville D, Cieuta C, Picard F, Prud'homme JF, Bate L, Sundquist A, Gardiner RM, Janssen GA, de Haan GJ, Kasteleijn-Nolst-Trenité DG, Bader A, Lindhout D, Riess O, Wienker TF, Janz D, Reis A. Genome search for susceptibility loci of common idiopathic generalised epilepsies. Hum Mol Genet 2000; 9: 1465-1472.
44. Sato KZ, Fujii T, Watanabe Y, Yamada S, Ando T, Kazuko F, Kawashima K. Diversity of mRNA expression for muscarinic acetylcholine receptor subtypes and neuronal nicotinic acetylcholine receptor subunits in human mononuclear leukocytes and leukemic cell lines. Neurosci Lett 1999; 266: 17-20.
45. Sharp AJ, Mefford HC, Li K, Baker C, Skinner C, Stevenson RE, Schroer RJ, Novara F, De Gregori M, Ciccone R, Broomer A, Casuga I, Wang Y, Xiao C, Barbacioru C, Gimelli G, Bernardina BD, Torniero C, Giorda R, Regan R, Murday V, Mansour S, Fichera M, Castiglia L, Failla P, Ventura M, Jiang Z, Cooper GM, Knight SJ, Romano C, Zuffardi O, Chen C, Schwartz CE, Eichler EE. A recurrent 15q13.3 microdeletion syndrome associated with mental retardation and seizures. Nat Genet 2008; 40: 322-328.
46. Sniezawska A, Dorszewska J, Rozycka A, Przedpelska-Ober E, Lianeri M, Jagodzinski PP, Kozubski W. MTHFR, MTR, and MTHFD1 gene polymorphisms compared to homocysteine and asymmetric dimethylarginine concentrations and their metabolites in epileptic patients treated with antiepileptic drugs. Seizure 2011; 20: 533-540.
47. Spielmann M, Reichelt G, Hertzberg C, Trimborn M, Mundlos S, Horn D, Klopocki E. Homozygous deletion of chromosome 15q13.3 including CHRNA7 causes severe mental retardation, seizures, muscular hypotonia, and the loss of KLF13 and TRPM1 potentially cause macrocytosis and congenital retinal dysfunction in siblings. Eur J Med Genet 2011; 54: 441-445.
48. Steinlein OK, Hoda JC, Bertrand S, Bertrand D. Mutations in familial nocturnal frontal lobe epilepsy might be associated with distinct neurological phenotypes. Seizure 2012; 21: 118-123.
49. Sugaya K, Giacobini E, Chiappinelli VA. Nicotinic acetylcholine receptor subtypes in human frontal cortex: changes in Alzheimer's disease. J Neurosci Res 1990; 27: 349-359.
50. Taske NL, Williamson MP, Makoff A, Bate L, Curtis D, Kerr M, Kjeldsen MJ, Pang KA, Sundqvist A, Friis ML, Chadwick D, Richens A, Covanis A, Santos M, Arzimanoglou A, Panayiotopoulos CP, Whitehouse WP, Rees M, Gardiner RM. Evaluation of the positional candidate gene CHRNA7 at the juvenile myoclonic epilepsy locus (EJM2) on chromosome 15q13-14. Epilepsy Res 2002; 49: 157-172.
51. Villiger Y, Szanto I, Jaconi S, Blanchet C, Buisson B, Krause KH, Bertrand D, Romand JA. Expression of an alpha7 duplicate nicotinic acetylcholine receptor-related protein in human leukocytes. J Neuroimmunol 2002; 126: 86-98.