Association Study of the 2-bp Deletion Polymorphism in Exon 6 of the *CHRFAM7A* Gene with Idiopathic Generalized Epilepsy

Agata Rozycka,¹ Jolanta Dorszewska,² Barbara Steinborn,³ Margarita Lianeri,¹
Anna Winczewska-Wiktor,³ Aleksandra Sniezawska,⁴ Kamila Wisniewska,¹ and Pawel P. Jagodzinski¹

There is evidence of linkage between the 15q13–q14 *locus*, containing the gene encoding the $\alpha 7$ subunit (*CHRNA7*) of the neuronal nicotinic acetylcholine receptor (nAChR) and its partially duplicated isoform (*CHRFAM7A*), and epilepsy. Additionally, a 2-bp deletion polymorphism (c.497–498delTG; rs67158670) in *CHRFAM7A*, resulting in a frame shift and truncation of the protein product, is associated with some neurological diseases. This study was designed to explore the possibility of an association of the c.497–498delTG polymorphism of *CHRFAM7A* with idiopathic generalized epilepsies (IGEs) in Polish children and young patients. The study included 197 IGE patients and 258 unrelated healthy individuals. The frequency of the *CHRFAM7A* c.497–498delTG polymorphism was determined in each group using heteroduplex analysis. An association between the c.497–498delTG polymorphism of *CHRFAM7A* and IGE was evidenced. It was demonstrated that the frequency of the *CHRFAM7A* 2-bp deletion carriers was significantly lower in the IGE patients than in the control group. The observed frequency of 2-bp deletion carriers was high in IGE subjects (64%), but significantly higher in control subjects (76%). Carriers of at least one copy of the –2 bp allele had halved their risk of IGE susceptibility (delTG/delTG and delTG/wild-type *versus* wild-type/wild-type: odds ratio = 0.55; 95% confidence intervals = 0.365–0.827; $p = 0.004$). Moreover, it has been demonstrated that this polymorphic variant is associated with the c.524–12_524–11insGTT variation (rs10649395) in intron 7 of *CHRFAM7A*. Our study substantiates the involvement of the $\alpha 7$ subunit of nAChR in the pathophysiology of IGEs and indicates that the *CHRFAM7A* c.497–498TG deletion or a nearby polymorphism may play a role in the pathogenesis of IGE. Further work should concentrate on ascertaining the exact mechanism of this polymorphism's effect and its relationship with IGE.

Introduction

IDIOPATHIC GENERALIZED EPILEPSIES (IGEs) belong to a group of epilepsies with no apparent structural brain damage or neurological abnormalities, and are mainly due to genetic factors (Perucca, 2001). Patients with IGEs are prone to recurrences of any of the following three seizure types, which occur both in childhood and in adolescence: absence seizures, myoclonic seizures, and primarily generalized tonic-clonic seizures. In keeping with the various types of epileptic seizures, clinical forms of common IGEs may include—childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), juvenile myoclonic epilepsy (JME), and epilepsy with generalized tonic-clonic seizures (EGTCS). However, these types of seizures follow complex inheritance

patterns, and an association among subgroups of IGE syndromes has also been observed.

In most cases, the identification of genes that underlies a predisposition to IGEs is difficult because of the clinical and genetic heterogeneity of this group of epilepsies, and only a few susceptibility genes have been identified so far. Among them, genes encoding neuronal nicotinic acetylcholine receptor (nAChR) subunits have emerged as candidate genes for inherited idiopathic epilepsies. Mutations of the genes coding for the nAChR $\alpha 4$ (*CHRNA4*), $\alpha 2$ (*CHRNA2*), and $\beta 2$ (*CHRN2*) subunits are associated with autosomal dominant nocturnal frontal lobe epilepsy (Phillips *et al.*, 2001; Rozycka *et al.*, 2003; Hoda *et al.*, 2009). Chromosomal regions harboring genes encoding subunits of the nAChRs were also screened for linkage to JME and the tested variants of

¹Department of Biochemistry and Molecular Biology, ²Laboratory of Neurobiology, Department of Neurology, Poznan University of Medical Sciences, Poznan, Poland.

Departments of ³Developmental Neurology, and ⁴Neurology, Poznan University of Medical Sciences, Poznan, Poland.

CHRNA4 conferred genetic susceptibility to this epileptic syndrome (Rozycka *et al.*, 2009). Suggestive evidence for this association has also been presented with regard to the *EJM2* locus (OMIM ID: 604827) within the region on chromosome 15q13-q14, in which the gene encoding the $\alpha 7$ subunit (*CHRNA7*) is located (Elmslie *et al.*, 1997; Taske *et al.*, 2002). Susceptibility loci for the common idiopathic epilepsies, comprising benign epilepsy of childhood with centrotemporal spikes and IGE subtypes, have also been mapped to the 15q13-q14 region (Neubauer *et al.*, 1998; Sander *et al.*, 2000; Jallon and Latour, 2005).

The human gene encoding the $\alpha 7$ subunit is partially duplicated, with both loci mapping to 15q13-q14, with ~1.5 Mb between the two copies (Riley *et al.*, 2002). Since the two related genes encoding the $\alpha 7$ subunit (*CHRNA7* and *CHRFAM7A*) are present in the human genome, two isoforms of the $\alpha 7$ subunit ($\alpha 7$ and dup $\alpha 7$, respectively) and their corresponding transcripts have been identified. The search for mutations of the $\alpha 7$ subunit gene is complicated, since exons 5 through 10 are duplicated and give rise to five new exons (D'-D-C-B-A) (Gault *et al.*, 1998) that frequently exist in either orientation, comprising a very common polymorphic inversion (Riley *et al.*, 2002; Flomen *et al.*, 2008).

The *CHRNA7* partial duplication is a relatively recent event that is unique to humans, with only *CHRNA7* and not *CHRFAM7A* present in other higher primates (Riley *et al.*, 2000; Flomen *et al.*, 2008). However, in humans, some chromosomes are missing the *CHRFAM7A* locus, and there are a few rare individuals who completely lack a copy of the hybrid gene (Gault *et al.*, 1998; Riley *et al.*, 2002; Flomen *et al.*, 2006; Sinkus *et al.*, 2009). Riley *et al.* (2002) suggested that the *CHRFAM7A* locus is the result of a primary duplication of the full-length *CHRNA7* gene, followed by a nonhomologous deletion of sequences contained in the more centromeric copy of *CHRNA7*. The absence of the *CHRFAM7A* gene on some chromosomes would represent a later deletion of the primary duplication event due to further recombination. An alternative explanation, suggested by Flomen *et al.* (2008), is that the absence of *CHRFAM7A* represents an ancestral sequence that did not undergo duplication of *CHRNA7*. Interestingly, *CHRFAM7A* exists in two orientations with reference to *CHRNA7* (Flomen *et al.*, 2008). Moreover, a large number of polymorphisms have been reported in this chromosomal region, which contains small insertions and deletions, segmental duplications, copy number variants (CNVs), and inverted repeats (Flomen *et al.*, 2008; Sharp *et al.*, 2008; Makoff and Flomen, 2009). Thus, numerous recombination events over time have created the complex and fragmented region observed today.

Recently, a 15q13.3 microdeletion syndrome (OMIM ID: 612001) was identified in 0.2–0.3% of individuals with mental retardation and epilepsy, as well as in schizophrenia, autism, and some other neuropsychiatric disorders (Sharp *et al.*, 2008; Spielmann *et al.*, 2011). The region on 15q13.3 that contains the 1.5-Mb deletion harbors at least seven genes, including the candidate gene for epilepsy, *CHRNA7*. Makoff and Flomen (2009) suggested that the polymorphic inversion of the *CHRFAM7A* gene would be more likely to predispose to the observed microdeletions, including deletion of the *CHRNA7* gene. The recurrent microdeletion at 15q13.3 has also been deemed as one of the genetic risk factors for common IGE syndromes, and was found in 1% of IGE pa-

tients, whereas it was not detected in controls (Helbig *et al.*, 2009). This association was confirmed in several independent IGE samples (Dibbens *et al.*, 2009; Mefford *et al.*, 2010; Muhle *et al.*, 2011). The particularly high expression of *CHRNA7* in the reticular thalamus (Breese *et al.*, 1997) indicates this gene's role in modulating thalamocortical pathways, which participate in the generation of the primarily generalized seizures seen in IGE (Blumenfeld, 2005). However, the underlying genetic alterations remain largely unknown in the vast majority of individuals with IGE (de Kovel *et al.*, 2010; Mulley *et al.*, 2011).

There is a common 2-bp deletion polymorphism (c.497–498delTG; rs67158670), with variability in its frequency between different ethnic groups (Riley *et al.*, 2000; Sinkus *et al.*, 2009). The –2bp allele is present only in the duplicated exon 6 of the *CHRFAM7A* gene and introduces a premature stop codon, causing a shift in the reading frame of the full-length *CHRFAM7A* transcript variant and probably leading to the generation of a nonfunctional protein product (Gault *et al.*, 1998). Another polymorphism, a 3-bp insertion (c.524–12_524–11insGTT; rs10649395), can be found in intron 7 of *CHRFAM7A* near the intron/exon border. This polymorphic variant has been previously demonstrated by Gault *et al.* (2003) to be in total association with the 2-bp deletion. The 3-bp insertion introduces additional pyrimidines into the splice acceptor site for exon 7, possibly increasing site use (Gault *et al.*, 2003).

The 2-bp polymorphism in exon 6 of *CHRFAM7A* is in strong linkage disequilibrium (LD) with the polymorphic inversion of *CHRFAM7A* and surrounding loci (Flomen *et al.*, 2008). On chromosomes containing the 2-bp polymorphism, *CHRFAM7A* almost always occurs in the same orientation as *CHRNA7*, whereas the wild-type (wt) *CHRFAM7A*, which does not contain the 2-bp polymorphism, is almost always in the opposite orientation (Flomen *et al.*, 2008). The *CHRFAM7A* 2-bp polymorphism is more prevalent in Caucasians than in African Americans, suggesting that the inversion of the gene occurred simultaneously or shortly before the 2-bp deletion (Sinkus *et al.*, 2009).

According to Raux *et al.* (2002) and supported by a recent study by Flomen *et al.* (2013), the 2-bp deletion polymorphism is a risk factor for auditory sensory gating deficit, which characterizes the majority of patients with chronic schizophrenia. As has been noted by Flomen *et al.* (2008), association with the 2-bp deletion of schizophrenia endophenotypes might be due to the orientation of the duplcon containing *CHRFAM7A*. Moreover, it has been shown that the presence of the 2-bp deletion in the *CHRFAM7A* gene may influence the risk of developing bipolar (BP) and major depressive (MD) disorders (Lai *et al.*, 2001; Hong *et al.*, 2004). Studies by Araud *et al.* (2011) and de Lucas-Cerrillo *et al.* (2011) have shown that the *CHRFAM7A* further decreases the protein's function when coexpressed with the $\alpha 7$ subunit, and that this could be prevented by the 2-bp deletion. Thus, copy number variation in *CHRFAM7A* and the presence of the 2-bp deletion are likely to be factors when considering the overall activity of $\alpha 7$ nAChR in human subjects. The functional impact of the *CHRNA7* partial duplication and its 2-bp deletion variant on the development of epileptic symptoms is yet to be determined.

The above findings point to the *CHRNA7* and *CHRFAM7A* genes as possibly being involved in the pathogenesis of

IGEs. Therefore, we set out to study the possibility of an association between the -2bp and $+3\text{bp}$ polymorphic variants of the *CHRFAM7A* and common IGE syndromes.

Methods

Subjects

The study group consisted of 455 participants (197 affected individuals and 258 healthy comparison subjects). IGE patients (85 males, 112 females) were collected from the Department of Neurology and Department of Developmental Neurology of the Poznan University of Medical Sciences in Poznan. All patients were Polish Caucasians aged 6–26 years, and fulfilled diagnostic classification criteria for IGE of the International Classification of Epileptic Syndromes (1989). Electroencephalographic (EEG) records were available for all individuals and showed a normal background activity with paroxysmal generalized spike-wave discharges, which represent the EEG hallmark of IGE. Seizure types in these individuals included typical absence seizures, myoclonic seizures, and primary generalized tonic-clonic seizures, all of them occurring at the typical age of onset.

The epilepsy sample comprised the following IGE syndromes: JME ($n=167$), EGTCS ($n=20$), and CAE/JAE ($n=10$). Individuals with a history of major psychiatric disorders or severe intellectual disability were excluded. Healthy blood donors, born in Poland, aged 15–35 and with no history of any epilepsy syndromes and neurological disorders served as controls. Written consent from all adult patients and legal guardians of the children were obtained, and the local Ethic Review Committee approved the study protocol.

Genotyping and sequencing of the *c.497–498delTG* and *c.524–12_524–11insGTT* polymorphisms

DNA used for genotyping of the delTG (rs67158670) and insGTT (rs10649395) polymorphisms was extracted from peripheral blood lymphocytes. To amplify the *CHRFAM7A* fragments harboring these polymorphisms, a common pair of the following primers was designed: TCTTCTGTTTCC ATCACCACACA (forward delTG) and GCTTCTTCCA GCGGTTAGTC (reverse delTG), and GGAGCCCTCGT TAGACAGAA (forward insGTT) and TTCATCTGCTGG GAAATCCT (reverse insGTT). The polymerase chain reaction (PCR) fragments, 226 and 240 bp in length, respectively, were subjected to polyacrylamide gel electrophoresis for

heteroduplex analysis in 10% acrylamide gel (500 V, 100 mA, 25 W for 60 min), and were visualized by silver staining.

The amplification produced a fragment of the wt allele and the polymorphic allele. A single band, and no heteroduplex band, indicated the absence of the delTG and insGTT alleles (Fig. 1). Thus, we have determined the presence or absence of the 2-bp deletion in each sample, but we did not quantify the copy number of the *CHRFAM7A*. This could be performed using TaqMan assay to evaluate the CNV of *CHRFAM7A* in samples without a 2-bp deletion (Flomen *et al.*, 2006). The fact that the primers are not specific for *CHRFAM7A* is the reason that our method cannot distinguish several individuals who have no copies or only one *CHRFAM7A* copy (*CHRFAM7A* null-allele). The result on our gel (Fig. 1) in each case would be the same as in the absence of the 2-bp deletion.

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 Sequencer; Applied Biosystems).

Statistical analysis

Comparison of genotype frequency was performed by the chi-square (χ^2) test in homozygous ($\text{delTG}/\text{delTG}$) or heterozygous (delTG/wt) patients carrying at least one copy of -2bp allele versus wt homozygous patients (wt/wt). The χ^2 test was also used for comparison of genotype frequency in female and/or male patients with epilepsy (EGTCS/CAE/JAE and JME together or JME separately) versus female and/or male healthy blood donors without epilepsy. Odds ratios (ORs) were calculated for all patients with epilepsy or JME separately, as were 95% confidence intervals (95% CI). The p -values ≤ 0.05 were considered significant.

Since the genotyping assay cannot distinguish between individuals with one or two copies of the 2-bp deletion in exon 6 because of high homology between the *CHRNA7* and *CHRFAM7A* genes, it was analyzed as a dominant allele. To overcome the complication resulting from the occasional presence of the *CHRFAM7A* null allele, genotype frequencies were estimated as for the diploid samples.

Results

Among the genotypes found in the control group, there were 197 individuals with the delTG genotype and 61 individuals

FIG. 1. Genotype variants with 2-bp deletion (samples: 2, 3, 4, 7, 8, 9, 12, 14–23) or no copies of the -2bp allele (samples 1, 5, 6, 10, 11, 13) in the *CHRFAM7A* gene. Since the genotyping assay cannot distinguish between individuals with one or two copies of the 2-bp deletion because of high homology between the *CHRNA7* and *CHRFAM7A* genes, heteroduplexes occur whenever at least one -2bp allele is present. Thus, all individuals had two wild-type alleles from *CHRNA7* and up to 2 alleles from *CHRFAM7A*, which may include 0, 1, or 2 2-bp deletion alleles. The amplification product with the 2-bp deletion was determined by the presence of a heteroduplex band, whereas a single 226 bp band and no heteroduplex band indicated the absence of the -2bp allele.

with the wt/wt genotype of *CHRFAM7A*. Among the genotypes found in the IGE group, the delTG allele was present in at least one copy in 126 patients and absent in 71 patients (Table 1). While studying the delTG polymorphism, we identified a 3-bp insertion (c.524-12_524-11insGTT) near the intron/exon border of the 3' end of intron 7. We expanded our analysis and primers to include this polymorphism. The insGTT polymorphic variant was found to be in total association with the delTG polymorphism, such that in all individuals harboring the delTG deletion (IGE patients and control subjects), the insGTT insertion was detected.

We found an association between the 2-bp deletion polymorphism in exon 6 of *CHRFAM7A* and IGE. We demonstrated that the frequency of the delTG genotype in the IGE patients was significantly lower than in the control group. The delTG genotype was present in 64% of subjects with IGE compared to 76% of controls, and wt homozygous genotype frequency was 36% in IGE patients compared to 24% in controls (Table 1). This significant relationship with the absence of the 2-bp deletion in exon 6 of *CHRFAM7A* was found in the entire IGE group ($p=0.004$) as well as in the JME subgroup ($p=0.002$) (Table 1).

The association between the IGE phenotype and the genotypic classification of delTG genotype or wt genotype resulted in significantly different distributions of the two groups of genotypes ($\chi^2=8.34$; $p=0.004$). The corresponding OR (delTG genotype *vs.* wt genotype) was 0.55 (95% CI=0.365–0.827) (Table 1). We then divided each group of subjects (i.e., subjects with epilepsy and controls) according

to gender and compared the two classes of genotypes within each subgroup. However, when analyzed separately, a similar effect was found (Table 1). Whereas the two subgroups were of proportionally similar size, the association between the wt genotype and the female IGE subgroup was statistically significant ($\chi^2=4.13$, $p=0.042$), with a considerable association trend between the wt genotype and the male IGE subgroup ($\chi^2=4.27$, $p=0.039$). The corresponding ORs (delTG genotype *vs.* wt genotype) in each subgroup were OR=0.57 (95% CI=0.327–0.983) and OR=0.53 (95% CI=0.286–0.971), respectively (Table 1).

Discussion

In our study, we demonstrated an association between the c.497–498delTG *CHRFAM7A* polymorphism and IGE. We showed that the absence of the studied 2-bp deletion (resulting in wt genotype) in the *CHRFAM7A* gene constitutes a susceptibility factor to IGEs. The proportion of delTG to wt carriers was higher in the controls as compared to the IGE individuals. Additionally, the proportion of JME subjects without the delTG allele was also increased (even to a greater extent) compared to that found in controls. Despite this, no significant difference in OR values (OR delTG genotype *vs.* wt genotype) was detected among the two subgroups by the χ^2 test for heterogeneity, suggesting that the delTG genotype may be a protective factor against IGEs, whatever the clinical status of the subject.

The 2-bp deletion polymorphism in exon 6, specific to *CHRFAM7A*, is very common in the human genome,

TABLE 1. DIFFERENCE IN THE GENOTYPE DISTRIBUTION OF THE *CHRFAM7A* c.497–498delTG POLYMORPHISM IN IDIOPATHIC GENERALIZED EPILEPSY PATIENTS AND CONTROL SUBJECTS

Gender	n	Genotype distribution absolute number (frequency) ^a		Genotype OR (95% CI) ^b delTG/delTG + delTG/wt <i>vs.</i> wt/wt
		wt genotype	delTG genotype	
F + M	w/o epilepsy	61 (0.24)	197 (0.76)	
	Total 258			
	w epilepsy	71 (0.36)	126 (0.64)	0.55 (0.365–0.827)
	Total 197			$p=0.004$; $df=1$; $\chi^2=8.34$
	EGTCS/CAE/JAE	8	22	
	JME	63 (0.38)	104 (0.62)	0.51 (0.334–0.782)
				$p=0.002$; $df=1$; $\chi^2=9.73$
	w epilepsy	39 (0.35)	73 (0.65)	0.57 (0.327–0.983)
M	Total 112			$p=0.042$; $df=1$; $\chi^2=4.13$
	EGTCS/CAE/JAE	5	10	
	JME	34 (0.35)	63 (0.65)	0.56 (0.317–0.993)
				$p=0.046$; $df=1$; $\chi^2=3.99$
	w/o epilepsy	28 (0.24)	88 (0.76)	
	Total 116			
	w epilepsy	32 (0.38)	53 (0.62)	0.53 (0.286–0.971)
	Total 85			$p=0.039$; $df=1$; $\chi^2=4.27$
	EGTCS/CAE/JAE	3	12	
	JME	29 (0.41)	41 (0.59)	0.45 (0.238–0.851)
				$p=0.013$; $df=1$; $\chi^2=6.14$

^aPresence of at least one 2-bp deletion, referred to as delTG genotype hereafter, as opposed to wild-type genotype, referred to as wt genotype hereafter.

^bORs were calculated for all patients with epilepsy or JME separately, as well as 95% CI. Comparison of genotype frequency was performed by chi-square (χ^2) test in homozygous (delTG/delTG) or heterozygous (delTG/wt) patients carrying at least one copy of –2 bp allele versus wt homozygous patients (wt/wt).

df, degree of freedom; w epilepsy, with epilepsy; w/o epilepsy, without epilepsy; EGTCS, epilepsy with generalized tonic-clonic seizures; CAE, childhood absence epilepsy; JAE, juvenile absence epilepsy; JME, juvenile myoclonic epilepsy; OR, odds ratios; 95% CI, 95% confidence intervals.

although its frequency varies among different ethnic groups, ranging from 10% in African Bantus up to 60% in Caucasians and 80% in Malays (Riley *et al.*, 2000), while the frequency of *CHRFAM7A* itself also varies among these populations (Sinkus *et al.*, 2009). Studies by Flomen *et al.* (2006) have indicated further variability in the presence of the *CHRFAM7A* null allele with a frequency of about 10%. All of this information indicates that, while commonly present, *CHRFAM7A* is not presented on every human chromosome 15 (Riley *et al.*, 2002). CNV at 15q13.3 contains a duplicon that frequently exists in either orientation, whereas the 2-bp deletion is likely to be part of a haplotype associated with the *CHRFAM7A* inversion. Since the 2-bp deletion polymorphism is a surrogate marker for this inversion polymorphism, the data from our assay make it clear that its frequency is also very high in the Polish population. Genotyping of the 2-bp deletion in healthy Polish subjects of Caucasian origin showed that the combined frequency of genotypes with one or both 2-bp deletion alleles is 76%. If it is assumed that our sample is in Hardy–Weinberg equilibrium, this equates to a 2-bp deletion allele frequency of ~51%, which is higher than that found for Caucasians in other studies (Gault *et al.*, 2003). In an earlier analysis in 34 patients with JME, Taske *et al.* (2002) revealed two patients in whom the *CHRFAM7A* gene was not detected. However, there is a plethora of structural genomic variants in the 15q13.3 region, and lack of identification of the PCR fragment related to the duplication might be due to a variation in the copy number of the distal segmental duplication (Szafranski *et al.*, 2010). As *CHRFAM7A* is located on an ~300 kb duplicon, the *CHRFAM7A* CNV may affect a much larger region than the gene itself (Flomen *et al.*, 2006). Therefore, any gene in this region would also have its copy number altered by this CNV and may potentially play a role in the phenotype. Finally, it is possible that the *CHRFAM7A* CNV is in LD with the functional polymorphism responsible for predisposition to JME. The importance of a proper genomic structure in this region (including the number of *CHRFAM7A* copies) in determining the phenotype of patients with JME remains not fully documented.

Our investigation suggests that the *CHRFAM7A* gene may be one of the candidate genes for IGEs and the lack of a copy of the 2-bp deletion polymorphism may be a susceptibility factor for these epileptic syndromes. A statistically significant ($p=0.004$, χ^2 test) lower frequency of the 2-bp deletion carriers in the IGE patients than in the controls suggests that a protective factor (either genetic or environmental), which increases the carrier status in controls is lacking in IGE subjects. There is considerable evidence implicating the 15q13–q14 region with the *CHRNA7/CHRFAM7A* gene cluster in two idiopathic epilepsies (Elmslie *et al.*, 1997; Neubauer *et al.*, 1998), which supports our results. This chromosomal region has also been implicated in the P50 sensory gating deficit (Freedman *et al.*, 2001). A French group reported an association between the 2-bp deletion and abnormal P50 auditory evoked potentials (Raux *et al.*, 2002), but failed to find an association between the 2-bp deletion and schizophrenia. Such a pattern that showed a strong linkage of this polymorphism to the P50 endophenotype, but only a weak linkage to schizophrenia within the same study, has been recently described by Flomen *et al.* (2013). These results, along with an association of the 2-bp deletion with

poor episodic memory (Dempster *et al.*, 2006), indicate the possibility that the *CHRFAM7A* inversion is connected to some endophenotypes of schizophrenia. Intriguingly, our study directly contradicts the finding of a higher frequency of the 2-bp deletion in the affected individuals, since we observed that the 2-bp deletion is less frequent in IGE patients than in controls, so it seems to provide a protective effect against IGE. This is in contrast to, for example, schizophrenia, where the deletion was found to be more common in patients. However, the association of IGE phenotypes with the presence of a wt *CHRFAM7A* has been evidenced and supports our hypothesis that the link between the *CHRFAM7A* 2-bp deletion polymorphism and IGE exists. We also cannot exclude that the 2-bp deletion might be in LD with the true causative DNA polymorphism, and consequently, either polymorphism linked to 15q13.3 could contribute to the pathogenesis of IGE.

Recent genome-wide scans for CNVs showed that a much rarer CNV at 15q13.3 is overrepresented in the common IGEs. In a population-based cohort study, Helbig *et al.* (2009) identified a 1.5-Mb microdeletion in the region encompassing *CHRNA7* in 12 out of 1,223 individuals with IGE, but not in 3,699 controls, and this association was confirmed in several independent IGE samples (Dibbens *et al.*, 2009; Mefford *et al.*, 2010; Muhle *et al.*, 2011). The 1.5-Mb deletion, which has previously been associated with mental retardation and seizures (Sharp *et al.*, 2008), contains at least seven genes, including *CHRNA7* as a candidate gene for epilepsy. The connection between the 15q13.3 deletion and common IGE symptoms strongly supports an involvement of *CHRNA7* in epileptogenesis. There are now two more well-characterized relatively common recurrent deletion CNVs at 15q11.2 and 16p13.11 associated with susceptibility to epilepsy with complex inheritance (de Kovel *et al.*, 2010), implying that other recurrent microdeletions may also be involved in epileptogenesis. In contrast, Mulley *et al.* (2011) found no evidence for the 15q13.3 microdeletion as a genetic modifier responsible for any of the genetic heterogeneity in a cohort of 151 families affected with epilepsy. Thus, the CNVs may act as modifiers in contributing to the phenotypic heterogeneity seen in families whose pedigrees suggest segregation of a major mutation.

The mechanisms by which loss of the 2-bp deletion in exon 6 of the *CHRFAM7A* confers the risk of IGE remain to be established. Since alternative splicing of *CHRFAM7A* has been observed, both the full-length transcript (NM_139320.1) and a splice variant lacking an internal exon (NM_148911.1) are expressed in the brain and peripheral tissues (Severance and Yolken, 2008; de Lucas-Cerrillo *et al.*, 2011). The N-terminally truncated products predicted by the largest open reading frames for each variant would lack the majority of the ligand binding domain, but they would retain the transmembrane region that forms the ion channel (de Lucas-Cerrillo *et al.*, 2011). The most likely effect of the 2-bp deletion would be to disrupt the reading frame to prevent translation of the full-length dup α 7 protein. However, it is unknown whether *CHRFAM7A* mRNA is translated; this would appear to be a post-translational effect. If translation does occur, dup α 7 may act in a dominant negative manner and disrupt formation of the mature α 7 homopentameric receptor (Raux *et al.*, 2002), which may in turn potentially be prevented by the 2-bp deletion. Thus, dup α 7 can act as a

dominant negative modulator of the $\alpha 7$ subunit's function, and may play a critical role in the downregulation of the receptor's function. The orientation of *CHRFAM7A* on the chromosome may also have functional consequences, as the DNA upstream elements of the likely location of the *CHRFAM7A* promoter would be altered by the inversion. If so, the inversion being in strong LD with the 2-bp deletion polymorphism could affect the expression of *CHRFAM7A* mRNA. This may be important in determining whether wt or inverted copies of *CHRFAM7A* are transcribed. Alternatively, *CHRFAM7A* mRNA expression may modulate *CHRNA7* mRNA expression by competing for transcription factors that bind to a sequence element in the shared part of each gene (Flomen *et al.*, 2008). Although most partially duplicated genes are regarded as nonfunctional, Korneev *et al.* (1999) have demonstrated that a partially duplicated gene may act as a natural antisense and may regulate gene expression in the nervous system. Such a scenario would require transcription of the antisense strand of *CHRFAM7A* to modulate the expression of *CHRNA7*.

Since every IGE and control subject with the 2-bp deletion also had the 3-bp insertion, it is certain that the c.524-12_524-11insGTT polymorphism is located in the *CHRFAM7A* gene as well. This polymorphic variant has been previously demonstrated to be associated with the 2-bp deletion by Gault *et al.* (2003). The 3-bp insertion in intron 7 introduces additional pyrimidines into the splice acceptor site for exon 7 and may lead to elongation of the *CHRFAM7A* transcript by increasing site use. According to the suggestions of Gault *et al.* (2003), exon 6 might be spliced out in the presence of the 2-bp deletion, leaving an exon 5/exon 7 junction, and the splice variant would leave the remainder of the coding sequence intact in the frame. This theoretically would have an interesting implication in view of the data that the 3-bp insertion polymorphism is in LD with the 2-bp deletion variant, and could be an interesting approach for future studies of polymorphism functions and their effect on *CHRFAM7A* transcription. Alternatively, genetic variations, other than the delTG or insGTT genotypes and affecting either *CHRFAM7A* or other genes in biological pathways controlling the IGE phenotype, are likely to be involved. This is consistent with the general view that many genes of small effect contribute to variation in complex traits such as IGE (Lucarini *et al.*, 2007).

In summary, we have determined that the 2-bp deletion in exon 6 of the *CHRFAM7A* gene is associated with IGE in that it appears to be a protective factor that is lacking in IGE subjects. The LD between the 2-bp deletion and 3-bp insertion in the *CHRFAM7A* gene is intriguing and could benefit from functional analysis of the presence and absence of these polymorphisms. Our results continue to support the existence of a locus on chromosome 15q13.3 that confers susceptibility to a broader IGE phenotype. However, the analyzed sequence variant in the positional candidate *CHRFAM7A* gene has a protective effect on IGE risk, which has not been identified so far in other diseases, but the presence of multiple segmental duplications in this region raises the possibility of undetected disease-causing genomic rearrangements. Although the 2-bp deletion polymorphism causes the frame shift in one of the *CHRFAM7A* transcripts, it is not necessarily causal. The *CHRFAM7A* is a fusion gene that may have no function itself. Considering the small sample size of our study, a large-scale association study of

common variants is needed to indicate whether there is some evidence supporting the linkage between the 15q13.3 chromosomal region and IGE.

Acknowledgments

Supported by grant No 502-01-01124182-07474, Poznan University of Medical Sciences. The editorial assistance of Professor Wieslaw H. Trzeciak is gratefully acknowledged.

Disclosure Statement

No competing financial interests exist.

References

- Araud, T., Graw, S., Berger, R., Lee, M., Neveu, E., Bertrand, D., and Leonard, S. (2011). The chimeric gene *CHRFAM7A*, a partial duplication of the *CHRNA7* gene, is a dominant negative regulator of $\alpha 7$ nAChR function. *Biochem Pharmacol* **82**, 904–914.
- Blumenfeld, H. Cellular and network mechanisms of spike-wave seizures. (2005). *Epilepsia* **46**, 21–33.
- Breese, C.R., Adams, C., Logel, J., Drebing, C., Rollins, Y., Barnhart, M., Sullivan, B., Demasters, B.K., Freedman, R., and Leonard, S. (1997). Comparison of the regional expression of nicotinic acetylcholine receptor $\alpha 7$ mRNA and [125I]- α -bungarotoxin binding in human postmortem brain. *J Comp Neurol* **387**, 385–398.
- de Kovel, C.G., Trucks, H., Helbig, I., Mefford, H.C., Baker, C., Leu, C., Kluck, C., Muhle, H., von Spiczak, S., Ostertag, P., Obermeier, T., Kleefuss-Lie, A.A., Hallmann, K., Steffens, M., Gaus, V., Klein, K.M., Hamer, H.M., Rosenow, F., Brilstra, E.H., Trenité, D.K., Swinkels, M.E., Weber, Y.G., Unterberger, I., Zimprich, F., Urak, L., Feucht, M., Fuchs, K., Möller, R.S., Hjalgrim, H., De Jonghe, P., Suls, A., Rückert, I.M., Wichmann, H.E., Franke, A., Schreiber, S., Nürnberg, P., Elger, C.E., Lerche, H., Stephani, U., Koeleman, B.P., Lindhout, D., Eichler, E.E., and Sander, T. (2010). Recurrent microdeletions at 15q11.2 and 16p13.1 predispose to idiopathic generalized epilepsies. *Brain* **133**(Pt1), 23–32.
- de Lucas-Cerrillo, A.M., Maldifassi, M.C., Arnalich, F., Renart, J., Atienza, G., Serantes, R., Cruces, J., Sánchez-Pacheco, A., Andrés-Mateos, E., and Montiel, C. (2011). Function of partially duplicated human $\alpha 7$ nicotinic receptor subunit *CHRFAM7A* gene: potential implications for the cholinergic anti-inflammatory response. *J Biol Chem* **286**, 594–606.
- Dempster, E.L., Touloupoulou, T., McDonald, C., Bramon, E., Walshe, M., Wickham, H., Sham, P.C., Murray, R.M., and Collier, D.A. (2006). Episodic memory performance predicted by the 2bp deletion in exon 6 of the 'alpha 7-like' nicotinic receptor subunit gene. *Am J Psychiatry* **163**, 1832–1834.
- Dibbens, L.M., Mullen, S., Helbig, I., Mefford, H.C., Bayly, M.A., Bellows, S., Leu, C., Trucks, H., Obermeier, T., Wittig, M., Franke, A., Caglayan, H., Yapici Z; EPICURE Consortium, Sander, T., Eichler, E.E., Scheffer, I.E., Mulley, J.C., and Berkovic, S.F. (2009). Familial and sporadic 15q13.3 microdeletions in idiopathic generalized epilepsy: precedent for disorders with complex inheritance. *Hum Mol Genet* **18**, 3626–3631.
- Elmslie, F.V., Rees, M., Williamson, M.P., Kerr, M., Kjeldsen, M.J., Pang, K.A., Sundqvist, A., Friis, M.L., Chadwick, D., Richens, A., Covanis, A., Santos, M., Arzimanoglou, A., Panayiotopoulos, C.P., Curtis, D., Whitehouse, W.P., and Gardiner, R.M. (1997). Genetic mapping of a major susceptibility

- locus for juvenile myoclonic epilepsy on chromosome 15q. *Hum Mol Genet* **6**, 1329–1334.
- Flomen, R.H., Breen, G., Collier, D., St Clair, D., and Makoff, A.J. (2006). Association study of CHRFAM7A copy number and 2bp deletion polymorphisms with schizophrenia and bipolar affective disorder. *Am J Med Genet B Neuropsychiatr Genet* **141**, 571–575.
- Flomen, R.H., Davies, A.F., Di Forti, M., La Cascia, C., Mackie-Ogilvie, C., Murray, R., and Makoff, A.J. (2008). The copy number variant involving part of the alpha7 nicotinic receptor gene contains a polymorphic inversion. *Eur J Hum Genet* **16**, 1364–1371.
- Flomen, R.H., Shaikh, M., Walshe, M., Schulze, K., Hall, M.H., Picchioni, M., Rijdsdijk, F., Toulopoulou, T., Kravariti, E., Murray, R.M., Asherson, P., Makoff, A.J., and Bramon, E. (2013). Association between the 2-bp deletion polymorphism in the duplicated version of the alpha7 nicotinic receptor gene and P50 sensory gating. *Eur J Hum Genet* **21**, 76–81.
- Freedman, R., Leonard, S., Gault, J.M., Hopkins, J., Cloninger, C.R., Kaufmann, C.A., Tsuang, M.T., Farone, S.V., Malaspina, D., Svrakic, D.M., Sanders, A., and Gejman, P. (2001). Linkage disequilibrium for schizophrenia at the chromosome 15q13–14 locus of the alpha7-nicotinic acetylcholine receptor subunit gene (*CHRNA7*). *Am J Med Genet* **105**, 20–22.
- Gault, J., Hopkins, J., Berger, R., Drebing, C., Logel, J., Walton, C., Short, M., Vianzon, R., Olincy, A., Ross, R.G., Adler, L.E., Freedman, R., and Leonard, S. (2003). Comparison of polymorphisms in the alpha7 nicotinic receptor gene and its partial duplication in schizophrenic and control subjects. *Am J Med Genet B Neuropsychiatr Genet* **123B**, 39–49.
- Gault, J., Robinson, M., Berger, R., Drebing, C., Logel, J., Hopkins, J., Moore, T., Jacobs, S., Meriwether, J., Choi, M.J., Kim, E.J., Walton, K., Buiting, K., Davis, A., Breese, C., Freedman, R., and Leonard, S. (1998). Genomic organization and partial duplication of the human $\alpha 7$ neuronal nicotinic acetylcholine receptor gene. *Genomics* **52**, 173–185.
- Helbig, I., Mefford, H.C., Sharp, A.J., Guipponi, M., Fichera, M., Franke, A., Muhle, H., de Kovel, C., Baker, C., von Spiczak, S., Kron, K.L., Steinich, I., Kleefuss-Lie, A.A., Leu, C., Gaus, V., Schmitz, B., Klein, K.M., Reif, P.S., Rosenow, F., Weber, Y., Lerche, H., Zimprich, F., Urak, L., Fuchs, K., Feucht, M., Genton, P., Thomas, P., Visscher, F., de Haan, G.J., Möller, R.S., Hjalgrim, H., Luciano, D., Wittig, M., Nothnagel, M., Elger, C.E., Nürnberg, P., Romano, C., Malafosse, A., Koeleman, B.P., Lindhout, D., Stephani, U., Schreiber, S., Eichler, E.E., and Sander, T. (2009). 15q13.3 microdeletions increase risk of idiopathic generalized epilepsy. *Nat Genet* **41**, 160–162.
- Hoda, J.C., Wanischek, M., Bertrand, D., and Steinlein, O.K. (2009). Pleiotropic functional effects of the first epilepsy-associated mutation in the human *CHRNA2* gene. *FEBS Lett* **583**, 1599–1604.
- Hong, C.J., Lai, I.C., Liou, L.L., and Tsai, S.J. (2004). Association study of the human partially duplicated alpha7 nicotinic acetylcholine receptor genetic variant with bipolar disorder. *Neurosci Lett* **355**, 69–72.
- International Classification of Epileptic Syndromes. (1989). Commission on Classification and Terminology of the International League Against Epilepsy. Proposal for revised classification of epilepsies and epileptic syndromes. *Epilepsia* **30**, 389–399.
- Jallon, P., and Latour, P. (2005). Epidemiology of idiopathic generalized epilepsies. *Epilepsia* **46**, 10–14.
- Korneev, S.A., Park, J.H., and O'Shea, M. (1999). Neuronal expression of neural nitric oxide synthase (nNOS) protein is suppressed by an antisense RNA transcribed from an NOS pseudogene. *J Neurosci* **19**, 7711–7720.
- Lai, I.C., Hong, C.J., and Tsai, S.J. (2001). Association study of nicotinic-receptor variants and major depressive disorder. *J Affect Disord* **66**, 79–82.
- Lucarini, N., Verrotti, A., Napolioni, V., Bosco, G., and Curatolo, P. (2007). Genetic polymorphisms and idiopathic generalized epilepsies. *Pediatr Neurol* **37**, 157–164.
- Mefford, H.C., Muhle, H., Ostertag, P., von Spiczak, S., Buysse, K., Baker, C., Franke, A., Malafosse, A., Genton, P., Thomas, P., Gurnett, C.A., Schreiber, S., Bassuk, A.G., Guipponi, M., Stephani, U., Helbig, I., and Eichler, E.E. (2010). Genome-wide copy number variation in epilepsy: novel susceptibility loci in idiopathic generalized and focal epilepsies. *PLoS Genet* **6**, e1000962.
- Makoff, A., and Flomen, R. (2009). Common inversion polymorphisms and rare microdeletions at 15q13.3. *Eur J Hum Genet* **17**, 149–150 (Letter).
- Muhle, H., Mefford, H.C., Obermeier, T., von Spiczak, S., Eichler, E.E., Stephani, U., Sander, T., and Helbig, I. (2011). Absence seizures with intellectual disability as a phenotype of the 15q13.3 microdeletion syndrome. *Epilepsia* **52**, e194–e198.
- Mulley, J.C., Scheffer, I.E., Desai, T., Bayly, M.A., Grinton, B.E., Vears, D.F., Berkovic, S.F., and Dibbens, L.M. (2011). Investigation of the 15q13.3 CNV as a genetic modifier for familial epilepsies with variable phenotypes. *Epilepsia* **52**, 139–142.
- Neubauer, B.A., Fiedler, B., Himmelein, B., Kampfer, F., Lassker, U., Schwabe, G., Spanier, I., Tams, D., Bretscher, C., Moldenhauer, K., Kurlmann, G., Weise, S., Tedroff, K., Eeg-Olofsson, O., Wadelius, C., and Stephani, U. (1998). Centrottemporal spikes in families with rolandic epilepsy: linkage to chromosome 15q14. *Neurology* **51**, 1608–1612.
- Perucca, E. (2001). The management of refractory idiopathic epilepsies. *Epilepsia* **42**, 31–35.
- Phillips, H.A., Favre, I., Kirkpatrick, M., Zuberi, S.M., Goudie, D., Heron, S.E., Scheffer, I.E., Sutherland, G.R., Berkovic, S.F., Bertrand, D., and Mulley, J.C. (2001). *CHRNA2* is the second acetylcholine receptor subunit associated with autosomal dominant nocturnal frontal lobe epilepsy. *Am J Hum Genet* **68**, 225–231.
- 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., and Campion, D. (2002). The 22bp 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* **7**, 1006–1011.
- Riley, B., Makoff, A., Mogudi-Carter, M., Jenkins, T., Williamson, R., Collier, D., and Murray, R. (2000). Haplotype transmission disequilibrium and evidence for the linkage of the *CHRNA7* gene region to schizophrenia in Southern African Bantu families. *Am J Med Genet* **96**, 196–201.
- Riley, B., Williamson, M., Collier, D., Wilkie, H., and Makoff, A. (2002). A 3-Mb map of a large segmental duplication overlapping the alpha 7-nicotinic acetylcholine receptor gene (*CHRNA7*) at human 15q13-q14. *Genomics* **79**, 197–209.
- Rozycka, A., Skorupska, E., Kostyrko, A., and Trzeciak, W.H. (2003). Evidence for S284L mutation of the *CHRNA4* in a white family with autosomal dominant nocturnal frontal lobe epilepsy. *Epilepsia* **44**, 1113–1117.
- Rozycka, A., Steinborn, B., and Trzeciak, W.H. (2009). The 1674⁺¹¹C>T polymorphism of *CHRNA4* is associated with juvenile myoclonic epilepsy. *Seizure* **18**, 601–603.

- Sander, T., Schulz, H., Saar, K., Gennaro, E., Riggio, M.C., Bianchi, A., Zara, F., Luna, D., Bulteau, C., Kaminska, A., Ville, D., Cieuta, C., Picard, F., Prud'homme JF, Bate, L., Sundquist, A., Gardiner, R.M., Janssen, G.A., de Haan, G.J., Kasteleijn-Nolst-Trenité, D.G., Bader, A., Lindhout, D., Riess, O., Wienker, T.F., Janz, D., and Reis, A. (2000). Genome search for susceptibility loci of common idiopathic generalised epilepsies. *Hum Mol Genet* **9**, 1465–1472.
- Sharp, A.J., Mefford, H.C., Li, K., Baker, C., Skinner, C., Stevenson, R.E., Schroer, R.J., Novara, F., De Gregori, M., Ciccone, R., Broome, A., Casuga, I., Wang, Y., Xiao, C., Barbacioru, C., Gimelli, G., Bernardina, B.D., Torniero, C., Giorda, R., Regan, R., Murday, V., Mansour, S., Fichera, M., Castiglia, L., Failla, P., Ventura, M., Jiang, Z., Cooper, G.M., Knight, S.J., Romano, C., Zuffardi, O., Chen, C., Schwartz, C.E., and Eichler, E.E. (2008). A recurrent 15q13.3 microdeletion syndrome associated with mental retardation and seizures. *Nat Genet* **40**, 322–328.
- Severance, E.G., and Yolken, R.H. (2008). Novel alpha7 nicotinic receptor isoforms and deficient cholinergic transcription in schizophrenia. *Genes Brain Behav* **7**, 37–45.
- Sinkus, M.L., Lee, M.J., Gault, J., Logel, J., Short, M., Freedman, R., Christian, S.L., Lyon, J., and Leonard, S. (2009). A 2-base pair deletion polymorphism in the partial duplication of the alpha7 nicotinic acetylcholine gene (*CHRFAM7A*) on chromosome 15q14 is associated with schizophrenia. *Brain Res* **1291**, 1–11.
- Spielmann, M., Reichelt, G., Hertzberg, C., Trimborn, M., Mundlos, S., Horn, D., and Klopocki, E. (2011). 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* **54**, 441–445.
- Szafranski, P., Schaaf, C.P., Person, R.E., Gibson, I.B., Xia, Z., Mahadevan, S., Wiszniewska, J., Bacino, C.A., Lalani, S., Potocki, L., Kang, S.H., Patel, A., Cheung, S.W., Probst, F.J., Graham, B.H., Shinawi, M., Beaudet, A.L., and Stankiewicz, P. (2010). Structures and molecular mechanisms for common 15q13.3 microduplications involving *CHRNA7*: benign or pathological? *Hum Mutat* **31**, 840–850.
- Taske, N.L., Williamson, M.P., Makoff, A., Bate, L., Curtis, D., Kerr, M., Kjeldsen, M.J., Pang, K.A., Sundqvist, A., Friis, M.L., Chadwick, D., Richens, A., Covanis, A., Santos, M., Arzimanoglou, A., Panayiotopoulos, C.P., Whitehouse, W.P., Rees, M., and Gardiner, R.M. (2002). Evaluation of the positional candidate gene *CHRNA7* at the juvenile myoclonic epilepsy locus (EJM2) on chromosome 15q13–14. *Epilepsy Res* **49**, 157–172.

Address correspondence to:

Agata Rozycka, PhD

Department of Biochemistry and Molecular Biology

Poznan University of Medical Sciences

6 Swieczkiego Street

Poznan 60-781

Poland

E-mail: arozycka@umed.poznan.pl

Received for publication December 31, 2012; received in revised form August 8, 2013; accepted August 12, 2013.