**PRODH mutations and hyperprolinemia in a subset of schizophrenic patients**

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The increased prevalence of schizophrenia among patients with the 22q11 interstitial deletion associated with DiGeorge syndrome has suggested the existence of a susceptibility gene for schizophrenia within the DiGeorge syndrome chromosomal region (DGCR) on 22q11. Screening for genomic rearrangements of 23 genes within or at the boundaries of the DGCR in 63 unrelated schizophrenic patients and 68 unaffected controls, using quantitative multiplex PCR of short fluorescent fragments (QMPSF), led us to identify, in a family including two schizophrenic subjects, a heterozygous deletion of the entire PRODH gene encoding proline dehydrogenase. This deletion was associated with hyperprolinemia in the schizophrenic patients. In addition, two heterozygous PRODH missense mutations (L441P and L289M), detected in 3 of 63 schizophrenic patients but in none among 68 controls, were also associated with increased plasma proline levels. Segregation analysis within the two families harboring respectively the PRODH deletion and the L441P mutation showed that the presence of a second PRODH nucleotide variation resulted in higher levels of prolinemia. In two unrelated patients suffering from severe type I hyperprolinemia with neurological manifestations, we identified a homozygous L441P PRODH mutation, associated with a heterozygous R453C substitution in one patient. These observations demonstrate that type I hyperprolinemia is present in a subset of schizophrenic patients, and suggest that the genetic determinism of type I hyperprolinemia is complex, the severity of hyperprolinemia depending on the nature and number of hits affecting the PRODH locus.

**INTRODUCTION**

The 22q11 deletion, which represents the most frequent interstitial deletion, with an incidence of 1 in 4000 (for a review see 1), has been associated with a wide phenotypic spectrum, including DiGeorge syndrome (DGS; MIM 188400) and the velo-cardio-facial syndrome (VCFS; MIM 192430), characterized by aplasia or hypoplasia of the thymus and the parathyroid glands, conotruncal cardiac defects, typical facial appearance, cleft palate, and learning disabilities. Most DGS and VCFS patients have a common 3 Mb deletion (2). Several lines of evidence (for a review see 3) indicate that a susceptibility gene for schizophrenia is likely to be located on chromosome 22q11: first, there is an increased prevalence of schizophrenia (25–30%) among patients with the 22q11 interstitial deletion (4); second, and reciprocally, an increased prevalence of the 22q11 DiGeorge syndrome chromosomal region (DGCR) deletion (0.3–2%) has been detected by fluorescence in situ hybridization (FISH) in schizophrenic patients (5,6), suggesting that this deletion represents a genetic subtype of schizophrenia (7); third, linkage studies have provided suggestive, although inconclusive, arguments for a schizophrenia locus on chromosome 22q11 (8).

Here we report the identification of a deletion of 350 kb within the DGCR region in a family including two schizophrenic subjects. This deletion, which involves the entire PRODH locus, was associated with hyperprolinemia in the schizophrenic patients. In addition, we identified in three schizophrenic patients two rare PRODH missense mutations that were also associated with high plasma proline levels.

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RESULTS

Genomic rearrangements of the DGCR in schizophrenic patients

We first screened 63 unrelated schizophrenic patients and 68 unaffected controls for a total or partial genomic rearrangement of DGCR. To this end, we used quantitative multiplex PCR of short fluorescent fragments (QMPSF; Fig. 1), a method based on simultaneous amplification of multiple short sequences under quantitative conditions (9–11). A total of 23 genes, including 20 genes that had previously been located within the 3 Mb DGCR region (TUBA8, USP18, PRODH, DGCR5, DGCR2, GSCL, HIRA, NLVCF, UFD1L, PNUTL1, TBX1, GNB1L, COMT, ARVCF, RANBP1, PRODH pseudogene, ZNF74, PIK4CA, SNAP29 and UBE2L3) and 3 genes at the boundaries (CECR1, VPREB1 and BCR), were analyzed. While QMPSF detected heterozygous deletions of the genes located within the DGCR region in 14 DiGeorge patients (Fig. 1B) and revealed that TUBA8, USP18, DGCR5 and UBE2L3 were in fact located outside the DGCR, this pattern was not found in any schizophrenic patient or control subjects. However, QMPSF revealed a heterozygous deletion affecting the PRODH gene in one familial schizophrenia case (Fig. 1C) and a duplication of PRODH (data not shown) in two subjects (one patient and one control). Subsequent QMPSF analyses, covering all 15 exons of PRODH, indicated that the rearrangements found in the three individuals involved the entire gene (see the deletion in Fig. 1D). Additional QMPSF revealed that the heterozygous 22q11 deletion, whose size was estimated to 350 kb, also involved the DGCR6 and DGS-A loci (data not shown).

Effects of PRODH rearrangements on prolinemia levels

The PRODH gene encodes proline dehydrogenase (oxidase), a mitochondrial enzyme that ensures the conversion of proline to Δ1-pyrroline-5-carboxylate (P5C), a rate-limiting step in proline catabolism. Measurement of plasma proline levels revealed that both individuals with a PRODH duplication had normal prolinemia, whereas the schizophrenic patient with the heterozygous deletion had hyperprolinemia (538 µmol/l). The patient’s schizophrenic sister (Fig. 2A: family F1) had the same deletion as well as an increased proline level (338 µmol/l). In contrast, the asymptomatic mother of these two schizophrenic patients, who also carried the deletion, had normal prolinemia (172 µmol/l).

Proline levels and PRODH genotype in DiGeorge patients

The observation of family F1 indicates that a heterozygous deletion of the PRODH gene is not sufficient per se to produce hyperprolinemia, and is reminiscent of the report that only approximately half of DiGeorge patients have hyperprolinemia (12). Indeed, we measured plasma proline levels in nine DiGeorge children, with the large deletion confirmed by QMPSF, and found hyperprolinemia in only six of them (mean 355 µmol/l, range 295–532 µmol/l). Thus, additional factors, either genetic or environmental, modulate proline levels in patients with a heterozygous deletion of the PRODH gene.

We sequenced exons 3–15 of the haploid PRODH allele (Table 1) in these nine DiGeorge patients, and failed to detect any variation in the six patients with high proline levels. Therefore, if a genetic modifying factor is present in these DiGeorge patients, it must lie either within the PRODH non-coding region or at a different locus.

Screening for PRODH nucleotide variations in schizophrenic patients

We next screened schizophrenic patients without detectable heterozygous genomic rearrangement for genetic variations of the PRODH gene. Sequencing of the PRODH coding region in eight schizophrenic patients revealed several DNA nucleotide variations located within different exons. The distribution of these variations was then examined in the 63 schizophrenic patients and 68 controls (Table 2). Two rare mutations (L441P and L289M) were detected in patients but not in controls. The L441P mutation, which was detected only in one schizophrenic proband, affects a highly conserved residue. We analyzed the segregation of this variation in the relatives of the affected proband (Fig. 2B: family F2). In this family, two schizophrenic subjects carried this mutation and had abnormal plasma proline levels (Table 3).

A compound heterozygous mutation L441P/R431H was detected in the relative with the highest level of prolinemia (694 µmol/l). The L289M substitution detected in two schizophrenic patients with mild hyperprolinemia (345 and 377 µmol/l) was found associated, in one patient, with the R431H substitution and, in the other, with the E521R substitution (Tables 2 and 3).

The status of the R453C substitution (Table 2), which also affects a conserved residue, is less clear. In family F1 (Fig. 2A), harbouring the PRODH heterozygous deletion, the presence in trans of the R453C mutation in one patient was associated with a higher prolinemia level (Table 3). In addition, this mutation was found in association with the A455S substitution in another patient with slightly increased prolinemia (312 µmol/l). This mutation was also detected in two controls with normal proline levels, but without additional nucleotide variations (Table 3).

PRODH nucleotide mutations in severe type I hyperprolinemia

To further document the relationship between PRODH mutations and hyperprolinemia, we next genotyped two unrelated children suffering from seizure related to type I hyperprolinemia (13, and unpublished data) with high levels of plasma proline (on average 800 and 1255 µmol/l, respectively). QMPSF detected no genomic rearrangement of the PRODH locus, but sequence analysis led us to identify in both patients a homozygous L441P mutation, associated with a heterozygous R453C mutation in the first case (Table 3). These observations confirm the deleterious effect of the L441P substitution. Furthermore, the clustering in cis of two rare PRODH variations in one patient might contribute to the severity of the phenotype.
Figure 1. Detection of 22q11 genomic deletions by QMPSF. In each panel, the electropherogram of the subject (in red) was superposed on that of a control (in blue). The vertical axis displays fluorescence in arbitrary units and the horizontal axis indicates the size in base pairs. (A), (B) and (C) correspond to a QMPSF covering four genes within DGCR (PRODH, UFD1L, ARVCF and HIRA). (D) corresponds to a QMPSF covering 10 exons of the PRODH gene. The MSH2 amplicon is used to normalize the different electropherograms. (A) Subject without deletion. (B) DiGeorge patient. Heterozygous deletions of the genes can easily be detected by a 50% reduction of the corresponding peak height (indicated by an arrow). (C) and (D) F1 schizophrenic proband with a heterozygous PRODH deletion. In panel (C), the PRODH amplicon corresponds to exon 3. In panel (D), exons 1, 2, 4, 5, 9–12, 14 and 15 of the PRODH gene were analyzed. An exonic fragment specific to the PRODH pseudogene (pPRODH) was included. Exons 9–12 are present both in the PRODH gene and in the pseudogene. Their deletion therefore resulted in a 25% decrease of the fluorescence (dots).
DISCUSSION

Taken together, these findings demonstrate that type I hyperprolinemia (MIM 239500), characterized by a deficiency of proline dehydrogenase activity and usually considered as a benign condition in childhood (14), is present in a subset of schizophrenic patients. Interestingly, homozygous truncating mutation of the PRODH gene in mice results both in hyperprolinemia and in deficit in prepulse inhibition (PPI) of the acoustic startle response—a sensory-gating impairment.
Interestingly, it was recently reported that several relatively mild functional consequences on enzyme activity may carry clusters of several DNA variations, each of them with proline levels was not assessed (18). It will therefore be populations of distinct ethnic origin, but the correlation with the DGCR region, are associated with schizophrenia in several levels are probably not simply homozygotes for a single more complex. Some individuals with abnormal plasma proline (especially moderate hyperprolinemia) could in fact be even suggest that the mode of inheritance of type I hyperprolinemia has been considered so far to be autosomal recessive (14), but some heterozygotes have been shown to have a moderate increase in plasma proline level (17). Our results including three homozygous substitutions. In association with a deletion of the PRODH locus in one patient (Fig. 2: family F1) and with the A455S substitution in a second one. Including two homozygous substitutions. In association with an R431H substitution in one patient and with an E521R substitution in a second one. Including three homozygous substitutions. In addition, two frequent polymorphisms, A167V and R185W, located within exons 4 and 5 respectively, were also detected.

associated in humans with several psychiatric conditions, including schizophrenia (15). However, greater deficit of PPI not associated with hyperprolinemia has been reported in mice bearing a large heterozygous 22q11 deletion, suggesting that another gene modulating the PPI phenotype is located within this chromosomal region (16).

In the present study, which suggests a possible correlation between schizophrenia and alteration of the proline pathway, prolinemia was not systematically assessed in patients and controls. Quantifying precisely the magnitude of the association between hyperprolinemia and schizophrenia will require systematic measurement of plasma proline levels in a large population of patients and controls. These studies, which are underway, require careful assessment of prolinemia under standardized conditions, since plasma proline levels are subject to diurnal variation.

This study also shows that distinct molecular alterations of the PRODH gene (either deletion and/or different missense mutations), resulting in abnormal plasma proline levels, are present in our sample of patients. Inheritance of type I hyperprolinemia has been considered so far to be autosomal recessive (14), but some heterozygotes have been shown to have a moderate increase in plasma proline level (17). Our results suggest that the mode of inheritance of type I hyperprolinemia (especially moderate hyperprolinemia) could in fact be even more complex. Some individuals with abnormal plasma proline levels are probably not simply homozygotes for a single deleterious PRODH mutation or compound heterozygotes, but may carry clusters of several DNA variations, each of them with relatively mild functional consequences on enzyme activity. Interestingly, it was recently reported that several PRODH exon 11 (exon 12 according to our numbering) variations, which probably arise as a result of gene conversion events between the PRODH gene and a non-functional pseudogene located within the DGCR region, are associated with schizophrenia in several populations of distinct ethnic origin, but the correlation with proline levels was not assessed (18). It will therefore be important to determine the functional consequences of the different PRODH genotypes on the plasma proline levels.

The exact link between a defect in the proline pathway and psychiatric phenotypes remains to be characterized. The PRODH enzyme ensures the conversion of proline into Δ1-pyrroline-5-carboxylate (P5C), a precursor of two important neuromediators, glutamate and γ-aminobutyric acid (GABA). A direct neurotoxic effect of L-proline on hippocampal neurons has already been demonstrated (19). In addition, proline potentiates glutamate transmission in rat hippocampal neurons, and may have, at high concentrations, excitotoxic effects leading to cellular losses (20). Interestingly, smaller gray matter volumes in several brain structures including hippocampus, have been reported in schizophrenic patients (for a review see 21). Furthermore, similar abnormalities are present, at least in part, in children with DiGeorge syndrome (22). Whatever the exact mechanism involved, our results suggest that control of prolinemia should be of therapeutic interest in a subset of schizophrenic patients.

### MATERIALS AND METHODS

#### Subjects

After informed consent, 63 Caucasian schizophrenic patients and 68 healthy controls matched for age, sex and ethnicity were included in the study. Familial data were systematically recorded, and affected first-degree relatives were also included in families of probands in which rare PRODH mutations were found. All patients fulfilled the DSMIII criteria for schizophrenia (23). Diagnoses of schizotypal personality in relatives were made using the SSP diagnostic interview (24).

#### Determination of proline levels

Plasma proline levels were determined in the morning, after overnight fasting. All samples were analyzed in the same expert.

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### Table 2. Distribution of PRODH variations in 63 unrelated schizophrenic probands and 68 controls

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Exon</th>
<th>Probands (n=63)</th>
<th>Controls (n=68)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L289M</td>
<td>8</td>
<td>2(^d)</td>
<td>0</td>
</tr>
<tr>
<td>R431H</td>
<td>12</td>
<td>9(^d)</td>
<td>12(^d)</td>
</tr>
<tr>
<td>L441P</td>
<td>12</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>R453C</td>
<td>12</td>
<td>2(^d)</td>
<td>2</td>
</tr>
<tr>
<td>A455S</td>
<td>12</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>A472T</td>
<td>12</td>
<td>5</td>
<td>1(^f)</td>
</tr>
<tr>
<td>E521R</td>
<td>14</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

\(^a\)All substitutions were heterozygous unless otherwise stated.
\(^b\)PRODH exons were numbered according to the alignment of mRNA (GenBank accession no. U79754) with the corresponding genomic contig (GenBank accession no. NT_011519).
\(^c\)In association with an R431H substitution in one patient and with an E521R substitution in a second one.
\(^d\)Including two homozygous substitutions.
\(^e\)Including three homozygous substitutions.

### Table 3. Correlation between PRODH genotypes and plasma proline levels

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Proline level (μmol/l) n &lt; 290</th>
</tr>
</thead>
<tbody>
<tr>
<td>L441P/L441P</td>
<td>1255</td>
</tr>
<tr>
<td>L441P/L441P + R453C</td>
<td>800 (range 413–1745)</td>
</tr>
<tr>
<td>L441P/R431H(^h)</td>
<td>694</td>
</tr>
<tr>
<td>Del/R453C(^e)</td>
<td>538</td>
</tr>
<tr>
<td>L289M/wt; E521R/wtd</td>
<td>377</td>
</tr>
<tr>
<td>L441P/wtb(^f)</td>
<td>360</td>
</tr>
<tr>
<td>L289M/wt; R431H/wtd</td>
<td>345</td>
</tr>
<tr>
<td>Del/wtf</td>
<td>338</td>
</tr>
<tr>
<td>R453C/wt; A455S/wtd</td>
<td>312</td>
</tr>
<tr>
<td>R453C/wtf</td>
<td>221</td>
</tr>
<tr>
<td>R453C/wtf</td>
<td>179</td>
</tr>
<tr>
<td>Del/wtf</td>
<td>172</td>
</tr>
</tbody>
</table>

\(^h\)Proline levels indicated in (13).
\(^f\)Detected in the F2 family.
\(^e\)Detected in the F1 family.
\(^d\)The phase of the two substitutions was not determined.
\(^c\)Detected in a control subject.
laboratory using ion exchange chromatography on a BIONTRONIK LC 3000 system. Subjects were considered as hyperprolinemic if plasma proline levels were above normal values (85–290 μmol/l for adults and 95–235 μmol/l for children, respectively). None of the hyperprolinemic subjects was alcoholic, and all had normal hepatic function.

**QMPSF**

Short exonic fragments (160–310 bp) of CECR1, TUBA18, USP18, PRODH, DGCR5, DGCR2, GSCL, HIRA, NLVCF, UFD1L, PNU1L1, TXB1, GNB1L, COMT, ARVC, RANBP1, PRODH pseudogene, ZNF74, PIK4CA, SNAP29, UBE2L3, VPREB1 and BCR were PCR-amplified in four independent multiplexes. The 15 exons of PRODH were covered using two separate multiplexes. An additional fragment, corresponding to exon 3 of MSH2 located on chromosome 2, was co-amplified, as a control, in each multiplex PCR. The forward primer of each pair was 5′-labeled with the 6-FAM fluorochrome. Sequences of primers used for QMPSF and PCR conditions are available upon request. After electrophoresis for 3 h on an automated sequencer (PE Applied Biosystems, Foster City, CA), data were analyzed using the Gene Scanner Model 672 Fluorescent Fragment Analyzer (PE Applied Biosystems). Electropherograms were superposed on those generated from control DNA by adjusting to the same level the peaks obtained for the control amplicon, and the heights of the corresponding peaks were then compared among the different samples.

**Sequence analysis**

Exons 3–15 of PRODH were PCR-amplified using the primers listed in Table 1. PCR was performed in a final volume of 50 μl containing 100 ng of DNA, 1 μM of each primer, 200 μM of dNTP, 0.5 units of Taq polymerase (Abgene, Epsom, UK), 1.5 mM MgCl₂, 67 mM Tris–HCl pH 8.8, 16 mM (NH₄)₂SO₄ and 0.01% Tween-20. After purification by low-melt agarose gel electrophoresis, PCR products were directly sequenced on a gel electrophoresis, PCR products were directly sequenced on an Applied Biosystems Model 377 automated sequencer. The accession number for the genomic contig containing the PRODH gene is NT_011519.

**REFERENCES**


