Loss of imprinted genes and paternal SUR1 mutations lead to hyperinsulinism in focal adenomatous hyperplasia

J.-C. FOURNET (1, 2), V. VERKARRE (1, 2), P. DE LONLAY (1, 2, 3), J. RAHIER (6), F. BRUNELLE (4), J.-J. ROBERT (3), C. NIHOUL-FÉKETÉ (5), J.-M. SAUDUBRAY (3), C. JUNIEN (1)
1) INSERM UR 383 Hôpital Necker-Enfants Malades, Clinique Maurice Lamy, 149, rue de Sèvres, 75743 Paris Cedex 15, France.
(2) Department of Pathology.
(3) Department of Pediatrics.
(4) Department of Radiology.
(5) Department of Surgery, Hôpital Necker-Enfants Malades, Paris France.
(6) Department of Pathology, University Hospital St-Luc of Louvain, Brussels, Belgium.

SUMMARY - Two types of histopathological lesions, a focal adenomatous hyperplasia of islet cells of the pancreas in about 30% of operated sporadic cases, and a diffuse form can be observed in congenital hyperinsulinism, or Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI). In sporadic focal forms, specific losses of maternal alleles (LOH) of the imprinted chromosomal region 11p15, restricted to the hyperplastic area of the pancreas, were observed. Similar mechanisms are observed in embryonal tumors and in the Beckwith-Wiedemann syndrome which is also associated with neonatal but transient hyperinsulinism. However this region also contains the sulfonylurea receptor (SUR1) gene and the inward rectifying potassium channel subunit (KIR6.2) gene, involved in recessive familial forms of PHHI, but not known to be imprinted. We now report somatic reduction to hemizygosity or homozygosity of a paternal SUR1 constitutional heterozygous mutation, in five patients with a focal form of PHHI. Thus this somatic event (LOH) which leads both to b cell proliferation and to hyperinsulinism can be considered as the somatic equivalent, restricted to a microscopic focal lesion, of constitutional uniparental disomy associated with unmasking of a heterozygous parental mutation leading to a somatic recessive disorder.
INTRODUCTION

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI; OMIM 256450) is a glucose-metabolism disorder characterised by unregulated secretion of insulin and profound hypoglycemia. The incidence of PHHI is 1/50,000 live births (1, 2) in which 95% of the cases are sporadic. Treatment of patients with diazoxide and/or somatostatin analogs is not always effective necessitating an intervention such as pancreatectomy (3). Rare familial forms may be caused by recessive or dominant defects in four different genes: 1) the sulfonylurea receptor gene (SUR1) (4, 5, 6); 2) the inward rectifying potassium channel subunit gene (KIR6.2) (7, 8); 3) the glutamate dehydrogenase gene (GLUD-1) (2) and 4) the glucokinase gene (GK) (9). Two types of histopathological lesions are associated with PHHI despite there being one standard clinical presentation: a focal form, FoPHHI, and a diffuse form, DiPHHI (10-12).

FoPHHI, which represents approximately 30% of operated cases (13), is characterised by focal hyperplasia of islet-like cells, including hypertrophied insulin cells with giant nuclei. In DiPHHI, all the islets of Langerhans throughout the pancreas are irregular in size and contain distinctly hypertrophied insulin cells. These two forms can be distinguished by pancreatic venous sampling (14). Peroperative extemporaneous histological examination is performed to determine whether subtotal or partial pancreatectomy is required (12).

Hyperinsulinemia with hyperplasia of islets of Langerhans may be associated with several overlapping syndromes predisposing to tumors including the Beckwith-Wiedemann syndrome (BWS) (MIM.130650) (15). The 11p15.5 chromosome region involved in BWS contains an imprinted domain including several imprinted genes, characterized by monoallelic expression (16, 17, 18, 19). These include a) four maternally-expressed genes: H19, a candidate tumor suppressor gene; P57KIP2 (20), a negative regulator of cell proliferation (21); KVLQT1, the gene coding for the potassium channel involved in the long QT syndrome (19); and HASH2, a transcription factor (22) and also, b) one paternally-expressed gene: IGF2, the insulin-like growth factor II (17, 23). However some genes, in this domain, including KVLQT1, and the insulin gene, INS, may escape functional imprinting in some tissues or developmental stages (24). Alteration of imprinting in BWS in most embryonal tumors and even in adult tumors can result in unbalanced expression affecting either one gene or the whole imprinted domain by loss of the maternal chromosome or relaxation of genomic imprinting (15, 25).

The intriguing similarity between islet cell hyperplasia and tumorigenesis prompted us to investigate whether the genes in the 11p15 region are involved in sporadic cases of PHHI. As it is possible that hyperplasia of islet-like cell clusters is clonal in origin we searched for loss of alleles in the 11p15 region in fifteen cases of FoPHHI and six cases of DiPHHI. We found that focal islet cell hyperplasia in FoPHHI is associated with the loss of maternal alleles in the 11p15 region (26). In contrast constitutional heterozygosity was retained in six cases with DiPHHI. Similar mechanisms are observed in embryonal tumors and in the Beckwith-Wiedemann syndrome which is also associated with neonatal but transient hyperinsulinism. However the 11p15 chromosomal region also contains the SUR1 and the KIR6.2 genes involved in recessive familial forms but not known to be imprinted. Although the parental bias in loss of maternal alleles did not argue in favor of their direct involvement, the LOH may also unmask a recessive mutation leading to persistent hyperinsulinism. We now report somatic reduction to hemizygosity or homozygosity of a paternal SUR1 constitutional heterozygous mutation, in five patients with a focal form of PHHI. Thus this somatic event which leads both to b cell proliferation and to hyperinsulinism can be considered as the somatic equivalent, restricted to a microscopic focal lesion, of constitutional uniparental disomy associated with unmasking of a heterozygous parental mutation leading to a somatic recessive disorder.

PATIENTS AND METHODS

We studied only patients with neonatal PHHI onset (within 72 hours of birth) who were diazoxide resistant. Patients were screened by selective pancreatic venous sampling (PVS) by transparietal portal catheterisation peroperative surgical examination and analysis of extemporaneous frozen sections to identify cases, nearly 30%, where a focal lesion of the islet cells allowed us to perform partial pancreatectomy (14). A complete relief of hyperinsulinemic
hypoglycemia was obtained and the post-operative plasma glucose levels were strictly normal after a mean follow-up of 33.4 months (21-45 months) after surgery avoiding diabetes. Histological diagnosis of focal adenomatous hyperplasia was performed on extemporaneous frozen sections as described and further confirmed after fixation and paraffin embedding (27). PCR analysis was performed for LOH: 15 microsatellite markers for chromosome 11 were used to genotype paired samples of DNA extracted from frozen samples of normal pancreas and hyperplastic islet cells, leukocytes of patients and their parents. PCR-SSCP and sequence analysis. PCR primer pairs used to amplify coding for the NBF2 (nucleotide binding fold 2) and NBF1 domain of the SUR1 gene were as described previously. PCR and SSCP (Single Strand Conformation Polymorphism) analyses were performed and samples that contained aberrantly migrating fragments were directly sequenced.

RESULTS

Pancreatic histopathology

Twenty one infants with neonatal diazoxide-resistant PHHI undergoing pancreatectomy were investigated. Fifteen cases were FoPHHI and six cases DiPHHI as assessed by selective pancreatic venous sampling coupled with peroperative surgical examination and analysis of extemporaneous frozen sections. Partial or near total pancreatectomy specimens from all patients were analysed. The two forms gave clearly different patterns. The adenomatous hyperplasia or so-called FoPHHI was characterized by focal hyperplasia of some islet-like cells, including hypertrophied insulin secreting cells with giant nuclei. All islets outside the lesion had an apparently normal aspect. In contrast, in the DiPHHI cases all the islets of Langerhans throughout the pancreas were irregular in size and contained distinctly hypertrophied insulin cells. DNA was extracted from acutely sliced frozen samples of hyperplastic (H), and apparently normal (P) pancreas. The focal lesion represented between 50 to 80 % of the cells.

Loss of maternal alleles of imprinted genes in FoPHHI

To examine whether hyperinsulinism was associated with a loss of heterozygosity (LOH) in the 11p15 region, twenty-one infants with sporadic PHHI resistant to diazoxide and who underwent pancreatectomy were investigated. Selective pancreatic venous sampling coupled with peroperative surgical examination and analysis of extemporaneous frozen sections allowed us to identify fifteen cases with FoPHHI and six cases with DiPHHI. Patients with PHHI were genotyped for 15 markers along the short arm of chromosome 11. Paired samples of DNA extracted from frozen sections of normal pancreas and hyperplastic islet cells, and from leukocytes, were investigated. LOH was observed in islet cell hyperplasia samples from all 15 patients with FoPHHI. The same polymorphic loci on 11p were examined for LOH in paired constitutional DNA and abnormal pancreas DNA from six DiPHHI patients. No LOH was detected. Thus unlike FoPHHI, constitutional heterozygosity was consistently maintained in cases of DiPHHI. LOH was often observed as partial losses of alleles. This may have been due to contamination of islet cell hyperplasia samples with exocrine pancreas cells some of which, in spite of careful selection on frozen sections, contaminated the samples used for DNA preparation.

To identify the parental origin of the chromosome lost in FoPHHI cases, the parents were genotyped using polymorphic microsatellite markers covering the whole length of chromosome 11. In all fifteen FoPHHI cases, the allele lost in hyperplastic islet cells was of maternal origin. Since LOH was often observed as partial losses, imbalance between the two alleles (Fo5 and Fo8) rather than total loss (Fo10) allowed us to determine the maternal origin of the allele lost in FoPHHI. To determine the extent of the LOH, we genotyped the DNA from paired samples of normal pancreas or leukocytes and of hyperplastic tissue using 21 microsatellite markers covering the whole length of chromosome 11. Isoallelicism extended over the entire chromosome in three cases (Fo3, Fo4 and Fo5). In four cases (Fo1, Fo2, Fo7 and Fo8), LOH did not extend to the telomere of the long arm. The smallest region of overlap of LOH was restricted to the region from the most telomeric marker, D11S922, to D11S899 in case Fo8. This somatic event is consistent with a proliferative monoclonal lesion. To test whether this LOH only affected chromosome 11 we also genotyped paired DNA samples of normal and hyperplastic tissue from all FoPHHI patients using 8 polymor-
phic markers for different loci or regions, some of which have been implicated in insulinoma. Germline heterozygosity was retained for all these markers. The only exception was the MEN1 locus on 11q13, consistent with the complete loss of maternal chromosome 11 in 5 out of 15 patients.

Detection of heterozygous mutations in the SUR1 paternal allele

The most commonly deleted region contained the SUR1 (28) and KIR6.2 (29) genes that code for two subunits of the K+ATP channel and are involved in familial recessive PHHI (4, 5, 7). To understand whether the LOH may also unmask a recessive phenotype responsible for hypersecretion of insulin we looked for deleterious mutations in the SUR1 gene. As most published mutations were located in the NBF2 (nucleotide binding fold 2) domain, we studied the exons coding for this domain and the exons coding for the NBF1 domain. Using SSCP analysis we detected a mobility shift by electrophoresis in constitutional DNA from leukocytes (L) samples and from normal pancreas (P) samples from five out of twelve patients, Fo1, Fo9, Fo11 and Fo12. In all four cases the mobility shift was also found in leukocyte DNA from their respective fathers (F). In agreement with the loss of maternal alleles, the signal of the mutant band inherited from the father was enhanced in the DNA from the hyperplastic lesion (H) while the signal of the normal wild type band inherited from the mother was greatly diminished. The same pattern was observed for all four patients, Fo1, Fo9, Fo11 and Fo12 for exons 35, 37, 33 and 37 respectively.

Characterization of the SUR1 gene mutations

Mutations in the SUR1 gene displaying such band shifts were identified by nucleotide sequence analysis of PCR products amplified in independent reactions from leukocyte DNA samples. All four patients were constitutionally heterozygous for 3 new missense mutations.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Region</th>
<th>Nucleotide substitution</th>
<th>Codon</th>
<th>Amino acid substitution</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fo11</td>
<td>exon 33</td>
<td>nt 4058 G&gt;C</td>
<td>1353</td>
<td>Arg&gt;Pro</td>
<td>R1353P</td>
</tr>
<tr>
<td>Fo1</td>
<td>exon 35</td>
<td>nt 4291 C&gt;T</td>
<td>1421</td>
<td>Arg&gt;Cys</td>
<td>R1421C</td>
</tr>
<tr>
<td>Fo9 and Fo12</td>
<td>exon 37</td>
<td>nt 4480 C&gt;T</td>
<td>1494</td>
<td>Arg&gt;Try</td>
<td>R1494W</td>
</tr>
</tbody>
</table>

Nucleotide and codon positions are according to the full-length human SUR1 cDNA sequence incorporating the alternative splice form of exon 17 (GenBank accession no L78208, L78224).

In all four cases, the father was also constitutionally heterozygous for the same mutation found in the proband. A total of 212 chromosomes from a population of 106 unrelated individuals were tested for the presence of these newly described mutations. In this population and in other individuals reported in the literature, no such missense mutations were detected, precluding a polymorphism. Furthermore in all four cases an arginine, a basic amino acid, was replaced by a non-polar amino acid such as proline (1352), cysteine (1420), or tryptophan (1493), increasing the likelihood that the mutation would result in a change in the protein conformation. Finally, comparison of homologous sequences of five different members of the ABC (ATP Binding Cassette) protein superfamily revealed that positions 1352, 1420 and 1493 are well-conserved and code for an arginine or another basic amino acid. Taken altogether these data strongly suggest that these missense mutations are deleterious. For the eight remaining patients screening of the remaining exons of the SUR1 gene and of the KIR6.2 gene is underway.

Homozygous SUR1 mutations in DiPHHI

In total, screening for mutations of the NBF2 and NBF1 domains of SUR1 in 94 PHHI patients led to the discovery of 19 mutations: 7/26 in FoPHHI cases, 9/42 in diffuse forms, 3/26 in non operated cases. All mutations were found on the paternal chromosome in FoPHHI patients while in at least two DiPHHI patients both maternal and paternal mutations were identified. Thus unlike FoPHHI, homozygous mutations were found in cases of DiPHHI.
DISCUSSION

We describe for the first time the occurrence of a recessive endocrine disorder which is due to somatic reduction to hemizygosity or homozygosity of a paternally-inherited mutation limited to a focal hyperplastic lesion in four patients. The minimal region of LOH includes candidate genes such as the SUR1 gene and the KIR6.2 gene in 11p15.1 and the imprinted domain H19-IGF2-HASH2-KVLQT1-P57KIP2 in 11p15.5 (fig. 1).

In the focal lesion a loss of maternal alleles therefore resulted simultaneously in two different consequences, hyperinsulinism and proliferation: 1) the reduction to hemi- or homozygosity of the paternal defective allele of the SUR1 gene with loss of the wild-type maternal allele responsible for hyperinsulinism. Hyperinsulinism is the direct consequence of loss of function of the $K^+\text{ATP}$ channel as already shown by patch clamp studies (30). In all cases the father carried the mutant allele therefore excluding the hypothesis of preferential paternal de novo germline mutation; 2) the loss of maternal alleles includes the cluster of imprinted genes in the 11p15.5 region (31) comprising growth factors such as IGF2 (17) and tumor suppressor genes, such as H19 (32) and P57KIP2 (18). This necessarily leads to altered expression of these and other genes in the imprinted domain, suggesting that unbalanced expression of the corresponding gene products may give rise to the increase in proliferation of $b$ cells, a striking feature of focal adenomatous hyperplasia, not observed in the diffuse form (27). Such altered expression of imprinted genes is already well-documented for embryonal tumors and remarkably in Wilms’ tumor. This is consistent with the lesion being a tumorigenic process as suggested by its morphological features.

Thus maternal LOH with somatic reduction to homozygosity of the paternal SUR1 mutation can be considered as the somatic equivalent, restricted to this microscopic focal lesion, of constitutional uniparental disomy (UPD) associated with unmasking of a heterozygous parental mutation leading to a recessive disorder (33). The genetic alteration causing FoPHHI may thus be similar to that causing BWS but is limited to the clonal proliferation originating from one islet cell precursor. In BWS, however, hyperinsulinemia is transient but it is unclear whether the lesions are focal or diffuse. As demonstrated by antibodies against proliferating cell nuclear antigen (PCNA), a nuclear antigen marker for cells in S phase, proliferating cells are present in focal lesions but absent from diffuse lesions (34). There are several examples of abnormal phenotypes associated with UPD which can result both from the presence of imprinted genes on the chromosome involved in this non-Mendelian inheritance mechanism and/or from reduction to homozygosity of a mutation associated with an autosomal recessive disorder (33).

The mechanism involved in FoPHHI is similar to that recently reported for type I autosomal dominant polycystic kidney disease (ADPKD) (35). In contrast to this recurrent loss of 16p in PKD, the somatic
loss of 11p15 probably occurs very rarely in heterozygous individuals as demonstrated by 1) the rarity of relapse after partial pancreas resection; 2) in individuals heterozygous for SUR1 mutations, hyperinsulinism occurs sporadically with no other case in the family while half of the children of the father and possibly other relatives must be carriers of the same mutation in the SUR1 gene. Thus focal forms of PHHI may occur in population with a high incidence of PHHI associated with SUR1 mutations, as Saudi Arabsians, but are probably undiagnosed. However in order to define more precisely the risk of occurrence in these populations, but also the risk of recurrence in families with a sporadic case, a systematic search for the mutation in the proband and in relatives would have to be undertaken, together with a retro- or prospective search for focal lesions.

Despite having similar clinical presentation, an important diagnostic goal is to distinguish patients with focal adenomatous hyperplasia of islet cells from those with the diffuse abnormality because management strategies differ significantly: medical treatment of patients with diazoxide and/or somatostatin analogs is frequently ineffec
tive necessitating a 95% pancreatectomy in diffuse forms but only partial pancreatec
tomy in focal forms, avoiding iatrogenic diabetes (36, 2, 12). According to a mean follow-up of 6 years in twenty-two patients with FoPHHI, the somatic nature of the second hit explains that it is possible to per
torm only partial pancreatectomy limited to the lesion, avoiding the occurrence of a iatrogenic diabetes.

REFERENCES
gnosis value of pancreatic venous sampling corre
15. JUNIEN C. Beckwith-Wiedemann syndrome, tumor
24. YUAN L. QIAN N. TYCKO B. An extended region of bi-allelic gene expression and rodent-human syn


