Somatic Deletion of the Imprinted 11p15 Region in Sporadic Persistent Hyperinsulinemic Hypoglycemia of Infancy Is Specific of Focal Adenomatous Hyperplasia and Endorses Partial Pancreatectomy

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Abstract

Sporadic persistent hyperinsulinemic hypoglycemia of infancy (PHHI) or nesidioblastosis is a heterogeneous disorder characterized by profound hypoglycemia due to inappropriate hypersecretion of insulin. An important diagnostic goal is to distinguish patients with a focal hyperplasia of islet cells of the pancreas (FoPHHI) from those with a diffuse abnormality of islets (DiPHHI) because management strategies differ significantly. 16 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 10 cases with FoPHHI and 6 cases with DiPHHI. We show here that in cases of FoPHHI, but not those of DiPHHI, there was specific loss of maternal alleles of the imprinted chromosome region 11p15 in cells of the hyperplastic area of the pancreas but not in normal pancreatic cells. This somatic event is consistent with a proliferative monoclonal lesion. It involves disruption of the balance between monoallelic expression of several maternally and paternally expressed genes. Thus, we provide the first molecular explanation of the heterogeneity of sporadic forms of PHHI such that it is possible to perform only partial pancreatectomy, limited to the focal somatic lesion, so as to avoid iatrogenic diabetes in patients with focal adenomatous hyperplasia. (*J. Clin. Invest.* 1997. 100:802–807.) Key words: hyperinsulinism • nesidioblastosis • potassium channel • Beckwith-Wiedemann syndrome • loss of alleles

Introduction

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI)[1] (MIM.256450), formerly known as nesidioblastosis, is a glucose-metabolism disorder characterized by dysregulation of insulin secretion and profound hypoglycemia. Prompt recognition and correction of hypoglycemia are necessary to prevent permanent damage to the developing brain (1). Treatment commonly involves use of drugs, including diazoxide and somatostatin analogs, which inhibit insulin secretion (2). Often, however this is not effective and pancreatectomy is required. Two types of histopathological lesions are associated with PHHI despite having similar clinical presentation: a focal form (FoPHHI) and a diffuse form, (DiPHHI) (3–5). FoPHHI, $\sim$ 30% of all cases, is characterized 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, and peroperative extemporaneous histological examination determines whether subtotal or partial pancreatectomy is required (6).

The great majority of PHHI cases (95%) are sporadic with an estimated incidence of 1/50,000 live births, but in countries with substantial inbreeding the incidence may be as high as 1/2,500. In rare familial autosomal recessive forms of PHHI, mutations in the high affinity sulfonylurea receptor (SUR) gene and in the inward rectifying potassium channel subunit (KIR6.2) gene, which are adjacent chromosomal loci in 11p15.1, were identified (7–11). The β cell K^+_{ATP} channel comprises a heterodimeric complex of at least two proteins encoded by the SUR and KIR6.2 genes. Neither of these proteins has K^+_{ATP} channel activity in isolation, but the two proteins together in a number of systems reconstitute K^+_{ATP} channel currents (12). Acutely isolated β cells from Caucasian infants suffering from sporadic PHHI, one with FoPHHI, and four with DiPHHI lack K^+_{ATP} channel activity (13). However, no germline mutations have been found in the SUR or KIR6.2 genes of these sporadic cases. It is likely therefore that loss of K^+_{ATP} channel activity is a common endpoint of a number of different pathological mechanisms, the result of which is loss of normal β cell stimulus secretion coupling. Thus, the origins of heterogeneity of pancreatic β cell morphology in the various forms of sporadic PHHI remain elusive, although the lack of functional K^+_{ATP} channels is a common finding.

Hyperinsulinemia with hyperplasia of islets of Langerhans may be associated with several overlapping syndromes predis-

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1. *Abbreviations used in this paper:* BWS, Beckwith-Wiedemann syndrome; DiPHHI, diffuse form of PHHI; FoPHHI, focal form of PHHI; KIR6.2, potassium channel subunit gene; LOH, loss of heterozygosity; PHHI, persistent hyperinsulinemic hypoglycemia of infancy; SUR, sulfonylurea receptor; TSC, Bourneville tuberous sclerosis.

posing to tumors including the Beckwith-Wiedemann syndrome (BWS) (MIM.130650) (14). The 11p15.5 chromosome region involved in BWS contains an imprinted domain including several imprinted genes, characterized by monoallelic expression. These include (*a*) four maternally expressed genes: H19, a candidate tumor suppressor gene (15, 16); P57KIP2, a potent tight-binding inhibitor of several G1 cyclin/Cdk complexes, and a negative regulator of cell proliferation (17, 18); KVLQT1, the gene coding for the potassium channel involved in the long QT syndrome, which also encompasses five BWS chromosomal rearrangement breakpoints, a balanced translocation from a rhabdoid tumor, and a region conferring tumor supressor activity (19, 20); and (h)ASH2, a transcription factor (21); and also (*b*) one paternally expressed gene, IGF2, the insulinlike growth factor II (22, 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). Thus, genomic imprinting may involve *cis*-acting gene regulation on a scale larger than that of a single gene. Alteration of imprinting in BWS (25, 26), in most embryonal tumors (27–30), and even in adult tumors (31) can result in unbalanced expression affecting either one gene (25) or the whole imprinted domain by loss of the maternal chromosome or relaxation of genomic imprinting (32–38).

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 isletlike cell clusters is clonal in origin, we searched for loss of alleles in the 11p15 region in 10 cases of FoPHHI and 6 cases of DiPHHI. We found that focal islet cell hyperplasia in FoPHHI is associated with the loss of maternal alleles in the 11p15 region. In contrast, constitutional heterozygosity was retained in six cases with DiPHHI.

Methods

Patients. We studied only patients with neonatal PHHI onset (within 72 h of birth) who were diazoxide resistant. We administered 15 mg/kg per d diazoxide in three doses/day for at least 5 d. Diazoxide efficacy was defined as the complete normalization of blood glucose levels (> 3 mmol/liter) measured before and after each meal in patients fed normally, after stopping intravenous glucose and any other medication for at least five consecutive days. Two confirmed glucose levels lower than 2.5 mmol/liter in a 24-h period lead us to consider the patients as unresponsive to diazoxide. Patients were screened by selective pancreatic venous sampling by transparietal portal catheterization (6), 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 with complete relief of hyperinsulinemic hypoglycemia, avoiding diabetes.

PCR analysis. Fresh tissues were frozen in liquid nitrogen and stored at −80°C until DNA isolation. Frozen hyperplastic tissue and normal pancreas were pulverized in a frozen mortar and genomic DNA was extracted as previously described (39). PCR was performed with 16 ng of DNA, standard buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, 1.5 mM $MgCl_2$, 0.1% Triton, and 0.01% gelatin), 10 pmol of each primer, and 30 mM of each dNTP. We used a denaturing step at 95°C for 5 min followed by 26–34 cycles of a denaturation step at 95°C for 15 s, an annealing step at 55°C for 30 s, and an elongation step at 72°C for 1 min. A final extension step at 72°C for 5 min was also performed. The Taq polymerase (GIBCO-BRL, Cergy-Pontoise, France) was added directly to the reaction mixture and amplification was performed using a Gene Amp PCR system 9600 (Perkin Elmer Cetus, Montigny-le-Bretonneux, France). Microsatellite markers for chromosome 11 were from telomere to centromere: D11S922, D11S1758, D11S1760, D11S1338, D11S932, D11S569, D11S1334 in 11p15.5; D11S926 in 11p15.4; D11S1307, D11S899, D11S902, D11S921, D11S928 in 11p15.1; D11S1324 in 11p14; D11S1360 in 11p14 to 11p12; and D11S987, D11S916, D11S901, D11S1365, D11S925, D11S934 in 11q. For other chromosomal loci, the following markers were used: D3S1297, D3S1263, D9S756, D8S277, D8S262, D18S57, D16S407, and D16S405 (40, 41).

1 μl of each amplification product was loaded on a 10% polyacrylamide gel and run at 60 W and the DNA was transferred to a hybond N+ membrane (Amersham France, Les Ulis, France). One of the two primers was labeled using the 3′ terminal extension kit (Boehringer Mannheim, Meylan, France) and used to probe the membrane for hybridization at 42°C for at least 3 h in Amasino solution (42). The membrane was then washed at room temperature for 10 min in 2× SSC, 0.1% SDS, and 5 min in a fresh 2× SSC, 0.1% SDS solution. X-Omat or Biomax films (Eastman-Kodak Co., Marne-la-Vallée, France) were used for autoradiography.

Results

Pancreatic histopathology. 16 infants with neonatal diazoxide-resistant PHHI undergoing pancreatectomy were investigated. 10 cases were FoPHHI and 6 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 analyzed as previously described (5, 43). The two forms gave clearly different patterns. The adenomatous hyperplasia or socalled FoPHHI was characterized by focal hyperplasia of some isletlike cells, including hypertrophied

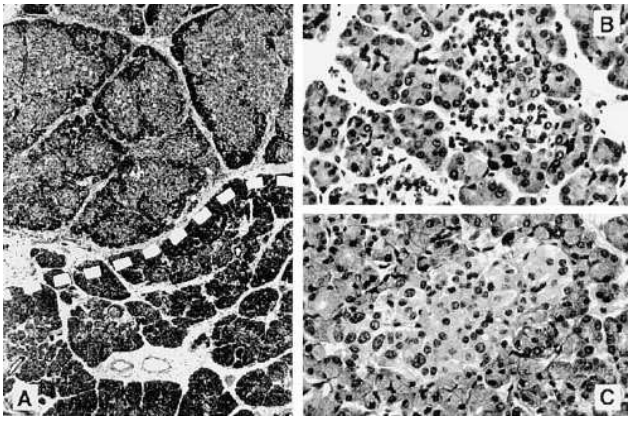

Figure 1. Histopathological findings in PHHI patients with the focal and diffuse types of lesion. (*A*) Focal lesion (FoPHHI), adenomatous hyperplasia (*top*) adjacent to normal pancreatic parenchyma (*bottom*). Toluidin blue, 40×. (*B*) Focal lesion, resting islet outside the tumor. The cytoplasm is difficult to distinguish and there is no nuclear abnormality. Toluidin blue, 400×. (*C*) Diffuse lesion (DiPHHI), all the islets are composed of large β cells with abundant cytoplasm and some have an abnormal nucleus. Numerous β cell nuclei are larger and more prominent than normal. Toluidin blue, 400×. In the FoPHHI cases, the normal (*A, bottom,* and *B*), and focal adenomatous (*A*, *top*) areas were identified histologically on frozen sections of pancreas so that DNA and RNA could be prepared from each area. According to a frozen section, the frozen sample was cut along the dotted line, as illustrated, to isolate the adenomatous hyperplasia from the normal pancreas area.

insulin-secreting cells with giant nuclei (Fig. 1 *A*). All islets outside the lesion had an apparently normal aspect (Fig. 1 *B*). In contrast, in the DiPHHI cases, all the islets of Langerhans throughout the pancreas were irregular in size and contained distinctly hypertrophied insulin cells (Fig. 1 *C*). DNA was extracted from acutely sliced frozen samples of hyperplastic (H), and apparently normal (P) pancreas. The focal lesion represented between 50 and 80% of the cells.

Loss of 11p maternal alleles in FoPHHI. To examine whether hyperinsulinism is associated with a loss of heterozygosity (LOH) in the 11p15 region, the 16 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 10 patients with FoPHHI (Fig. 2). We also genotyped girl twins discordant for FoPHHI (case Fo6). The two girls shared 10 genotypes and are therefore monozygotic twins (data not shown). The DNA from the islet cell hyperplasia samples, normal pancreas, and leukocyte DNA from the affected girl were compared with the DNA from the leukocytes of her normal twin sister and their parents. A loss of chromosome 11 markers was found only in the islet cell hyperplasia sample, evidence of a postzygotic event leading to hyperplasia in the pancreas of only one of the twins. 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 (data not shown). Thus, unlike FoPHHI, constitutional heterozygosity was consistently maintained in cases of DiPHHI. LOH was often observed as partial losses of alleles (Fig. 2). 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 10 FoPHHI cases, the allele lost in hyperplastic islet cells was of maternal origin (Fig. 3). 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. Isoallelism 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 D11S921 in case Fo7 (Fig. 4).

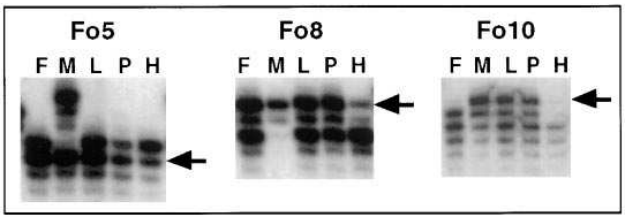

Figure 3. Maternal origin of 11p15 allelic loss in focal hyperplasia of three FoPHHI patients. Representative autoradiographs of the D11S1307 allele distribution in hyperplastic pancreas (*H*), normal DNA from leukocytes (*L*), and normal pancreas (*P*) from the patients Fo5, Fo8, and Fo10, and constitutional DNA of their father (*F*) and mother (*M*). The arrow indicates the maternal allele lost in hyperplastic pancreas. Since focal hyperplasia samples were contaminated by exocrine pancreas, the loss of maternal allele is only partial in Fo5 and Fo8 and almost complete in Fo10.

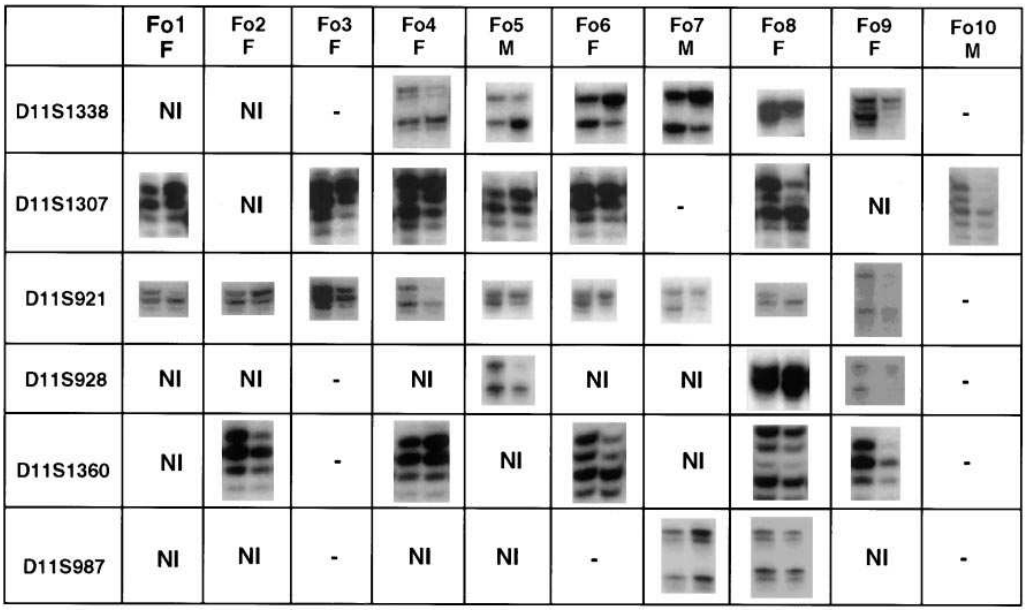

Figure 2. Loss of alleles (LOH) at loci on chromosome 11 in focal hyperplasia of the pancreas. LOH in hyperplasia samples was detected as loss or decrease of the signal intensity of polymorphic microsatellite markers amplified by PCR from pancreas hyperplasia (*right lane*) using normal DNA from normal pancreas as the reference (*left lane*) for each of the 10 FoPHHI patients. NI, not informative; F, female; M, male. Since focal hyperplasia samples were contaminated by exocrine pancreas, imbalance between the paternal and maternal alleles rather than complete loss of one allele was observed in most samples.

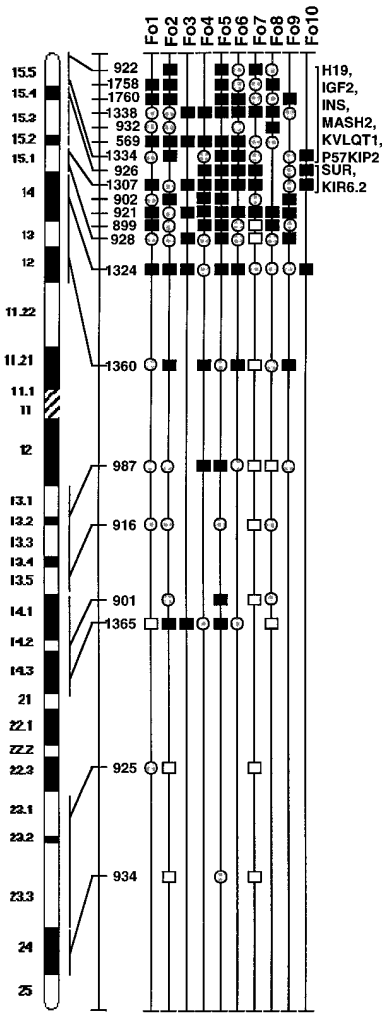

Figure 4. Diagrammatic representation of chromosome 11 LOH in FoPHHI patients. (*open square*), no LOH; (*shaded circle*), non-informative; (*solid square*), LOH.

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 eight polymorphic markers for different loci or regions, some of which have been implicated in insulinoma. They included the von Hippel Lindau locus in 3p25-26 (D3S1297, D31263), the multiple endocrine neoplasia locus in 11q13 (D11S916), the Bourneville tuberous sclerosis (TSC) locus in 9q34 (TSC1) (D9S759), and markers in 16p13 (TSC2) (D16S407, D16S405), in 8pter-p23.1 (D8S277), on chromosome 8 (D8S262), and in 18q12.2-q12.3 (D18S57) (44–46). Germline heterozygosity was retained for all these markers (data not shown). The only exception was the multiple endocrine neoplasia locus on 11q13, consistent with the complete loss of maternal chromosome 11 in 5 of 10 patients.

Discussion

We show that focal hyperplasia of islet cells of the pancreas is a monoclonal proliferative lesion characterized by loss of maternal 11p alleles only in the hyperplastic islet cells in 10/10 cases of FoPHHI. This is consistent with the lesion being a tumorigenic process, as suggested by its morphological features. That this lesion occurred somatically was further demonstrated by LOH in one monozygotic girl twin discordant for FoPHHI. Heterozygosity was maintained at five non-chromosome 11 regions including 3p25 (von Hippel Lindau), 9q34 (TSC1), and 16p13 (TSC2), possibly involved in pancreas hyperplasia (44–46). LOH at the multiple endocrine neoplasia locus and at the WT1 loci was found in four (Fo2, Fo3, Fo4, and Fo5) and six (Fo2, Fo3, Fo4, Fo5, Fo6, and Fo9) FoPHHI cases, respectively. This LOH is probably not specific since it involved markers along the whole length of chromosome 11 in 3 of 10 FoPHHI patients (Fo3, Fo4, and Fo5), suggesting the loss of a complete chromosome 11. This specific bias in maternal LOH associated with FoPHHI contrasts with DiPHHI. We found maintenance of heterozygosity in all six DiPHHI cases studied, providing the first evidence for different molecular causes of these two forms of PHHI.

Thus, FoPHHI is a new example of maternal LOH or uniparental paternal disomy. Previously documented examples include complete and incomplete forms of BWS or hemihypertrophy, nonsyndromic overgrowth associated with malignancy, and several embryonal tumors (14, 26, 47). BWS is sometimes associated with neonatal hyperinsulinemia. 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. We found the LOH to be restricted to the FoPHHI lesion. The LOH must therefore have occurred at a late stage during embryogenesis in a precursor cell common to all the islet cells constituting the hyperplasia. As demonstrated by antibodies against proliferating cell nuclear antigen, a nuclear antigen marker for cells in S phase, proliferating cells are present in focal lesions but absent from diffuse lesions (43).

The minimal region of LOH in 11p15 in FoPHHI patients is between markers D11S922 in 11pter to D11S921 in 11p15.1, as defined in patient Fo7. This region includes candidate genes such as the SUR gene and the KIR6.2 gene in 11p15.1 (7–11) and the imprinted domain H19-IGF2-HASH2-KVLQT1-P57KIP2 in 11p15.5 (15, 18, 19, 22, 24, 28). However, as the SUR and KIR6.2 genes lie in a region not known to be imprinted (between D11S1334 and D11S902), the parental bias in loss of maternal alleles does not argue in favor of their direct involvement. Moreover in familial PHHI, inheritance of recessive mutations in the SUR and KIR6.2 genes, both from the mother and the father, implies that both alleles of the two genes are active. Hyperinsulinemic hypoglycemia may affect only one of the monozygotic twins (49, 50). The case of the twins discordant for FoPHHI, one of which showed a somatic maternal LOH, but only in hyperplastic islet cells, strongly argues against the inheritance of constitutional recessive mutations in the SUR and KIR6.2 genes causing FoPHHI. Although it could be hypothesized that the twins were heterozygotes for a mutation in one of the two genes, reduction to homozygosity in only one of the twins also implies a somatic event.

In contrast, the genes lying in an imprinted domain over 800-kb long, in distal 11p15.5, are more likely candidate genes. IGF2 gene expression and that of H19 are mutually exclusive and methylation of the H19 promoter regulates the expression of the two genes with a parent-specific imprint (51, 52). The maternal LOH at 11p15 associated with FoPHHI could indeed

involve the loss of a maternally expressed allele of either one of the candidate tumor suppressor genes H19 (16) and P57KIP2 or the region conferring tumor suppressor activity within the KVLQT1 gene (17, 20), and/or the duplication of the paternally expressed IGF2 allele, a growth factor that lies between the H19 and P57KIP2 genes (53). Our findings for FoPHHI are reminiscent of recent data provided by an oncogene-induced tumorigenesis model of pancreatic β cells in mice, which supplies further evidence that IGF2 plays a central role in pancreatic tumorigenesis (54, 55). Disruption of imprinting and focal activation of Igf2 in the pancreas of these mice led to the development of pancreatic islet hyperplasia and carcinoma. Presumably, the proliferation of immature structures, with consequent hyperplasia, is a result of the mitogenic and antiapoptotic effects of Igf2 (56, 57). In FoPHHI, the imbalance between IGF2 and H19, rather than the upregulation of IGF2 alone, may therefore be responsible for pancreatic hyperplasia in FoPHHI, as previously proposed in a Wilms' tumor model (30). Analysis of this model led to the suggestion that normal cell growth requires coordinate expression of both H19, a putative tumor suppressor gene, and IGF2, an autocrine growth factor. Abolition of H19 expression and/or upregulation of IGF2 may therefore cause increased cell growth.

There is growing indirect evidence that the insulin gene may be imprinted (58). Thus, the relationship between insulin gene expression and imprint defects associated with PHHI should be investigated. Recently, the KVLQT1 gene, which causes the familial cardiac defect long-QT, was shown to encompasse all five BWS chromosomal rearrangement breakpoints plus one rhabdoid tumor breakpoint and a region conferring tumor suppressor activity (19). Interestingly, KVLQT1 is imprinted, with preferential expression of the maternal allele and with relaxation of imprinting in some normal tissues, predominantly heart. We are currently investigating whether KVLQT1 is expressed and imprinted in normal pancreas, as loss of the maternally expressed KVLQT1 might also lead to focal hyperplasia. Finally, the link between the altered expression of these genes and the lack of functional K^+_{ATP} channels, observed in both forms of PHHI (13), may help decipher the cascade of events in FoPHHI. All 10 cases of FoPHHI were diazoxide resistant, and, as recently shown, diazoxide responsiveness is dependent upon preservation of the COOH terminus of SUR (10).

The relevance of histopathological differences between FoPHHI and DiPHHI has been a matter of debate (3, 5, 6, 59, 60). Our finding of specific loss of 11p15 maternal alleles in FoPHHI clearly indicates different molecular events associated with the two forms. Thus, despite the common clinical presentation of PHHI, these two lesions are clearly two different nosological entities. Differential diagnosis based on catheterism and histopathological examination can avoid complete pancreatectomy and thus iatrogenic diabetes in a large proportion of PHHi patients who can be cured by partial pancreatectomy (6). Moreover, this 11p15 alteration may lead to new biological diagnostic procedures to help in the management of PHHI. Finally, because the risk of recurrence in FoPHHI patients is much lower than in rare cases with a homozygous constitutional mutation in the SUR and KIR6.2 genes, identification of a somatic loss of 11p alleles limited to the resected hyperplasia might be valuable during genetic counseling to reassure parents that the case was sporadic.

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