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Molecular and immunohistochemical analyses of the focal form of congenital hyperinsulinism

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Congenital hyperinsulinism is a rare pancreatic endocrine cell disorder that has been categorized histologically into diffuse and focal forms. In focal hyperinsulinism, the pancreas contains a focus of endocrine cell adenomatous hyperplasia, and the patients have been reported to possess paternally inherited mutations of the *ABCC8* and *KCNJ11* genes, which encode subunits of an ATP-sensitive potassium channel (K_{ATP}). In addition, the hyperplastic endocrine cells show loss of maternal 11p15, where imprinted genes such as *$p57^{kip2}$* reside. In order to evaluate whether all cases of focal hyperinsulinism are caused by this mechanism, 56 pancreatectomy specimens with focal hyperinsulinism were tested for the loss of maternal allele by two methods: immunohistochemistry for $p57^{kip2}$ ($n = 56$) and microsatellite marker analysis ($n = 27$). Additionally, 49 patients were analyzed for K_{ATP} mutations. Out of 56 focal lesions, 48 demonstrated clear loss of $p57^{kip2}$ expression by immunohistochemistry. The other eight lesions similarly showed no nuclear labeling, but the available tissue was not ideal for definitive interpretation. Five of these eight patients had paternal K_{ATP} mutations, of which four demonstrated loss of maternal 11p15 within the lesion by microsatellite marker analysis. All of the other three without a paternal K_{ATP} mutation showed loss of maternal 11p15. K_{ATP} mutation analysis identified 32/49 cases with paternal mutations. There were seven patients with nonmaternal mutations whose paternal DNA material was not available, and one patient with a mutation that was not present in either parent's DNA. These eight patients showed either loss of $p57^{kip2}$ expression or loss of maternal 11p15 region by microsatellite marker analysis, as did the remaining nine patients with no identifiable K_{ATP} coding region mutations. The combined results from the immunohistochemical and molecular methods indicate that maternal 11p15 loss together with paternal K_{ATP} mutation is the predominant causative mechanism of focal hyperinsulinism.
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Infants with congenital hyperinsulinism were once believed to have abnormal pancreatic development associated with persistence of packets of islet cells budding off ducts, termed nesidioblastosis.[1] Observations based on immunohistochemical investigations have shown that nesidioblastosis, as defined above, is a common feature of the pancreas in normoglycemic neonates and infants,[2–4] and nesidioblastosis by itself is not considered the underlying histologic basis of congenital hyperinsulinism. Recent studies on the molecular basis of the disease have disclosed specific genetic defects in the regulation of insulin secretion.[5] Patients with mutations in glucokinase (GK, MIM.138079),[6] glutamate dehydrogenase (GLUD1, MIM.138130),[7] and short-chain L-3-hydroxyacyl coenzyme A dehydrogenase (SAHAD, MIM 601609)[8] usually respond to medical therapy with diazoxide. The most severe form of hyperinsulinism unresponsive to diazoxide is presumed to be associated with defects in a β-cell ATP-sensitive potassium channel, K_{ATP},[9,10] because

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diazoxide suppresses insulin release through its action at the K_{ATP}. The K_{ATP} is a hetero-octamer composed of two subunits encoded by two separate genes, *ABCC8* (formerly *SUR1*, MIM.600509) and *KCNJ11* (formerly *Kir6.2*, MIM.600937) that reside on the same locus of chromosome 11p15.

Two histologically and genetically distinct groups are recognized among patients with K_{ATP} defects, diffuse hyperinsulinism and focal hyperinsulinism.[11] Diffuse hyperinsulinism is characterized by the presence of enlarged islet cell nuclei throughout the pancreas, and is due to recessive mutations in *ABCC8*[12,13] or *KCNJ11*.[14,15] All β cells are functionally abnormal, and infants with diffuse hyperinsulinism require near total pancreatectomy to achieve control over blood sugar levels. Focal hyperinsulinism, on the contrary, can be cured by resection of a focus of adenomatous hyperplasia (Figure 1a and b).[16,17] The lesion is present within an otherwise normal pancreas with islet cell nuclei of normal size.

The focal type of congenital hyperinsulinism has been demonstrated to arise in individuals who have a germline mutation in the paternal allele of *ABCC8* or *KCNJ11*. Focal proliferation of β cells occurs when there is a somatic loss of the maternally derived 11p15 in one of the β cells.[18,19] The acquired event results in expression of the mutated paternal allele, and thereby a functional loss of the K_{ATP}. In the sub-band harboring the *ABCC8* and *KCNJ11* loci, there are imprinted genes whose products are involved in regulation of cell proliferation, such as H19, $p57^{kip2}$, and IGF2.[20–22] H19 and $p57^{kip2}$ are expressed on the maternal allele, whereas IGF2 is expressed on the paternal allele. $p57^{kip2}$ has been shown to lead to cell cycle arrest by acting as an inhibitor of G1 cyclin/cyclin-dependent kinase complexes.[23,24] $p57^{kip2}$ expression is demonstrated in the pancreatic endocrine cells, and the fraction of β cells expressing $p57^{kip2}$ does not vary during development.[25] While dysregulation of insulin secretion by β cells results from the abnormal K_{ATP} activity, it has been hypothesized that the endocrine cell proliferation that characterizes focal hyperinsulinism is due to an imbalance of the imprinted genes located at 11p15.

Screening and sequencing of *ABCC8* and *KCNJ11* genes have identified more than 40 mutations causing congenital hyperinsulinism. No 'hot spots' for mutations are present; most mutations are unique to the respective family, except for two mutations common in the Ashkenazi Jewish population.[15] In the focal form of hyperinsulinism, our results and those of others are consistent and demonstrate that coding region mutations in the paternal allele are identified in 60–70% of the patients.[26] Since K_{ATP} mutations have not always been identified in patients with the histologically focal form, we questioned whether all cases of focal hyperinsulinism are caused by the same mechanism.

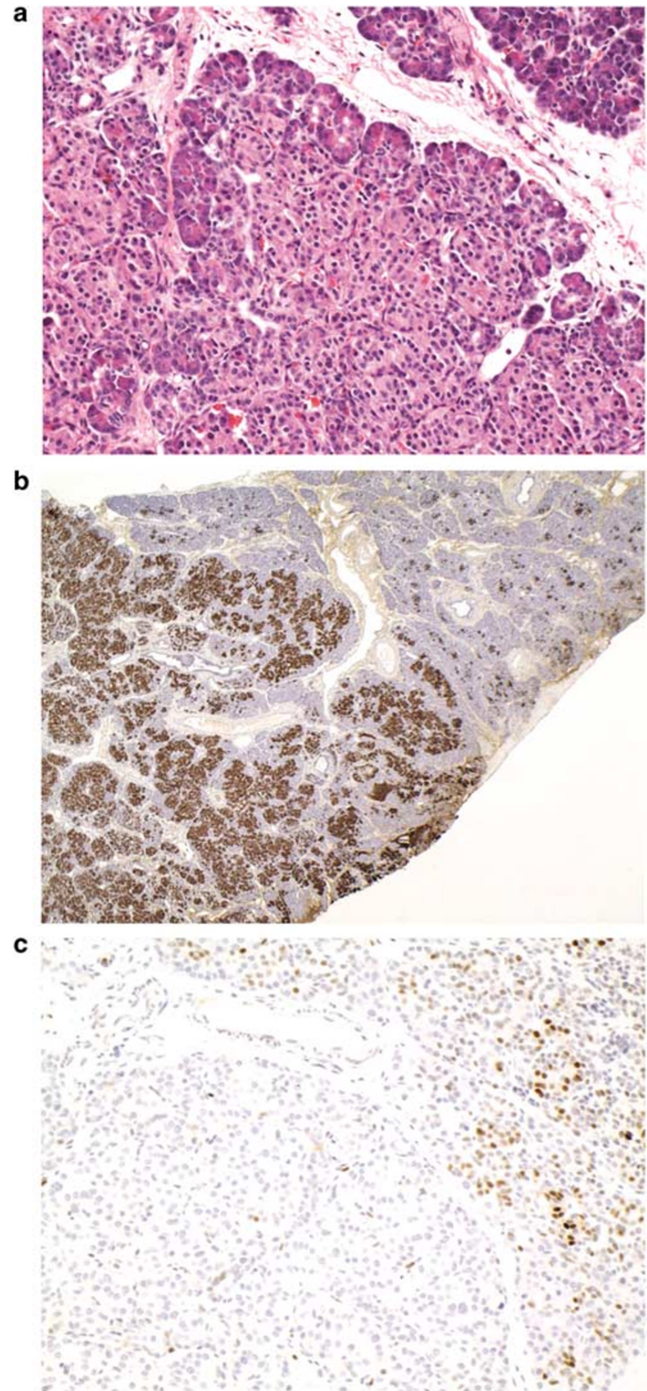

Figure 1 Photomicrographs of the focal form of congenital hyperinsulinism, case no. 19. (**a**) Endocrine cells occupy more than 40% of lobules in a focus of adenomatous hyperplasia (focal hyperinsulinism). Hematoxylin and eosin, original magnification $\times$200. (**b**) Immunohistochemistry for insulin, original magnification $\times$20. (**c**) Immunohistochemistry for $p57^{kip2}$, original magnification $\times$200. Endocrine cell nuclei within the focus of adenomatous hyperplasia are negative for $p57^{kip2}$, while islet cell nuclei in the normal area of the pancreas are positive. A small number of duct epithelial cells and acinar cells also show positive labeling.

In order to answer this question, 56 pancreatectomy specimens with a focal lesion were analyzed. We used immunohistochemistry for $p57^{kip2}$ to

demonstrate loss of gene expression on the maternal allele[27] and microsatellite marker analysis to document directly the loss of maternal 11p15 region in pancreata with focal lesions.

Materials and methods

Patients

A total of 125 children underwent partial or near-total pancreatectomy for treatment of congenital hyperinsulinism at the Children's Hospital of Philadelphia between January 1, 1990 and May 31, 2004. In children who had pancreatectomy after October 1, 1998 (101 cases), intraoperative frozen section analysis was performed to guide the extent of surgery.[17] The pancreatic specimens were subsequently routinely processed, and, using previously described criteria,[28] one of the following diagnoses was assigned: diffuse hyperinsulinism, focal hyperinsulinism, or equivocal (when histologic features did not meet the criteria). In all, 57 patients were identified to have the focal form, but one case was excluded from the study because the focus of adenomatous hyperplasia was exhausted during additional sectioning of the paraffin-embedded tissue. Age at the time of initial surgery ranged from 13 to 431 days (mean = 96 days). Long-term follow-up information (at least 10 months post-surgery) including formal evaluation of fasting adaptation was available for 31 patients. Thirteen patients were cured, 12 were adequately controlled, and six remained hypoglycemic, including four who underwent additional surgery. Terms to describe the outcome (cure and adequate control) have been defined previously.[17] Short-term informal postoperative outcome information was available in 16 patients. Seven patients were cured, eight became medically manageable, but one patient remained hypoglycemic and underwent an additional pancreatic resection. Written informed consent was obtained from parents of the children. The study was approved by the Children's Hospital of Philadelphia institutional review board.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks were sectioned at a thickness of 5 μm and were placed on Superfrost Plus (silanated) slides (Fisher, Pittsburgh, PA, USA). They were heated at 60°C for 20 min, deparaffinized in xylene, and hydrated in a graded series of alcohols. Immunohistochemistry for p57^{kip2} was performed with an avidin–biotin horseradish peroxidase complex system using the Vectastain Elite ABC kit (PK-6100, Vector Laboratories, Burlingame, CA, USA), diaminobenzidine tetrahydrochloride (Dako, Carpinteria, CA, USA), and Dako Autostainer Universal Staining System with hematoxylin as counterstain. Primary mouse monoclonal antibody, p57^{kip2} Ab-6 (Lab Vision Corporation, Fremont, CA, USA), secondary biotinylated anti-guinea-pig IgG (BA700, Vector laboratories, Burlingame, CA, USA), and normal goat serum (S1000, Vector Laboratories, Burlingame, CA, USA) were used. Antigen retrieval was carried out using a microwave oven. Negative controls consisted of substitutions of buffer for the primary antibody. Nuclear labeling of the lesional islet cells and the islet cells in the surrounding normal pancreas was recorded. When the nuclear counter stain by hematoxylin was completely obscured by the reaction (brown color), the reaction was interpreted as strong. When the counter stain could still be seen, the reaction was interpreted as moderate. When there was only faint or equivocal brown color on the nuclei, the reaction was interpreted as weak. Pancreatic tissue obtained from a normoglycemic individual was used to compare the labeling intensity among separate runs. For comparison, immunohistochemistry for p57^{kip2} was also performed on 10 cases with the diffuse form of congenital hyperinsulinism.

K_{ATP} Mutation Analysis and Microsatellite Haplotype Analysis

Genomic DNA from patients and their parents was isolated from 3 ml of peripheral blood using the PUREGENE blood kit (Gentra Systems, Minneapolis, MN, USA). Mutations for *ABCC8* and *KCNJ11* genes were screened and identified by conformation-sensitive gel electrophoresis and by direct sequencing as described previously.[28,29] Paraffin-embedded blocks containing a focus of adenomatous hyperplasia and unaffected pancreas were selected for each case. The tissue was sectioned at a thickness of 4–5 μm, and was placed on Superfrost Plus (silanated) slides (Fisher, Pittsburgh, PA, USA). On average, 10–15 sections from the lesional areas and normal areas were prepared. DNA was extracted from both areas with the PUREGENE Cell and Tissue kit (Gentra Systems, Minneapolis, MN, USA). Patient and parent genomic DNA from leukocytes, patient lesional tissue DNA, and patient normal tissue DNA were amplified for microsatellite markers (D11S909, D11S921, D11S902, and D11S899) using fluorescent labeled forward primers and True Allele PCR Premix (Perkin-Elmer, Boston, MA, USA). All these markers lie on chromosome 11p flanking the *ABCC8/KCNJ11* locus. The primer sequences for these markers were obtained from the Genome Database website (http://www.gdb.org/). Additional 4 base pair (GAAA)*n* and 2 base pair (CA)*n* repeats were chosen from intron 7 and intron 10, respectively, of the *ABCC8* gene. Primer sequences were 5′-GCGACACAGCAAGACTCTG-3′ and 5′-CTCCCTA ACTCCTTTCTTT-3′ for the intron 7 repeat, and 5′-

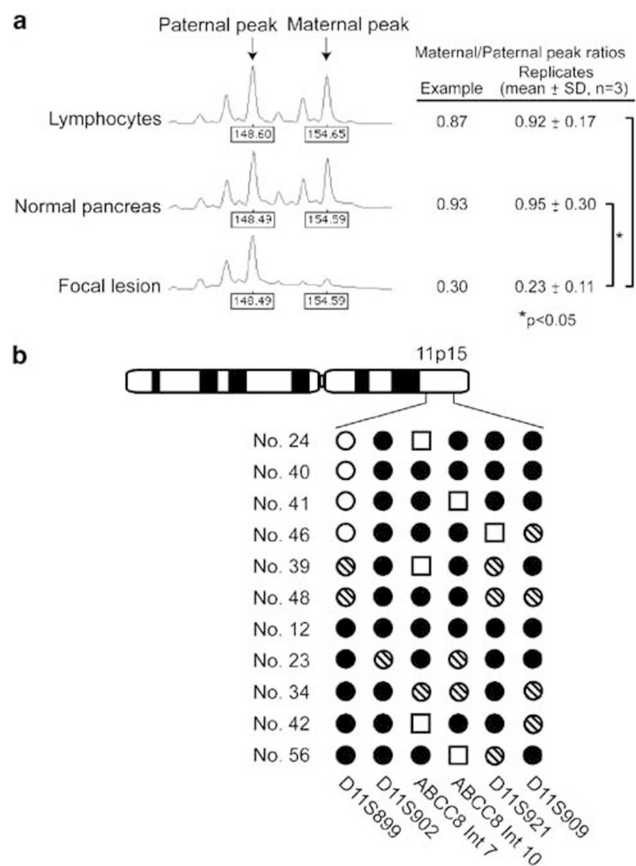

Figure 2 11p15 microsatellite marker analysis. (**a**) Microsatellite marker, *ABCC8* intron 10, patterns in patient no. 19, showing loss of the maternal 154 bp allele in DNA from the focal lesion compared to lymphocytes or adjacent normal pancreas. (**b**) Representative focal lesions showing loss of maternal 11p15, including two cases with no identifiable mutation (nos. 42 and 46) and a case with a *de novo* mutation (no. 40). Solid black circles represent maternal allelic loss. Open circles represent no significant allelic loss. Shaded circles represent uninformative markers. Open squares represent failure of PCR amplification.

GCTTTTGACACAACAGGAAGAT-3′and 5′-GGCTG GGAGAGTAGAATCATCAG-3′ for the intron 10 repeat. The PCR products were run on an ABI 377 sequencer and analyzed using the ABI Prism Genescan3.0 (Perkin-Elmer, Boston, MA, USA) and ABI Prism Genotyper2.1 (Perkin-Elmer, Boston, MA, USA) software to determine the peak sizes and peak areas for the specific marker allele. The ratio of the maternal to paternal peak areas for the lesion was compared to the same ratio for the peaks from the normal pancreatic tissue (or lymphocytes, if normal tissue was limited in quantity), as shown in Figure 2a. Each marker was run at least in triplicate and the ratios for normal and lesional tissue were compared using the Student's *t*-test. The maternal allele was deemed lost when the focal lesion ratio was less than the normal tissue ratio, with $P<0.05$ considered as significant.

Results

Demonstration of Loss of p57^{kip2} Expression by Immunohistochemistry

Pancreatectomy specimens from patients with focal hyperinsulinism usually include a focus of endocrine cell adenomatous hyperplasia surrounded by normal pancreas (Figure 1a and b). This is to achieve clear surgical margins. Immunohistochemistry reaction with p57^{kip2} antibody labeled islet cell nuclei within the area of normal pancreas (Figure 1c). Not all islet cell nuclei labeled strongly, which was expected because p57^{kip2} is an inhibitor of G1 cyclin/cyclin-dependent kinase complexes, and its expression depends on the cell cycle stage in the individual cells. When there were moderately and strongly labeled endocrine cell nuclei in the islets of the normal portion of the pancreas, p57^{kip2} reaction was interpreted as positive ('+' in Table 1). A small number of the pancreatic duct epithelial cells and acinar cells showed moderate and strong nuclear labeling. Some of the foci of endocrine adenomatous hyperplasia had ill-defined borders and were interdigitating. Within such lesions, positively labeled duct epithelial cells and acinar cells were seen between the hyperplastic endocrine cells. In general, the labeling strength was in the moderate range rather than in the strong range in tissue sections that were previously frozen for the purpose of intraoperative analysis.

Out of 56 pancreatic specimens containing a focus of adenomatous hyperplasia, 48 showed clear loss of p57^{kip2} expression within the lesion by immunohistochemistry (Table 1). Endocrine cells within the adenomatous foci lacked the p57^{kip2} labeling that is seen in the islet cell nuclei of the normal surrounding pancreas from the same individual ('+' in Table 1 and Figure 1c). The focal lesions and normal pancreas were present either on a single slide processed in a same cassette (43 specimens), or on separate slides which had been processed simultaneously (five specimens). When only previously frozen, pancreatic tissue used for intraoperative frozen section analysis was available for the adenomatous areas, normal pancreas was also chosen from the blocks of previously frozen tissue (frozen section controls). In the remaining eight pancreatic specimens (nos. 5, 6, 12, 22, 24, 34, 35, and 44), although the endocrine cells forming an adenomatous focus similarly did not display p57^{kip2} nuclear labeling, the results were interpreted as not definitive for one of three reasons: (1) p57^{kip2} labeling in the normal areas was weak, as defined in the Materials and methods, possibly due to prior freezing, (2) there was only a narrow margin of normal tissue around the lesion, and only a small number of p57^{kip2}-positive islet cells were identified, and (3) the lesion itself was too small and crushed to conclude that the nuclei were negative for the p57^{kip2} reaction.

Table 1 *ABCC8* and *KCNJ11* mutations, p57[kip2] expression in pancreatic tissue, and the result of microsatellite marker analysis of 56 patients with the labeling focal form of congenital hyperinsulinism

Patient no.	K_{ATP} *mutations*[a]	*Nuclear labeling of p57*[kip2]		*Microsatellite marker analysis at 11p15*	*Remarks on histology*
		Lesion	*Islets in normal area*		
1	g3992-9a/−	−	+	ND	
2	R1494Q/−	−	+	ND	
3	V21D/−	−	+	ND	
4	g3992-9a/−	−	+	ND	
5	3576 del g/−	Small lesion	+	ND	
6	R74W/−	−	Small normal area and weak	Loss of maternal allele	
7	C717X/−	−	+	Loss of maternal allele	
8	1874 del c/−	−	+	ND	
9	Q954X/−	−	+	ND	
10	g3992-9g/−	−	+	Loss of maternal allele	
11	E501K/−	−	+	Loss of maternal allele	
12	R136L[b]/−	−	Weak	Loss of maternal allele	
13	c2924-9a/−	−	+	Loss of maternal allele	Focal lesion occupies large area of pancreas
14	g3992-9a/−	−	+	ND	
15	3084 del g/−	−	+	ND	
16	R302H[b]/−	−	+	Loss of maternal allele	
17	g3992-9a/−	−	+	ND	
18	536-539 del atgg/−	−	+	ND	
19	R1215W/−	−	+	Loss of maternal allele	
20	R999X/−	−	+	ND	
21	L1350Q/−	−	+	ND	
22	G1401R/−	−	Weak	Loss of maternal allele	
23	g2041-21a/−	−	+	Loss of maternal allele	
24	G7R/−	−	Weak	Loss of maternal allele	
25	g3992-9a/−	−	+	Loss of maternal allele	Rare nonadjacent large islet cell nuclei
26	g3992-9a/−	−	+	ND	
27	Q954X/−	−	+	ND	
28	delF1388/−	−	+	ND	
29	Q472X/−	−	+	ND	
30	G40D[b]/−	−	+	Loss of maternal allele	
31	S116P[b]/−	−	+	ND	
32	g3992-9a/−	−	+	ND	
33	g2116+1t, nonmaternal	−	+	ND	
34	A101D[b], nonmaternal	−	Small normal area	Loss of maternal allele	Focal lesion occupies large area of pancreas
35	F27S, nonmaternal	−	Weak	Loss of maternal allele	
36	G1379R, nonmaternal	−	+	ND	
37	1631 del t, nonmaternal	−	+	ND	
38	R1215W, nonmaternal	−	+	Loss of maternal allele	
39	L503P, nonmaternal	−	+	Loss of maternal allele	
40	F686S, *de novo*	−	+	Loss of maternal allele	
41	1332+4 del c, maternal[c]	−	+	Loss of maternal allele	
42	−/−	−	+	Loss of maternal allele	
43	−/−	−	+	ND	
44	−/−	Small lesion	+	Loss of maternal allele	
45	−/−	−	+	Loss of maternal allele	
46	−/−	−	+	Loss of maternal allele	
47	−/−	−	+	ND	
48	−/−	−	+	Loss of maternal allele	
49	−/−	−	+	ND	
50	ND	−	+	ND	
51	ND	−	+	ND	
52	ND	−	+	Loss of maternal allele	Rare nonadjacent large islet cell nuclei
53	ND	−	+	Loss of maternal allele	Focal lesion occupies large area of pancreas

Table 1 Continued

Patient no.	K_{ATP} mutations[a]	Nuclear labeling of p57[kip2]		Microsatellite marker analysis at 11p15	Remarks on histology
		Lesion	Islets in normal area		
54	ND	–	+	ND	
55	ND	–	+	ND	
56	ND	–	+	Loss of maternal allele	

[a]Nucleotide and codon positions of the *ABCC8* mutations are according to the full-length human *ABCC8* (*SUR1*) cDNA sequence incorporating the alternatively spliced form of exon 17 (Genebank Accession no. L78224). Codon positions of *KCNJ11* mutations are according to the human *KCNJ11* (*Kir6.2*) sequence (Genebank Accession no. D50582). The paternal mutations are listed on left of /, and the maternal on the right, when known.
[b]*KCNJ11* mutations.
[c]See text for detail.
ND: test not done.

All 10 pancreatic specimens studied from patients with diffuse hyperinsulinism did not show loss of p57[kip2] labeling of the islet cell nuclei (data not shown). Among them, five patients had *ABCC8* mutations and one had *KCNJ11* mutations.

Microsatellite Marker Analysis

Haplotyping by microsatellite markers was performed in 27 cases of focal hyperinsulinism. Selective loss of the maternal 11p region was demonstrated in all focal lesions by significant decrease ($P<0.05$) in maternal/paternal peak ratios of the PCR products amplified from the lesional tissue compared to those obtained from the normal pancreas or lymphocytes (Table 1). Peaks for one marker (*ABCC8* intron 10) obtained from case no. 19 and other representative results are shown in Figure 2a and b, respectively.

Among these 27 patients were one with a *de novo* mutation (no. 40), five with no identifiable K_{ATP} mutations (nos. 42, 44, 45, 46, and 48), and three without mutation analysis data (nos. 52, 53, and 56) (see also below). One patient with a maternally inherited *ABCC8* intronic single base deletion (no. 41) also showed maternal loss of the 11p15 region (see Discussion).

K_{ATP} Mutation Analysis

K_{ATP} mutation analysis was conducted in 49 patients. Thirty-two patients had either an *ABCC8* or a *KCNJ11* mutation that was inherited from the father (Table 1). Seven other patients possessed mutations that were not from the mother, but paternal DNA material was not available. One individual (no. 40) was found to have an F686S point mutation which was not identified in either parent. This mutation is considered to have occurred *de novo*. No changes were detected in the coding and flanking regions of *ABCC8* and *KCNJ11* in eight cases screened by conformation-sensitive gel electrophoresis. A nucleotide deletion (1332 + 4 del c) was identified in intron 8 of the maternal *ABCC8* in patient no. 41. The deletion was initially detected by conformation-sensitive gel electrophoresis. These particular abnormal bands were not seen in 50 normoglycemic individuals by the same method (data not shown). Direct sequencing further confirmed the absence of the change in 25 normoglycemic individuals.

Five of the eight cases whose p57[kip2] immunohistochemical results were not definitive as described in the previous section had paternally inherited *ABCC8* mutations (nos. 5, 6, 22, and 24) or a *KCNJ11* mutation (no. 12), and two others had nonmaternal mutations (nos. 34 and 35). The one remaining case had no identifiable coding region mutations (no. 44). Microsatellite marker analysis demonstrated loss of maternal 11p15 in the focal lesion of this patient (see above).

Discussion

Focal hyperinsulinism has been proposed to arise through a two-hit mechanism that includes loss of heterozygosity for the maternal chromosome 11p and reduction to hemizygosity of the paternally derived allele with K_{ATP} channel mutations. Since K_{ATP} mutations are not always identified in patients with histologically focal form of hyperinsulinism, we questioned whether all cases of focal hyperinsulinism result from the same mechanism. In order to answer this question, this study was conducted utilizing morphologic and molecular techniques.

In total, 56 pancreatic specimens were analyzed by immunohistochemistry for the p57[kip2] nuclear protein, which is imprinted with expression from the maternal allele. All but eight focal lesions demonstrated clear loss of p57[kip2]. Nevertheless, we were able to demonstrate that these eight cases without definitive p57[kip2] results had at least one strand of molecular evidence that the lesion arose through the same pathway. Loss of the 11p15 region

was directly documented by microsatellite marker analysis in seven of the eight cases with or without paternal K_{ATP} mutations. For the one remaining patient (no. 5), the focal lesion was so small that no lesional tissue was left in the paraffin block to perform microsatellite marker analysis. This patient, however, did have a paternally inherited K_{ATP} mutation.

Mutation analysis showed that one patient had a *de novo* K_{ATP} mutation, that is, the mutation was not present in either parent's DNA. The focal lesion of this patient showed a clear loss of $p57^{kip2}$ labeling and loss of maternal 11p15 microsatellite markers. There were eight patients in whom we could not identify any K_{ATP} coding and flanking region mutations. Seven of them showed a clear loss of $p57^{kip2}$ expression, and five lesions (including one, no. 44, with a crushed lesion too small to conclude a $p57^{kip2}$ expression loss) demonstrated a significantly reduced peak of the maternal 11p15 markers. These data strongly support the contention that focal islet cell adenomatous proliferation is caused by loss of maternally imprinted genes, even in patients with *de novo* mutations and patients with no identifiable mutations.

One patient (no. 41) revealed to have a single base deletion in intron 8, 4 bp 3′ to the exon/intron junction. No other base changes were identified by direct sequencing of all exons. This single base deletion was initially considered a candidate for the disease-causing mutation as it could alter the splicing mechanism. However, immunohistochemistry for $p57^{kip2}$ and microsatellite marker analysis were both consistent with loss of the maternally derived 11p15, compatible with the usual mechanism for the focal form of congenital hyperinsulinism (ie, loss of the maternal allele, leading to exposure of a paternal disease-causing mutation). As this deletion in intron 8 was not identified in 50 normoglycemic individuals, it may represent a rare sequence variant of uncertain significance. This example is instructive in that results from two separate methods supplemented a result by a third, and the combination of all three modalities was necessary to interpret the alterations.

The sensitivity of conformation-sensitive gel electrophoresis to identify mutations is generally 90%.[30] As there was a small chance that some K_{ATP} mutations escaped identification by this method, PCR products were subsequently directly sequenced for two of eight conformation-sensitive gel electrophoresis-negative patients, disclosing no mutations. Intronic mutations that can alter splicing may not be detected if they reside outside the PCR-amplified flanking region. Another possibility to be considered is that patients may have acquired a somatic mutation in the pancreatic endocrine cells during an earlier stage of development. In this scenario, there would still be a need for a second genetic event to lose a maternally derived allele in order to develop a focal lesion. Mutation analysis of the endocrine cells within the lesion would be necessary to test this hypothesis.

As indicated in Table 1, there were five focal lesions that had unusual histologic features. While the majority of foci of adenomatous hyperplasia are small and measure less than 1.0 cm in diameters,[26] three lesions (nos. 13, 34, and 53) were large and occupied almost the entire pancreas. There were two other cases (nos. 25 and 52) in which rare large islet cell nuclei were present in the pancreas nonadjacent to the focus of adenomatous hyperplasia (focal lesion). However, because some focal lesions have ill-defined borders and proliferating endocrine cells extend processes into the adjacent normal pancreas, it is possible that the large islet cell nuclei in the nonadjacent pancreas are still part of the same lesion. Although the large nuclei thought to be 'outside' the lesion may, indeed, be part of the same lesion, we do not currently know the significance of these large nuclei. Similar cases have been observed in other series.[17,28] Nevertheless, in all of the five focal hyperinsulinism cases with unusual histology, we demonstrated loss of the maternal 11p15 region by microsatellite marker analysis. Therefore, we infer that focal hyperinsulinism with some unusual histologic features are due to the proposed pathway.

In summary, all 56 focal hyperinsulinism patients and their pancreatic islet cell adenomatous lesions analyzed by immunohistochemical and molecular techniques showed at least one piece of evidence consistent with the proposed pathogenesis of the disease, that is, paternal K_{ATP} mutation paired with maternal 11p15 loss. The results indicate that this is the predominant causative mechanism of the focal form of hyperinsulinism.

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