CLINICAL STUDY

Diabetes caused by insulin gene (*INS*) deletion: clinical characteristics of homozygous and heterozygous individuals

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Abstract

Background: Mutations of the preproinsulin gene (*INS*) account for both permanent neonatal diabetes (PND) and adult-onset diabetes. The molecular mechanism of complete *INS* deletion has recently been published and we now add clinical data of homozygous and heterozygous subjects as well as the detailed mapping of the 646 bp deletion of the *INS* gene.

Methods: Location and size of the *INS* deletion was mapped in one case with PND and *INS* genotype of the whole family was further characterized by breakpoint-spanning PCR. The phenotype of monoallelic loss of *INS* was studied in 33 adult family members of a large consanguineous kindred with *INS* deletion.

Results: The 646 bp deletion was found in two individuals with PND that included exons 1 and 2 of the *INS* gene (chr11: g.2138434_2139080del646) and results in loss of approximately half of the preproinsulin protein. The two boys with homozygous *INS* deletion (D/D) presented with reduced birth weight, PND within the first 24 h of life and complete absence of C-peptide. Adult family members with the N/D had diabetes onset with earliest 25 years, while the oldest subject without diabetes was 45 years. *INS*-deletion-diabetes was initially treated with oral antidiabetic drugs but then transferred to insulin within 5–16 years. Overall, N/D-subjects ($n=11$) had a higher risk to develop insulin-dependent diabetes up to the fifth decade, if compared with normal subjects ($n=22$).

Conclusion: Complete loss of the human *INS* gene results in neonatal diabetes, while heterozygous *INS* deletion is a strong risk factor for developing insulin-dependent diabetes at adult age.

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Introduction

Diabetes mellitus is characterized by high blood glucose concentrations accompanied by insulin deficiency or impaired insulin function. Genetic factors that play a role in development of diabetes are increasingly recognized and are now established in diagnostic algorithms of autoantibody-negative diabetes (1, 2). Despite this, most diabetes genes act in a polygenetic fashion and few diabetes forms are caused by alterations of single genes (1). Detection and detailed characterization of these gene defects have improved our understanding of human pancreas and islet development and function. Furthermore, variation or mutation of the same gene can cause monogenic diabetes but also contribute to individual risk of the very common, polygenic type 2 diabetes (3–7). Recent studies indicated that autosomal dominant insulin gene mutations can cause permanent neonatal diabetes (PND) but also autoantibody-negative diabetes that is classified then as

type 1B or maturity-onset diabetes of the young (MODY)-type diabetes (8–10). The molecular mechanism behind these cases with a MODY-like presentation was suggested to be defective trafficking of proinsulin and increased endoplasmic reticulum (ER) stress (11). Recessive mutations of the preproinsulin (*INS*) gene have also been identified as a novel cause of neonatal diabetes and were the commonest cause of isolated PND in the offspring of consanguineous parents (12). In humans, complete loss or inactivation of the two insulin alleles clearly leads to insulin-deficient diabetes from birth, as it has been reported previously from our cases (12). But whether allelic depletion of *INS* has any human phenotype at all, remains controversial since inactivation of three of four mouse *INS* alleles (*Ins1*^{−/−}/*Ins2*^{−/+}) does not lead to diabetes during mouse lifespan (13).

In this study, we report on a large, consanguineous kindred with the deletion of exons 1 and 2 of the *INS* gene. The two boys with biallelic *INS* deletion had been

reported recently (12) and we now add mapping data of the 646 bp deletion resulting in loss of half of the preproinsulin protein and proximal parts of the insulin promoter. Furthermore, we report the phenotype of subjects with a heterozygous *INS* deletion and their risk of developing adult-onset and insulin-dependent diabetes.

Patients and methods

Subjects

The family studied originates from Lebanon and within this family two children with PND have been investigated for anomalies of known genes causing monogenic diabetes including *GCK*, *HNF1A*, *HNF4A*, *HNF1B*, *KCNJ11*, and *ABCC8* genes. As these showed normal sequences, the insulin gene was investigated and finally loss of the exons 1 and 2 of the *INS* gene has been identified as the underlying cause and was reported recently (12). Extended clinical follow-up studies on the two index patients with PND were performed at the referring Diabetes Centers. After the biallelic deletion was found in both cases with PND, first-degree relatives (siblings and parents) of one case agreed in clinical investigation including oral glucose tolerance test (OGTT) and HbA1c measurement. Furthermore, we report on a total of 33 adult family members that were available for clinical history, genetic testing, and at least one capillary HbA1c at the time of investigation. Informed consent was obtained from all studied individuals and the study has been approved by the local ethics committee of the Charité. OGTT have been performed in five family members according to a standardized protocol including baseline and post-glucose insulin and C-peptide measurements. Insulin

and C-peptide was measured by chemiluminescence enzyme immunoassay (Immulite 2000, Siemens, Munich, Germany).

Data and sample collection

Family studies have been performed partially in Lebanon, including collection of clinical data, capillary HbA1c-samples, and blood spots on filter paper for genetic testing. Those were done by one adult family member who was specifically trained to interview subjects on concomitant diseases and medication as well as to perform capillary blood tests and store samples appropriately. Classification of diabetes became difficult as onset of insulin deficiency and diabetes symptoms developed slowly and showed some variation between subjects. We classified those subjects as having diabetes if first, diabetes diagnosis was made by a physician or secondly, if diabetes was yet undiagnosed and HbA1c has been higher than the threshold of 6.1%, indicating at least relevant impaired carbohydrate metabolism. By this, we tried to compare diabetes status of family members living in European countries and Lebanon. We assume that we missed no cases with diabetes that required treatment or had a relevant HbA1c elevation.

Mutation screening

Samples for genetic testing have been collected as EDTA blood in cases followed in Melbourne and Berlin. In cases from Lebanon, capillary blood was collected on filter paper (Protein Saver 903, Whatman, Dassel, Germany) and DNA was subsequently isolated (DNA IQ Casework Sample Kit for Maxwell 16, Promega Corporation) and analyzed. The human *INS* gene was amplified in three exon-specific segments by PCR.

Figure 1 Pedigree of the family, genotype of tested subjects without deletion (N/N), with biallelic (D/D) or allelic (D/N) loss of *INS* are indicated. Filled symbols mark the two index cases with PND and hatched symbols those with adult-onset diabetes. The underage (< 18 years) subjects A4.2, A4.3, A4.4, and B4.6 are indicated in this pedigree to give a complete picture of the index families but they are not included into statistical analyses reported in Table 1. The insert shows gel electrophoresis of PCR products using primers spanning the 646 bp breakpoint as an example from one patient with neonatal diabetes and his siblings (upper band ~1000 bp and lower band ~350 bp).

Figure 2 Schematic representation of the proximal *INS* gene showing the deletion above the gene. Transcription factor binding sites of the proximal promoter region are indicated at their respective position; a part of the preproinsulin protein that is coded by exon 2 and lost by the deletion is indicated below the nucleotide sequence. The nomenclature of the deletion is based on the coding sequence where nucleotide 1 represents translational start site.

We used the following primers: for the noncoding exon 1, forward 5'-ggcaggggttgagaggtag-3' and reverse 5'-aatcctgagccacactgac-3'; for exon 2 forward 5'-gaagcatgtgggggtgag-3' and reverse 5'-gctgtcacttttaggacgtg-3'; and for exon 3 forward 5'-ccctgactgtgtctctctgt-3' and reverse 5'-agagagcgtggagagagctg-3'. We used the Mastercycler ep gradient S Amplification System (Eppendorf AG, Hamburg, Germany). The PCR conditions were: 94 °C 5 min, 30 cycles of 94 °C for 30 s, 59.5 °C for 30 s, and 72 °C for 30 s; 72 °C for 10 min and 10 °C for storage. Breakpoint-spanning PCR to discriminate between heterozygote deletion carriers was performed with the forward primer 5'-ccaggtcaccagacttta-3' and the reverse primer 5'-gaggaagaggtgctgacgac-3'. PCR products were 977 or 331 bp for normal or *INS* deleted alleles respectively. The PCR amplification system and conditions were: Mastercycler ep gradient S Amplification System (Eppendorf AG) and 94 °C 5 min, 30 cycles of 94 °C for 30 s, 58.8 °C for 30 s, and 72 °C for 2 min; 72 °C for 10 min and 10 °C for storage.

Statistical analysis

Clinical numeric data is given as median and range, where appropriate. Clinical characteristics of subjects were compared by Mann–Whitney *U* or χ^2 test (frequency of diabetes) by the statistical package PASW version 18 (Chicago, IL, USA).

Results

Identification of insulin gene deletion in the family

During the course of our studies of neonatal diabetes, we identified a consanguineous family (Fig. 1) in which diabetes at adult age appeared to segregate as a dominant trait, while neonatal diabetes occurred in a recessive way. Screening for *INS* mutation in these two cases resulted in a lack of the PCR product of the coding exon 2 and the noncoding exon 1, while exon 3 and the neighboring tyrosine hydroxylase gene (*TH*) were well amplified (11). Thus, sequencing with several primer sets within the region between *INS* and *TH* was performed to characterize size and exact

Table 1 Clinical features of the 33 adult family members screened for the *INS* deletion.

	Genotype	
	N/D	N/N
<i>n</i> (male/female)	11 (6/5)	22 (10/12)
Diabetes/all	5/11 ^{a,†}	3/22 ^a
Age (years)	44.5 (22–55)	37.7 (18–73.8)
Diabetes onset (years)	44.0 (25–47) [†]	65 (63–67)
BMI (kg/m ²)	23.1 (19.9–32.7)	25.1 (16.0–38.5)
HbA1c (%)	5.9 (4.8–12.3)*	5.2 (4.3–6.9)

Values are median (minimum–maximum), significant differences: **P* < 0.05, [†]*P* < 0.01 (N/N vs N/D with or without diabetes), ^a χ^2 test.

Table 2 Clinical characteristics and genotype of all eight subjects that developed diabetes.

Subject (sex)	Present age (y)	Geno-type	Most recent HbA1c (%)	Most recent BMI (kg/m ²)	Age at onset (y)	Diabetes
						Treatment
A3.2 (male)	54	D/N	12.3	21.5	33	33–34 y: diet; 34–49 y: OADs; from 49 y: insulin
A3.3 (male)	53	D/N	12.2	20.2	40	40–49 y: OADs; from 50 y: insulin
A3.4 (male)	51	D/N	6.5	26.2	42	42 y: OADs
A3.7 (female)	44	D/N	8.8	20.1	25	25–33 y: OADs; from 34 y: insulin
B3.1 (female)	50	D/N	6.9	27.8	47	47–50 y: OAD
2.2 (female)	74	N/N	6.7	32.3	65	Diet no OADs
B3.1.1 (male)	72	N/N	6.7	28.3	67	Diet no OADs
2.4 (male)	64	N/N	6.6	38.5	63	Diet no OADs

OADs, oral antidiabetic drugs.

location of the deletion. We found a small deletion of 646 bp including the proximal promoter region and exons 1 and 2 of the insulin gene (chr11: g.2138434_2139080del646). The deletion results in loss of all protein structures encoded by exon 2, namely signaling peptide, A-chain and half of C-peptide, but also of the proximal transcription factor binding sites G1, CRE3, CRE4, and the TATA box of the *INS* promoter (Fig. 2).

For family screening, a breakpoint-spanning PCR was established and we identified other subjects in the family with a heterozygous deletion of the *INS* exons 1 and 2. Overall, 33 adult family members with complete clinical data and HbA1c were analyzed to calculate the diabetes risk associated with the N/D genotype, including 22 cases without deletion and 11 cases with the N/D genotype (Table 1 and Fig. 1).

Patients with a homozygous *INS* gene deletion

Neonatal diabetes in the two cases with complete *INS* deletion was diagnosed within the first hours of life and treated directly with insulin (Table 2). In keeping with severe insulin deficiency, both children also had significant intrauterine growth retardation and birth weight was markedly reduced (Table 3). Following

insulin treatment, postnatal growth of both children was normal and caught up into growth percentiles of unaffected siblings. Apart from neonatal diabetes, neither boy had any other organ abnormality nor did both have impaired mental development.

Subjects with a heterozygous *INS* gene deletion

We tried to compare the diabetes status in family members living in Germany or in Lebanon respectively. The status 'diabetes' in this study was defined as diagnosis made by the physician (OGTT) or as HbA1c increased above 6.1% as the normal range cut off. This was largely applied to many family members from Lebanon, as they had not been regularly screened for diabetes. In the 33 adult family members that were available for genetic and clinical testing, diabetes was more frequent ($P < 0.05$) in those with N/D genotype (5 of 11) than with normal N/N (3 of 22) genotype (Table 1). Diabetic N/D-subjects had been diagnosed between 25 and 47 years of age and were initially treated with oral antidiabetic drugs (OADs). With a delay of 3–16 years, diabetes treatment was then changed to insulin in all cases with diabetes. Some cases with bad metabolic control also died of severe micro- and macro-vascular complications (subjects A

Table 3 Clinical and genetic characteristics of the two index families. Subjects presented are from Fig. 1.

	Subjects						
	B3.4	A3.9	A4.1	A4.2	A4.3	A4.4	B4.6
Genotype	N/D	N/D	N/D	N/N	N/N	D/D	D/D
Diabetes	No	No	No	No	No	PND	PND
Present age (y (sex))	44 (m)	37 (f)	21 (f)	17 (m)	8a (f)	12 (m)	6 (m)
HbA1c (%)	5.9	5.1	4.8	4.9	5.2	8.7	7.9
Fasting glucose (mg/dl)	96	76	74	82	70	166	220
Fasting insulin (mU/l)	8.7	4.5	6.2	9.7	4.4	<0.5 ^a	ND
HOMA-IR	2.1	0.8	1.1	2.0	0.8	ND	ND
ISI ^b	3.4	12.7	9.2	6.2	10.8	ND	ND
BMI (kg/m ² (s.d.))	27.3 (1.61)	22.8 (0.43)	19.9 (−0.6)	24.2 (0.85)	19.2 (1.6)	18.9 (0.3)	17.4 (0.5)
Birth weight (g (s.d.; GA))	ND	ND	2500 (−0.7; 36.0)	3170 (+1.3; 35.5)	3000 (+0.6; 36.5)	1650 (−3.0; 36.0)	1290 (−3.5; 35.0)
Present height (cm (s.d.))	160.3 (−3.0)	148.2 (−3.2)	147.8 (−3.3)	176.7 (−0.6)	122.3 (−0.4)	144.7 (−0.9)	115.8 (−0.5)

^aC-peptide, ND: not done, birth weights not known.

^bInsulin sensitivity index published by Matsuda & DeFronzo (20).

3.3 and A 3.7). The three subjects with normal genotype and diabetes (Fig. 1, subjects B 3.1.1, 2.2, and 2.4) had mild symptoms at onset and were diagnosed approximately two decades later than N/D-subjects (Tables 1 and 3).

Clinical investigation and OGTTs were performed in the index family with three members with N/D genotype and one index case with complete deletion (D/D; A4.4 in Fig. 1). The case with complete deletion (D/D) and neonatal diabetes from birth and displayed complete loss of C-peptide. On the contrary, the N/D-cases of the index family had no clinical signs of diabetes and displayed normal insulin sensitivity and insulin resistance as characterized by insulin sensitivity index (ISI) and HOMA-IR (Table 3).

As mentioned, birth weight was reduced in the D/D case as a result of reduced insulin secretion *in utero*, but whether heterozygous deletion carriers have a lower birth weight is not clear, as birth weights have not been documented in most adult family members born in Lebanon.

Conclusions

We report on a large family with subjects showing complete or heterozygous loss of the insulin gene. Complete loss results in neonatal diabetes, reduced birth weight and complete absence of C-peptide. Furthermore, heterozygous subjects have a relevant risk of developing insulin deficiency and diabetes with advanced age.

As reported recently, recessive *INS* mutations are a novel cause of permanent or transient neonatal diabetes (12). In humans, the phenotype of monoallelic loss of *INS* could be discussed controversial as in mice; a heterozygous null genotype at the *Ins2* locus is insufficient to cause diabetes, no matters whether alone or in combination with a homozygous null genotype at the *Ins1* locus (13).

In humans, some heterozygous mutations of the *INS* gene are known to cause diabetes, if the resulting, mutant proinsulin is misfolded and thus leading to increased ER stress and finally a dominant-negative effect on pancreatic survival β -cell function (11). Diabetes in these patients with heterozygous *INS* mutations shows a broad spectrum of clinical phenotypes suggesting that different *INS* mutations behave differently. Comparing these patients and *Ins1/Ins2* ko animal observations (13) the hypothesis was stressed that in humans, only dominant-negative *INS* mutations are able to cause diabetes, while loss of one allele was suggested to be without clinical impact (14, 15). Given the recessive inheritance, heterozygous offspring are much more frequent; however, less is known about the clinical impact of monoallelic impairment of the *INS* gene.

The two boys with biallelic *INS* deletion had undetectable C-peptide levels and absent insulin

function. Sequencing of the region between *TH* and *INS* gene indicated a very small 646 bp deletion that only affects parts of the insulin promoter, the noncoding exon 1 and the coding exon 2 of the *INS* gene. In detail, we have found that next to exons 1 and 2 basal promoter elements including the transcription factor binding sites G1, CRE3, CRE4, and the TATA box of the proximal *INS* promoter are lost. The basal ~ 100 bp promoter provides an assembly platform for the RNA polymerase II initiation complex and therefore next to exon 2, fundamental elements of regular *INS* transcription are also lost (16). CRE3 and CRE4 predominantly can interact with transcription factors activated by cAMP and diacylglycerol that can operate as activators, nonactivators, or suppressors (17). Mutagenesis experiments have shown that all four CRE sites of the human *INS* promoter are transcriptionally active (18). The human *INS* promoter G1 box (5'GTAGGGGA) binds the transcription factor Pur-1/MAZ and is specifically found in the primate *INS* promoter region (19).

Diabetes caused by heterozygous loss of one *INS* gene in our family was different from type 2 diabetes and characterized by normal or decreased body mass index (BMI) and early need of insulin treatment. Therefore, heterozygous subjects show characteristics of late-onset MODY-like or type 1B-like diabetes that resembles in some ways other monogenic diabetes forms that are associated with gene alterations of *GCK*, *HNF1A*, *HNF1B*, or *HNF4A*. This may suggest that sequencing and deletion analysis of *INS* should be included into diagnostic work up, if monogenic diabetes is assumed.

In summary, complete loss of the human *INS* gene results in neonatal diabetes, reduced birth weight and complete absence of C-peptide protein and insulin function. Furthermore, heterozygous *INS* deletion is a strong risk factor for developing diabetes at older age despite normal BMI. As heterozygous offspring might be frequent in a general population, genetic testing for *INS* gene alterations should be considered, if subjects are primarily insulin deficient and not obese.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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