Insulin gene mutations as cause of diabetes in children negative for five type 1 diabetes autoantibodies.

Riccardo Bonfanti¹, MD, Carlo Colombo², Bs, Valentina Nocerino³, Bs, Ornella Massa², PhD, Vito Lampasona⁴ PhD, Dario Iafusco⁵, MD, Matteo Viscardi⁴, MD, Giuseppe Chiumello¹, MD, Franco Meschi¹, MD, Fabrizio Barbetti², ³, ⁶, MD, PhD.

1  Department of Pediatrics, Scientific Institute H San Raffaele, Milan, Italy
2  Laboratory of Molecular Endocrinology, Bambino Gesù Pediatric Hospital, IRCCS, Rome, Italy,
3  Laboratory of Molecular Endocrinology and Metabolism, S Raffaele Biomedical Park Foundation, Rome, Italy,
4  Diagnostica e Ricerca H San Raffaele and Scientific Institute H San Raffaele, Milan, Italy,
5  2nd University of Naples, Naples, Italy,
6  Department of Internal Medicine, University of Tor Vergata, Rome, Italy

Corresponding author:
Fabrizio Barbetti, MD, PhD
E-mail: mody.2@libero.it

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**Objective.** Heterozygous, gain-of-function mutations of the insulin gene can cause permanent diabetes mellitus with onset ranging from the neonatal period through adulthood. Aim of our study was to screen the insulin gene in patients clinically classified as T1D, but negative to the search of T1D autoantibodies.

**Research design and methods.** We reviewed the clinical records of 326 patients with the diagnosis of T1D and identified 7 probands who had diabetes in isolation and were negative for the search of five T1D autoantibodies. We sequenced the *INS* gene in these 7.

**Results.** In 2 patients with diabetes onset at 2 y, 10 m and 6 y, 8 m, we identified the mutation G^{B8}S and a novel mutation in the pre(pro)insulin signal peptide (A^{Signal23}S).

**Conclusion.** Insulin gene mutations are rare in absolute terms in patients classified as T1D (0.6%) but can be identified after a thorough screening of T1D autoantibodies.
Mutations of insulin (INS) gene associated with neonatal/infancy onset diabetes cause sustained stress of the endoplasmic reticulum (ER-stress) which in turn trigger apoptosis of the pancreatic beta cell (1). In patients with insulin mutations with proteotoxic effect diabetes presents in isolation and in some individuals with onset of hyperglycemia well outside the neonatal period (1-4). As a consequence, individuals with INS gene mutations may be confused with patients with type 1, autoimmune diabetes (1-4).

**RESEARCH DESIGN AND METHODS**

We reviewed the clinical records of 326 patients with the diagnosis of diabetes (age range at diagnosis: 1-18 years) consecutively referred to the pediatric diabetes clinic of H S Raffaele Hospital during the years 2003-2006. Among these 326, we identified twentyfour patients who were negative to the search of all commonly used T1D autoantibodies (ICA, GADA, IA-2A, IAA) at the time of diagnosis. The cut-off (arbitrary units) was GADA <3, IA-2A <1, IAA <5; the thresholds for positivity in each assays corresponded to 99th percentiles of control subjects. From these 24 we excluded 4 patients with adolescent type 2 diabetes (Table 1). Among the remaining twenty patients we selected those with diabetes in isolation, who were tested for the novel T1D autoantibody against Zn transporter 8 (ZnT8A; cut off in arbitrary units: <12) (5). Seven patients who scored negative to all antibodies were analysed for insulin gene mutations by DNA direct sequencing, along with 4 ZnT8A+ patients (controls). When appropriate, mutation found were designated according to their position into the mature insulin chains (1).

**RESULTS**

In two patients we detected a heterozygous missense mutation of the INS gene: the already described G^{B8S} (or G32S) (2, 3) and a novel mutation resulting in a serine for an alanine in the twentythird aminoacid of the preproinsulin molecule: A^{Signal23S}; both mutations were confirmed by digestion with the appropriate restriction enzyme. DNA sequencing of the insulin gene of probands’ parents showed a normal sequence (i.e. the mutations arose as spontaneous mutations). No mutation was found in 200 controls with normal glucose tolerance or in ZnT8A+ patients.

The child bearing the G^{B8S} mutation was born after an uneventful pregnancy (39 weeks of gestation) with a birth weight of 2770 g (10th centile). At diabetes onset he was 2 years 10 months old, lean (BMI=16; 25th centile for corresponding age) and showed a detectable C peptide (0.49 ng/ml) that was lower, but still measurable after 2 years from diagnosis (0.34 ng/ml). Presently he is 6 years old, his insulin dose is 0.7 U Kg\(^{-1}\) d\(^{-1}\) with HbA1c of 8.7% (normal reference <6%). The individual with the A^{Signal23S} mutation (birth weight: 3,350 g) (25-50th centile) presented with typical symptoms of diabetes (polyuria, polydipsia) when he was 6 years 8 months old (HBA1c= 11% at diabetes onset). He was lean (BMI= 16.4; 50th centile). Insulin was started and continued for 6 months; during the following 2 years the patient went off/on insulin several times (a pattern that may resemble the so-called “honey moon” phase of T1D). C-peptide measured 11 and 24 months after onset of hyperglycemia was respectively 1.32 and 0.7 ng/ml. He is now 10 years old, on insulin therapy at the dose of 0.17 U Kg\(^{-1}\) d\(^{-1}\) with HbA1c of 6.4%.

**DISCUSSION**

Previously, heterozygous INS gene mutations had been detected in adult patients with so-called familial
hyperinsulinemia/hyper(pro)insulinemia who presented with variable phenotypes (mild diabetes or even hypoglycemia) and high serum levels of radioimmunoassayable insulin or proinsulin-like material. More recently, \textit{INS} mutations have been found associated with neonatal/infancy onset diabetes (1-3). We demonstrated that mutations with prototoxic effect are not secreted when expressed in HEK 293 cell line (1), and it is likely that also $S^{B8}$ and $S^{Signal_{23}}$ are retained in the ER. Nevertheless, the patient bearing the mutation in the signal peptide shows a milder clinical course and we cannot exclude that $S^{Signal_{23}}$ pre(pro)insulin may be partially processed and secreted. Present knowledge indicates that insulin mutations with a prototoxic effect cause apoptosis of the pancreatic beta cell (1), a process that in most patients takes several months from birth (1-3) and in some individuals, years (1-4, this report). Of note, only 6 patients among those reported in the papers by Stoy, Edghill, Molven and Colombo (1-4) were diagnosed within the first 4 weeks of birth (i.e. the time interval still in use to define the neonatal period) while most of them (more than 40) were diagnosed in the first year of life (infancy). Thus, we believe that classifying these patients as having “Permanent Neonatal Diabetes Mellitus” (PNDM) is misleading and that this definition should be abandoned in favor of “Monogenic Diabetes of Infancy” (MDI), as previously suggested by our group (1). Indeed, at least 12 patients with insulin gene mutations had the diagnosis of diabetes during childhood or adulthood (1-4, this report), making the neonatal onset an exception.

The Italian proband bearing the $G^{B8S}$ (G32S) mutation had the diagnosis of diabetes at about 3 years of age, approximately two years later than patients carrying the same mutation described by Stoy (2). Presently, it is not clear why patients with the same \textit{INS} gene mutation, even from the same family, can present with diabetes either during infancy, childhood or adulthood (1-4). It is tempting to speculate that the apoptotic process in some of these patients may be modulated/slurred by the individual capacity of degrading misfolded insulin (by a process known as endoplasmic reticulum-associated degradation, ERAD). Another intriguing hypothesis, not mutually exclusive with the previous one, could be that some beta cell regeneration may take place in some individuals and not in others. The observation that in these two patients (and in others previously described) (1) insulin secretion was still detectable two years after diabetes onset suggests that any of these mechanisms could be at work.

In conclusion, insulin gene mutations are rare in absolute terms in patients clinically classified as T1D (2/326 or 0.6%), but can be identified after a thorough screening of T1D autoantibodies.
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Table 1.

Table title: Clinical criteria used for classification of new cases of diabetes with onset during childhood/adolescence.

| Clinical diagnosis | Type 1 diabetes | Type 2 diabetes | Monogenic diabetes in isolation | HNF1 beta (MODY 5) | Wolfram syndrome | Post CMV infection |
|--------------------|-----------------|-----------------|---------------------------------|-------------------|-----------------|-------------------|
| Number of patients | 309             | 4               | 7                               | 2                 | 3               | 1                 |
| T1D autoantibodies (ICA, GADA, IA-2A, IAA, ZnT8*) | At least 1 | None | None | None | None | None |
| BMI                | Not considered  | >90 centile    | Normal, low                     | Normal, low       | Normal, low     | Normal, low       |
| C-peptide (ng/ml)  | Low/un-detectable | >1.5†          | Low, normal or high             | <1.5              | <1.5            | <1.5              |
| Age range at diagnosis of diabetes | 1-18y | 12-15y | 2y, 7m-15y, 4m | 12-14y | 10-14y | 13y |
| Features other than diabetes | none | Hypertension dislipidemia | none | Renal disease. Pancreas hypoplasia at ultrasound or NMR. Abnormal liver enzymes. | Optic atrophy/ diabetes insipidus/ deafness. | Documented perinatal CMV infection, growth retardation, deafness. |

Table footnote: * Four patients were negative to ICA, GADA, IA-2A and IAA, but positive to ZnT8A. † At least two determinations 6 months apart. NMR= nuclear magnetic resonance. CMV= cytomegalovirus.