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Clinical and molecular genetics of neonatal diabetes due to mutations in the insulin gene

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Abstract Over the last decade our insight into the causes of neonatal diabetes has greatly expanded. Neonatal diabetes was once considered a variant of type 1 diabetes that presented early in life. Recent advances in our understanding of this disorder have established that neonatal diabetes is not an autoimmune disease, but rather is a monogenic form of diabetes resulting from mutations in a number of different genes encoding proteins that play a key role in the normal function of the pancreatic beta-cell. Moreover, a correct genetic diagnosis can affect treatment and clinical outcome. This is especially true for patients with mutations in the genes *KCNJ11* or *ABCC8* that encode the two protein subunits (Kir6.2 and SUR1, respectively) of the ATP-sensitive potassium channel. These patients can be treated with oral sulfonylurea drugs with better glycemic control and quality of life. Recently, mutations in the insulin gene (*INS*) itself have been identified as another cause of neonatal diabetes. In this article, we review the role of *INS* mutations in the pathophysiology of neonatal diabetes.

Keywords ER stress · Insulin · Maturity-onset diabetes of the young · Neonatal diabetes · Type 1 diabetes · Unfolded protein response

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1 Introduction

Insulin plays a key role in the regulation of glucose homeostasis with diabetes resulting from insulin deficiency, whether complete deficiency as in type 1 diabetes or relative deficiency as in type 2 diabetes. A genetic basis for diabetes is well established with some forms resulting from a mutation in a single gene (monogenic forms of diabetes) and others from a combination of common, and possibly rare, genetic variants acting together with environmental factors to affect risk of disease (polygenic forms of diabetes) [1]. Insulin was the first protein to be completely sequenced [2] with the amino acid sequence of bovine insulin determined by Sanger and his colleagues from 1951–1955. The amino acid sequence of human insulin was reported by Nicol et al. in 1960 and human proinsulin (the single-chain precursor to insulin) by Oyer et al. in 1971 [3–5]. The human preproinsulin cDNA and complete amino acid sequence of preproinsulin were identified by Bell et al. in 1979 and the cloning and the characterization of the human insulin gene by Bell et al. in 1980 [6, 7].

Studies of human insulin from nondiabetic and diabetic patients suggested that differences in sequence could be a genetic cause of diabetes but definitive proof was lacking (reviewed in Kimmel et al. 1967) [8]. The first mutant human insulin protein was identified by Tager and colleagues in 1979 and subsequently mutant proinsulin in 1981 in patients with hyperinsulinemia and hyperproinsulinemia, respectively, both very rare conditions with only 15 families reported to date [9–12]. Moreover, most patients with hyperinsulinemia or hyperproinsulinemia did not develop overt diabetes. These studies suggested that if mutations in the human insulin gene were a cause of diabetes, they were very rare and unlikely to be a significant factor contributing to disease. This view has

now changed based on recent studies from our laboratory and others reporting the identification of many new mutations in the insulin gene in humans and mice [13–19]. Mutations in the human insulin gene (*INS*) are a common cause of permanent neonatal diabetes mellitus (PNDM). They can also result in diabetes diagnosed outside infancy with a diagnosis of type 1b diabetes or maturity-onset diabetes of the young. They are also the cause of diabetes in two mouse models–the Akita and Munich mouse. Clinical studies of these patients and molecular and cell biological studies of the mutant insulin, preproinsulin and proinsulin proteins are providing new insight into the pathophysiology of diabetes as well as insulin biosynthesis [18, 19].

2 Molecular structure of insulin and insulin biosynthesis

Insulin is the major biosynthetic and secretory product of the beta cell accounting for 20% of total mRNA (100–200,000 insulin mRNA molecules/cell), 10% of total cellular protein and 50% or more of total protein synthesis when maximally stimulated corresponding to 1.3×10^6 molecules of insulin/min [20, 21].

The initial product of translation of human insulin mRNA is a single chain precursor molecule, preproinsulin, a protein of 110 amino acid residues including the signal peptide of 24 amino acid residues. The emerging signal peptide first interacts with the signal recognition particle in the cytosol. This complex then interacts with the translocon on the ER membrane which targets the nascent proinsulin peptide chain into the ER. The signal peptide is rapidly cleaved and degraded and proinsulin then folds in the lumen of the ER forming the characteristic disulfide bonds of the insulin molecule. In proinsulin, the C-peptide bridges the B-chain and the A-chain via two pairs of basic amino acids (Arg-Arg and Lys-Arg, respectively) (Fig. 1). In mature insulin, the C-peptide and the four basic residues have been excised and the B- and A-chains are connected covalently only by the two disulfide bonds between the B7-A7 and B19-A20 cysteines. A third intrachain disulfide bond connects A6 and A11 cysteines. The folding of proinsulin and disulfide bond formation are catalyzed by several classes of ER chaperone proteins. Only the fully folded native proinsulin protein can normally progress from the ER to the Golgi compartments. After transitioning through the Golgi apparatus, the proinsulin is concentrated and sorted into immature secretory granules in the transGolgi network. The proteolytic processing of proinsulin to insulin and C-peptide takes place mainly in the maturing secretory granules by cleavage sequentially at the B-chain-C-peptide junction by proconvertase (PC) 1/3 and carboxypeptidase E (CPE) yielding des-31,32-proinsulin and then at the C-peptide-A-chain junction by PC2 and CPE yielding insulin and C-peptide. Three molecules of Arg and one molecule of Lys are released during this process. Insulin and C-peptide are stored in the mature secretory granules and secreted in equimolar amounts [22].

3 Insulin mutations in monogenic forms of diabetes

3.1 Insulin mutations and diabetes

Insulin gene mutations as a cause of diabetes were identified independently by ourselves (Støy et al. 2007) and by Colombo et al. (2008) [13, 17]. We carried out a linkage analysis in a three-generation family with PNDM of unknown etiology. The results suggested that the putative diabetes gene was located on either chromosome 2, 3, 6 or 11. Cataloguing the genes under the linkage peaks of each of these chromosomes revealed two candidate genes—*NEUROD1* and *INS*. There were no mutations in the coding region of *NEUROD1*. Sequencing of *INS* revealed the missense mutation G32S in affected family members suggesting that an *INS* mutation was responsible for PNDM in this family. The association of *INS* mutations with PNDM was confirmed by the identification of mutations in 15 additional probands. Interestingly, the majority of the mutations were not inherited but were *de novo* in origin. All were in the heterozygous state. Colombo et al. [17] used a candidate gene approach based on the fact that a mutation in the *Ins2* gene (the mouse homolog of the insulin gene in human) was the cause of diabetes (described as maturity-onset diabetes of the young) in the Akita mouse model of diabetes [18]. They identified seven different *INS* mutations in 9 probands with PNDM.

Mutations (in the heterozygous state) in *INS* have now been reported in 66 probands with a variety of diagnoses including PNDM, infancy-onset diabetes (i.e. diabetes diagnosed before 12 months of age), type 1b diabetes (i.e. non-autoimmune type 1 diabetes (T1DM)), maturity-onset diabetes of the young (MODY), and early-onset type 2 diabetes (T2DM) (Figs. 1–2 and Table 1) [13–17, 23–28]. There are multiple affected family members in 23/66 families. Of the 107 carriers in these families, 104 have diabetes and three have normal glucose tolerance.

In the large Exeter cohort of patients with PNDM diagnosed before 6 months of age, 14% of patients born to non-consanguineous parents were found to carry a heterozygous *INS* mutation. The incidence of *INS* mutations in patients diagnosed with diabetes outside the neonatal period is less well defined, but is estimated to be less than 2%. The mutations in 18/66 (27%) of the probands were inherited from an affected parent whereas in the remaining 48 probands (73%) the mutation was *de novo* in origin. In one family, two affected children with the same mutation were born to unaffected parents, neither of them carriers, suggesting that one of the parents was a germline mosaic.

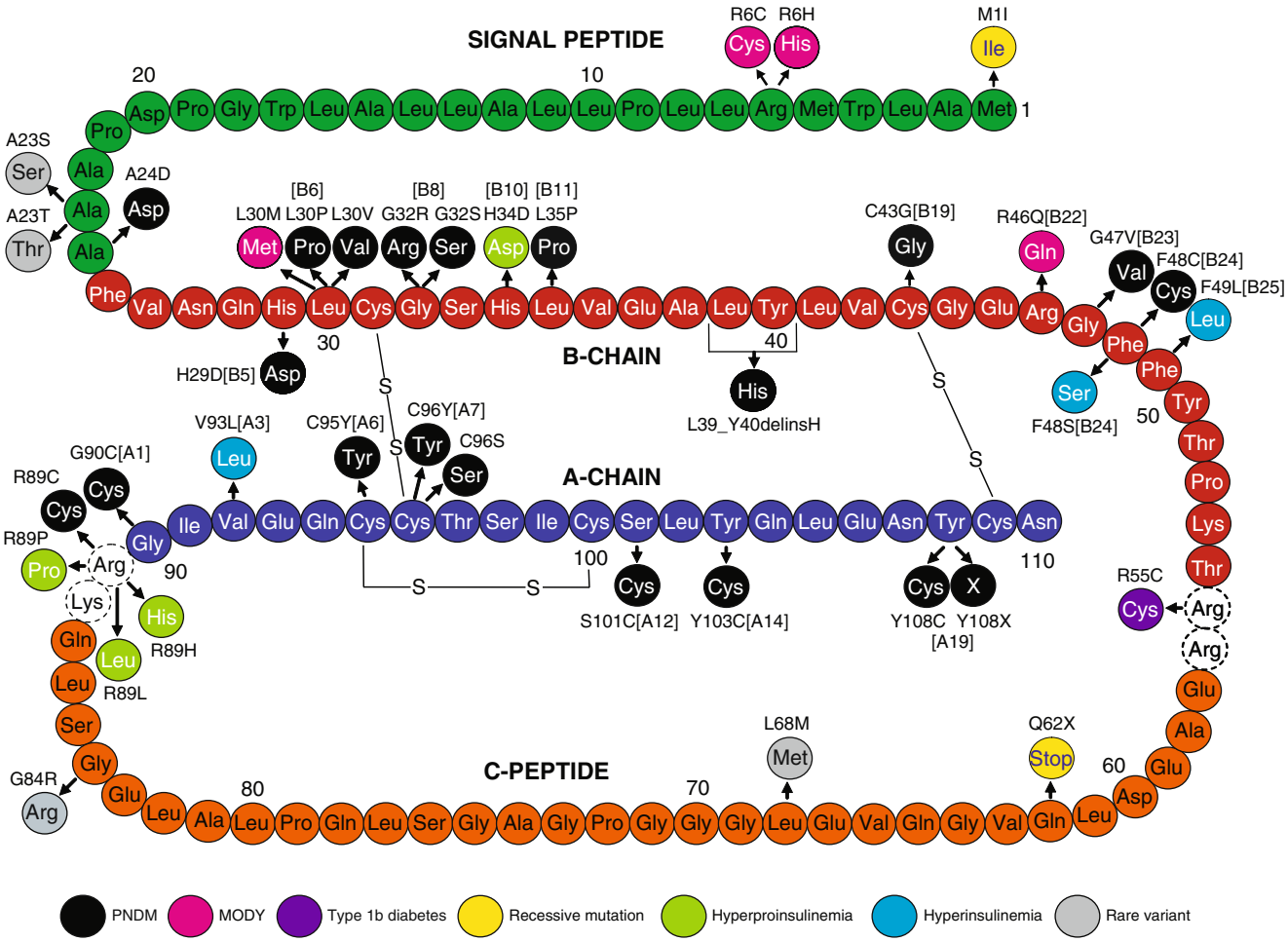

Fig. 1 Diagrammatic representation of the amino acid sequence of human preproinsulin (signal peptide–green, B-chain–red, C-peptide–orange, A-chain–dark blue) indicating sites of mutations identified in patients with diabetes as well as hyperinsulinemia and hyperproinsulinemia. Mutations shown in black disrupt proinsulin folding and/or disulfide bond formation leading to permanent neonatal diabetes mellitus (PNDM); mutations in light blue do not impair folding but are associated with reduced insulin receptor binding potency (hyperinsulinaemia); mutations in light green are associated with hyperproinsulinaemia and either impair proteolytic processing to insulin or, in the case of H34D, aggregation and sorting into dense-core granules of the regulated secretory pathway. Mutations in pink and purple were found in patients with a diagnosis of maturity-onset diabetes of the young (MODY) and type 1b diabetes, respectively. The R55C mutation has been found in patients with a diagnosis of type 1b diabetes as well as MODY. The mutations shown in gray (A23S, A23T, L68M and G84R) are rare variants without functional effects on proinsulin/insulin biosynthesis. The mutations shown in yellow are recessive mutations that affect insulin biosynthesis (mutation of the translation initiation Met) or cause the synthesis of a nonsense protein (Q62X)

The diabetes-associated mutations lead to the synthesis of a structurally abnormal preproinsulin or proinsulin protein. The mutations are located in the signal peptide, the B-chain and A-chain regions and the pairs of basic residues that flank the C-peptide. Those located in the signal peptide impair the function of this domain of preproinsulin including absent or altered cleavage of the signal peptide. The mutations in proinsulin affect formation of the disulfide bonds linking the B-chain and A-chain as well as the intra-A-chain disulfide bond thereby leading to a misfolded proinsulin molecule that is retained in the ER impairing normal beta-cell function and resulting in cell death [17, 19, 29–31].

Garin et al. [32] recently reported recessive mutations in *INS* resulting in neonatal diabetes. These mutations affect proinsulin biosynthesis *per se* and function as null mutations, doing so by several different mechanisms. One mutation results in deletion of exons 1 and 2 of the insulin gene. There are several mutations located in the promoter region of the gene that result in decreased transcription. A mutation in the region encoding the 3′-untranslated region of the mRNA affects polyadenylation and mRNA stability. Other mutations affect translation of insulin mRNA by mutation of the translation initiation codon, or lead to synthesis of a truncated proinsulin molecule (Figs. 1 and 3).

Homozygous mutations in *INS* have been reported in 15 families/21 patients. These patients were diagnosed with PNDM (67% of patients), type 1b diabetes (9% of patients)

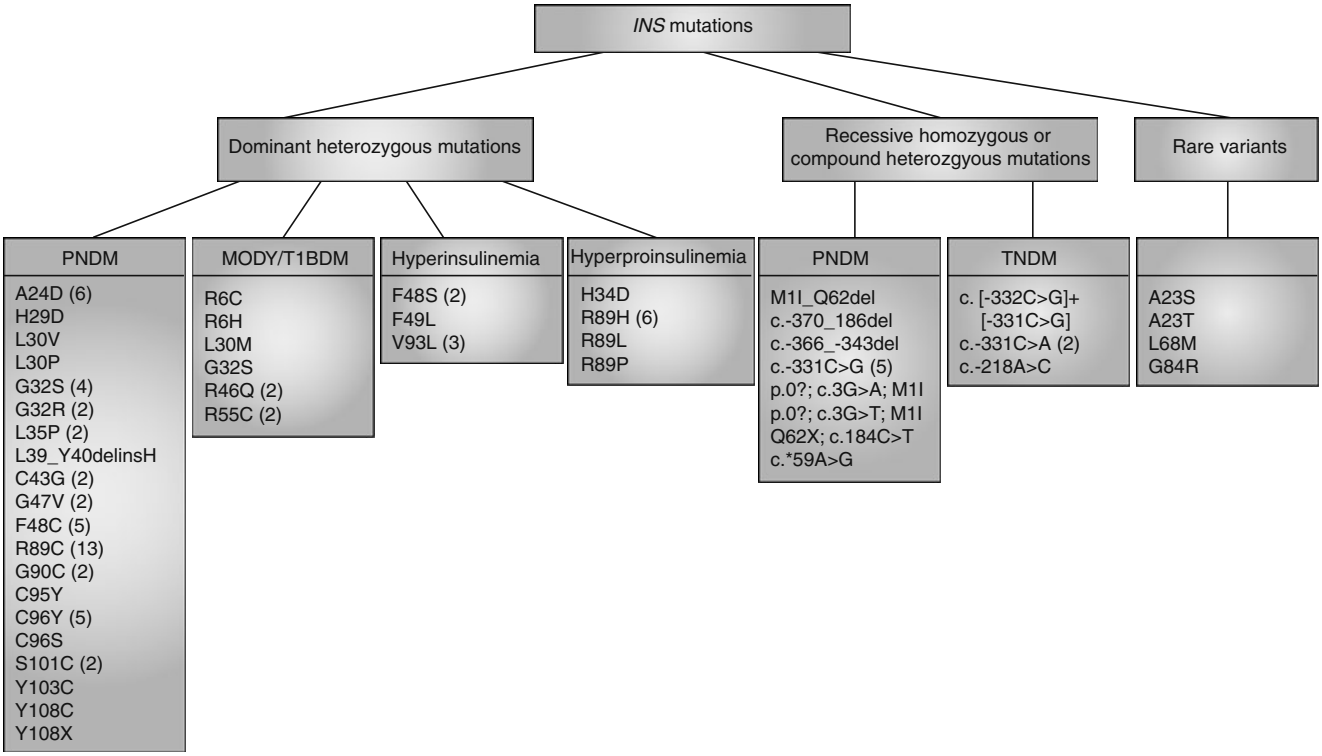

Fig. 2 Summary of human insulin gene mutations and disease phenotype. The numbers in brackets indicate the number of probands with that specific mutation. PNDM, permanent neonatal diabetes mellitus; TNDM, transient neonatal diabetes mellitus; MODY, maturity-onset diabetes of the young; T1BDM, type 1b diabetes mellitus

and, in contrast to heterozygous *INS* mutation carriers, with transient neonatal diabetes mellitus (TNDM) (24% of patients). Homozygous *INS* mutations are the most common cause of PNDM without extra-pancreatic features in patients born to consanguineous parents (~32%), but are a rare cause of neonatal diabetes in patients born to non-consanguineous parents (~1%) [32].

3.2 Characteristics of mutations

All the mutations in the preproinsulin protein reported to date are shown in Fig. 1. In addition to the mutations associated with diabetes, hyperinsulinemia or hyperproinsulinemia, there are four mutations that are unlikely to be pathogenic: A23S, A23T, L68M and G84R [14, 23, 28] (Figs. 1 and 2). The mutation A23S has been reported to be associated with type 1b diabetes. However, this site is not highly conserved amongst preproinsulin sequences and is Ser in the chimpanzee sequence strongly suggesting that it does not lead to diabetes (Fig. 4). Functional studies indicate that G84R does not affect proinsulin biosynthesis suggesting that the putative diabetes-associated mutation is not pathogenic [30].

The dominantly-acting diabetes-associated mutations involve 19/110 amino acids of preproinsulin and include 23 missense mutation, one nonsense mutation and one insertion/deletion mutation. They are located in all regions of the preproinsulin molecule except the C-peptide: signal peptide (3 mutations), B-chain (12 mutations), A-chain (8 mutations) and pairs of basic amino acids that flank the C-peptide (2 mutations). The latter mutations are found at the enzymatic cleavage site between the B-chain and C-peptide (R55C) and the C-peptide and A-chain (R89C). Other mutations of Arg89 have been described in patients with hyperproinsulinemia. Thirteen mutations were identified in more than one proband (Fig. 2, Table 1) and mutations at five codons (A24D, G32S/R, F48C, R89C, and C96Y/S) account for 56% (37/66) of all mutations identified to date. Six residues are sites for different amino acid substitutions: R6C/H, A23S/T, L30M/P/V, G32S/R, C96Y/S and Y108C/X. Five mutations were identified in families where all affected family members were diagnosed with diabetes outside the neonatal period or infancy (R6C, R6H, L30M, R46Q and R55C) highlighting the different effects of these various mutations on pancreatic beta-cell function. As noted above, A23S/T, L68M, and G84R are most likely rare variants that are not pathogenic and patients carrying these variant are not included in the genotype/phenotype analyses described below.

Ten different homozygous *INS* mutations have been reported to date [32]. Five homozygous mutations have been found in regions of *INS* affecting transcription or

Table 1 Insulin gene mutations and diabetes

Location of mutation	Mutation		Homozygous/ Heterozygous	Probands/ Patients	Phenotype/Diagnosis (Number of subjects)	Reference
	Protein change	Nucleotide change				
Promoter, signal peptide, B-chain, C-peptide	–	c.−370-?_186+?del	Homozygous	1/2	PNDM	[32]
Promoter	–	c.−366_−343del	Homozygous	1/2	PNDM	[32]
Promoter	–	c.−331 C>A	Homozygous	2/2	TNDM	[32]
Promoter	–	c.−331 C>G	Homozygous	5/9	PNDM (6), TNDM (1), T1BDM (2)	[32]
Promoter	–	c.−332 C>G+c.−331 C>G	Compound heterozygous	1/1	TNDM	[32]
Promoter	–	c.−218 A>C	Homozygous	1/1	TNDM	[32]
Signal peptide	p.0? (M1I)	c.3 G>T	Homozygous	1/1	PNDM	[32]
Signal peptide	p.0? (M1I)	c.3 G>A	Homozygous	1/1	PNDM	[32]
Signal peptide	R6C	c.16 C>T	Heterozygous	1/3	MODY	[14]
Signal peptide	R6H	c.17 G>A	Heterozygous	1/4	MODY/ T2DM / GDM / Normal	[26]
Signal peptide	A23S	c.67 G>T	Heterozygous	1/1	T1BDM*	[23]
Signal peptide	A23T	c.67 G>A	Heterozygous	1/4	T2DM (2) / IGT (1) / Normal*	[28]
Signal peptide	A24D	c.71 C>A	Heterozygous	6/8	PNDM (7) / T1BDM (1)	[13, 14, 16]
B-chain	H29D	c.85 C>G	Heterozygous	1/1	PNDM	[14]
B-chain	L30V	c.88 C>G	Heterozygous	1/1	PNDM	[17]
B-chain	L30M	c.88 C>A	Heterozygous	1/6	MODY (5) / Normal	[26]
B-chain	L30P	c.89 T>C	Heterozygous	1/1	PNDM	[17]
B-chain	G32S	c.94 G>A	Heterozygous	5/8	PNDM (7) / T1BDM (1)	[13, 14, 23, 24]
B-chain	G32R	c.94 G>C	Heterozygous	2/5	PNDM (4) / MODY (1)	[13, 14]
B-chain	L35P	c.104 T>C	Heterozygous	2/2	PNDM	[14, 17]
B-chain	L39_Y40delinsH	c.114_118 TCTCT>AC	Heterozygous	1/1	PNDM	[17]
B-chain	C43G	c.127 T>G	Heterozygous	2/3	PNDM (2) / T2DM (1)	[13, 14]
B-chain	R46Q	c.137 G>A	Heterozygous	2/6	MODY	[15, 27]
B-chain	G47V	c.140 G>T	Heterozygous	2/2	PNDM	[13, 14]
B-chain	F48C	c.143 T>G	Heterozygous	5/6	PNDM	[13, 14]
C-peptide/B-chain junction	R55C	c.163 C>T	Heterozygous	2/5	T1BDM (2) / MODY (3)	[15, 26]
C-peptide	Q62X	c.184 C>T	Homozygous	1/1	PNDM	[32]
C-peptide	L68M	c.202 C>A	Heterozygous	1/1	T2DM*	[14]
C-peptide	G84R	c.250 G>A	Heterozygous	1/1	PNDM*	[14]
C-peptide/ A-chain junction	R89C	c.265 C>T	Heterozygous	13/20	PNDM (15) / T1BDM (5)	[13, 14, 16, 17, 24]
A-chain	G90C	c.268 G>T	Heterozygous	2/2	PNDM	[13, 14]
A-chain	C95Y	c.284 G>A	Heterozygous	1/1	PNDM	[17]
A-chain	C96Y	c.287 G>A	Heterozygous	5/7	PNDM (6) / T1BDM (1)	[14, 16, 25]
A-chain	C96S	c.287 G>C	Heterozygous	1/1	PNDM	[14]
A-chain	S101C	c.302 C>G	Heterozygous	2/3	PNDM	[14]
A-chain	Y103C	c.308 A>G	Heterozygous	1/1	PNDM	[14]
A-chain	Y108C	c.323 A>G	Heterozygous	1/2	PNDM	[14]
A-chain	Y108X	c.324 C>G	Heterozygous	1/1	PNDM	[17]
3′ UTR	–	c.59 A>G	Homozygous	1/1	PNDM	[32]

Mutations associated with hyperinsulinemia and hyperproinsulinemia are not included in this table

*Uncertain pathogenicity. TIBDM, type 1b diabetes mellitus; T2DM, type 2 diabetes mellitus; GDM, gestational diabetes mellitus; IGT, impaired glucose tolerance; 3′ UTR, 3′-untranslated region

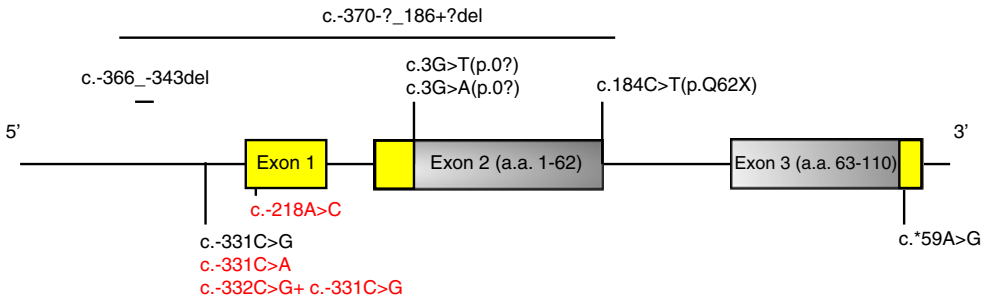

Fig. 3 A schematic of the human insulin gene showing the locations of mutations in the homozygous or compound heterozygous state in probands with PNDM and TNDM. Mutations involving coding regions are shown above the gene while those involving non-coding regions are shown below the gene. The protein coding regions of the gene are shown in gray and the regions encoding the 5′- and 3′-untranslated regions of insulin mRNA in yellow. The red and black text indicates mutations identified in probands with TNDM and PNDM, respectively. Exon 2 encodes amino acids (a.a.) 1–62 and exon 3 encodes amino acids 63–110 of the preproinsulin molecule. Adapted from Garin et al. [32]

mRNA processing: three single base substitutions and a 24-base pair deletion are located in the promoter and one single base substitution is in the 3´-untranslated region (3′-UTR). One patient was a compound heterozygote for two promoter mutations. Four homozygous mutations have been identified that affect the coding regions of *INS*: two different mutations that result in mutation of the translational initiation site (M1I), a large deletion of exons 1 and 2 (M1_Q62del) and a nonsense mutation in the C-peptide region (Q62X). The site in the promoter at c.-331 seems to be a mutational hotspot, with seven probands identified with mutations at this site (c.-331C>G and c.-331C>A).

3.3 Clinical characteristics of patients with PNDM due to heterozygous (dominant-negative) insulin mutations

The majority of patients with heterozygous mutations in *INS* were diagnosed with diabetes before 6 months of age (85% in the Exeter cohort). However, in contrast to the majority of patients with mutations in *KCNJ11* or *ABCC8*, *INS* mutations have also been found in patients diagnosed from 6–12 months of age. Ninety-six percent of patients with a heterozygous *INS* mutation in the Exeter cohort were diagnosed with diabetes in the first year of life. The age-at-diagnosis of diabetes (excluding patients with the mutations R6C/H, L30M, R46Q and R55C) varies from 0–1,560 weeks with a median of 11 weeks in the Exeter cohort and 26 weeks in the remaining patients. In contrast to the Akita mouse where there appears to be a difference in age-at-onset of diabetes between male and female mice (with males having an earlier onset with more severe hyperglycemia, at least on a C57BL/6 background) [18], there is no difference in age-at-diagnosis between male and female human carriers of a heterozygous *INS* gene mutation. Both male and female human carriers present with severe beta-cell failure at diagnosis including, in some instances, diabetic ketoacidosis or severe symptomatic

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                    10        20        30        40        50        60        70        80        90        100       110
                    |         |         |         |         |         |         |         |         |         |         |
          Human     MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQ-VGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN 110
        Gorilla     MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQ-VGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN 110
      Chimpanzee    MALWMRLLPLLVLLALWGPDPASAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQ-VGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN 110
      Orangutan     MALWMRLLPLLALLALWGPDPAQAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQ-VGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN 110
Crab-eating Macaque MALWMRLLPLLALLALWGPDPAPAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDPQ-VGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN 110
    Vervet Monkey   MALWMRLLPLLALLALWGPDPVPAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDPQ-VGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN 110
        Mouse-2     MALWMRFLPLLALLFLWESHPTQAFVKQHLCGSHLVEALYLVCGERGFFYTPMSRREVEDPQ-VAQLELGGGPGAGDLQTLALEVAQQKRGIVDQCCTSICSLYQLENYCN 110
         Rat-2      MALWIRFLPLLALLILWEPRPAQAFVKQHLCGSHLVEALYLVCGERGFFYTPMSRREVEDPQ-VAQLELGGGPGAGDLQTLALEVARQKRGIVDQCCTSICSLYQLENYCN 110
        Rabbit      MASLAALLPLLALLVLCRLDPAQAFVNQHLCGSHLVEALYLVCGERGFFYTPKSRREVEELQ-VGQAELGGGPGAGGLQPSALELALQKRGIVEQCCTSICSLYQLENYCN 110
        Cattle      MALWTRLRPLLALLALWPPPPARAFVNQHLCGSHLVEALYLVCGERGFFYTPKARREVEGPQ-VGALELAGGPG-----AGGLEGPPQKRGIVEQCCASVCSLYQLENYCN 105
          Pig       MALWTRLLPLLALLALWAPAPAQAFVNQHLCGSHLVEALYLVCGERGFFYTPKARREAENPQ-AGAVELGGGLGG--LQALALEGPPQKRGIVEQCCTSICSLYQLENYCN 108
          Dog       MALWMRLLPLLALLALWAPAPTRAFVNQHLCGSHLVEALYLVCGERGFFYTPKARREVEDLQ-VRDVELAGAPGEGGLQPLALEGALQKRGIVEQCCTSICSLYQLENYCN 110
          Cat       MAPWTRLLPLLALLSLWIPAPTRAFVNQHLCGSHLVEALYLVCGERGFFYTPKARREAEDLQ-GKDAELGEAPGAGGLQPSALEAPLQKRGIVEQCCASVCSLYQLEHYCN 110
        Chicken     MALWIRSLPLLALLVFSGPGTSYAAANQHLCGSHLVEALYLVCGERGFFYSPKARRDVEQPL-VSSPLRG---EAGVLPFQQEEYEKVKRGIVEQCCHNTCSLYQLENYCN 107
          Frog      MALWMQCLPLVLVLLFSTPNT-EALANQHLCGSHLVEALYLVCGDRGFFYYPKIKRDIEQAQ-VNGPQDN---ELDGMQFQPQEYQKMKRGIVEQCCHSTCSLFQLENYCN 106
       Zebrafish    MAVWLQAGALLVLLVVSSVSTNPGTP-QHLCGSHLVDALYLVCGPTGFFYNPK--RDVEPLLGFLPPKSAQETEVADFAFKDHAELIRKRGIVEQCCHKPCSIFELQNYCN 108
                    **      .*: :* .   .  .  *********:*******  **** *   *: *                    *****:*** . **:::*::***
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Fig. 4 Multiple sequence alignment of preproinsulin from fish to human. The ClustalW2 alignment was obtained using the online interface at EBI (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The sequence of the signal peptide is shown in green letters, the B-chain in red, the C-peptide in orange and A-chain in blue. The basic amino acids that flank the C-peptide and are the sites of proteolytic processing are shown in black letters. The conserved Cys residues are highlighted in yellow. Asterisks (*) indicate identical amino acids in all the sequences shown in this alignment, colons (:) indicate conserved residues and periods (.) indicate semi-conserved amino acids. Note that these do not represent all the known preproinsulin sequences but rather are a selection of representative sequences

hyperglycemia, a sign of almost complete insulin deficiency. The majority of patients are treated with insulin in full replacement doses [14].

Mutation carriers are born small for gestational age with median birth weights from 2,700–3,087 g (6th to 27th percentile) most likely reflecting in utero insulin deficiency. Interestingly, male carriers have been reported to have more severe growth retardation than female carriers [14].

More than half of patients with diabetes due to a heterozygous *INS* mutation have been screened for the presence of beta-cell auto-antibodies (anti-GAD65, islet cell antibodies (ICA) and insulin antibodies). There were no detectable auto-antibodies except for anti-insulin antibodies in some patients, most likely secondary to long-standing insulin therapy. The majority of patients had no residual beta-cell function as evidenced by low or undetectable basal or stimulated C-peptide levels. These measurements were in most cases performed months or years after the diagnosis of diabetes. However in one study, the C-peptide levels were determined at onset of diabetes and in most patients at one or two other times. These data suggest a gradual and progressive decline in beta-cell function over time. Six of 11 patients had normal, and in some instances, high C-peptide level at diagnosis of diabetes, declining levels at the repeat measurements and finally undetectable C-peptide months or years after the onset of diabetes [17].

Neurological dysfunction is a key feature of the phenotype of some patients with PNDM due to mutations in *KCNJ11* or *ABCC8* [33–35]. Patients with *INS* mutations do not have other associated extra-pancreatic features. Some patients have complications secondary to longstanding diabetes such as neuropathy and retinopathy. Signs of insulin resistance (e.g. the presence of acanthosis nigricans) have been noted in some overweight patients [13].

3.4 Clinical characteristics of patients with MODY

A subgroup of *INS* mutations is found exclusively in patients diagnosed with diabetes outside infancy and early childhood: R6H, R6C, L30M, R46Q and R55C. These patients fulfill traditional MODY criteria: non-obese, diagnosis generally <25 years of age and a family history of diabetes consistent with autosomal dominant inheritance. The diabetes is non-ketotic and the patients are treated with diet, oral hypoglycemic agents (OHA) or insulin. The majority of patients have residual beta-cell function as evidenced by detectable C-peptide levels. The extent of beta-cell failure could also be progressive, as some patients have declining C-peptide levels over time. One exception to the generally mild phenotype of this group of patients is a Norwegian family with the R55C mutation. The proband and her mother were diagnosed with diabetes under the dramatic setting of diabetic ketoacidosis and they now both require insulin in full replacement doses. A French family with the same mutation has mild, non-ketotic diabetes. This variation is presumably due to differences in genetic background and its effect on diabetes phenotype. Carriers of the R6H, R6C, L30M, R46Q and R55C mutations appear to have a normal birth weight (median, 4,000 g). However, birth weight in this group may be influenced by the presence of diabetes in the mothers and more studies are required to clarify the effect of these mutations on birth weight [14, 15, 26, 27].

The relationship between genotype (i.e. specific *INS* mutation) and phenotype is beginning to emerge from the studies to date. Some mutations (R6C/H, L30M, R46Q and R55C) are associated with a later age-at-diagnosis and a milder clinical course with patients maintaining adequate glycemic control on diet or oral hypoglycemic agents alone. However, the majority of *INS* mutations results in diabetes in the neonatal period and requires treatment with replacement doses of insulin. There can be, however, differences in the age-at-diagnosis of diabetes between carriers with the same mutation even within the same family indicating the role of other factors (genetic and nongenetic) in modifying the effect of the mutant protein on beta-cell function.

3.5 Clinical characteristics of patients with neonatal diabetes due to homozygous or compound heterozygous mutations

The phenotype of patients with diabetes due to a homozygous (or compound heterozygous) mutation in *INS* is characterized by severe intrauterine growth retardation (birth weight, <1 percentile) and diabetes most likely reflecting severe insulin deficiency in the pre- and postnatal life, respectively. The majority of patients are diagnosed with diabetes in the first days or weeks of life. Again, there are no extra-pancreatic manifestations.

The diabetes in this group of patients can be permanent or transient. The patients with PNDM appear to have lower birth weights (median SD scores for birth weight −3.9 vs. −1.8, $P=0.03$) and diabetes diagnosed earlier in life (2 days vs. 24 days, $P=0.04$) when compared to patients with TNDM. The mutations c. −366_−343del, c.3G>A (p.0?), c.3G>T (p.0?), c.184C>T (p.Q62X), c.−370−?186+?del) and c.*59A>G) appear to be associated with PNDM whereas the mutations at c.−218 and c.−331 have been identified in patients with both PNDM ($n=6$) and TNDM ($n=5$) as well as type 1b diabetes ($n=2$).

Interestingly, the carrier parents (i.e. heterozygous for the mutation) of patients who are homozygous or compound heterozygous for an *INS* mutation have normal glucose tolerance indicating that a single insulin allele is sufficient to provide the insulin required to maintain normal glycemia.

4 Insulin gene mutations and diabetes in animal models

Two mouse models of diabetes due to dominantly-acting heterozygous insulin gene mutations have been described, the Akita (*Ins2*^{C96Y}) and the Munich (*Ins2*^{C95S}) mouse [18, 19]. The C96Y mutation has also been reported in humans. The mutation C95S has not yet been described suggesting additional human mutations will be found. Hyperglycemia and hypoinsulinemia develop from the fourth week of life. Male mice have a more severe phenotype with pronounced polydipsia, polyuria, growth retardation and high mortality rates. By contrast, female mice have only mild hyperglycemia and normal life expectancy. This sexual dimorphism remains unexplained, but could be related to protective effects of estrogen.

Akita mice have reduced numbers of beta cells and reduced pancreatic insulin content. There is no evidence of inflammation of the islets as seen in islets from patients with type 1 diabetes. Electron microscopic studies of beta cells from diabetic Akita mice reveal severe organelle dysfunction with enlarged ER, swollen mitochondria, prominent lysosomes and a reduced number of secretory granules. These findings are consistent with a block in the progression of mutant proinsulin (and possibly some normal proinsulin) from the ER to the Golgi apparatus accompanied by ER stress. The accumulation of misfolded mutant proinsulin in the ER leads to activation of the unfolded protein response (UPR) including expression of the pro-apoptotic *CHOP* gene. In this regard, the development of diabetes is delayed by 8–10 weeks in Akita mice lacking *CHOP* highlighting the role of the UPR and apoptosis in beta-cell death in this model. Further studies of the Akita and Munich mouse models could lead to a better understanding of the pathophysiology of diabetes due to expression of a mutant proinsulin protein and perhaps novel approaches for treating this form of diabetes [31, 36].

5 Pathophysiology of insulin mutations

5.1 Structure/function studies of insulin mutations

Studies of the effects of mutations that affect processing of preproinsulin to proinsulin or lead to misfolding of proinsulin have shown that the mutant proteins accumulate in the ER and are poorly secreted (Fig. 5). The expression

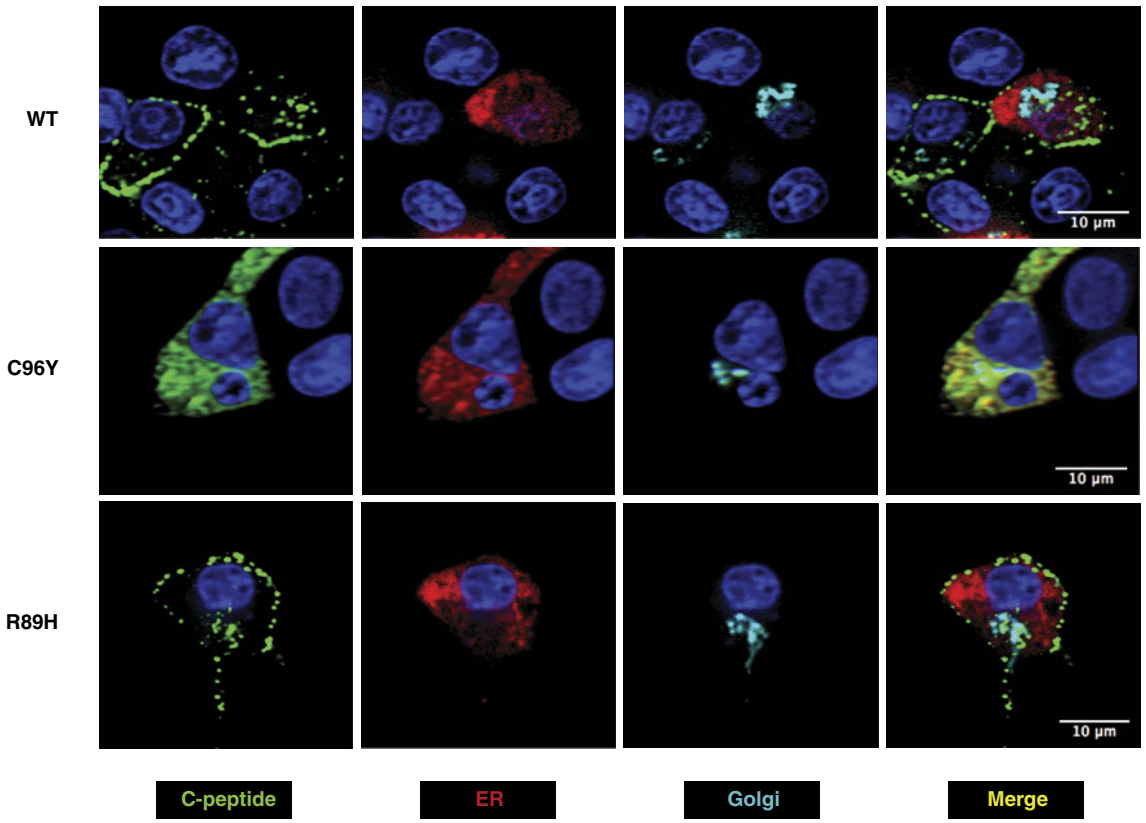

Fig. 5 Subcellular localization of wild-type (WT) and mutant human proinsulin expressed in rat INS-1 insulinoma cells [30]. The location of human C-peptide immunoreactive protein is shown in green, ER is shown in red and the Golgi marker in cyan. Nuclei were stained with DAPI (blue). The WT and R89H hyperproinsulinemia mutant proinsulins are localized to secretory granules. By contrast, the C96Y PNDM-associated mutant proinsulin is localized to the expanded ER and is not transported from the ER to the Golgi and packaged into secretory granules

of the mutant proteins also leads to induction of the UPR and in time apoptosis. The mutant proteins also appear to exert a dominant-negative effect on the synthesis and secretion of native proinsulin due to induction of the UPR and consequent attenuation of translation [17, 29, 30].

Hodish et al. (2010) have established an elegant transgenic model to study the effect of the C96Y mutation on insulin biosynthesis *in vivo* by crossing transgenic mice expressing GFP-tagged proinsulin with Akita mice. They show that the mutant (C96Y) proinsulin blocks native proinsulin biosynthesis followed by loss of pancreatic beta-cells. Thus, the aggregation of both mutant and native proinsulin in the ER likely triggers ER stress [37].

5.2 Pathophysiology of homozygous insulin gene mutations

In contrast to the dominantly-acting mutations, the mutations found in the homozygous state result in diabetes through reduced insulin biosynthesis *per se*. These mutations affect insulin biosynthesis by distinct mechanisms including gene deletion, mutation of the translation initiation site, synthesis of a nonsense protein, impaired transcription due to mutations of critical transcription factor binding sites in the promoter, and impaired mRNA polyadenylation leading to insulin mRNA instability, all resulting in severely impaired insulin production, but without ER stress. These patients presumably have otherwise normal beta cells and the transient nature of the diabetes in some of these patients is consistent with this view [32].

6 Insulin gene mutations and familial syndromes of hyperproinsulinemia and hyperinsulinemia with mild diabetes

A subgroup of mutations in *INS* causes mild diabetes or impaired glucose tolerance by another mechanism. These mutations lead to the synthesis of an insulin with reduced biological activity or prevent processing of proinsulin to insulin leading to hyperinsulinemia or hyperproinsulinemia, respectively. In contrast to the *INS* mutations associated with PNDM, the beta cells are able to synthesize and secrete these molecules, thus indicating that these variants of *INS* do not *per se* lead to cellular or organelle dysfunction.

6.1 Hyperinsulinemia

Three rare missense mutations, F48S (insulin Los Angeles), F49L (insulin Chicago), and V93L (insulin Wakayama) (Figs. 1 and 2) have been identified in patients with phenotypes ranging from normal glycemia to mild diabetes and concomitant hyperinsulinemia [9, 38–43]. These mutations affect amino acids involved in insulin receptor binding (B24-Phe, B25-Phe and A3-Val) and as a consequence the mutant insulins have reduced insulin receptor affinity and biological activity. Mutation carriers have hyperinsulinemia, but impaired glucose tolerance or overt diabetes only develops in adults with insulin resistance. When diabetes develops, it is mild and satisfactory glycemic control can be achieved with diet, oral hypoglycemic agents or small doses of insulin.

6.2 Hyperproinsulinemia

Four rare heterozygous missense mutations in *INS* have been identified in patients with a disorder characterized by hyperproinsulinemia and a variable degree of glucose intolerance: three mutant forms of proinsulin that involve the C-peptide-A chain junction (R89H (proinsulin Tokyo), R89L (proinsulin Kyoto) and R89P) and a mutation in the B-chain, H34D (proinsulin Providence) [9–12, 44–49]. The glucose intolerance varies from normal glucose tolerance to mild diabetes that can in most cases be managed with diet only.

The substitution of Arg-89 for His, Leu or Pro at the C-peptide-A-chain junction (the site of proteolytic cleavage of des-31, 32 proinsulin to insulin and C-peptide) interferes with the action of the prohormone convertase PC2 and as a consequence, these patients have high levels in the circulating of a proinsulin-like material that has been cleaved only at the B-chain-C-peptide junction. The *INS* mutation H34D causes hyperproinsulinemia by another mechanism. A proportion of newly synthesized Asp-B10 proinsulin is secreted unprocessed in a glucose-independent manner via an unregulated pathway and enters the circulation as Asp-B10 proinsulin whereas the majority enters the regulated secretory pathway and is processed and secreted as Asp-B10 insulin. The reason for the missorting of Asp-B10 proinsulin is unclear but might be related to the higher biological potency of both Asp-B10 proinsulin and insulin relative to native proinsulin and insulin which increases its binding to recycling insulin receptors in the beta cell leading to its uncontrolled release and/or lysosomal degradation [50]. Perhaps, Asp-B10 proinsulin, thus, reduces the requirement for release of normally processed insulin (including Asp-B10 insulin) by the regulated pathway.

7 Conclusion

Mutations in the insulin gene are associated with diverse phenotypes from hyperinsulinemia and hyperproinsulinemia to neonatal diabetes. These mutations (in both the heterozygous and homozygous state) are a common cause of neonatal diabetes. They can also be found in patients with a diagnosis of type 1b diabetes or MODY. A correct genetic diagnosis in these patients is important for correct classification of their

disease as well as for purposes of genetic counseling. Currently, there is no specific treatment for these patients apart from insulin-based regimens when this is required but future research may identify approaches for addressing the cellular stress resulting from the biosynthesis of a mutant protein and thereby preserving beta-cell function and allowing the normal allele to provide the insulin necessary for maintaining normal glucose tolerance.

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