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Association of insulin gene VNTR *INS -23/Hph1 A>T (rs689)* polymorphism with type 1 diabetes mellitus in Egyptian children

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Abstract

Background: Type1 diabetes mellitus (T1DM) has a multi-factorial pathogenesis; the interplay between genetic susceptibility and environmental factors is thought to provide the fundamental element for the disease. Apart from HLA, more than 50 genetic variants are associated with T1DM. *INS -23/Hph1 A>T* (rs689) is one of the effective loci with inconsistent reports in the literature. Accordingly, this study was designed to define the frequencies of *INS -23/Hph1 A>T* polymorphism and its association with T1DM in Egyptian diabetic children and their non-diabetic family members as compared to healthy controls.

Methods: Using polymerase chain reaction-restriction fragment length polymorphism methodology, analysis of *insulin* gene VNTR polymorphism was performed for 496 samples (91 patients, 179 parents, 130 siblings, and 96 controls); parents and siblings were apparently healthy.

Results: *INS* genotypes and allele frequencies were comparable between patients, non-diabetic siblings, and parents ($p = 0.97$ and 0.77, respectively). However, the *TT/AT* genotype and *T* allele were over-presented in the three family groups compared to controls ($p = 0.0015$ and 0.0029, respectively).
Comparing patients to controls, the *T* allele is considered a risk factor for the development of TIDM (OR 2.56, 95% CI 1.42–4.62, $p = 0.0017$).
INS -23/Hph1 A>T polymorphism showed concordance between patients and their mothers (Kappa = 0.446, $p = 0.000$) but not with their fathers (Kappa = 0.031, $p = 0.765$).

Conclusions: *INS -23/Hph1 A>T* gene polymorphism was shown to be a risk factor for the development of TIDM. This is in agreement with some and in disagreement with other reports. Studies of risk susceptibility factors have to be carried out locally in each community; results cannot be extrapolated from one ethnic group to another.

Keywords: Type 1 Diabetes Mellitus, TIDM, IDDM2, *INS-23*

Background

Type1 diabetes mellitus (TIDM) is one of the most common chronic childhood illnesses [1]. T1DM results from autoimmune destruction of the insulin-producing beta cells in the pancreas [1]. People with a family history of T1DM and type2 diabetes mellitus (T2DM) are six and three times more likely, respectively, to develop these diseases than unrelated individuals [2]. T1DM has a multi-factorial pathogenesis; the interplay between genetic susceptibility and environmental factors is thought to provide the fundamental element for the disease [3]. Alleles or genetic variants associated with T1DM provide either susceptibility to or protection from the disease within a given environmental background [3]. HLA genes have the highest effect on risk susceptibility to TIDM [4]. However, more than 50 loci outside the human leukocyte antigen (HLA) region have been confirmed to affect T1DM [4]. The genetic makeup with the balance between susceptibility and protection alleles determines the age of onset of T1DM [5]. Next in importance to

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HLA, polymorphisms in the noncoding region of the *insulin* (*INS*) gene (IDDM2) contribute to susceptibility to or protection from T1DM [6]. This locus includes a variable nucleotide tandem repeat (VNTR) mini-satellite located at the 5′ end of the *insulin* gene [6]. There are three main VNTR classes defined by the VNTR size: class I (26–63 repeats), class II (approximately 80 repeats), and class III (140–200 repeats) [7]. Polymorphisms within the *insulin* gene are in linkage disequilibrium with the VNTR region and can therefore be used as markers for these gene variants [7]. The *223HphI A>T* single-nucleotide polymorphism (SNP), a polymorphic sequence in the *INS* gene, was found to be in strong linkage disequilibrium with the VNTR alleles; the *A* allele is in linkage with the short (class I), and the *T* allele is in linkage with the long (class III) VNTR allele [8]

The literature shows inconsistency as regards the association of the *A* and *T* alleles with risk susceptibility to TIDM. In some reports, the *A* allele is claimed to be associated with risk of developing diabetes and the *T* allele is claimed to be protective [8–12]. Other reports indicate that *T* allele is the one associated with risk susceptibility [13–15] while others deny an impact of either on risk susceptibility to TIDM [16–20].

Risk susceptibility, in general, reflects an interaction between genetic and environmental factors [3]. Ethnic variations in the relative frequency of different alleles as well as exposure to a different local environment lead to variable contribution of a given gene polymorphism to risk susceptibility to one disease or the other [3]. Hence, results cannot be extrapolated from one population to another and such studies have to be locally performed in each community.

Studies performed in Egypt on genetic background of T1DM, including studies of the research team, have so far addressed mainly the contribution of the HLA region and *CTLA-4* [21–23]. Only one previous study, performed on 25 subjects, addressed the role of *INS* gene in risk susceptibility to TIDM [24].

In the current study, The *INS -23/Hph1 A>T* (rs689) was tested in diabetic children, non-diabetic siblings, and parents as compared to normal controls. This may help, together with other genetic information, to predict the possibility of a child, born to a diabetic family, to develop the disease leading to early effective therapy and better management of diabetic children.

Methods

On account of the controversy of literature, this study was designed to define the frequencies of *INS -23/Hph1 A>T* polymorphism and its association with T1DM in Egyptian diabetic children and their non-diabetic family members as compared to healthy controls.

The work was carried out in accordance with the Helsinki Declaration for experiments involving human beings. The study was approved by the Institutional Review Board of the National Cancer Institute, Cairo University (Approval No 200809003.2 on October 28, 2010), and a written informed consent was obtained from all participants or their guardians.

Subjects

The study included 496 subjects: 91index cases with type 1 diabetes mellitus, 179 parents, 130 siblings, and 96 control healthy subjects; parents and siblings were apparently healthy. Nine families were with a single parent; the missing parent either refused to share, was divorced, or unavailable. Eighty-nine (89) families have only one diabetic proband and seven (7.3%) have two or more diabetic probands; only one was included in statistical analysis. All patients are attending an outpatient clinic in the Diabetes Endocrinology and Metabolism Pediatrics Unit, at Cairo University Children's Hospital. Patient's ages at the time of the study ranged from 1.5 to < 18 years with a mean of 8.6 ± 4 and a median of 9 years. The age of onset ranged from 1 year to 12.6 years with a mean of 5.3 ± 3.6 and a median of 5 years. Seventeen cases have age of onset ≤ 1 year; patients with age of onset ≤ 6 months were excluded as 95% of patients with diabetes onset < 6 months of age have a monogenic origin [25]. Complete physical examination, anthropometry, and examination for any associated conditions, congenital anomalies, or complications were routinely done for every patient.

The following information was obtained:

1. Age of onset for index case (by definition < 18 years).
2. Age of onset for other siblings (if any).
3. History of diabetes mellitus in the family occurring at any age.
4. History of other autoimmune diseases in the family including, among others, autoimmune thyroiditis, pernicious anemia, and Addison's disease.

DNA analysis and PCR-RFLP testing for *INS -23/Hph1 A>T*

Genomic DNA was extracted from peripheral blood using the salting out technique [26]. *INS-23/Hph I* polymorphism was determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method [27]. The PCR reaction mixture contained 50 ng genomic DNA, 0.5 μM of each primer (forward: 5′-AGC AGG TCT GTT CCA AGG-3′ and reverse: 5′-CTT GGG TGT GTA GAA GAA GC-3′), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM of each dNTP, and 0.75 units Taq DNA polymerase (Promega, USA) in a final volume of 25 μl. The cycle consisted

of initial denaturation for 2 min at 95 °C, 32 cycles at 94 °C for 20 s for denaturing, 15 s at 57 °C for annealing, and 30 s at 72 °C for extension with final extension at 72 °C for 10 minutes. The PCR products were digested with the *Hph I* restriction enzyme at 37 °C for 18 h. After digestion, products were electrophoresed on 2.5% agarose gel and visualized by ethidium bromide staining. The *T* and *A* alleles could be distinguished as bands of 231 plus 129 bp and 191 plus 129 bp, respectively (Fig. 1).

Statistical analysis

SPSS version 17.0 was used for data management. Proportions were compared using the chi-square and Fisher exact tests. Odds ratio of genotype(s) was calculated with 95% confidence interval. Parametric and non-parametric tests compared the means of two or more than two independent groups (*t* test and ANOVA).

Hardy–Weinberg equilibrium (HWE) was evaluated using chi-square test to estimate the study quality. Genotype and allele frequencies were calculated for the described SNPs. The groups were compared using the $\chi 2$ test to analyze the statistical significance of the difference in allelic distribution of various polymorphisms in patients and controls. Measurement of agreement Kappa between patients and both parents was calculated using Cohen Kappa coefficient. *P* value was considered significant at 0.05 level.

Results

Frequencies of *rs689* in the different groups are shown in Table 1.

The genotype distribution was comparable between patients, parents, and non-diabetic siblings (p = 0.97). *AA* was over-represented in the controls while *TT and AT* were over-represented in the three family groups compared to controls (p = < 0.0015 and 0.0029, respectively).

The distribution of the *A* and *T* alleles is comparable between patients, parents, and non-diabetic siblings (p = 0.77). The *T* allele is over-represented in the three diabetic family groups as compared to the control group (p = 0.0029). Comparing patients to normal controls, the *T* allele is considered a risk factor for the development of TIDM (OR 2.56, 95% CI 1.42–4.62, p = 0.0017).

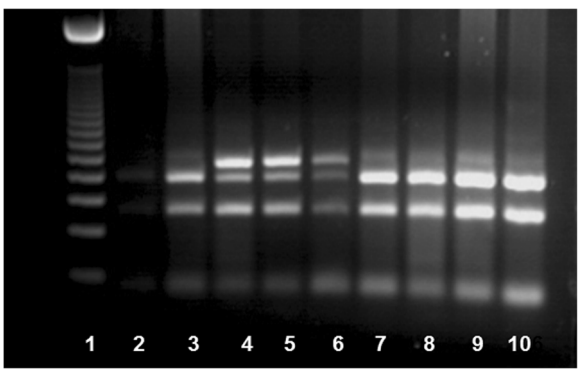

Fig. 1 *INS -23 Hph1 A>T* polymorphism. Lane 1: 50 bp ladder, lanes 3 and 7–10: Wild type (*AA*) 191 and 129 bp; lanes 4-6: heterozygous (*AT*) 231, 191, and 129 pb

By analysis of *INS -23/Hph1 A>T* polymorphisms in each parent (father and mother) and comparing it with patients, the *AA* allele was inherited from the mother in 34 cases and from the father in 25 cases, the *AT* allele was inherited from the mother in 23 cases and from the father in 15 cases while the *TT* allele was inherited from the mother or the father in one case each (Table 2).

There was concordance between patients and their mothers (concordance rate 58/82, measure of agreement Kappa = 0.446, p = 0.000) but not with their fathers (concordance rate 41/81, measure of agreement Kappa = 0.031, p = 0.765) (Table 2).

There was no association between *INS -23/Hph1 A>T* polymorphisms in patients and other autoimmune diseases, e.g., Hashimoto's thyroiditis, rheumatoid arthritis, and rheumatic heart disease or Crohn's disease (P = 0.23).

Discussion

Apart from MHC (IDDMI), more than 50 Non-HLA genes were reported to contribute to risk susceptibility to TIDM [4]. Among these, *INS* gene (IDDM2) is claimed to be the most effective; *INS* gene, also, plays an important role in the progress of other endocrine diseases [28].

Polymorphisms within the *insulin* gene are in linkage disequilibrium with the VNTR region and were used as a surrogate marker for the gene variants in several studies [10, 11, 29, 30].

In the current study, we analyzed the *223HphI A>T* in 91 diabetic children and their family members as well as 96 normal controls. We included parents and siblings as they are sharing, at least partly, the genotype(s) with the patients; the parents are not diabetic and the siblings are not diabetic so far. Our results indicate that *T* allele carriers are at 2.56 folds risk to develop TIDM.

In agreement with our results, Peterson et al. [15] reported a 2.44 folds increased risk associated with the *INS*rs689 *T* allele. Other studies had the same conclusion with a 4.5 folds risk associated with homozygous *TT* genotype [13] and 4.02, 4.4, and 6.96 folds risk in three Israeli Jewish populations [14].

Other reports, however, deny any impact of *INS*rs689 polymorphism on risk susceptibility to TIDM [16–20]. The study of Benedek et al. [14] that documented the association of *T* allele with ITD in three Jewish populations failed to prove that association in the Israeli Arabs which highlights the effect of ethnicity. They attributed their finding to the higher frequency of the VNTR class

Table 1 Genotype and allele frequency of *INS -23 A>T* in type 1 diabetes mellitus families

Group	No	*INS 23* Genotype				*T* Allele	HW[a]
		Wild AA	Hetero AT	Homo TT	AT + TT	No %	p
Type 1 Diabetics	91	53 58.24	36 37.36	2 4.4	38 41.758	40/182 21.97	0.143
Non-diabetic siblings	130	70 53.85	56 43.08	4 3.08	60 46.15	64/260 24.62	0.067
Parents	179	102 56.98	73 40.78	6 3.35	79 44.13	85/358 23.74	0.1
Control	96	78 81.25	17 17.71	1 1.04	18 18.75	19/192 9.895	0.945

Hetero heterozygous, *Homo* homozygous
[a]Hardy Weinberg for genotype distribution

I variant allele 814, among Arabs, that does not predispose to T1D when paternally inherited [31].

In contrast to our results, a meta-analysis performed by Zhang et al. [32] documented the *A* allele of the-*23HphI* polymorphism as the risk allele and the *T* as the protective allele.

The only previous Egyptian study is also in contrast with our results showing the *A* allele of the-*23HphI* polymorphism as the risk allele and the *T* as the protective allele. However, the sample size was too small with 25 cases and 20 controls; in their series, all patients had class I and all controls had class III. Also all family members had class III except for three mothers with class I with no comment on the distribution of wild, hetero, and homozygous in the different groups [24].

A mechanistic explanation of the association has been proposed, as the *A* T1DM-risk allele, suggested in some studies, is claimed to relate to lower levels of insulin transcription in thymus leading to non-effective deletion of insulin-specific auto-reactive T cells [9, 29, 33–35]. This explanation may be questioned from several aspects. First, the genotype claimed to be associated with lower levels of insulin transcription in the thymus is the one associated with higher level of production in the pancreas. This leads to the second point of consideration; breaking of central tolerance is not the only mechanism in autoimmune diseases but peripheral tolerance also plays a major role [36]. Breaking of peripheral tolerance is affected by many environmental factors including viral infections and others with marked variability in different communities.

Another potential explanation of the discrepancy is that most studies, including ours, did not determine the subclasses of VNTR I and VNTR III. For instance the assumed protective role of VNTR III is claimed to be attributed to VNTR IIIA/IIIA [10]. Also, VNTR class I variant allele 814 does not predispose to T1D when paternally inherited [31].

Our study showed maternal rather than paternal concordance with TIDM children (Kappa = 0.446, p = 0.000 and Kappa = 0.031, p = 0.765, respectively). This could be also contributing to the discrepancy in the literature as the parental source of inheritance affects the impact of different genes on risk susceptibility [7, 30, 31].

Apart from this mechanistic theory, risk susceptibility associated with gene polymorphisms is largely affected by ethnic variations including the relative genotype frequency in different populations which varies from one community to another [12, 13, 16, 33, 34, 37].

Table 2 Concordance of *INS -23/Hph1 A>T* polymorphisms between type 1 diabetic children and their parents

Mother	Patient				Father	Patient			
	AA	AT	TT	Total		AA	AT	TT	Total
AA	*34*	8	0	42	AA	*25*	19	0	44
AT	12	*23*	1	36	AT	20	*15*	0	35
TT	1	2	*1*	4	TT	1	0	*1*	2
Total	47	33	2	82	Total	46	34	1	81

Figures in italic = number of cases sharing *INS -23/Hph1 A>T* polymorphisms with their parents. Concordance rate with mothers: 58/82, Kappa value = 0.446, p = 0.000. Concordance rate with fathers: 41/81, Kappa value = 0.031, p = 0.765

Conclusion

We report that the *INS -23/Hph1 A>T* polymorphism is a significant risk susceptibility locus to TIDM in Egyptian children; carriers of the *T* allele are at 2.56 folds risk to develop the disease. These results are in agreement with some and in disagreement with other previous studies. The discrepancy of results may also indicate that more than one mechanism is involved with central tolerance vs. peripheral tolerance playing the major role. In general, this highlights the importance of performing susceptibility studies locally; findings from one ethnic group or population cannot be extrapolated to others. Limitations of this study include the relatively small number of patients and control groups. Also, further studies integrating different TIDM susceptibility genes are highly recommended.

Abbreviations
TIDM: Type1 diabetes mellitus; T2DM: Type2 diabetes mellitus; INS: Insulin; IDDM: Insulin-dependent diabetes mellitus; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; VNTR: Variable nucleotide tandem repeat; SNP: Single-nucleotide polymorphism

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Authors' contributions
AMK made the work design, supervised the data analysis, and finalized the manuscript. GT participated in the molecular genetic testing. SH participated in the molecular genetic testing and drafted the manuscript. ERR participated in the molecular genetic testing and revised the manuscript. MFM participated in the study design, collected the patient's samples, and integrated the clinical and laboratory data. MMH, MA, NEB, HMB, and AI participated in the study design, followed the patients clinically, and supplied the clinical data. NS supervised the clinical part and finalized the clinical data. All authors approved the manuscript

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Availability of data and materials
All data are included in the manuscript

Ethics approval and consent to participate
The work was carried out in accordance with the Helsinki Declaration for experiments involving human beings. The study was approved by the Institutional Review Board of the National Cancer Institute, Cairo University (Approval No 200809003.2 on October 28th 2010), and a written informed consent was obtained from all participants or their guardians.

Consent for publication
NA

Competing interests
The authors declare that they have no competing interest.

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References
1. Gillespie KM (2006) Type 1 diabetes: pathogenesis and prevention. CMAJ. 175: 165–170
2. Dorman JS, Bunker CH (2000) HLA-DQ locus of the human leukocyte antigen complex and type 1 diabetes mellitus: aHuGE review. Epidemiologic Reviews 22:218–227
3. Erin EB, Eisenbarth GS (2008) Extreme genetic risk for type 1 A diabetes in the post genome era. J Autoimmun 3:11–16
4. Lempainen J, Härkönen T, Laine AP, Knip M, Ilonen J, The Finnish Pediatric Diabetes Register (2013) Associations of polymorphisms in non HLA loci with autoantibodies at the diagnosis of type 1 diabetes: INS and IKZF4 associate with insulin autoantibodies. Pediatric Diabetes 14:490–496
5. Devendra D, Liu E, Eisenbarth GS (2004) Type 1 diabetes: recent developments. BMJ 328:750–754
6. Concannon P, Erlich HA, Julier C, Morahan G, Nerup J, Pociot F et al (2005) Type 1 Diabetes Genetics Consortium. Type 1 diabetes: evidence for susceptibility loci from four genome-wide linkage scans in 1,435 multiplex families. Diabetes 54:2995–3001
7. Bennett ST, Lucassen AM, Gough SCL, Willenberg HS, Meyer G, Penna-Martinez M et al (1995) Susceptibility to human type 1 diabetes is determined by tandem repeat variation at the insulin minisatellite locus. Nat Genet 9:284–293
8. Howson JMM, Walker NM, Smyth DJ, Todd JA, Type I Diabetes Genetics Consortium (2009) Analysis of 19 genes for association with type I diabetes in the Type I Diabetes Genetics Consortium families. Genes Immun 10(Suppl 1):74–84
9. Awata T, Kawasaki E, Ikegami H, Kobayashi T, Maruyama T, Nakanishi K et al (2007) Insulin gene/IDDM2 locus in Japanese type 1 diabetes: contribution of class I alleles and influence of class I subdivision in susceptibility to type 1 diabetes. J Clin Endocrinol Metab 92:1791–1795
10. Laine AP, Holmberg H, Nilsson A, Ortqvist E, Kiviniemi M, Vaarala O et al (2007) Finnish Paediatric Diabetes Registry Two insulin gene single nucleotide polymorphisms associated with type 1 diabetes risk in the Finnish and Swedish populations. Dis Markers 23:139–145
11. Ramos-Lopez E, Lange B, Kahles H, Meyer G, Penna-Martinez M, Reisch N et al (2008) Insulin gene polymorphisms in type 1 diabetes, Addison's disease and the polyglandular autoimmune syndrome type II. BMC Med Genet 9:65
12. Chung H, Yang S, Shin C, Park KS, Lee YA, Kim JH et al (2010) The association of variable number of tandem repeats of the insulin gene with susceptibility to type 1 diabetes among Korean subjects. Diabetes Metab Res Rev 26:474–480
13. Barratt BJ, Payne F, Lowe CE, Hermann R, Healy BC, Harold D et al (2004) Remapping the insulin gene/IDDM2 locus in type 1 diabetes. Diabetes 53: 1884–1889
14. Benedek G, Brautbar C, Vardi P, Sharon N, Weintrob N, Zung A et al (2009) Effect of polymorphism in insulin locus and HLA on type 1 diabetes in four ethnic groups in Israel. Tissue Antigens 73:33–38
15. Pettersen E, Skorpen F, Kvaløy K, Midthjell K, Grill V (2010) Genetic heterogeneity in latent autoimmune diabetes is linked to various degrees of autoimmune activity: results from the Nord-Trøndelag Health Study. Diabetes 59:302–310
16. Raha O, Sarkar BN, Bhaskar LVKS, Veerraju P, Chowdhury S, Mukhopadhyay S et al (2011) Insulin (INS) promoter VNTR polymorphisms: interactions and association with type 1 diabetes mellitus in Bengali speaking patients of Eastern India. Diabetologia Croatica 40:99–106
17. Steck AK, Wong R, Wagner B, Johnson K, Liu E, Romanos J, Wijmenga C et al (2012) Effects of non-HLA gene polymorphisms on development of islet autoimmunity and type 1 diabetes in a population with high-risk HLA-DR, DQ genotypes. Diabetes 61:753–758
18. Matsumoto C, Awata T, Iwamoto Y, Kuzuya T, Saito T, Kanazawa Y (1994) Lack of association of the insulin gene region with type 1 (insulin-dependent) diabetes mellitus in Japanese subjects. Diabetologia 37:210–213
19. Awata T, Kurihara S, Kikuchi C, Takei S, Inoue I, Ishii C, Takahashi K et al (1997) Evidence for association between the class I subset of the insulin gene minisatellite (IDDM2 locus) and IDDM in the Japanese population. Diabetes 46:1637–1642
20. Kawaguchi Y, Ikegami H, Shen GQ, Nakagawa Y, Fujisawa T, Hamada Y et al (1997) Insulin gene region contributes to genetic susceptibility to, but may not to low incidence of, insulin-dependent diabetes mellitus in Japanese. Biochem Biophys Res Commun 233:283–287
21. Kamel AM, Mira MF, Mossallam GI, Ebid GTA, Radwan ER, Aly Eldin NH et al (2014) Lack of association of CTLA-4 +49 A/G polymorphism with predisposition to type 1 diabetes in a cohort of Egyptian families. EJMHG 15:25–30
22. Saleh HM, Rohowsky N, Leski M (2008) The CTLA4 -819 C/T and+49 A/G dimorphisms are associated with Type 1 diabetes in Egyptian children. Indian J Hum Genet 14:92–98
23. Mosaad YM, Elsharkawy AA, El-Deek BS (2012) Association of CTLA-4 (+49A/G) gene polymorphism with type 1 diabetes mellitus in Egyptian children. Immunol Invest 41:28–37
24. Abo El-Ella SSA, Shaltout AA, Tawfik MM, Deeb M, DM EL-L, Khatab ES et al (2011) Non HLA genetic markers association with type-1 diabetes mellitus. EJMHG 12:39–47
25. Vaxillaire M (2009) Monogenic forms of diabetes mellitus: an update. Endocrinol Nutr 56S4:26–29
26. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215
27. Xu Y, Wei Z, Zhang Z, Xing Q, Xing Q, Hu P, Xal Z et al (2009) No association of the insulin gene VNTR polymorphism with polycystic ovary syndrome in a Han Chinese population. Reprod Biol Endocrinol 7:141

28. Stead JD, Buard J, Todd JA, Jeffreys AJ (2000) Influence of allele lineage on the role of the insulin minisatellite in susceptibility to type 1 diabetes. Hum Mol Genet 9:2929–2935
29. Desai M, Zeggini E, Horton VA, Owen KR, Hattersley AT, Levy JC et al (2006) The variable number of tandem repeats upstream of the insulin gene is a susceptibility locus for latent autoimmune diabetes in adults. Diabetes 55:1890–1894
30. Bennett ST, Todd JA (1996) Human type 1 diabetes and the insulin gene: principles of mapping polygenes. Annu Rev Genet 30:343–347
31. Bennett ST, Wilson AJ, Esposito L, Bouzekri N, Undlien DE, Cucca F et al (1997) Insulin VNTR allele-specific effect in type 1 diabetes depends on identity of untransmitted paternal allele. The IMDIAB Group. Nat Genet 17:350–352
32. Zhang N, Huang W, Dong F, Liu Y, Zhang B, Jing L et al (2015) Insulin gene VNTR polymorphisms -2221MspI and -23HphI are associated with type 1 diabetes and latent autoimmune diabetes in adults: a meta-analysis. Acta Diabetol. 52:1143–1155
33. Pugliese A, Zeller M, Fernandez A Jr, Zalcberg LJ, Bartlett RJ, Ricordi C et al (1997) The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat Genet 15:293–297
34. Ikegami H, Noso S, Babaya N, Hiromine Y, Kawabata Y (2008) Genetic basis of type 1 diabetes: similarities and differences between east and west. Rev Diabet Stud. 5:64–72
35. Alizadeh BZ, Koeleman BP (2008) Genetic polymorphisms in susceptibility to type 1 diabetes. Clin Chim Acta 387:9–17
36. Jeker LT, Bour-Jordan HI, Jeffrey A (2012) Breakdown in peripheral tolerance in type 1 diabetes in mice and humans. Cold Spring Harb Perspect Med 2: a007807. https://doi.org/10.1101/cshperspect.a007807
37. Pugliese A, Miceli D (2002) The insulin gene in diabetes. Diabetes Metab Res Rev 18:13–25

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