Insights Into the Molecular Mechanism for Type 2 Diabetes Susceptibility at the KCNQ1 Locus From Temporal Changes in Imprinting Status in Human Islets

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The molecular basis of type 2 diabetes predisposition at most established susceptibility loci remains poorly understood. KCNQ1 maps within the 11p15.5 imprinted domain, a region with an established role in congenital growth phenotypes. Variants intronic to KCNQ1 influence diabetes susceptibility when maternally inherited. By use of quantitative PCR and pyrosequencing of human adult islet and fetal pancreas samples, we investigated the imprinting status of regional transcripts and aimed to determine whether type 2 diabetes risk alleles influence regional DNA methylation and gene expression. The results demonstrate that gene expression patterns differ by developmental stage. CDKN1C showed monoallelic expression in both adult and fetal tissue, whereas PHLD2A, SLC22A18, and SLC22A18AS were biallelically expressed in both tissues. Temporal changes in imprinting were observed for KCNQ1 and KCNQ1OT1, with monoallelic expression in fetal tissues and biallelic expression in adult samples. Genotype at the type 2 diabetes risk variant rs2237895 influenced methylation levels of regulatory sequence in fetal pancreas but without demonstrable effects on gene expression. We demonstrate that CDKN1C, KCNQ1, and KCNQ1OT1 are most likely to mediate diabetes susceptibility at the KCNQ1 locus and identify temporal differences in imprinting status and methylation effects, suggesting that diabetes risk effects may be mediated in early development. Diabetes 62:987–992, 2013

The translation of established type 2 diabetes risk variants into an improved understanding of disease pathology is challenging. Progress has been made primarily at the few loci where causal alleles are coding (1–4), but disease mechanisms are unclear for the majority of loci mapping outside coding regions. The KCNQ1 locus harbors at least two independent regions of association with type 2 diabetes risk (intron 10 and intron 15), both acting through impaired islet function (5–11). KCNQ1 itself encodes the K,7.1 voltage-gated potassium channel subunit, which is expressed in human B-cells (12) but plays an uncertain role in insulin secretion. Neither patients with cardiac arrhythmia caused by KCNQ1 mutations nor KCNQ1-null mice demonstrate hyperglycemia or glucose intolerance (13,14), whereas KCNQ1 knockdown in human islets does not alter insulin secretion (15).

In accordance with the location of KCNQ1 at the imprinted 11p15.5 region, associated alleles at both signals confer disease risk only when maternally inherited (16). It has been demonstrated, primarily through studies of the syntenic region of mouse chromosome 7, that regional gene expression is regulated by differential methylation at the promoter of KCNQ1 overlapping transcript 1 (KCNQ1OT1), a nontranslated antisense RNA that regulates maternal-specific expression of downstream genes (17) (Fig. 1).

Disruption of genomic architecture at the 11p15.5 chromosomal region has a well-established role in Beckwith-Wiedemann syndrome (BWS), a congenital overgrowth syndrome often associated with hypoglycemia (18). Furthermore, unbalanced placental expression of two regional genes, PHLD2A and CDKN1C, is associated with intrauterine growth retardation (19). We hypothesize that type 2 diabetes risk may be mediated through disruption of methylation and imprinted gene expression within the imprinted cluster. In this study, we perform the first assessment of 11p15.5 regional imprinting in adult human islets and fetal pancreas and investigate the effect of risk genotype status on DNA methylation and imprinted gene expression.

RESEARCH DESIGN AND METHODS

Islet and fetal pancreas isolation and DNA/RNA extraction. Human islets of deceased donors of European descent were obtained (with research consent) from the Oxford Diabetes Research & Wellness Foundation Human Islet Isolation Facility (n = 30) and the Human Tissue Laboratory at Lund University Diabetes Centre (n = 42). Fetal pancreas samples (n = 18) were obtained with informed consent and ethical approval from the North West Regional Ethics Committee. All islet preparations were >80% pure, with RNA integrity numbers >7. Donor and purity details are provided in Supplementary Table 1. DNA and RNA were extracted using TRIzol (Life Technologies).

cDNA synthesis. cDNA was generated through random-primed first-strand synthesis from 1 μg RNA in accordance with standard protocols, including treatment with DNase 1.

Genotyping. Type 2 diabetes-associated single-nucleotide polymorphisms (SNPs) (rs231362 and rs2237895) were selected as the lead SNPs (strongest evidence for association) in each of the independent signals. Reporter coding SNPs for imprinting analysis were selected to have the highest possible minor allele frequencies, maximizing heterozygous samples capable of differentiating mRNA products from homologous chromosomes. Genotyping was performed...
FIG. 1. Schematic representation of imprinting control [as described in (17)] and type 2 diabetes-associated SNPs at 11p15.5. A: Chromosome 11:2,450,000–2,960,000. The closed circle at the DMR represents a high level of methylation; the open circle represents a low level of methylation. The transcribed sequence is shown in dark gray, and untranscribed is shown in light gray. Arrows indicate direction of transcription. B: Chromosome 11:2,650,000–2,880,000. The smaller region is distinguished by exonic (boxes) and intronic regions of KCNQ1 (dark gray), a region of KCNQ1OT1 transcription (light gray), and relative positions of top disease-associated SNPs (rs231362, chr11:2,691,471, and rs2237985, chr11:2,857,194). All genomic coordinates are b37/hg19 (graphics not to scale).
FIG. 2. A: Imprinting status of genes in the 11p15.5 cluster CDKNIC. Fragment analysis traces demonstrate monoallelic (imprinted) expression of CDKNIC in adult islet (top) and fetal pancreas (bottom) samples. Left: genomic DNA, with the two size peaks characteristic of a heterozygote for del171APVA highlighted. Right: cDNA from the same heterozygous samples. In both cases, only one size peak is evident, indicating the presence of mRNA from only one chromosome. Every sample heterozygous for del171APVA at the gDNA level appeared homozygous at the cDNA level, indicating monoallelic expression. B: Imprinting status of genes in the 11p15.5 cluster KCNQ1, KCNQ1OT1, PHLD2, SLC22A18, and SLC22A18AS in the samples available for study (Fig. 3). There was also no detectable relationship between rs231362 risk allele number and total gene expression (Supplementary Fig. 5) or between methylation at any of the five tested sites and total gene expression (Supplementary Fig. 5) or between methylation at any of the five tested sites and total gene expression (Supplementary Fig. 5). Power for this analysis was reduced by undetermined risk allele parent of origin. We also examined the effect of risk genotype on allele-specific expression levels but identified no impact on the balance of expression between chromosomes for any tested gene (P > 0.2).

DISCUSSION

There is compelling evidence that diabetes risk at the KCNQ1 locus is mediated through a gene with imprinted expression (16). By demonstrating that PHLD2, SLC22A18, and SLC22A18AS are biallelically expressed in both adult and fetal pancreas and islets, we show that they are unlikely to be involved in a proximal molecular mechanism for diabetes risk. Likewise, any diabetes susceptibility

rs2237895 was nominally associated (P < 0.1) with changes in methylation status at three of the sites tested and at differing developmental stages (Fig. 3). At the diagnostic DMR region and CTCL binding site, methylation was higher (7.3 and 5.6%, P = 0.02 and 0.08, respectively) in fetal samples homozygous for the risk allele than in fetal nonrisk homoygotes. This effect was not apparent in adult islets, where risk genotype had no effect on methylation levels at these same sites (P > 0.25). Conversely, an effect was seen only in adult tissues at the PLGL1 binding site. Here, methylation was 1.6% higher in risk genotype homoygotes than in nonrisk homoygotes (P = 0.006) in adult islets, but no effect was seen in fetal pancreas (P = 0.30). No effects were identified from the top disease-associated SNP within intron 10 of KCNQ1 (rs231362, P > 0.1) (Supplementary Fig. 3) or from either SNP at the two candidate assays (P > 0.1) (Supplementary Fig. 4).

**Type 2 diabetes risk genotype and total and allele-specific gene expression.** We identified no relationship between rs2237895 risk allele number and total gene expression of KCNQ1, KCNQ1OT1, CDKNIC, PHLD2, SLC22A18, or SLC22A18AS in the samples available for study (P > 0.1 for all genes) (Fig. 4). There was also no relationship between rs231362 risk allele number and total gene expression (Supplementary Fig. 5) or between methylation at any of the five tested sites and total gene expression (P > 0.1 in all cases). Power for this analysis was reduced by undetermined risk allele parent of origin. We also examined the effect of risk genotype on allele-specific expression levels but identified no impact on the balance of expression between chromosomes for any tested gene (P > 0.2).
mechanism working through KCNQ1 or KCNQ1OT1 is likely to be early in islet development because these transcripts are imprinted in fetal pancreas but not in adult islets.

The cyclin-dependent kinase inhibitor CDKN1C (encoding p57KIP2), imprinted at both developmental time points, emerges as a particularly strong regional candidate. CDKN1C is expressed by 30–40% of β-cells in healthy individuals but never concurrently with the Ki67 marker of cell proliferation (23). CDKN1C expression is abolished in the hyperproliferative pancreatic lesions of focal hyperinsulinemia, suggesting a key role in regulating pancreatic β-cell proliferation (23). Loss-of-function mutations in CDKN1C cause BWS and hypoglycemia, whereas gain-of-function mutations in the PCNA-binding domain have recently been shown to cause congenital undergrowth (18,24). A higher level of DNA methylation in individuals carrying more risk alleles is consistent with a disease model of reduced KCNQ1OT1 transcription, diminished repressive histone modifications (25), and increased CDKN1C expression, leading to impaired islet proliferation or development.

We demonstrate that risk genotype status is related to DNA methylation in a developmentally variable manner. Although the relationship between genotype and methylation is statistically stronger in adult than in fetal samples (probably attributable to improved power from a larger sample size), the magnitude of effect appears larger in fetal samples. It is noteworthy that the true allele-specific effect size is likely to be underestimated because data were obtained by pyrosequencing PCR products amplified from both chromosomes. Further work in larger numbers of human islet samples, when they become available, will be required to explore in more detail the relationship between DNA methylation and expression.

By use of the largest cohort of human islets currently available, we have performed the first assessment of imprinting status at 11p15.5 in human adult islets and fetal pancreas. The data provide insights into the complexity of imprinting at 11p15.5, highlighting the necessity of performing functional studies in relevant tissues and at appropriate developmental stages. The data have significant implications for the molecular mechanism by which associated variants in this region exert their effect on diabetes risk.

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FIG. 4. Total expression according to rs2237895 type 2 diabetes risk genotype. Box plots show the effect of risk allele number (x-axis) on total mRNA expression level (y-axis), separated by tissue type. Boxes represent quartiles; whiskers encompass values within 1.5 times the interquartile range. There was no evidence for an effect of risk allele number on expression levels of any of the tested genes in either tissue type ($P > 0.05$ in all cases). T2D, type 2 diabetes.
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M.E.T., D.J.G.M., A.P.M., C.M.L., M.I.M., and A.L.G. designed the project. P.R.J. and L.C.G. provided human islet samples. N.H. provided fetal pancreas samples. M.E.T., M.D.N., and A.B. performed the research. M.E.T., M.I.M., and A.L.G. analyzed the data. M.E.T., M.I.M., and A.L.G. wrote the manuscript. D.J.G.M., M.D.N., A.P.M., C.M.L., A.B., N.H., and L.C.G. contributed to the manuscript. A.L.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Hamming KS, Soliman D, Matemisz LC, et al. Coexpression of the type 2 diabetes susceptibility gene variants KCNJ11 E23K and ABCC8 S1306A alter the ATP and sulfonylurea sensitivities of the ATP-sensitive K(+) channel. Diabetes 2009;58:2419–2424

2. Nicolson TJ, Bellomo EA, Wijesekara N, et al. Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. Diabetes 2009;58:2070–2083

3. Rees MG, Wincovitch S, Schultz J, et al. Cellular characterisation of the GCKR P446L variant associated with type 2 diabetes risk. Diabetologia 2012;55:114–122

4. Beer NL, Tribble ND, McCulloch LJ, et al. The P446L variant in GCKR associated with fasting plasma glucose and triglyceride levels exerts its effect through increased glucokinase activity in liver. Hum Mol Genet 2000;18:4081–4088

5. Tsai FJ, Yang CF, Chen CC, et al. A genome-wide association study identifies susceptibility variants for type 2 diabetes in Han Chinese. PLoS Genet 2010;6:e1000847

6. Unoki H, Takahashi A, Kawaguchi T, et al. SNPs in KCNQ1 are associated with susceptibility to type 2 diabetes in East Asian and European populations. Nat Genet 2008;40:1098–1102

7. Voight BF, Scott LJ, Steinthorsdottir V, et al.; MAGIC investigators; GIANT Consortium. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. Nat Genet 2010;42:579–589

8. Yasuda K, Miyake K, Horikawa Y, et al. Variants in KCNQ1 are associated with susceptibility to type 2 diabetes mellitus. Nat Genet 2008;40:1092–1097

9. Holmkvist J, Banasik K, Andersen G, et al. The type 2 diabetes associated minor allele of rs2237895 KCNQ1 associates with reduced insulin release following an oral glucose load. PLoS ONE 2009;4:e6572

10. Jonsson A, Isomaa B, Tuomi T, et al. A variant in the KCNQ1 gene predicts future type 2 diabetes and mediates impaired insulin secretion. Diabetes 2009;58:2409–2413

11. Tan JT, Nurbaya S, Gardner D, Ye S, Tai ES, Ng DP. Genetic variation in GCKR P446L variant associated with type 2 diabetes risk. Diabetologia 2003;46:1046–1002

12. Jespersen T, Grunnet M, Olesen SP. The KCNQ1 potassium channel: from gene to physiological function. Physiology (Bethesda) 2005;20:408–416

13. Pan Q, Ma J, Zhou Q, et al. KCNQ1 loss-of-function mutation impairs gastric acid secretion in mice. Mol Biol Rep 2010;37:1329–1333

14. Rosengren AH, Braun M, Mahdi T, et al. Reduced insulin exocytosis in human pancreatic β-cells with gene variants linked to type 2 diabetes. Diabetes 2012;61:1726–1733

15. Kong A, Steinthorsdottir V, Masson G, et al.; DIAGRAM Consortium. Parental origin of sequence variants associated with complex diseases. Nature 2009;462:868–874

16. Thakur N, Tiwari VK, Thomassin H, et al. An antisense RNA regulates the bidirectional silencing property of the KCNQ1 imprinting control region. Mol Cell Biol 2004;24:7855–7862

17. Weksberg R, Shuman C, Beckwith JB. Beckwith-Wiedemann syndrome. Eur J Hum Genet 2010;18:8–14

18. McMin J, Wei M, Schauf N, et al. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. Placenta 2006;27:490–540

19. Mackay DJ, Callaway Jl, Marks SM, et al. Hypomethylation of multiple imprinted loci in individuals with transient neonatal diabetes is associated with mutations in ZFPP7. Nat Genet 2008;40:949–951

20. Arboleda VA, Lee H, Parnaik R, et al. Mutations in the PCNA-binding domain of CDKN1C are associated with susceptibility variants for type 2 diabetes in Han Chinese. PLoS Genet 2008;4:e1000847

21. Kassam SA, Arieh I, Thornton PS, et al. p57Kip2 expression in normal islet cells and in hyperinsulinism of infancy. Diabetes 2001;50:2763–2769

22. Arboleda VA, Lee H, Parnaik R, et al. Mutations in the PCNA-binding domain of CDKN1C cause IMGae syndrome. Nat Genet 2012;44:788–792

23. Redrup L, Branco MR, Perdeaux ER, et al. The long noncoding RNA Kcnq1ot1 organises a lineage-specific nuclear domain for epigenetic gene silencing. Development 2009;136:525–530