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REVIEW

Understanding Genetic Heterogeneity in Type 2 Diabetes by Delineating Physiological Phenotypes: *SIRT1* and its Gene Network in Impaired Insulin Secretion

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■ Abstract

Type 2 diabetes (T2D) is a chronic metabolic disease which shows an exponential increase in all parts of the world. However, the disease is controllable by early detection and modified lifestyle. A series of factors have been associated with the pathogenesis of diabetes, and genes are considered to play a critical role. The individual risk of developing T2D is determined by an altered genetic background of the enzymes involved in several metabolism-related biological mechanisms, including glucose homeostasis, insulin metabolism, the glucose and ion transporters involved in glucose uptake, transcription factors, signaling intermediates of insulin signaling pathways, insulin production and secretion, pancreatic tissue development, and apoptosis. However, many candidate genes have shown heterogeneity of associations with the disease in different populations. A possible approach to resolving this complexity and understanding genetic heterogeneity is to delineate the physiological phenotypes one by one as studying them in combination may cause discrepancies in association studies. A systems biology approach involving regulatory proteins, transcription factors, and microRNAs is one way to understand and identify key factors in complex diseases such as T2D. Our earlier studies have screened more than 100 single nucleotide polymorphisms (SNPs) belonging to more than 60 globally known T2D candidate genes in the Indian population. We observed that genes invariably involved in the activity of pancreatic β-cells provide susceptibility to type 2 diabetes (T2D). Encouraged by these results, we attempted to delineate in this review one of the commonest physiological phenotypes in T2D, namely impaired insulin secretion, as the cause of hyperglycemia. This review is also intended to explain the genetic basis of the pathophysiology of insulin secretion in the context of variations in the *SIRT1* gene, a major switch that modulates insulin secretion, and a set of other genes such as *HHEX*, *PGC-a*, *TCF7L2*, *UCP2*, and *ND3* which were found to be in association with T2D. The review aims to look at the genotypic and transcriptional regulatory relationships with the disease phenotype.

Keywords: type 2 diabetes · genetic heterogeneity · SIRT1 · insulin secretion · gene network · micro RNA · single nucleotide polymorphism

1. Introduction

The growing morbidity and mortality associated with T2D is reflected by an increasing trend observed over the last few years. As per the International Diabetes Federation update (IDF Diabetes Atlas, 5th Edition, 2012), 370 million people in the world are living with diabetes. This accounts for a worldwide prevalence of 8.3%, with 10.5% in North America and the Caribbean, 10.9% in the Middle East and North Africa, 6.7% in Europe, 9.2% in South and Central America, 4.3% in Africa, 8.7% in South-East Asia, and 8.0% in the Western Pacific Region. There are approximately another 50% undiagnosed cases in the world as well. The IDF figures also reflect that half of the

people who die from diabetes are under the age of 60. This trend has shown up in every part of the world, with alarming indicators for South-East-Asia (including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, and Thailand). A 2-3 fold increase in the prevalence of the disease is expected by the year 2030 (country and regional data on diabetes, WHO), despite the knowledge that the disease is largely preventable by a modified lifestyle.

Increasing incidence of T2D during last few years points to the role of environmental factors in the development of the disease, while familial clustering and twin studies have confirmed a genetic component [1, 2]. Recent literature supports a critical share of genetic component in the pathogenesis of T2D. The relationship between genetic and environmental factors in the development of T2D is complex. For instance, environmental factors may be responsible for the initiation of cell damage or other metabolic abnormalities, while genes may regulate the rate of progression to diabetes. In some cases, genetic factors may provide a background for environmental factors to start processes favoring disease development.

2. Genetic background: a critical component in T2D etiology and impaired insulin secretion

Positive family history confers a 2.4-fold increased risk for T2D; 15-25% of first-degree relatives of patients with T2D develop impaired glucose tolerance or diabetes [3]. The life-time risk (at age 80 years) for T2D has been calculated to be 38% if one parent has T2D [3]. If both parents are affected, the prevalence of T2D in the offspring is estimated to approach 60% by the age of 60 years [4]. This shows that a strong genetic component is present in the etiology of the T2D, but it is considered to be genetically heterogeneous. Various genetic studies have helped in identifying the susceptibility and modifier genes.

2.1 Candidate genes and genome-wide studies: delineating susceptibility factors in T2D

Genome-wide linkage and association studies and studies of candidate genes with a possible role in pathogenesis of the disease have resulted in a brief catalogue of about 100 genes (supplementary **Table A1**, in the Appendix). Variations of these genes have been described, and correlations between genotype and disease phenotype have been drawn. These studies have provided strong evidence of an association of the genes involved in β-cell development, proliferation, function, and apoptosis [5-13]. Other associated genes are involved in

Abbreviations:

COUP-TF1	chicken ovalbumin upstream promoter transcription factor 1
E1A	adenovirus early region 1A
E2F1	E2F transcription factor 1 (retinoblastoma-associated protein 1)
EP300	E1A-binding protein p300
FoxO	forkhead box O
HHEX	hematopoietically-expressed homeobox protein
HIC1	hypermethylated in cancer 1
IDF	International Diabetes Federation
JNK	c-Jun N-terminal kinase
LCR-F1	locus control region factor 1
MafA	V-maf avian musculoaponeurotic fibrosarcoma oncogene Homolog A
miR	microRNA
mt-ND3	mitochondrially encoded NADH dehydrogenase 3
MTPN	myotrophin
NAD	nictotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
ND3	NADH dehydrogenase 3
NF	necrosis factor
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
OR	odds ratio
P53	tumor protein p53
Pbx1a	pre-B-cell leukemia homeobox 1a
PDK1	pyruvate dehydrogenase kinase, isozyme 1
PDX-1	pancreatic and duodenal homeobox 1
PGC-1α	PPARG coactivator 1α
PPAR	peroxisome proliferator-activated receptor
PPARG	peroxisome proliferator-activated receptor gamma
RNA	ribonucleic acid
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
SIRT1	sirtuin (silent mating type information regulation 2 homolog) 1
SREBP-1a	sterol regulatory element-binding protein 1a
STZ	streptozotocin
T2D	type 2 diabetes
TCF7L2	transcription factor 7 like 2
TF	transcription factor
TFBS	TF-binding site
TSS	transcription start site
UCP2	uncoupling protein 2
YY1	yin yang 1

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glucose homeostasis [5, 7, 14], insulin secretion, resistance, and function [5, 7, 12-23], membrane transporters [5, 24], transcription factors (TFs) [25-27], and energy sensing and fat metabolism [7, 8, 10, 12, 24, 28-30].

2.2 Problems with large genetic studies: genetic heterogeneity vs. heterogeneity of results

One of the most important aspects in understanding the complex disease is its genetic heterogeneity [31]. However, many of the candidate genes have shown heterogeneity in their associations with the disease in different populations, leaving the question of genetic heterogeneity still open. One of the reasons for the different results seems to be associated with the study design, in particular that of large meta-analyses that pool T2D cases of different ancestry or traits and assess them with a generalized intermediate phenotype of hyperglycemia, despite the fact that the phenotype can differ because of a variety of unrelated reasons within the body physiology or environment.

2.3 Resolving the question of genetic heterogeneity and assignable phenotypes

It may be worth defining and resolving the genetic etiology responsible for the level of subphenotypes. However, this is a very difficult task due to the complexity and heterogeneity of the disease. Yet, delineating to the best possible level of phenotypic classification may be helpful in achieving a better understanding of the disorder. As a starting approach for case-control comparisons, patients may be subdivided into the following four stereotypical phenotypes:

1. Insulin resistance in signaling pathways and defects in insulin-mediated glucose uptake in the muscle.
2. Impaired insulin secretion due to dysfunction of pancreatic beta-cells.
3. Disruption of secretory function of adipocytes and impaired insulin action in liver.
4. Association with microvascular (retinopathy-cataract, impaired vision, nephropathy-renal failure, peripheral neuropathy-sensory loss, and motor weakness) and macrovascular (coronary circular complications, i.e., myocardial infarction, and cerebral circulation complication, i.e., stroke) complications.

The specific genetic background of an individual may provide a strong basis for the development of one of these metabolic conditions. In previous studies, we have observed an association of genes involved in impaired insulin secretion as the cause of hyperglycemia, providing the genetic basis for one of the simplest physiological phenotypes in T2D, which is prevalent in the ethnic groups from northern India [29, 32-34], and which is a subject of focused discussion in this review.

3. Oxidative stress, impaired insulin secretion, and T2D

Oxidative stress is a widely accepted contributor to the development and progression of hyperglycemia in diabetes and its complications, usually accompanied by increased production of free radicals or impaired antioxidant defenses or both [35, 36]. The production of reactive oxygen species (ROS) is enhanced by multiple sources, including the electron transport chain in mitochondria [37], nonenzymatic glycosylation reaction [38], and membrane-bound NADPH oxidase [39]. ROS production decreases insulin gene expression and thus insulin secretion. Oxidative stress-mediated post-translational loss of several transcription factors, including pancreatic and duodenal homeobox 1 (PDX-1) and V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), have been associated with reduced insulin gene expression [40-44]. ROS production has also been associated with the pathophysiology of insulin resistance and decreased insulin function, which involves a complex network of insulin signaling (including JNK and Akt signaling pathways), by disrupting insulin-induced cellular redistribution of insulin receptor substrate-1 [45] and phosphatidylinositol 3-kinase [46], and beta-cell dysfunction caused by apoptosis of pancreatic beta-cells, known as beta-cell glucotoxicity. The genetic background that may support a situation equivalent to oxidative stress [32, 33] and inefficiency in antioxidant defenses could qualify as a relevant candidate in understanding and controlling T2D.

3.1 Sirtuins in oxidative stress: SIRT1 putative functions and role in insulin secretion

Sirtuins represent a conserved family of nictotinamide adenine dinucleotide (NAD)-dependent deacetylases and ADP-ribosyltransferases that influence the production and detoxification of ROS [34]. They have gained attention in recent years as several human SIRT proteins, including SIRT1 to SIRT7, have evolutionarily conserved and nonconserved functions at different cellular locations [47].

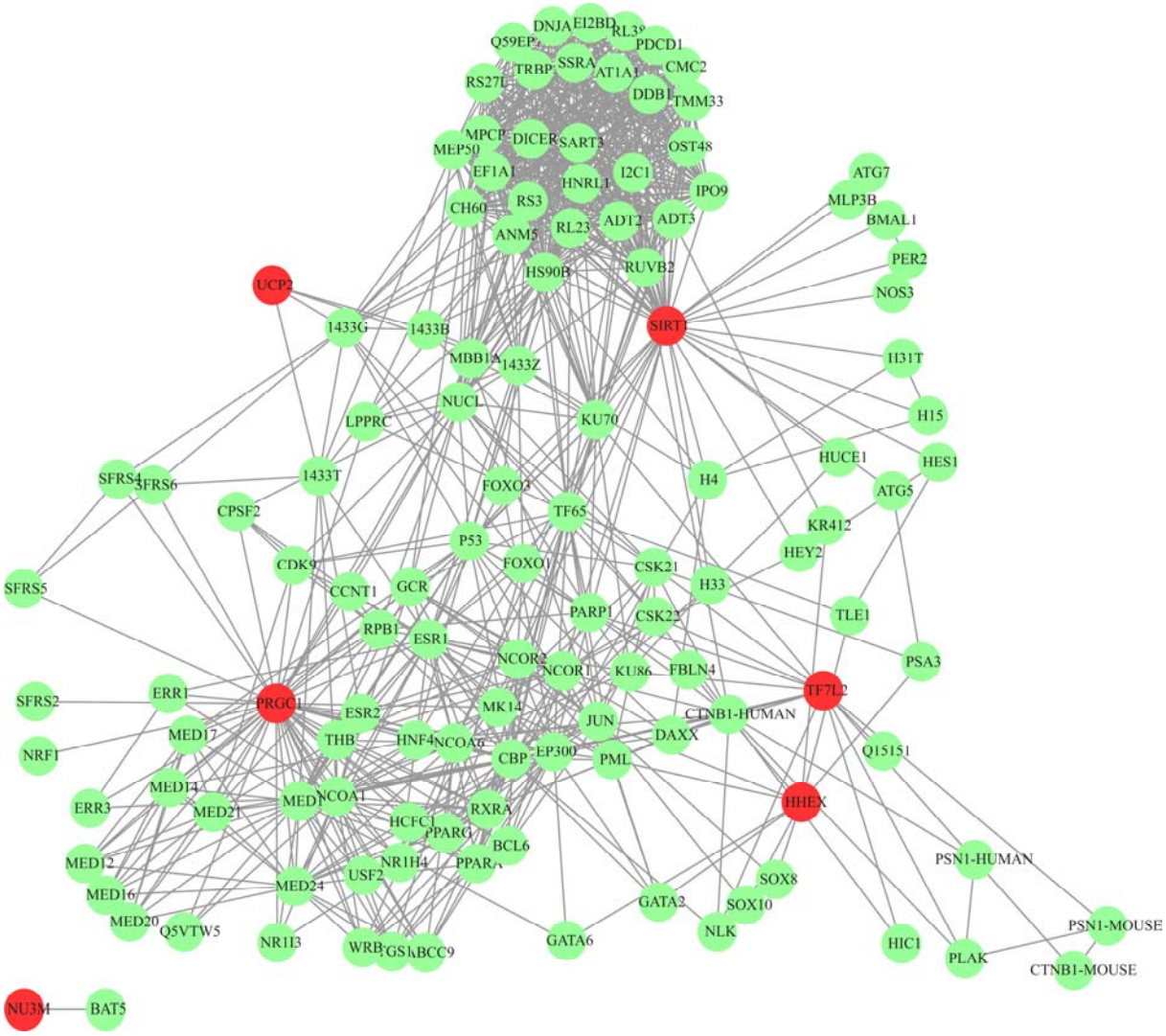

Figure 1. Protein-to-Protein interaction network. The figure shows an expanded view of the network imported from Cytoscape, where nodes represent proteins and edges physical interactions. Red-colored nodes highlight the proteins of interest, green-colored nodes highlight the interacting proteins, and edges are represented by gray colored lines.

SIRT1, SIRT6, and SIRT7 have been detected in different subnuclear localizations, where SIRT6 and SIRT7 were associated with heterochromatic regions and nucleoli, respectively. SIRT3, SIRT4, and SIRT5 have been localized in mitochondria.

SIRT1 is involved in a series of critical cellular functions, including circadian rhythms, glucose-lipid metabolism, stress resistance, mitochondrial biogenesis, chromatin silencing, apoptosis, and inflammation (reviewed in [48]). It has been argued that its versatile ability is due to its role as a regulator of Wnt signaling pathways. The regulatory activity of SIRT1 is achieved through three dishevelled proteins that act as critical messengers for more than 19 Wnt ligands [49]. SIRT1 may be involved in the regulation of adipose tissue inflammation and glucose homeostasis, both of which are important etiologic components of insulin resistance and T2D. It may also participate in the regulation of insulin secretion [50] and protection of pancreatic β-cells from oxidative stress [51]. ROS cause the relocalization and degradation of SIRT1, while downstream effects of SIRT1 can decrease or increase ROS resistance, and/or directly decrease ROS production. Therefore, it is legitimate to wonder whether SIRT1 depletion could be an adaptive mechanism to promote oxidative stress resistance.

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SIRT1 also acts as a major switch to modulate insulin secretion and regulate gene expression of uncoupling protein 2 (UCP-2) and peroxisome proliferator-activated receptor gamma (PPARG) coactivator 1α (PGC-1α) [29, 32], among others. UCP2 is repressed by SIRT1 in beta-cells of the pancreas and PGC-1α is activated in the liver, under starvation and calorie restriction conditions [50].

3.2 SIRT1 variants and diabetes susceptibility

A number of variants have been reported in the *SIRT1* gene. All variations (rare and common) that may have a functional role were compiled using the 'FuncPred' tool from the National Institute of Environmental Health Sciences (NIEHS), NIH (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm), available as supplementary material from the publisher.

SIRT1 genetic variations have been associated with various phenotypes in diabetes and the metabolic syndrome. Variations rs7895833 and rs1467568 have been associated with BMI and risk of obesity, with a combined odds ratio (OR) = 0.87 (0.77-0.97), p = 0.02, and OR = 0.82 (0.73-0.92), p = 0.0009, respectively [52]. The *SIRT1* variations rs10509291 and rs7896005 were nominally associated with type 2 diabetes and a decrease in acute insulin secretion in Pima Indians (OR = 1.25 (1.05-1.48), p = 0.01 and OR = 1.17 (1.02-1.34), p = 0.02, respectively) [53]. In our previous study, one of the *SIRT1* promoter variations, rs12778366, along with the background of reported risk genotype combinations of mt-ND3, PGC-1α, and UCP2-866, showed a very high effect size, with corrected OR = 8.91, p = 6.5×10^{-11} (compared with the protective genotype combinations). The risk level was considerably lower in the genotype background of TX (OR = 6.68; p = 2.71×10^{-12}) and CX (OR = 3.74; p = 4.0×10^{-3}). Also, regulatory variations in PGC-1α and UCP2 as well as structural variation in mitochondrial complex ND3 (involved in the electron transport chain and oxidative phosphorylation) in combination with *SIRT1* variation provided either risk or protection, depending on the genetic status [34].

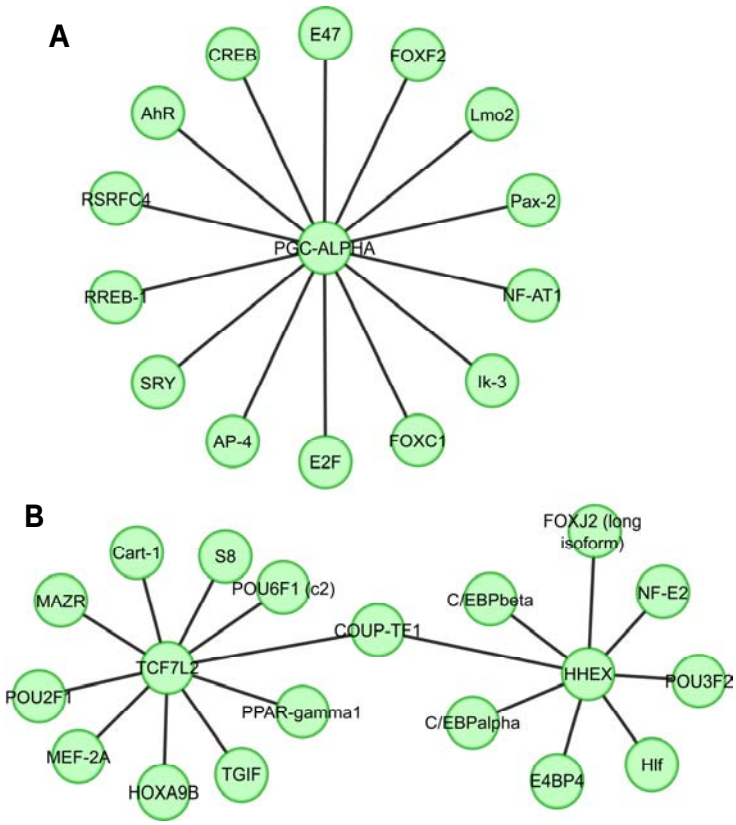

Figure 2. Transcription factor-to-gene transcriptional regulatory network. The transcription factor binding was predicted; it is shown in a network (**A**). TCF7L2- and HHEX-regulating transcription factors were identified; the network shows COUP-TF1 regulating both genes (**B**).

If the pathogenesis of hyperglycemia is understood in the context of hypoinsulinemia and low glucose mobilization in pancreatic beta-cells over a period of stressful life-style, such as irregular eating habits or hunger following a diet, we hypothesize that this may result in a hyperinsulinemic response to regulate glucose in the circulation to maintain homeostasis. Continuation of such a life-style in the context of T2D susceptibility genomic variation would result in the failure of beta-cells to produce sufficient insulin and to compensate for the long abuse of the body physiology. This situation may result in T2D depending on the frequency of the recurrent insult imposed on the body. It has been reported that skipping meals induces people to ingest more calories; 31% high-calorie food was consumed after short-term deprivation, a life-style increasingly common in society today [54]. It is estimated that 10-15% of T2D patients behave in this way. The combination of genetic background regarding *SIRT1*, *PGC-1α*, *TCF7L2*, *HHEX*, *UCP2*, and *mtND3*, genetic associations of transcription

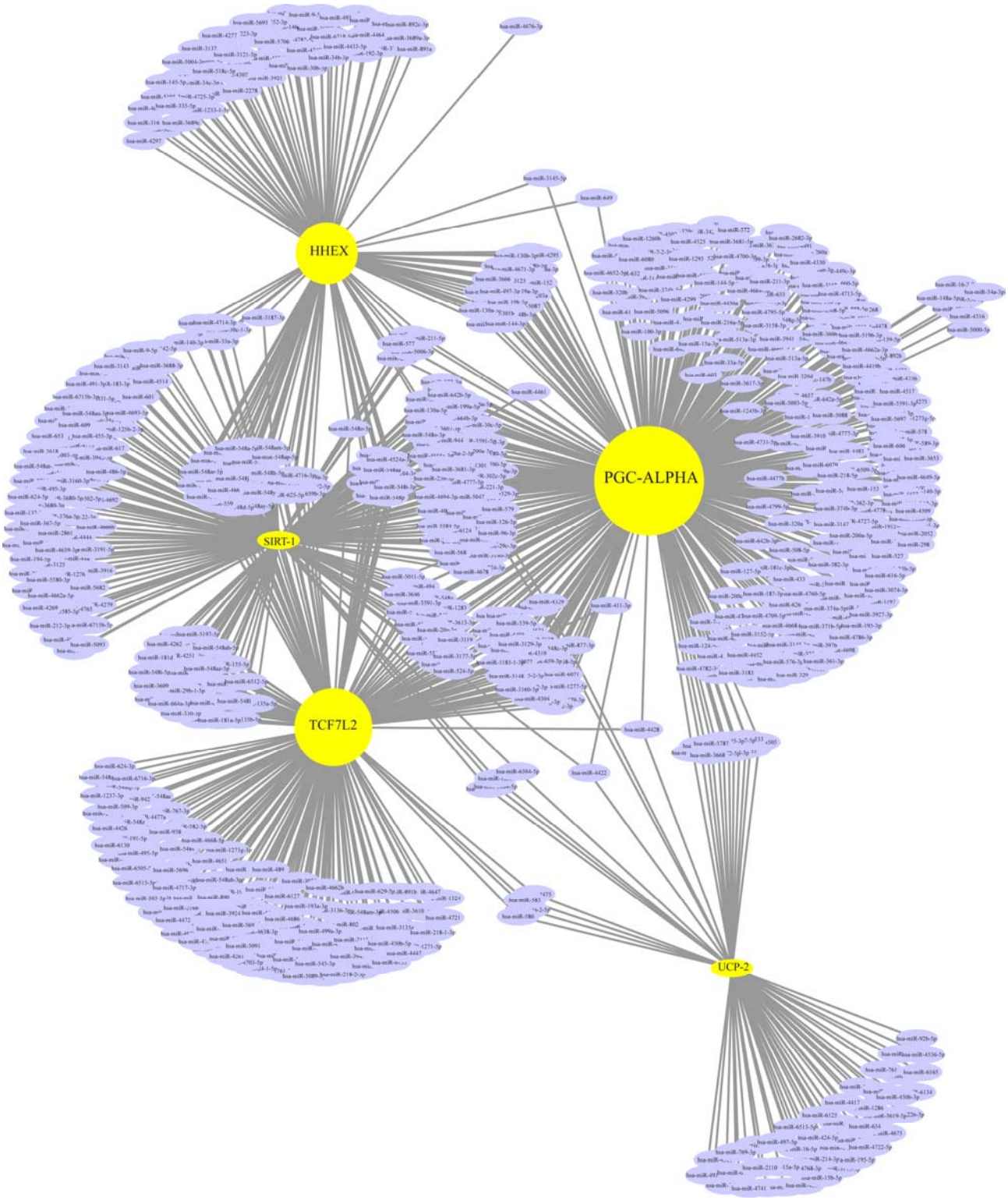

Figure 3. MicroRNA-to-gene post-transcriptional regulatory network. PGC-α, HHEX, SIRT-1, TCG7L2, and UCP-2 hubs are represented in yellow color. These are the genes of interest. Grey-colored nodes highlight their regulating microRNAs. Nodes and edges are illustrated by filled circles and green lines, respectively. The network is constructed based on the microRNA target prediction results.

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factors, including TCF7L2 and HHEX, which control insulin secretion, irregular dietary patterns, and unhealthy lifestyle is the driving force in the pathogenesis of diabetes and its vascular complications [5, 6, 12, 23, 55, 56]. Previous studies have also emphasized the importance of understanding the complex interactions between different regulatory factors, such as proteins, transcription factors, and microRNAs in T2D [57, 58].

4. Networks and pathways of associated genes in T2D: methodology and impaired insulin secretion

In our previous studies, the genes *HHEX*, *PGC-a*, *TCF7L2*, *UCP2*, and *ND3* were in association with T2D, and were observed to interact directly or indirectly with *SIRT1* [29, 32-34]. To learn more about the interaction of these genes with the related proteins, we constructed a biological network which included both transcriptional regulatory relationships and integrated protein interactome.

4.1 Construction of a protein-protein interaction network

The protein-protein interaction network for each gene was constructed computationally using the Cytoscape plug-in called APID2NET. APID2NET is a new tool that works with Cytoscape to allow the exchange of unified interactome data by querying the APID (Agile Protein Interaction Data Analyzer) server to provide interactive analysis of protein-to-protein interaction (PPI) networks. The program is designed to visualize, explore, and analyze the proteins and interactions retrieved, including the annotations and attributes associated to them, such as: GO terms, InterPro domains, experimental methods that validate each interaction, Pubmed IDs, UniProt IDs, etc. The tool provides interactive graphical representation of the networks with all Cytoscape capabilities, plus new automatic tools to find concurrent functional and structural attributes for all protein pairs in a network [59].

4.2 Scale-freeness topology of the networks

Biological networks are characterized by topological features that establish their scale-freeness property. Protein interaction networks also exhibit a scale-free geometry, where the nodes are not uniformly populated with neighbors. Not all the nodes of these networks follow the rule of having an average number of links per node. Most of the nodes have few partners, while a few nodes, also called 'hubs', interact with many partners.

The power law process is used to estimate the parameters and validate the network models with their scale-freeness property. Usually, R-squared values closer to 1 indicate higher correlation and a stronger linear relationship between the data variables. Here, the R-squared values follow this rule as well, emphasizing that the networks are scale-free, i.e., they are unevenly populated with hubs and less dense nodes. Biological networks are found to be very sensitive to the removal of hub proteins. Here, nodes could be established by a protein-TF-microRNA regulatory interaction (**Figures 1-3**), where the highly interacting nodes are called hubs. After the network analysis, we observed that the hub proteins are communicating with many other significant proteins involved in several pathways that are reported to be affected in T2D. We propose that further biochemical investigations need to be conducted on the removal of these hub proteins to provide better understanding of the roles played by these proteins in the pathophysiology of T2D [60].

4.3 Transcription factor: target relationships developed from the UCSC genome browser

To analyze the transcriptional regulation of these genes we extracted the TF binding using the UCSC genome browser. The tables were downloaded from the UCSC table browser retriever tool. We retrieved two files, TFbsConFactors.txt and TFbsConsSites.txt, using the following options:

1. Clade: Mammal
2. Genome: Human
3. Assembly: Feb.2009 (GRCh37.hg19)
4. Group: Regulation
5. Track: TFBS Conserved
6. Table: tfbsConsSites and tfbsConsFactors

TFbsConsSites provides chromosomal coordinates of TF-binding sites (TFBSs) on human, mouse, and rat genes; TFbsConfactors.txt provides the Uniprot IDs of TFs. Further, Uniprot Ids were converted to NCBI gene IDs via BioMart. The homologs of mouse and rat TFs were identified using NCBI's HomoloGene data base through which we compiled the human TF-to-TFBS relationships. The promoter region of each gene (from 2 kb upstream of the transcription start site (TSS) to 0.5 kb downstream of the TSS) was scanned for the TFBSs, and identified in the above TF-to-TFBS re-

lationships. When an occurrence of a certain TFBS was found, the corresponding TF was linked with that gene. In this way, we developed a set of TF-to-gene regulatory relationships [61].

4.4 microRNA: target relationships

Candidate microRNA (miR) mature sequences were downloaded from miRBase, release 19, the microRNA database. We retrieved the 3'UTR sequence of T2D genes and TF from the UCSC table browser retriever tool. We predicted miR targets for T2D genes using miRandaV3.3a, and constructed the miR-gene and miR-TF relationship network using Cytoscape.

4.5 Candidate TF-to-miR regulatory relationships from the UCSC genome table analysis

Transcription factors and microRNAs were the main regulators of gene expression. We collected the TF regulatory relationship from the UCSC genome table browser (http://genome.ucsc.edu/), and downloaded the two files, TFbsConFactors.txt and TFbsConsSites.txt, from UCSC hg19. TFbsConsSites provided predicted chromosomal coordinates of TF-binding sites (TFBSs) on human, mouse, and rat genes, while TFbsConfactors.txt linked the internal TF increments to nucleotide IDs. These nucleotide IDs were further converted into NCBI gene IDs via BioMart (http://www.ebi.ac.uk/biomart/) and NCBI's HomoloGene data base. The data file was used to find the human homolog of mouse and rat TFs, which enabled us to compile an enlarged set of human TF-to-TFBS relationships.

We retrieved the 1600 miRs from MiRBase, release 19, August 2012. For all miRs, approximately 60% were embedded in protein-coding gene regions, and located on the same strand of the host gene, i.e., intragenic, whereas the others were located outside protein-coding gene regions, i.e., intergenic. For intragenic miRs, we assumed that they have the same transcriptional factors as their host genes, considering co-transcription of intragenic miRs and hosts. Also, from UCSC hg19, we downloaded intragenic miR coordinate information (refmicroRNA.txt file) from the UCSC ID of the transcripts, which specified the chromosomal locations of the miRs. The promoter region of each miR (from 2 kb upstream of the TSS to 0.5 kb downstream of the TSS) was scanned for the TFBSs identified in the above TF-to-TFBS relationships. If an occurrence of a certain TFBS was found, the corresponding TF was linked with that miR. In this way, we developed a set of TF-to-miR regulatory relationships. On this basis, candidate TF-to-target miR relationships were established for intragenic miRs [62].

4.6 Protein-to-protein interactions and impaired insulin secretion

The query in the protein-to-protein interaction network was conducted with the Cytoscape plug-in called APID2NET for each protein (SIRT1, PGC-1α, TCF7L2, HHEX, and UCP2). The analysis showed an involvement of 127 proteins with 969 interactions (including self-loop) and 127 proteins with 914 interactions (excluding self-looping) (**Figure 1**).

SIRT1 interacted with a larger number of proteins than the other four proteins (PGC-1α, TCF7L2, HHEX, and UCP2), namely with 51 proteins including 512 interactions. This was followed by PGC-α (45 nodes, 218 edges), TCF7L2 (16 nodes, 39 edges), HHEX (11 nodes, 20 edges), and UCP2 (5 nodes, 5 edges). EP300 emerged as the central node connected with 30 nodes through 135 edges, which included SIRT1, PGC-1α, and TCF7L2 (**Figure 1**). Recently, EP300 has been identified through a gene regulatory network cascade as a key regulator in T2D; it was linked to oxidative stress as molecular mechanism in the pathogenesis of T2D and hypertension, and generated the co-regulated network of insulin signaling [63]. β-catenin interaction with TCF7L2 and nuclear co-activators, such as EP300, has been known to result in the simulation of Wnt or β-cat/TCF downstream target gene transcription [64, 65].

In the protein-to-protein interaction analysis, SIRT1 and PGC-1α were identified as hubs in the network. In the gene set enrichment analysis (GSEA), genes playing a role in oxidative phosphorylation were found to be enriched [66]. The expression of these genes was shown to be co-regulated with a modest reduction (~20%) in the expression of transcriptional co-activator PGC-1α, which is involved in mitochondrial biogenesis. PGC-1α also correlated with this co-regulated expression. The reduced expression of PGC-1α and 1β is also co-regulated with the reduced expression of nuclear respiratory factor (NRF) regulated oxidative phosphorylation genes in both insulin resistant and diabetic subjects [66]; this was also demonstrated in a parallel study in Mexican Americans. *TCF7L2*, a commonly studied T2D gene in genome-wide association studies (GWAS) [67], is observed to have a strong association in diverse

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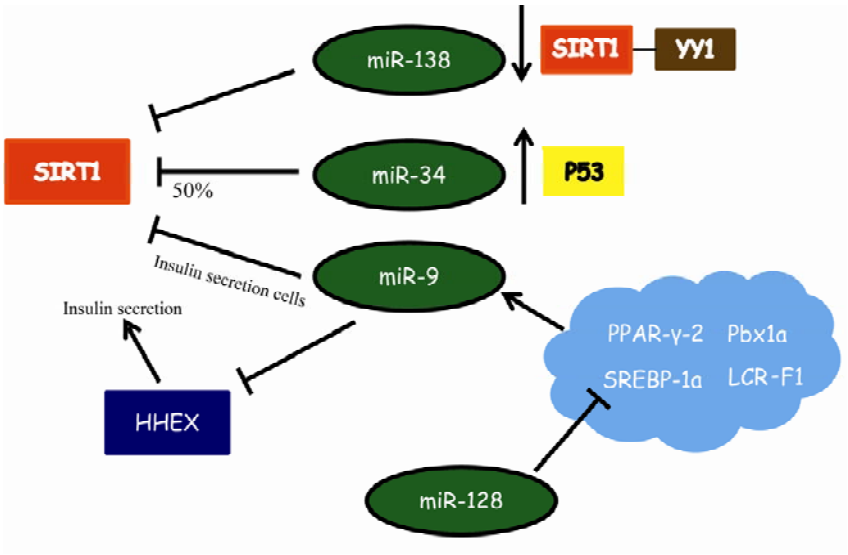

Figure 4. Role of microRNAs and their interaction with SIRT1 in the regulation of insulin secretion. Green-colored ellipses represent the microRNA nodes, their edges are illustrated by black lines. Genes are shown in boxes, grouped genes in a cloud box. Directional lines denote upregulation or downregulation of genes.

ethnic groups of T2D cases [68]. Recently, in an interaction analysis, TCF7L2 was reported to interact with 11 proteins. Furthermore, two new pathways (PPAR and adipocytokine signaling pathway) were shown to be associated with T2D, and *PPARG* was observed to be one of the top-ranking T2D risk genes in the protein interaction network [67].

4.7 Transcriptional regulation analysis and impaired insulin secretion

The *SIRT1* promoter may be activated by E2F1 and HIC1 during cellular stress [69, 70]. E2F1 directly binds to the *SIRT1* promoter at a consensus site located at bp position -65, and appears to regulate the basal expression level of SIRT1 [70]. Two HIC1-binding sites have been assigned to base pair positions -1116 and -1039 within the SIRT1 promoter [69]. In addition, two functional p53-binding sites (-178 bp and -168 bp), which normally suppress *SIRT1* expression, have been identified [71]. SIRT1 deacetylates various transcriptional factors, including P53, PGC-1α, forkhead box O (FoxO), and NF-κB [72-74]. To construct the transcriptional regulatory network, we identified the TF-binding site in the promoter region of these genes, and constructed 34 nodes with 32 edges, in which chicken ovalbumin upstream promoter TF1 (COUP-TF1) turned out to be a common regulator for *HHEX* and *TCF7L2* (**Figure 2**).

4.8 SIRT1: microRNA analysis and impaired insulin secretion

The role of miRs in T2D has long been established [75]. Since then, many miRs have been identified as critical components in the pathogenesis of the disease that has a polysystemic nature. Involvement of miRs paves the way to understanding the genetic basis of the disease and to designing novel diagnostic, prognostic, and curative approaches [76]. miR-375 is one of the important miRs that regulate the secretion of insulin. By de-expression of its targets Mtpn and PDK1, it induces insulin secretion [75, 77-79] and increases death by lipoapoptosis, as it regulates cell viability and proliferation by its secretion in β-cells. Deletion of this miR causes severe insulin-deficient diabetes in *ob/ob* mice [75, 77, 79-86].

miR-9 was observed to be expressed in pancreatic development; it caused impairment of insulin secretion and upregulation of cardiomyocytes in STZ-induced diabetic mice [81, 87]. miR-124a upregulated by glucose was shown to regulate the insulin exocytosis pathway; it caused escalated insulin release when no glucose was accessible, and reduced the secretion of glucose-induced insulin [87-89]. It was proposed that these effects are due to the targeting of Foxa2, which in turn downregulates Sur-1, Kir6.2, and Pdx1, with Pdx1 directly regulating the expression of the insulin gene [87]. miR-34a was shown to increase in response to 'palmitate', causing β-cells to be more susceptible to apoptosis and inhibition of nutrient-induced insulin secretion [88]. miR-34a is upregulated in the liver of STZ-induced diabetic mice [90]. Interestingly, two other miRs, miR-7 [91] and miR-375 [82], are expressed both in brain and β-cell islets. β-cells and neurons share similar secretion mechanisms, and are responsive to signals in the bloodstream, including glucose and insulin. Nearly 20 miRs are experimentally identified to target SIRT1 [92].

In our studies, we concentrated on well-known miRs that target SIRT1, and on those that are targeted by SIRT1 (**Figure 4**). MiR-138, miR-34a,

and miR-9 were identified as a few of the important small RNAs, which target SIRT1. SIRT1 expression is only ≈50% decreased in the presence of miR-34a [93]. P53 increases the expression of miR34a, resulting in a decrease in SIRT1 expression and a decrease in P53 activity [93]. It is reported that miR-138 and SIRT1 are mutually negative feedback regulators, where SIRT1 suppresses the expression of miR-138 at transcriptional level by directly binding to its upstream regulatory region. Axotomy induced upregulation; deacetylase activity of SIRT1 is needed for downregulation of miR-138 in response to axotomy [94]. SIRT1 also regulates miRs through other transcription factors. MiR-134 is suppressed by SIRT1 via a repressor complex containing the transcription factor YY1 [95]. Recently, miR-9 has been identified to regulate the expression of SIRT1 in insulin-secreting cells [96].

With the abundance of data suggesting involvement of miRs in T2D, we explored this scenario computationally using our experimentally proven set of genes [29, 32-34]. Our miR-gene regulatory analysis identified miR-9-3p (mature miR from the 3' arm of the miR-9) that targets HHEX which is also known to control insulin secretion (**Figure 3**). We predicted the transcription factors that controlled the expression of miR-9. We first searched miR-9 in the miR base and found it located at three loci, chromosome 1 (hsa-mir-9-1), chromosome 5 (hsa-mir-9-2), and chromosome 15 (hsa-mir-9-3). For our analysis, we took only two miR-9 loci (hsa-miR-9-1 and has-miR-9-2), which were similar to mouse miR-9 located at chromosome 3 and 13 (mmu-miR-9-1 and mmu-miR-9-2). Both miR-9-1 and miR-9-2 of humans are intronic miRs located within C1orf61 and LINC00461, respectively. Generally, it is assumed that intragenic miRs have a common promoter with their host genes, and are expressed simultaneously [97-99]. In both cases, these miRs and their respective genes carried common transcription factors.

Through ENCODE data analysis, we found four TFs (Pbx1a, PPARG-2, SREBP-1a, LCR-F1) regulating miR-9-1. Interestingly, three of these TFs had shown their involvement in insulin secretion. β-cell-specific overexpression of active SREBP-1a impaired insulin secretion and modestly impaired glucose tolerance [100]. It is also known that miR-128-2 decreases the expression of SREBP1 in cell lines independent of the SIRT1 status [101], which provides an insight into the miR-to-miR regulation present in insulin secretion. PPARG overexpression suppresses the energy-consuming exocytotic process of insulin secretory granules, at least in part, through reduction of ATP production by increased UCP-2 protein in β-cells, where UCP-2 gene transcription is upregulated by PPARG [102]. $Pdx1^{+/-}$ mice have impaired insulin secretion and increased β-cell apoptosis, factors contributing to β-cell failure during T2D [103, 104]. Also, from our analysis we predict that LCT-F1 and a further three TFs play a novel role in insulin secretion by controlling the expression of miR-9, which regulates the expression of SIRT1 and our predicted target *HHEX*. We also identified a direct role of *SIRT1*-targeting miRs; they target other important genes such as *UCP2*, *PGC-1α*, *TCF7L2*, and *HHEX*.

5. Conclusions

Insulin resistance is an intricate disorder, and affects a series of biological mechanisms, including signaling pathways and metabolic processes. In recent times, the key challenge has been to identify the signaling pathways or biochemical processes that play a significant role in T2D. Hyperglycemia caused by impaired insulin secretion could be one such, simple, physiological phenotype in T2D to be delineated for genotype relationship and appropriate intervention.

In this review, we have analyzed and discussed a set of genes found in association with T2D, and we have attempted to recognize these genes for their role in insulin secretion. These key regulatory genes identified in T2D include *SIRT1*, *HHEX*, *PGC-ALPHA*, *TCF7L2*, *UCP2*, and *ND3*. In a computational analysis which aimed to evaluate the interaction between these genes, EP300 emerged as a central node connected with 30 other nodes through 135 edges, which included *SIRT1*, *PGC-1α*, and *TCF7L2*. EP300 has been identified as a key regulator in T2D through a gene-regulatory network cascade, with oxidative stress as the molecular mechanism leading to T2D and hypertension [63]. β-catenin interaction with *TCF7L2* and nuclear co-activators such as EP300 is known to result in the stimulation of Wnt or -β-cat/TCF downstream target gene transcription [64, 65]. In the protein-to-protein interaction analysis, SIRT1 and *PGC-1α* were identified as hubs in the network. We also identified new TFs and microRNAs that apparently regulate important key regulators in T2D. This finding provides an opportunity to identify new regulatory feedback loop mechanisms, and to generate a better understanding of the complex T2D disease.

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In conclusion, our interaction analysis approach provides an important perspective on the understanding of genetic heterogeneity in complex disorders such as T2D, and on resolving its complexity. It may also help in delineating physiological phenotypes that may otherwise act as confounding factors and result in discrepancies in association studies when analyzed in combination without further differentiation. In contrast, delineating insulin secretion as a physiological phenotype of T2D shows precise association with the variant genotypes of the studied genes implicated for the phenotype.

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■ Appendix

Table A1. Genes involved in the pathogenesis of type 2 diabetes, and their association with insulin resistance

Gene	Chromosome	Pathway	Kind of study	Reference for association with T2D	Reference for association with glucose level and/or HOMA-B
ADAMTS9*	3p14.1	Enzyme	DIAGRAM meta-analysis; candidate gene study	Zeggini et al. (2008) [24]; Waters et al. (2010) [13]	
ADCY5*	3q21.1	Insulin secretion	Meta-analysis (of 21 GWAS), follow-up study		Dupuis et al. (2010) [7]
ADRA2A*	10q25.2	Insulin secretion	Meta-analysis (of 21 GWAS), follow-up study		Dupuis et al. (2010) [7]
AP3S2*	5q26.1	Signal transduction	GWAS	Kooner et al. (2011) [12]	
BCL2*	18q21.3	Anti-apoptotic protein	Multi-ethnic meta-analysis	Saxena et al. (2012) [5]	
BCL11A*	2p16.1	Zinc finger protein	Combined GWAS and follow-up meta-analysis	Voight et al. (2010) [14]	
C2CD4A-4B	15q22.2	Nuclear factor	Meta-analysis (21 GWAS), follow-up study; GWAS and meta-analysis; GWAS	Shu et al. (2010) [21]; Yamakuchi et al. (2010) [105]	Dupuis et al. (2010) [7]
CAPN10	2q37.3	Inhibitor of insulin secretion	Genome-wide linkage study	Horikawa et al. (2000) [16]	
CDC123	10p13	Cell cycle regulation	DIAGRAM; GWAS and meta-analysis; candidate gene study	Zeggini et al. (2008) [24]; Shu et al. (2010) [21]; Waters et al. (2010) [13]	
CDKAL1	6p22.3	Insulin response	GWAS; candidate gene study; multi-ethnic meta-analysis	Saxena, et al. (2007) [9]; WTCCC (2007) [55]; Zeggini et al. (2007) [30]; Scott, et al. (2007)[10]; Waters et al. (2010) [13]; Saxena et al. (2012) [5]	
CDKN2A-B	9p21	Islet development	GWAS; candidate gene study; multi-ethnic meta-analysis	Scott, et al. (2007)[10]; Waters et al. (2010) [13]; Saxena, et al. (2007) [9]; Saxena et al. (2012) [5]; Zeggini et al. (2007) [30]; Parra et al. (2011) [27]	
CENTD2	11q13.4	Glucose homeostasis and insulin secretion	Combined GWAS and follow-up meta-analysis; multi-ethnic meta-analysis	Voight et al. (2010) [14]; Saxena et al. (2012) [5]	
CHCHD2P9	9q21.31	Transcription factor	Combined GWAS and follow-up meta-analysis	Voight et al. (2010) [14]	
CRY2	11p11.2	Mammalian circadian pacemaker	Meta-analysis (21 GWAS), follow-up study		Dupuis et al. (2010) [7]
DGKB-TMEM195	7p21.2	Signal transduction	Candidate gene study		Dupuis et al. (2010) [7]; Ramos et al. (2011) [26]

DUSP9	Xq28	Insulin action and beta cell function	Combined GWAS and follow-up meta-analysis	Voight et al. (2010) [14]	
EXT2-ALX4	11p12-p11	Cell development and function	GWAS	Sladek et al. (2007) [6]	
ENPP1/PCSK1/PC1	6q22-q23	Insulin resistance and glucose intolerance	Genome-wide linkage study	Meyre et al. (2005) [17]; Ali et al. (2013) [106]	
FADS1	11q12.2-q13.1	Fatty acid biosynthesis	Meta-analysis (21 GWAS), follow-up study		Dupuis et al. (2010) [7]
FITM2-R3HDML	20q13.12	Fat storage-inducing transmembrane protein	East Asian meta-analysis,	Cho et al. (2011) [8]	
FOLH1	11p11.2	Type II transmembrane glycoprotein	Meta-analysis; multi-ethnic meta-analysis	Saxena et al. (2012) [5]	
FTO	16q12.2	Fat-mass and obesity related gene	GWAS; candidate gene study; genome-wide linkage study	Zeggini et al. (2007) [30]; Frayling et al. (2007) [28]; Scott et al. (2007) [10]; Ali et al. (2013) [106]	
GATAD2A	19p13.11	Transcriptional repressor	Meta-analysis; multi-ethnic meta-analysis	Saxena et al. (2012) [5]	
GCC1-PAX4	7q32.1	Member of the paired box family of transcription factors	East Asian meta-analysis	Cho et al. (2011) [8]	
GCK	7p15.3-p15.1	Fasting blood glucose levels	Multi-ethnic meta-analysis	Saxena et al. (2012) [5]	Dupuis et al. (2010) [7]
GCKR [§,]*	2p23	glucose transport and sensing	Candidate gene study; multi-ethnic meta-analysis	Saxena et al. (2012) [5]	Dupuis et al. (2010) [7]; Ramos et al. (2011) [26]
G6PC2	2q24.3	glucose-stimulated insulin secretion	FUSION study; candidate gene study		Dupuis et al. (2010) [7]; Chen et al. (2008) [69]; Ramos et al. (2011) [26]
GIPR*	19q13.3	Insulin secretion	Meta-analysis; multi-ethnic meta-analysis	Saxena et al. (2012) [5]	Saxena et al. (2012) [5]
GLIS3	9p24.2	Cell proliferation and development	East Asian meta-analysis, meta-analysis (21 GWAS), follow-up study	Cho et al. (2011) [8]	Dupuis et al. (2010) [7]
GRB14*	2q22-q24	Signal transduction	GWAS	Kooner et al. (2011) [12]	
HHEX	10q23.33	β-cell development or function	First GWAS by French group; candidate gene study; multi-ethnic meta-analysis	Saxena et al. (2007) [9]; Scott et al. (2007) [10]; Sladek et al. (2007) [6]; WTCCC (2007) [55]; Steinthorsdottir et al. (2007) [11]; Zeggini et al. (2007) [30]; Waters et al. (2010) [13]; Ali et al. (2013) [106]; Saxena et al. (2012) [5]	
HLA-DQB1[‡]	6p21.3	Autoimmune mechanisms	Meta-analysis	Saxena et al. (2012) [5]	
HMGA2	12q15		Combined GWAS and follow-up meta-analysis; multi-ethnic meta-analysis	Voight et al. (2010) [14]; Saxena et al. (2012) [5]	
HMG20A	15q24	Neuronal differentiation	GWAS	Kooner et al. (2011) [12]	

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HNF1A/1B	12q24.2	Transcription factor	Combined GWAS and follow-up meta-analysis; multi-ethnic meta-analysis	Voight et al. (2010) [14]; Winckler et al. (2005) [25]; Parra et al. (2011) [27]; Saxena et al. (2012) [5]	
HNF4A	20q13.12	Pancreatic beta-cell function and impaired insulin secretion	Genome-wide linkage study; GWAS	Love-Gregory et al. (2004) [15]; Kooner et al. (2011) [12]	Kooner et al. (2011) [12]
IDE	10q23-q25	Insulin degradation and hyperglycemia	GWAS	Sladek et al. (2007) [6]; Zeggini et al. (2007) [30]; Ali et al. (2013) [106]	
IGF1*	12q23.2	Glucose homeostasis and insulin resistance	Meta-analysis (21 GWAS), follow-up study		Dupuis et al. (2010) [7]
IGF2BP2	3q27.2	Beta-cell function	GWAS; candidate gene study; multi-ethnic meta-analysis	Saxena et al. (2007) [9]; Scott et al. (2007) [10]; Zeggini et al. (2007) [30]; Yamauchi et al. (2010) [105]; Waters et al. (2010) [13]; Saxena et al. (2012) [5]	
IRS1*	2q36	Insulin resistance and hyperinsulinemia	Combined GWAS and follow-up meta-analysis; candidate gene study; multi-ethnic meta-analysis	Voight et al. (2010) [14]; Saxena et al. (2012) [5]; Rung et al. (2009) [18]	Ramos et al. (2011) [26]
JAZF1	7p15.2-p15.1	Transcriptional repressor	DIAGRAM study; candidate gene study; multi-ethnic meta-analysis	Zeggini et al. 2007 [30]; Waters et al. (2010) [13]; Saxena et al. (2012) [5]	
KCNJ11	11p15.1	Insulin secretion	GWAS by French gr.; UK group; candidate gene study; multi-ethnic meta-analysis	Saxena et al. (2007) [9]; Scott et al. (2007) [10]; Sladek et al. (2007) [6]; WTCCC et al. (2007) [55]; Saxena et al. (2012) [5]; Waters et al. (2010) [13]	
KCNK16	6p21.2-p21.1	Insulin secretion	East Asian meta-analysis	Cho et al. (2011) [8]	
KCNQ1	11p15.5	Insulin secretion	Combined GWAS and follow-up meta-analysis; candidate gene study; multi-ethnic meta-analysis	Yasuda et al. (2008) [19]; Tsai et al. (2010) [20]; Yamauchi et al. (2010) [105]; Parra et al. (2011) [27]; Unoki et al. (2008) [22]; Voight et al. (2010) [14]; Been et al. (2011) [107]; Waters et al. (2010) [13]; Saxena et al. (2012) [5]	
KLF14	7q32.3	Insulin sensitivity	Combined GWAS and follow-up meta-analysis	Voight et al. (2010) [14]	
MADD	11p11.2	Insulin secretion	Meta-analysis (21 GWAS), follow-up study		Dupuis et al. (2010) [7]
MAEA	4p16.3	Not known	East Asian meta-analysis	Cho et al. (2011) [8]	
MTNR1B	11q21-q22	Glucose homeostasis	Combined GWAS and follow-up meta-analysis; candidate gene study	Voight et al. (2010) [14]	Dupuis et al. (2010) [7]; Bouatia-Naji et al. (2009) [108]; Lyssenko et al. (2009) [109]
NOTCH2*	1p13-p11	A type 1 transmembrane receptor	DIAGRAM study	Zeggini et al. (2008) [24]	
PEPD	19q13.11	Insulin secretion	East Asian meta-analysis	Cho et al. (2011) [8]	
PPARG	3p25	Insulin resistant	GWAS; candidate gene study; multi-ethnic meta-analysis	Saxena et al. (2007) [9]; Scott et al. (2007) [10]; WTCCC (2007) [55]; Waters et al. (2010) [13]; Saxena et al. (2012) [5]; Rai et al. (2007) [29]; Steinthorsdottir et al. (2007) [11]	

PRC1	15q26.1	Cytokinesis	Combined GWAS and follow-up meta-analysis	Voight et al. (2010) [14]	
PROX1	1q41	β-cell development	Meta-analysis (21 GWAS), follow-up study		Dupuis et al. (2010) [7]
PSMD6	3p14.1	Protein degradation	East Asian meta-analysis	Cho et al. (2011) [8]	
PTPRD	9p23-p24.3	Insulin Signalling	GWAS	Tsai et al. (2010) [20]	
SIRT1	10q21.3	Energy sensing molecule	Candidate gene study	Rai et al. (2007) [29]	
SLC2A2(GLUT2)	3q26.1-q26.2	Glucose transport and sensing	Meta-analysis (21 GWAS), follow-up study; meta-analysis	Saxena et al. (2012) [5]	Dupuis et al. (2010) [7]
SLC30A8	8q24.11	Insulin production and secretion	First GWAS by French group; candidate gene study; multi-ethnic meta-analysis	Saxena et al. (2007) [9]; Scott et al. (2007) [10]; Sladek et al. (2007) [6]; Steinthorsdottir et al. (2007) [11]; Zeggini et al. (2007) [30]; Waters et al. (2010) [13] ; Saxena et al. (2012) [5]	Dupuis et al. (2010) [7]; Ramos et al. (2011) [26]
SLC39A4	8q24.3	Tissue development and BMP/TGF-β signaling	Meta-analysis	Saxena et al. (2012) [5]	
SPRY2	13q31.1	Signal transduction	GWAS and meta-analysis	Shu et al. (2010) [21]	
SREBF1	17p11.2	Transcription factors	Meta-analysis; multi-ethnic meta-analysis	Saxena et al. (2012) [5]	
SRR	17p13	Insulin secretion	GWAS	Tsai et al. (2010) [20]	
ST6GAL1	3q27-q28	Insulin action and cell surface trafficking	GWAS	Kooner et al. (2011) [12]	Kooner et al. (2011) [12]
TCF7L2	10q25.3	Insulin secretion	First GWAS by French group; GWAS; candidate gene study; multi-ethnic meta-analysis; genome-wide linkage study	Saxena et al. (2007) [9]; Scott et al. (2007) [10]; Sladek et al. (2007) [6]; Grant et al. (2006)[56]; Ali et al. (2013) [106]; WTCCC (2007) [55]; Steinthorsdottir et al. (2007) [11]; Yamauchi et al. (2010) [105]	Dupuis et al. (2010) [7]; Ramos et al. (2011) [26]
THADA	2p21	Apoptosis	DIAGRAM study; candidate gene study; multi-ethnic meta-analysis	Zeggini et al. (2008) [24]; Waters et al. (2010) [13]; Saxena et al. (2012) [5]	
TH/INS	11p15.5	Glucose homeostasis	Multi-ethnic meta-analysis	Saxena et al. (2012) [5]	
TMEM163	2q21.3	Insulin secretion	GWAS	Tabassum et al. (2012) [23]	
TP53INP1	8q22	Nuclear factor	Combined GWAS and follow-up meta-analysis	Voight et al. (2010) [14]	
TSPAN8	12q14.1-q21.1	Receptor	DIAGRAM study	Zeggini et al. (2008) [24]	
UCP2	11q13	Insulin secretion	Candidate gene study	Rai et al. (2007) [29]	
UBE2E2	3p24.2	insulin biosynthesis, secretion and signaling	GWAS	Yamauchi et al. (2010) [105]	
VPS26A	10q21.1	Transporter	GWAS	Kooner et al. (2011) [12]	
WFS1	4p16.1	Renal function	Candidate gene study; multi-ethnic meta-anal.	Waters et al. (2010) [13]; Saxena et al. (2012) [5]	
ZBED3	5q13.3	Zinc finger (ZF) protein family	Combined GWAS and follow-up meta-analysis	Voight et al. (2010) [14]	
ZFAND6	15q25.1	ZF protein family	Combined GWAS and follow-up meta-analysis	Voight et al. (2010) [14]	
ZFAND3	6pter-p22.3	ZF protein fam.	East Asian meta-analysis	Cho et al. (2011) [8]	
ZMAT4	8p11.21	ZF protein family	Candidate gene study		Ramos et al. (2011) [26]

Legend: References [1-109]. ‡ T1D, § Serum triglycerides, * Insulin level and/or HOMA-IR. *Abbreviations*: BMP - bone morphogenetic protein, DIAGRAM - Diabetes Genetics Replication And Meta-analysis, GWAS - genome-wide association study, T1D - type 1 diabetes.

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