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Advances and challenges in biomarker development for type 1 diabetes prediction and prevention using omic technologies

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Abstract

Importance of the field—Biomarkers are essential for the identification of high risk children as well as monitoring of prevention outcomes for type 1 diabetes (T1D).

Areas covered in this review—This review discusses progress, opportunities and challenges in biomarker discovery and validation using high throughput genomic, transcriptomic and proteomic technologies. The authors also suggest potential solutions to deal with the current challenges.

What the reader will gain—Readers will gain an overview of the current status on T1D biomarkers, an integrated review of three omic technologies, their applications and limitations for biomarker discovery and validation, and a critical discussion of the major issues encountered in biomarker development.

Take home message—Better biomarkers are still urgently needed for T1D prediction and prevention. The high throughput omic technologies offer great opportunities but also face significant challenges that have to be solved before their potential for biomarker development is fully realized.

Keywords

Biomarkers; Prediction; Prevention; Type 1 Diabetes

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease resulting from the poorly understood interactions between susceptibility genes, the environment and the immune system. It has been projected that the incidence of T1D will continue to increase with an average rate of 3% per year (1-4) in underdeveloped and developing countries. In attempts to curtail this rising incidence, novel prediction and prevention strategies are urgently needed. The long silent prodromal period before the onset of clinical disease offers many opportunities for the prevention of T1D. The disease can be prevented in numerous ways in NOD mouse models. Although various prevention trials have been conducted in humans, no breakthroughs have yet been made in preventing the disease. Possible reasons for this failure include the difficulties of accurately identifying a sufficiently large high risk population at the early stages of the disease, the inability to conduct a large numbers of clinical trials, poorly understood etiology of the disease, and heterogeneity of the disease pathogenesis. Therefore, prevention tailored for the whole at-risk population may not be effective and personalized prevention strategies based on one's own risk and etiology may prove to be more efficient.

Biomarkers play essential roles for both the identification of the high risk population and more importantly for tailoring and monitoring of therapies for the disease. T1D results from a cascade of molecular, cellular and metabolomic changes starting at a very early stage in life, probably

in utero. Therefore, biomarkers for T1D may be derived from a variety of sources that can be grouped into six major categories: metabolomic, autoantibodies, immune cells, proteomic, transcriptomic, and genetic (Fig. 1). Before the clinical onset, diabetes can be accurately diagnosed with several glucose tolerance tests and hemoglobin A1c levels. Although these metabolomic changes remain the golden standard for pre-diabetes diagnosis, these changes occur at late stages of the disease process and have relatively little value for disease prevention. Earlier prediction of T1D became possible three decades ago through the use of islet cell autoantibodies (ICA). The discovery of autoantibodies against specific islet autoantigens such as insulin and GAD (Glutamate dehydrogenase) and the continuously improving assays for these autoantibodies has allowed significant increase in the predictive value of these autoantibodies (5-7). However, islet autoantibodies also have major limitations. Most importantly, the appearance of islet autoantibodies marks a relatively late stage in disease development. Because T1D prevention may be more effective before active autoimmune response, identification of the high risk population before the appearance of islet autoantibodies would be of great value. Furthermore, islet autoantibodies are not implicated in the disease pathogenesis and are not useful for assessing therapeutic outcomes.

A number of studies have also suggested that T1D patients are defective in the number and/or function of immune cells including CD4⁺ regulatory T cells (8,9), antigen presenting cells (10,11) and NKT cells (12). Furthermore, autoreactive T cells against islet-cell antigens are increased in T1D patients (13). These and other cellular changes have great potential to become excellent biomarkers for T1D prediction and prevention as they are likely implicated in the disease pathogenesis and may occur before the onset of islet autoantibodies. The development of such biomarkers also faces great challenges that have been reviewed elsewhere (13) and will not be discussed here.

It is well known that the interaction between susceptibility genes and environmental factors result in a cascade of changes in gene and protein expression levels. Therefore, susceptibility genes, gene expression, protein expression and post-translational modifications (PTM) are rich sources of biomarkers for T1D. Such biomarkers have great potential as they may occur early in the disease process and can be easily and cheaply assayed. The developments in high throughput technologies in the last two decades have opened the door to a variety of exciting advances with regards to biomarker discovery. The extensive set of omic methodologies can be used to capture the molecular changes occurring at various levels of an organism and during the entire disease progression process. The knowledge gained through these technologies should provide new biomarkers for T1D prediction and prevention. In this review, we will discuss progress, opportunities and challenges in the discovery and validation of T1D biomarkers using genetic, transcriptomic and proteomic technologies.

2. T1D susceptibility genes as risk factors for T1D

A genetic predisposition to T1D has been suggested by the familial clustering of the disease and demonstrated by a variety of genetic studies. Earlier studies indicated that the strongest genetic susceptibility for T1D is encoded within the HLA class II region and accounts for approximately 50% of the familial clustering (14). Genome-wide linkage analysis suggested that a large number of susceptibility genes may be implicated in T1D. Recently, genome-wide association studies (GWAS) have become a method of choice for mapping genes involved in many complex diseases including inflammatory bowel disease (15,16), multiple sclerosis (17,18), type 2 diabetes (19-22), and type 1 diabetes (23-30). To date GWAS have identified over 750 regions associated with more than 148 traits (GWAS Catalog www.genome.gov/gwastudies). The number of genomic regions associated with each disease phenotype is quite large. Often, the regions showing association harbor many genes that

potentially could contribute to the observed disease phenotypes. It has also become apparent that some of the associated regions are shared by multiple diseases with similar etiology (31).

Table 1 summarizes the genomic intervals identified for T1D in multiple GWAS studies. The main finding of these GWAS studies is the large number of associated intervals with variable degrees of association evidence. An intrinsic problem with GWAS is the potentially high false-positive rate due to the large number of markers analyzed in the studies (32). The intervals with evidence in multiple studies are likely to contain susceptibility genes while others may be spurious associations. Among the large numbers of associated intervals, the overall evidence for the intervals in Table 1 is generally convincing. Other intervals remained to be confirmed in additional studies. Although GWAS are typically conducted on a relatively large sample sizes, the earlier GWAS were designed to detect variants of relatively larger effect size. Various studies have shown that as sample size in GWAS increases, the number of loci that are able to be identified and validated will also increase. It is also likely that studies of different populations will identify more associated intervals. The early GWAS also employed simpler statistical methods and assume independence between variants. These approaches do not account for the interactions that are occurring between multiple loci contributing to disease risk. Future GWAS should take the limitations of the earlier GWAS into consideration and may allow for the discovery of additional associations.

GWAS also confirmed that the strongest genetic susceptibility genes for T1D were encoded in the human leukocyte antigen (HLA) region. All non-HLA genes appear to have very weak contribution to T1D susceptibility. The previously confirmed disease genes such as *INS* and *PTPN22* are among the stronger non-HLA T1D susceptibility genes (33-36). The newly discovered T1D association intervals generally have weaker contribution. The weak effect of these susceptibility genes poses a serious challenge for the identification of the specific genes implicated in the disease. This task will require extensive sequencing and genotyping of large number of subjects for all genes in the associated intervals as well as functional characterization of the associated genes and variants. Identification of the specific disease genes and elucidation of the underlying functional mechanism will undoubtedly contribute valuable information for understanding the pathogenesis of T1D.

T1D susceptibility genes, particularly the HLA class II genes, have been widely used in population-based studies for the identification of high risk individuals. Although HLA genes allow the identification of a population at increased risk for the development of T1D, testing HLA genes alone lacks specificity, sensitivity and positive predictive value. It has been hoped that the identification of the non-HLA genes would increase the predictive value of genetic testing for T1D. It is believed by some investigators that the use of computer based modeling approaches with GWA data may be able to improve the assessment of disease risk (37). This may be possible only if significant gene-gene and/or gene-environment interactions occur. Although such interactions are certainly expected to occur in T1D, there is no available evidence to suggest that they will significantly improve our ability to accurately identify the high risk subjects with high specificity and sensitivity. We believe that one remaining hope to gain predictive value with T1D susceptibility genes is the possibility that rare variants with much larger effect on disease that are difficult to detect by GWAS will be identified by large scale sequencing studies. This hypothesis remains to be tested in future studies.

3. Gene expression as potential biomarkers for T1D

The microarray technologies for gene expression profiling have provided unparalleled opportunities for the discovery of gene expression changes associated with disease. The approach allows genome-wide characterization of genes implicated in disease pathogenesis as well as biomarkers for risk assessment, molecular classification of disease subtypes and

therapeutic monitoring. The approach has also been applied to the studies of T1D in both human patients and animal models. A major focus of the microarray studies has been on the elucidation of the immunological mechanism of the disease using the NOD mouse as a model. For example, our gene expression profiling in NOD mouse spleens identified two distinct groups corresponding to an immature (1-4 weeks) and mature (6-10 weeks) state. The rapid switch of gene expression occurring around 5 weeks of age defines a key immunological checkpoint for the development of disease (38). Analysis of three different tissues (pancreatic lymph nodes, spleen and peripheral blood cells) at six different stages defined a “road map” of gene expression profiles in NOD mice (39). Microarray technology was also successfully used to reveal the important roles of chemokines in T1D pathogenesis (40). Analysis of the CD4⁺ T cells from NOD congenic mice identified *Cd55* (*Daf1*) and *Acadl* as candidate genes for T1D (41). Microarray technology was also used to elucidate the role of regulatory T cells (42), to define a defect in central tolerance in NOD (43) and the molecular events associated with cyclophosphamide-induced diabetes (44). A series of studies in Dr. Eizirik’s laboratory has extensively characterized the gene expression profiles of pancreatic islet cells and cytokine-induced apoptosis (45-50).

Several studies attempted to characterize the gene expression profiles in T1D patients and/or prediabetic subjects (51,52). Our group conducted the first microarray study on peripheral blood cells (PBC) from human T1D patients and autoantibody-positive (AbP) subjects (51). The analysis resulted in the identification of over 100 genes found to be up-regulated in PBC of T1D and AbP subjects. Consistent with the studies in the NOD mice, many of the differentially expressed genes are involved in important immunological functions including antigen processing and presentation (e.g. *HLA-b,c*, *CD74*), cytotoxicity and apoptosis (e.g. *GZMB*, *GNLY*), and immune regulation (e.g. *MNDA*, *SELL*). Gene expression profiles in PBC of T1D patients were also characterized in two subsequent studies (53,54), each identifying a different set of genes as differentially expressed in T1D patients. Although the studies suggested a proinflammatory response in T1D patients, the specific genes identified in each study are non-overlapping. Microarray was also used to characterize gene expression in PBC from a small number of prediabetic subjects (52). This study suggested a down-regulation of genes involved antigen presentation, which contradicts the findings in our previous study with a slightly large sample size (51). Furthermore, microarray has been used as a readout system to assess serum protein differences in T1D and at-risk subjects (55) and this clever approach also indicates a proinflammatory state in T1D patients.

Review of the published literature on gene expression studies clearly indicates that the results are largely inconclusive and sometimes contradictory. Although the approach holds great promise that has yet to be realized, major improvement in the experimental design and conduct is necessary to obtain reliable and reproducible results. The first challenge for genomic studies on human T1D is the availability of study materials. It is evident that the most relevant materials to study T1D are cells from the pancreatic lymphnodes and pancreatic islets where the pathological events mainly take place. However, such samples are severely limited and have only become accessible with the establishment of the nPOD program (<http://www.jdrfnpod.org/index.php>). Therefore, all previous studies focused on PBC that only contain low frequencies of cells implicated in the disease process. PBC contain a very heterogeneous pool of immune cells and it is difficult and expensive to obtain sufficient number of the most relevant cells (regulatory T cells, dendritic cells and autoreactive cells, for example). Some attempts have been made to study cells purified from human PBC, e.g. monocytes (56,57). These studies confirmed an inflammatory profile in T1D patients, consistent with the results obtained from studying PBC. However, whether these genes can be used as biomarkers for disease prediction or therapeutic monitoring remains an open question.

A second major issue with the previous studies is the small sample sizes that range from a few subjects to a few dozens of subjects. Gene expression can be altered by a large number of factors including sex, age, genetic factors, diet, health status and others. Although investigators can and have attempted to match these covariates to their best ability, it is necessary to use large sample sizes to assess the impact of these variables. It is likely that the inconsistent results from different studies are mainly due to the small sample sizes used in all the previous studies. If the lessons from the genetic association studies can serve as guidelines, we believe that sample sizes in the thousands may also be required for gene expression studies.

A third major issue relates to the disease stages that are most relevant for gene expression studies. While most previous studies investigated T1D patients due to sample availability, the most relevant stages for studying both disease pathogenesis and biomarkers are the transitions from autoantibody-negative (AbN) to AbP and from AbP to clinical onset. Such studies require longitudinal samples from a very early time point, probably at birth, to disease onset. Ideally, RNA samples from different immune cell subsets should be banked for all subjects in large prospective cohorts such as the environmental determinants of diabetes in the young (TEDDY) (58). Joint analysis on gene expression, susceptibility genes and environmental factors using samples banked in TEDDY will undoubtedly offer the greatest power in defining the molecular events during T1D progression.

4. Serum proteins have great potential as T1D biomarkers

While proteomic studies are also limited by the availability of pathological tissues, the studies on serum proteins may reflect, at least partially, the events occurring in the pathological sites. Recent technological innovations have increased our potential to evaluate global changes in proteins in a comprehensive manner. This is especially true in mass spectrometry (MS)-based research where improvements, including ease-of-use, in high performance liquid chromatography (HPLC), column chemistries, instruments, software, and molecular databases have advanced the field of proteomics considerably. These technologies have been used in biomedical research including biomarker discovery, elucidation of molecular mechanisms and identification of drug targets. The major proteomic technologies in current research are summarized in Table 2 and Figure 2. These different techniques have been comprehensively reviewed elsewhere (59).

From the perspective of biomarker discovery, serum and plasma proteins are great sources for biomarkers but also pose a significant and well documented analytical challenge (60). The main challenge to overcome in proteomic research is that candidate biomarkers are present in trace amounts among a large background of non-relevant and abundant proteins. Diversity in the human proteome often gives rise to pluralities of structurally similar but functionally distinct proteins (61). Such micro-heterogeneity generally escapes proteomics discovery technologies and perhaps conventional immunoassays (61).

Several MS-based techniques have been developed and used to discover new biomarker candidates in a variety of human diseases including T1D. Two-dimensional gel electrophoresis (2DE) used to be the platform of proteomic discoveries for a long time. Separation in 2DE is based on isoelectric point (pI) as a first dimension and SDS-PAGE as second dimension. Despite widespread use, 2DE has been plagued by low resolution in that only those high abundant proteins are visible on the gels. This issue has been resolved by the addition of a third dimension (3DE) based on the hydrophobicity of the proteins (59,62). Introducing hydrophobic properties in 3DE resolves the issue of low resolution of 2DE; however, it remains to be a very labor intensive platform. A new proteomic tool, called Mud-PIT based 2D-HPLC, coupled with MS/MS, has resolved some of the issues related to proteomic studies (63-67).

Since proteins are involved in all cellular processes, their cumulative expression profile reflects the specific activity of cells. Early proteomic studies in relation to T1D were conducted in the 1980s using 2D-based techniques and described the changes in the protein expression patterns in islets from mice (68,69). A large effort was dedicated to insulin production and secretion as a part of normal islet physiology. Today no proteomic data is available based on human materials directly studying beta-cell destruction and development of T1D. Most proteomic data are derived from animal models and cell lines (70-72). One study has used SELDI-TOF MS to identify autoantibodies to glial fibrillary acidic protein in both NOD mice and human patients (73). Another study has described the global protein expression in the whole human pancreas allowing for the creation of a reference 2D electrophoresis map of 302 proteins. Although useful for understanding components involved, due to the fact that the proteins identified were from both endocrine and exocrine tissue, it is difficult to use the data for T1D pathogenesis research (74). This gap in our understanding has begun to be filled by a recent study, which identified 6873 proteins from pooled islets, with a role in development of diabetes (69). A number of islet-derived β -cell lines (75) and animal models (76) have contributed to our understanding of the pathogenesis of T1D. Proteomics has been applied in studies of differentiating β -cells, cytokine exposed islets, dietary manipulated islets, and in transplanted islets. A detailed account of proteomic studies on pancreas and pancreatic islets is described in previous reports (77,78). Although various studies have revealed a complex and detailed picture of the protein expression profiles many functional implications remain to be answered. What has been ascertained to this point is a rather detailed picture of protein expression in β -cell lines, islets, and transplanted islets both *in vitro* and *in vivo* (71,72,77). Some of the identified proteins in these proteomic discoveries are listed in Table 3. The available data indicate that the β -cell is an active participant in its own destruction during diabetes development. Furthermore, as is the case seen in genomics, no single protein alone seems to be responsible for the development of diabetes. Rather, the cumulative pattern of changes seems to be what favors a transition from dynamic stability in the unperturbed β -cell to dynamic instability and eventually to β -cell destruction (69,75,76).

Beta-cell destruction is proposed to be the result of a dominant Th-1 like cytokine profile at the time of disease onset (79-81), others have countered by proposing a loss of immune response at the time of T1D onset (82,83) in response to cytokine and autoantigen stimulation. Ryden et. al. (84) obtained PBMC from T1D patients and high-risk children, and stimulated them with the autoantigen GAD 65 and mitogen phytohaemagglutinin (PHA). Cytokines and chemokines were detected in cell-culture supernatants by protein microarray in relation to clinical outcome (C-peptide). Increased secretion of IFN- γ , IL-2, TNF- α (Th-1 response) was observed in high-risk children, when PBMC were stimulated, either spontaneously or with GAD-65. This Th-1 dominance was associated with a high risk (40%) of developing T1D within 5 years. PHA stimulation lead to increased secretion of cytokine IL-5 (Th-2 response) in PBMC from the high-risk children, indicating that still healthy high-risk individuals have the ability to switch a Th1-like profile into a more protective Th2-like profile in the presence of the autoantigens GAD₆₅ and insulin (81). PBMC from newly diagnosed T1D children has increased TGF- β and IL-10 secretion (a Th-3 response), upon stimulation, irrespective of the mode. TGF- β is directly involved in generation of FOXP3⁺ Treg cells (85). Interleukin-10 (IL-10) is a regulatory cytokine that plays a central role in controlling inflammatory processes, and IL-10-secreting T cells may constitute an additional mechanism that are responsible for peripheral tolerance (86,87). Although the protective Th-3 response is high at the onset of T1D, the reduced function of the Treg cells reported by us and others (9), is insufficient to counter the dominant Th-1 response leading to the destruction of β -cells.

Identification of surrogate biomarkers predictive of those at high risk for developing T1D would be beneficial, particularly if such surrogate biomarkers result in higher sensitivity and specificity, better positive predictive value, or earlier detection of at-risk subjects. Biomarker

development for T1D using proteomic tools has been slow but is increasing rapidly. A pilot proteomic analysis of human plasma and serum from a subset of controls and T1D patients enrolled in the Diabetes Autoantibody Standardization Program identified Alpha-2-glycoprotein 1 (zinc), Clusterin, Corticosteroid-binding globulin, Lumican, and Serotransferrin as putative biomarkers (88). Our own global peptide finger print approach using SELDI-TOF MS identified 146 protein/peptide peaks. Validation on the test dataset showed 82.8% specificity and 76.2% sensitivity in prediction of T1D from AbN controls (89). However, the identity of these proteins from SELDI study is difficult to determine and validate. A recent study identified transthyretin, apolipoprotein A1, apolipoprotein C1 and cystatin C as markers for diabetic nephropathy (90).

Despite some progress in discovering proteomic biomarker candidates for T1D, no new biomarkers approaching the predictability of islet autoantibodies have been published to date. This sad truth is due, in addition to the difficulties in biomarker discovery, to a series of technical and biological issues related to biomarker validation. The proteomic discovery platforms discussed earlier are usually not suitable for the validation studies that require large numbers of samples. In this regard, the sandwich immunoassays (ELISA and bead-based Luminex assays) are excellent platforms and tools of choice for validation of protein biomarker candidates due to their robustness and high-throughput capabilities in terms of measuring large numbers of samples. These techniques have already been widely used to study candidate serum proteins including cytokines, chemokines, soluble forms of various receptors and inflammatory mediators. The literature has suggested that all studies only analyzed one or a few serum proteins and generally suffered from small sample size and therefore the results were difficult to replicate (89). Our extensive unpublished results indicate that serum is an excellent source for T1D biomarkers but their validation requires thousands of cross-sectional and prospective samples.

5. Expert Opinion

T1D is a disease for which there are fortunately excellent biomarkers for disease prediction. Impending disease can be accurately identified before the appearance of clinical symptoms using metabolic tests (OGTT and IVGTT) and/or hemoglobin A1c. Furthermore, children at high risk for the development of T1D can be identified using a combination of multiple autoantibodies against pancreatic islet cell antigens. These tests are routinely used in clinical studies and patient care. Improvement on the assays, addition of new islet autoantibodies and delineation of the antibody subclasses may further enhance the utility of the islet autoantibody tests. However, these existing biomarkers do not fully meet the need for T1D prediction and prevention due to the imperfect positive predictive value and more importantly the relative late appearance of autoantibodies as well as the lack of causal relationship with disease pathogenesis.

There is still an urgent need for better and earlier biomarkers for T1D prediction and prevention. Successful prevention of the disease requires the identification of high risk populations at the earliest time possible and before the appearance of islet autoantibodies. Furthermore, surrogate biomarkers are needed to access the outcomes of prevention therapies in early stages. Due to the long asymptomatic period for diabetes, it is too expensive and time consuming for clinical trials to wait for the final clinical outcome. The lack of suitable surrogate biomarkers for T1D has severely hampered progress in clinical trials. As discussed in this review, the high throughput omic technologies have offered new opportunities to develop such biomarkers. However, new biomarkers with the potential to fundamentally change diabetes research and care are yet to come due to the many challenges that are being, or will be, resolved.

The first major challenge for all biomarker development programs is the availability of biological samples. It has become very clear that biomarker validation will require thousands of samples irrespective of the technologies and the type of molecules. Furthermore, samples from large cohorts prospectively monitored at high intensity must be used to validate the biomarker candidates. This most difficult challenge is being addressed by several international consortia such as TEDDY and TrialNet. Retrospective analysis of the banked samples should significantly improve the experimental design and outcomes. The second challenge for T1D biomarker development concerns further improvement of technologies used for discovery and validation. In this regard, technologies for genetic and transcriptomic studies are quite mature and the costs are rapidly reducing. However, proteomic analysis still faces severe challenges in both the discovery and validation platforms. Finally, biomarker development programs have to solve the computational challenge. Cumulative evidence suggests that no single biomarker can provide adequate power for T1D or other complex diseases due to the multifactorial nature of the diseases. Therefore, biomarkers for T1D have to rely on the combination of multiple markers. As a result, the simultaneous consideration of genetic, transcriptomic, proteomic, other omic and cellular changes occurring during disease progression will be required for accurate assessment of disease risks and monitoring of therapeutic outcomes. Advanced computational and statistical analyses are needed to develop and validate multivariate models as biomarkers. The development of multivariate models requires the solving of two statistical issues: first, selecting an optimal subset of markers (a single multivariate model) from all available sets of variables with which to make predictions; and second, predicting the phenotypic statuses based on the selected subset of markers. As progress is being made in all three challenge areas, we anticipate that new and improved biomarkers will become available for T1D in the near future.

Article Highlights

- Currently available biomarkers allow the identification of at-risk subjects but are not useful for therapeutic monitoring.
- New biomarkers are urgently needed for early disease prediction and therapeutic monitoring.
- High throughput genetic, transcriptomic and proteomic technologies offer great opportunities for T1D biomarker discovery.
- Better biomarkers are yet to be discovered and validated due to technical and biological challenges.
- Significant effort needs to be devoted to the collection of samples from large prospective cohorts, improvement on technological platforms for both biomarker discovery and validation, and development of computational technologies that integrate multiple types of biomarkers.

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Figure 1.

Natural history of Type 1 Diabetes with molecular, cellular and metabolic events during disease progression. All T1D patients probably have a genetic predisposition that does not change over time. The appearance of islet autoantibodies mark two important transitions, one being from AbN to AbP, and the other being from AbP to T1D. Molecular and cellular events associated with these transitions may serve as T1D biomarkers.

Figure 2.

Phases of Biomarker Discovery Pipeline. Each phase requires different technologies and experimental design. HPLC-MS (High precision liquid chromatography-mass spectrometry), 2DE (2-Dimension gel-electrophoresis), 3DE (3-Dimension gel electrophoresis), MRM-MS (Multiple-reaction monitoring-mass spectrometry).

Table 1

Summary of T1D genomic intervals identified by genetic mapping studies

WTCCC			Todd et al. 2007	Barrett et al. 2009	Other Studies						
SNP	Chr.	Gene of interest	P-value	OR	P-value	OR	P-value	OR	P-value	OR	Ref
rs2476601	1p13.2	PTPN22			1.7E-32	2.04	8.5E-85		8.7E-4	1.32	(26)
rs2269241	1p31.3	PGM1					5.9E-6	1.10			
rs3024505	1q32.1	IL10					2.2E-6	0.84			
rs2816316	1q31.2	RGS1					3.1E-5				
rs1990760	2q24.2	IFIH1	1.4E-10	0.86	3.8E-3	0.89	6.6E-9		0.027	0.849	(26)
rs1534422	2p25.1	NA					6.7E-6	1.08			
rs3087243	2q33.2	CTLA4					1.2E-15		0.02	1.191	(26)
rs11711054	3p21.31	CCR5					1.7E-5				
rs1051708	4p15.2	NA					2.8E-7				
rs4505848	4q27	IL2	6.3E-7	1.27			4.7E-13				
rs6897932	5p13.2	IL7R			0.095	0.93	0.026		6.9E-7	1.13	(91)
rs9268645	6p21.32	MHC					<10 ⁻¹⁰⁰				
rs11755527	6q15	BACH2					5.4E-8		0.0075	1.216	(26)
rs9388489	6q22.32	C6orf173					5.1E-8	1.17			
rs4948088	7p12.1	COBL					2.7E-6	0.77			
rs78043256	7p15.2	NA	3.3E-8	0.00051							
rs4729562	7q22	LOC646282							8.8E-5	0.81	(91)
rs7020673	9p24.2	GLIS3					1.9E-9	0.88	2.9E-4	1.30	(26)
rs947474	10p15	PRKCQ					1.2E-7				
rs1225130	10p15.1	IL2RA					1.3E-13		4.6E-4	1.30	(26)
rs10509540	10q23.31	C10orf59					6.9E-9	0.75			
rs7111341	11p15.5	INS					4.4E-48		4.3E-9	0.622	(26)
rs3764021	12p13	CLEC2D	7.1E-5	0.64	0.0267	0.93					
rs4763879	12p13.31	CD69					2.8E-7	1.09			
rs773107	12q13	SUOX,							2.8E-5	1.25	(27)
rs10876864	12q13	ERBB3							8.3E-5	1.33	(26)

SNP	Chr.	Gene of interest	WTCCC		Todd et al. 2007		Barrett et al. 2009		Other Studies	
			P-value	OR	P-value	OR	P-value	OR	P-value	OR
rs1701704	12q13	CDK2					9.8E-6	1.25		(27)
rs2292239	12q13	ERBB3	1.4E-9	0.77	1.8E-14	1.28	2.2E-25			
rs17696736	12q24	C12orf30	7.2E-14	1.37	1.8E-6	1.16			8.7E-8	0.86 (91)
rs3184504	12q24.12	SH2B3					2.8E-27			
rs1465788	14q24.1	NA					1.4E-8	0.86		
rs941578	14q32.2	DLK1	0.049	0.9			0.00042	1.09	9.8E-7	0.9 (92)
rs3825932	15q24	CTSH					7.7E-8			
rs4788084	16p11.2	IL27					5.2E-8	0.86		(91)
rs1244426	16p12.3	NA					2.0E-6	1.1		
rs12708716	16p13	KIAA0350	1.28×10^{-8}	0.77	7.07×10^{-9}	0.83	2.2E-16		1.0E-6	0.63 (26)
rs7202877	16q23.1	NA					5.7E-11	1.28		
rs1695693	17p13.1	NA					3.2E-6	0.92		
rs2290400	17q12	ORMDL3					1.3E-7	0.87		
rs7221109	17q21.2	NA					9.9E-8	0.95		
rs2542151	18p11	PTPN2	8.4E-8	1.33	3.3E-10	1.29				
rs1893217	18p11.21	PTPN2								
rs763361	18q22	CD226			1.5E-5	1.18	3.6E-15			
rs2304256	19p13.2	TYK2	2.6E-3	0.84			1.2E-5		1.4E-10	0.86 (92)
rs425105	19q13.32	NA					1.5E-7	0.86		
rs2281808	20p13	NA					5.0E-7	0.9		
rs2232613	20q11	NA			0.738	0.98			8.1E-5	1.11 (91)
rs5753037	22q12.2	NA					1.8E-14	1.1		
rs229541	22q13	C1QTNF6					2.1E-7			
rs2664170	Xq28	NA					3.0E-5	1.16		

NA-Not Available

Table 2

Current technologies in proteomics for identification of marker proteins

Proteomics Approach	Sample Prep	Technology	Detection	Number of Molecules	Multiplex	Number of Samples	Detection limit	Pro's/Cons
ELISA	None to Minimal	Immunoassay	Absorbance	1	No	Unlimited	mid pg/ml to high mg/ml	High throughput Only 96 wells
Multiplex	None to Minimal	Luminex-200	Fluorescence	upto 100 molecules/well	Yes	Unlimited	mid pg/ml to high mg/ml	High throughput 96 wells
		FlexMAP 3D	Fluorescence	upto 500 molecules/well	Yes	Unlimited	mid pg/ml to high mg/ml	High throughput, 96 and 384 well, Assay availability
		Flow cytometry	Fluorescence	upto 20 molecules/well	Yes	Unlimited	mid pg/ml to high mg/ml	High throughput 96 wells
		Antibody arrays	Fluorescence	1-200 spots/slide	Yes	2	low pg/ml to ng/ml	Integrated view of a single pathway
2D	Low Salt buffers Protein labelling	IEF SDS-PAGE	Fluorescence MS	abundant proteins	Duplex	Limited	1000-1200 spots	Protein map Time and labor intensive Interference by abundance proteins
2D-HPPLC MS	Extensive prep: Digestion of proteins Removal of High Abundance proteins	HPPLC MS	MS	Unlimited	Available	Limited	Low abundant	deeper scan of the proteome Sophisticated Instrumentation Steep learning curve
3D	Protein labeling 1st Separation by IEF 2nd Separation by RPHPLC 3rd Separation by SDS-PAGE	IEF HPPLC SDS-PAGE Fluorescence MS	Fluorescence MS	3000-5000	Single Plex Duplex	Limited	Low abundant	Deep scan Extremely time and labor intensive

Table 3
Proteomic studies identifying unique proteins associated with pancreatic islets or beta-cells

	Tissue/Cell	Model	Species	Platform	Up	Down	Identified Protein	# of Proteins	Ref
1	Pancreas	Rough endoplasmic reticulum in normal and acute pancreatitis	Rat	ITRAQ, MS	3	3	pancreatic triacylglycerol lipase precursor, Erp27, and prolyl 4-hydroxylase beta polypeptide, fibrinogen alpha, beta and gamma chains	NA/469	(93)
2	INS-1	11.6 and 30mM Glucose	Cell Line	SILIA, MS			fatty acid synthase, E-FABP, CRABP-1, Glucose-6-phosphate isomerase, Cytosolic acetyl-CoA acetyltransferase	NA/816	(94)
3	Pancreas	Development of Pancreas	Porcine	2DE			Pdx1, Ptf1a, Pax4		(95)
4	Islets	Islet	murine	LC-MS				7014/6873	(69)
5	Islets	Glucose stimulation	murine	LC-MS	77	65	Prdx3, DJ-1 (Park7), Sod2, VAMP-2, Syt4, Rab3b	1487/NA	(69)
6	beta-TC-6	Endoplasmic reticulum, Glucose toxicity	Cell Line	2D, MS	4	12	Erp46	46/23	(96)
7	INS-1	Identify the potential islet molecules related to pancreatic cancer-associated diabetes	Cell Line	2D, MS	10	5	Prp19, HMOX1 and GRP78		(97)
8	Islets	Type-2 diabetes	Human	FTIR-MS, SELDI			Multiple pathways		(98)
9	Panc-1	MHC peptidome	Cell Line	LC-MS				131/	(99)
10	NIT-1	Secretory Vesicles	Cell Line	LC-MS			Secretory Proteins	270/	(100)
11	RNAKT-15	Islet differentiation	Cell Line	Microarray, LC-MS					(101)
12	Human Islets, MIN-6	ER Stress and beta-cell apoptosis		2DE			Carboxypeptidase E		(102)