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GENETIC AND FUNCTIONAL ANALYSES OF DIABETIC NEPHROPATHY WITH FOCUS ON CHROMOSOME 3q

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To my family with love

献给我挚爱的家人

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ABSTRACT

Diabetic nephropathy (DN) is a complex disease affected by both genetic and environmental factors. Genetic and functional analyses of the susceptibility and/or resistance genes in DN may provide useful information for better understanding the pathogenesis and further developing novel therapeutic approaches in this disease.

This thesis concerns about genetic and functional analyses of the candidate genes, which are selected from a region in chromosome 3q linked to DN. Genetic association studies are performed mainly with type 1 diabetes (T1D) patients with or without DN. They come from European descent and are selected from Genetics of Kidney Disease in Diabetes (GoKinD) collection. Gene expression analyses at both mRNA and protein levels were examined with kidney tissues of *db/db* mice. The *ADIPOQ* gene is located in chromosome 3q27.3. A promoter single nucleotide polymorphism (SNP) rs266729 in the gene was found to be associated with DN in female T1D patients. This polymorphism altered the sequence for SP1 binding site and subsequently caused the reduction of *ADIPOQ* promoter activity. The *MCF2L2* gene is located in chromosome 3q27.1. A non-synonymous SNP rs7639705 (Leu359Ile) was associated with DN in females. This polymorphism together with SNPs rs266729 in the *ADIPOQ* and rs11915160 in the *SOX2* (in chromosome 3q26.3) gene had combined effects on the association with DN in females. The *MME* gene is located in chromosome 3q25.1-25.2. A significant advance of *MME* expression at both mRNA and protein levels in kidney tissues of *db/db* mice were observed. Genetic polymorphisms rs3796268 and rs3773885 in the *MME* gene were found to be associated with DN in females. The *TRPC1* gene is located in chromosome 3q22-24. Although *TRPC1* mRNA expression in kidney tissues of *db/db* mice was significantly decreased in comparison with the controls, no association between the gene polymorphisms and DN was found.

Data from the studies in this thesis indicate that the *ADIPOQ, MCF2L2* and *MME* genetic polymorphisms but not *TRPC1* are associated with DN with the protective effect in female T1D patients among the GoKinD population. Thus, the evidence provided by this thesis may partially explain the linkage with DN in chromosome 3q. Further investigation on the studied genes and other candidates in the linkage region of this chromosomal arm will be interesting to explore the genetic association with gender-specificity and functional consequence in the development of DN.

Key words: Diabetic nephropathy, chromosome 3, single nucleotide polymorphism, genetic association, gene expression

LIST OF PUBLICATIONS

I **Zhang D**, Ma J, Brismar K, Efendic S, Gu HF. A single nucleotide polymorphism alters the sequence of SP1 binding site in the adiponectin promoter region and is associated with diabetic nephropathy among type 1 diabetic patients in the Genetics of Kidneys in Diabetes Study. *Journal of diabetes and its complications. 2009, 23(4), 265-272.*

II **Zhang D**, Efendic S, Bismar K , Gu HF. Effects of *MCF2L2*, *ADIPOQ* and *SOX2* genetic polymorphisms on the development of nephropathy in type 1 Diabetes Mellitus. *Submitted manuscript*

III **Zhang D**, Forsberg E, Efendic S, Bismar K, Gu HF. Genetic association study and functional analyses of the *MME* gene in type 1 diabetes patients with diabetic nephropathy. *Submitted manuscript*

IV **Zhang D**, Freedman BI, Flekac M, Santos E, Hicks PJ, Bowden DW, Efendic S, Brismar K, Gu HF. Evaluation of genetic association and expression reduction of *TRPC1* in the development of diabetic nephropathy. *American journal of nephrology. 2009, 29(3), 244-251*

Other Publication by the Same Author:

Ma J, **Zhang D**, Brismar K, Efendic S, Gu HF. Evaluation of the association between the common E469K polymorphism in the ICAM-1 gene and diabetic nephropathy among type 1 diabetic patients in GoKinD population. *BMC medical genetics. 2008, 27(9)47.*

CONTENTS

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACTN4	Actinin, alpha 4
ACR	Albumin (urine) /creatinine ratio
ADIPOQ	Adiponectin
ANP	Atrial natriuretic peptide
Bi-PASA	Bi-directional PCR amplification of specific allele
bp	Base pairs
CARS	Cysteinyl-tRNA synthetase
CDH13	Cadherin 13
CKD	Chronic kidney disease
cM	centimorgan
DASH	Dynamic allele specific hybridization
db	Diabetic gene
DH	Dbl-homology
DM	Diabetes mellitus
DN	Diabetic nephropathy
eGFR	Estimate glomerular filtration rate
ELMO1	Engulfment and cell motility 1
EGIR	European group on insulin resistance
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1
ESRD	End-stage renal disease
FRMD3	FERM domain containing 3
GFR	Glomerular filtration rate
GWAS	Genome-wide association study
GWS	Genome-wide linkage scan
GEF	Guanine-nucleotide exchange factor
GoKinD	Genetics of kidneys in diabetes
GTPase	Guanosine triphosphatase
ICAM-1	Intercellular adhesion molecule 1
KNG1	Kininogen 1
Mb	Megabase
MCF2L2	MCF.2 cell line derived transforming sequence-like2
MME	Membrane metallo-endopeptidase
MYH9	Myosin, heavy chain 9, non-muscle
MC	Mesangial cell
McSNP	Melting curve analysis of SNPs
NEP	Neutral endopeptidase
ND	Non-diabetic controls
PCR	Polymerase chain reaction
PPARγ	Peroxisome proliferator-activated receptor gamma
PVT1	Pvt1 oncogene
RAS	Renin-angiotensin system
RefSeq	Reference Sequence
RFLP	Restriction fragment length polymorphism

RhoGEF	Rho-family guanine-nucleotide exchange factor
SLC2A2	Solute carrier family 2 (facilitated glucose transporter) member 2
SLC12A3	Solute carrier family 12 (sodium/chloride transporters) member 3
SNP	Single-nucleotide polymorphism
SOX2	Sex determining region Y-box 2
SP1	Stimulatory protein 1
STZ	Streptozotocin
TAMRA	Tetramethylrhodamine
TDT	Transmission disequilibrium test
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TRPC1	Transient receptor potential channel 1
T1D	Type 1 diabetes
T2D	Type 2 diabetes
UAE	Urinary albumin excretion
UMOD	Uromodulin
UKPDS	U.K. prospective diabetes study
VEGF	Vascular endothelial growth factor
VPI	Vasopeptidase inhibitor
WESDR	Wisconsin epidemiologic study of diabetic retinopathy study
wk	Week
ZDF	Zucker diabetic fatty rats

1 INTRODUCTION

1.1 DIABETIC NEPHROPATHY

Diabetic nephropathy (DN) is one of the microvascular complications of diabetes mellitus (DM), in which patients exhibit persistent proteinuria, hypertension, declining renal function and increased premature mortality largely as a result of cardiovascular disease.

The natural history of DN is a process that progresses gradually over years. The process is categorized into stages based on the urinary albumin excretion (UAE) values. The cutoff values described below are according to the Diabetes Guidelines of American Diabetes Association (2009) [1]. The renal function reduction of DN begins with albuminuria leaving microalbuminuria level and entering the pathologic proteinuria range. The process is similar in both type 1 (T1D) and type 2 diabetes (T2D).

Table 1. Definitions of abnormalities in albumin excretion

Category	Spot collection* (μg/mg creatinine)	24h collection (mg/24h)	Timed collection (μg/min)
Normoalbuminuria	<30	<30	<20
Microalbuminuria (incipient DN)	30-299	30-299	20-199
Macroalbuminuria (overt DN)	≥300	≥300	≥200

*Note: Spot collection means a random spot urine sample, but first-void or other morning collections are preferred. Timed collection means 4-h or overnight collection.

Because of variability in urinary albumin excretion, two of three specimens collected within a 3- to 6-month period should be abnormal before considering a patient to have crossed one of these diagnostic thresholds. False positive includes exercise within 24 h, infection, fever, congestive heart failure, marked hyperglycemia, marked hypertension, pyuria, and hematuria which may elevate urinary albumin excretion over baseline values.

Nature history of T1D DN

As the earliest sign of the diabetic renal disorder, microalbuminuria appears within 5-15 years diabetic duration. In recent reports, the cumulative incidence of microalbuminuria over a lifetime is approximately 50%, with an increase rate around 2%-3% per annum [2]. Although initial studies suggested that about 80% of T1D subjects with microalbuminuria would progress to proteinuria [3, 4], more resent studies suggest that only 30%-40% of mircoalbuminuria patients will progress to macroalbuminuria [5]. That means around one third of microalbumiuria patients will revert to the normal albumin excretion stage; approximately another one third subjects will progress to macroalbuminuria with a peak incidence around the second decade of diabetic duration [2]. If untreated, with the progressive decline of glomerular filtration rate (GFR) over a further 10 years, almost all the patients with proteinuria develop end-stage renal disease (ESRD) or die prematurely of cardiovascular events.

Nature history of T2D DN

DN development in T2D subjects follows the similar course with T1D patients, but the proteinuria prevalence varies from 5% to 20% according to the ethnicity. Epidemiologic studies have found the DN is more prevalent and more rapidly develops among African-Americans, Asians and Native-Americans than in Caucasians [6]. Like in Pima Indians, more than 50% T2D develop proteinuria within 10-15 years of diabetes [7]. The prevalence is reported much lower in European descend. In the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), 24.8% American Caucasians had microalbuminuria and 20.5% had proteinuria during 12-year follow-up [8]. From the U.K. Prospective Diabetes Study (UKPDS), the prevalence of microalbuminuria and macroalbuminuria 10 years after T2D diagnosis was 25% and 5.3% respectively[9]. If without intervention, approximately a total of 30% T2D patients progress to overt DN and 20% develop ESRD after 20 years. Because of the high prevalence of T2D compared with T1D, majority of diabetic patients on dialysis are T2D.

1.2 END-STAGE RENAL DISEASE

DN is the single most common cause of chronic renal failure and accounts for significant morbidity and mortality worldwide. Once overt DN occurs, the GFR falls gradually. When GFR falls below 15 mL/min/1.73 m^2 (body surface area), the patient reaches the most advanced stage of chronic kidney disease (CKD) and the irreversible kidney function needs dialysis or transplantation treatment, which refers to ESRD.

Approximately 20%-30% of all diabetics will develop evidence of DN, but the risk of developing ESRD differs in different ethnic groups and different DM forms. In previous studies, the risk of ESRD was ever reported to be 13% at 20 years and 21.3% at 35 years after diagnosis of T1D in USA [10, 11]. Because of the availability of new treatment approaches and the more effective prevention strategies of DM and DN during the past decades, the declining risk of ESRD with T1D were reported in Finnish [12], Danish [13], Swedish [14], but not in Iceland [15] and German populations [16].

In T2D, 20%-30% patients with microalbuminuria progress to overt nephropathy, then ~20% will have progressed to ESRD after 20 years of overt nephropathy. Despite the similar prevalence of early stages of DN among different T2D populations, higher risk in development of ESRD has been extensively reported in Native-Americans, African-Americans, Hispanic/Latino-Americans and Asians than in Caucasians [7, 17]. The disparity gap in ESRD incidence appears much related to the genetic differences and sociocultural factors, which suggest that more in-depth studies, addressing specific issues, such as race, are needed to understand DN process fully.

1.3 GENETICS OF DIABETIC NEPHROPATHY

DN is a complex disease caused by both environmental and genetic factors. Risk factors including longer diabetic duration, hyperglycemia, hypertension, hyperlipidemia and smoking are considered being revolved in the disease pathogenesis. However,

familial aggregation of albuminuria, DN and ESRD has long been observed [18]. Study in sibling pairs also supported a strong genetic basis for DN risk [19]. The fact that some diabetic patients develop DN despite good glycemic control, whereas many do not develop kidney disease despite a long diabetic duration, can not be explained by the traditional risk factors [20, 21]. All the evidence suggests much of the risk for developing DN may be determined by hereditary factors. This realization has promoted the undertaking of multiple studies, yet to come to fruition, aimed at identifying the risk genetic factors.

There are two research approaches to map genes of complex diseases such as DN, linkage analysis and association study. Under a rare variant hypothesis, linkage analysis is performed based on extended families or affected sibling-pairs (Genome-Wide Linkage Scan, GWS). Under a common variant hypothesis, association study will be proposed based on case-control or families (transmission disequilibrium test, TDT) by using a dense marker map Genome-Wide Association Study (GWAS).

Figure 1. Category of Study Approaches

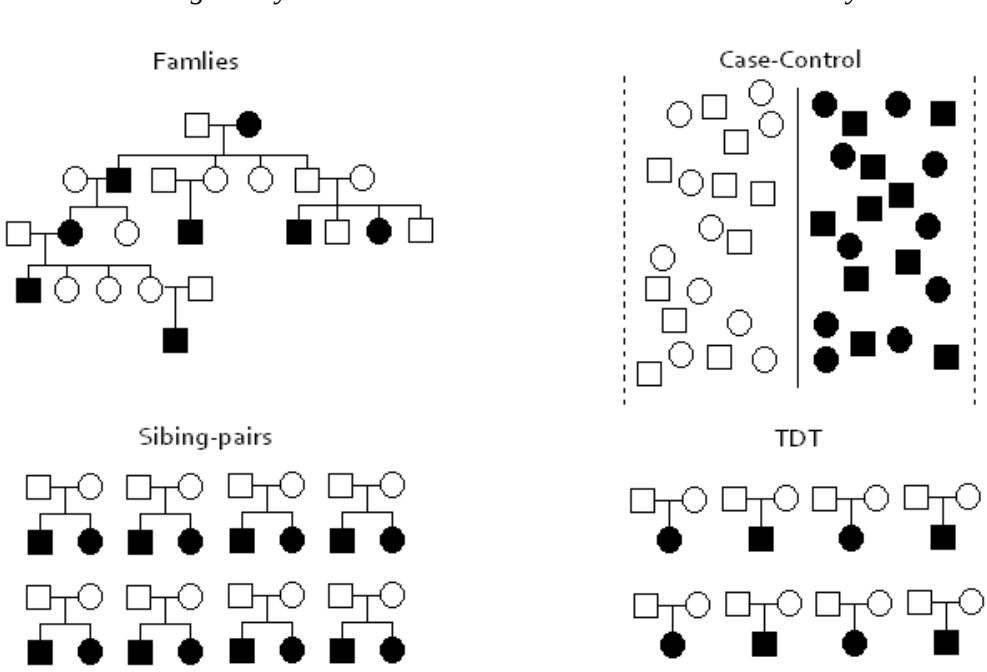


Figure 2. Another Category of Study Approaches

- *Top-down approach*
  - *Genome-Wide Linkage Scan (GWS)*
  - *Genome-Wide Association Study (GWAS)* (*free hypothesis*)
  - *Genome-Wide Sequencing (not yet feasible)*
- *Bottom-up approach*
  - *Candidate Gene Study*
  - *Candidate Sequencing* (*up-front hypothesis*)

Of course, as another point of view, we can also divide these approaches into other two parts based on free or up-front hypothesis [22]. But all these approaches are not isolated

from each other, new methods such as family based case-control study have generated by mutually intermingling. The improvement of genetic approaches to complex disorder thus will offer great potential to improve our understanding of pathophysiology, and the development of interventions and diagnostics.

1.3.1 Linkage analysis

Linkage analysis is a family-based approach using affected or discordant sib-pairs. This method attempts to identify a region of the genome that is transmitted within families along with the disease phenotype of interest. In this approach, highly polymorphic markers distributed across the genome are genotyped in large family pedigrees, and most common used markers are microsatellites. Single nucleotide polymorphisms (SNP)-based GWS were seldom reported till 2008. If the marker is more common in family members with DN, it indicates the presence of a nearby susceptibility variant going with the marker along the same chromosome region. For study DN in T1D patients, the analysis unit is often two generations with both parents enrolled (trios), while in T2D DN studies, the analysis units is often sibling pairs since the parents are generally unavailable when disease is detected. Whole genome scanning requires great effort and is time consuming due to the recruitment of large numbers of families, nevertheless, considerable GWS studies have been reported in DN.

The first GWS study has been published by Imperatore et al. in 1998 [23]. This study in Pima India population included 59 families with at least two siblings with T2D DN and analyzed a total of 503 microsatellites. The strongest evidence for linkage was observed on chromosome 7q and 3q. Almost at the same time, Moczulski and colleagues reported a 20cM region on Chr 3q containing the angiotensin II type 1 receptor gene with linkage to T1D DN [24]. From then on, more and more GWS studies were performed in different populations with presence of DN or ESRD. Till recently, GWS studies for chronic kidney disease or other renal function phenotypes such as albuminuria, serum creatinine, creatinine clearance and GFR were also published. Herein, we list the published GWS studies for DN and ESRD below (Table 2). Besides all the data listed on Table 2, Chistiakov et al. replicated the Chr 3q region in Russian Caucasians and confirmed the susceptibility locus linked with DN as previously described [25].

Table 2. GWS studies of diabetic nephropathy

	Year	Ethnics	Loci	Markers	Phenotypes	Authors
T1D DN	1998	Caucasian	3q147cM	D3S1308	Proteinuria and ESRD	Moczulski *et al.*[24]
	2007	Finnish	3q141cM	D3S3606-D3S3694	Proteinuria	Österholm et al.[19]
	2008	Caucasian	19q,2q,1q 6p 52cM 6q 142cM 3q 142cM	 rs1002200 rs1381768	Proteinuria and ESRD	Rogus *et al.*[26]
T2D DN	1998	Pima India	7q 3q	D7S500-D7S1804 D3S3053-D3S2427	Proteinuria and ESRD	Imperator *et al*.[23]
	2002	Turkish	18q	D18S469-D18S58	Proteinuria	Vardarli *et al*. [27]
	2003	African American	3q 7p 18q	D3S2460 D7S1820 D18S1364	ESRD	Bowden *et al*. [28]
	2005	African American	13q33.3 9q34.3 4p15.32 1q25.1	D13S796 D9S1826 D4S2639 D1S1589	ESRD	Freedman *et al*.[29]
	2006	Caucasian	5q 7q 22p	GATA67D03 GATA30D09B GATA11B12	ACR	Krolewski *et al*.[30]
	2006	Caucasian	2q 10q 18p 3q 7p	D2S1384 D10S2470-D10S677 D18S843 D3S1744 D7S3047-D7S3051	eGFR	Placha *et al*.[31]
	2007	Caucasian African American Mexican-American American American Indian	7q21.3 10p15.3 14q23.1 18q22.3 2q14.1 7q21.1 15q26.3	D7S2212-D7S821 D10S1435-D10S189 D14S587-D14S588 D18S1371-D18S1390 D2S410-D2S1328 D7S3046-D7S2212 D15S657-D15S642	Proteinuria and ESRD ACR	Iyengar *et al*.[32]
	2008	Caucasian African American	2p16 7q21 13q13 13q21-22 3p24-23 10p11	ATA47C04PD2S1352 D7S820/D7S821 D13S1493-D13S894 D13S1807-D13S800 D3S3038-D3S2432 D10S1208-D10S1221	eGFR ACR	Freedman *et al*.[33]

Note: ACR means urine albumin /creatinine ratio. eGFR means glomerular filtration rate estimated by creatinine or cystatin.

GWS ever offers great hope for identifying the major genetic components of DN. Considerable efforts have been expended conducting GWS for DN, however, few studies have achieved the levels of statistic evidence proposed for significance. It is because the linkage analysis is based on a rare variant hypothesis. The sample from affected sibling-pairs can avoid the population stratification, but also limited statistic power. Major limitations of linkage studies are relatively low power for complex disorders influenced by multiple genes, and the large size of the chromosomal regions shared among family members (often comprising hundreds of genes), in whom it can be difficult to narrow the linkage signal sufficiently to identify a causative gene.

1.3.2 Association study

Association study is another successful approach for genetic study in complex disease, which has been mostly performed in a case-control setting with unrelated affected

subjects compared with unrelated unaffected subjects. Two general approaches have been widely used: whole genome screen and candidate gene approach.

Genome Wide Association Study

Improvements in genotyping technologies have made GWAS affordable, to assay hundreds of thousands of SNPs and relate them to clinical conditions and measurable traits. In the past two years, there has been dramatic increase in genomic discoveries of complex human diseases, with nearly 100 loci for as many as 40 common diseases robustly identified and replicated in GWA studies [34]. Many of the genes which are previously unsuspected show the roles in the disease under study, and some in genomic regions contain no known genes. GWA studies for renal disease have been conducted using different markers involving microsatellites [35], gene-based SNPs [36, 37], SNPs indirectly distributed across the genome [38] or putatively functional SNPs [39]. Very recently, Pezzolesi et al. have reported a SNP-based GWAS for nephropathy in T1D, highlighting two loci (*FRMD3, CARS*) with strong association with DN [40]. A GWAS in ESRD of T2D patients was also performed and indentified *PVT1* gene on 8q24 with contribution to ESRD susceptibility in T2D [41]. A GWA meta-analysis by Köttgen and colleagues presented the *UMOD* locus has association with CKD, and the renal function trait GFR estimated by either creatinine or cystatin is associated with several loci [42]. GWA studies of other renal diseases or related traits such as lupus nephritis, gout, IgA nephropathy and blood pressure were reported with multiple novel loci [38, 43, 44]. Herein, all the GWA studies with tremendous bearings to nephropathy or renal function are listed below (Table 3).

Table 3. GWAS of renal diseases

Disease	Ethnics	Gene Symbol	Region	SNPs	Author and Reference
T1D DN	Caucasian	FRMD3	9q21-22	rs1888747 rs13289150	Pezzolesi *et al.* 2009[40]
		CARS	11p15.5	rs451041	
T1D DN	Irish	GTPBP4	10p15	D10S558 D10S1435	McKnight *et al.* 2006[35]
T2D DN	Japanese	SLC12A3 ELMO1	16q13 7p14	rs2289116 rs741301	Maeda *et al.* 2007[37]
ESRD of T2D	Pima Indian	PVT1	8q24	rs2720709 rs2648875	Hanson *et al.* 2007[41]
CKD	Caucasian	UMOD	16p12.3	rs12917707	Köttgen *et al.* 2009[42]
Estimated GFR (creatinine)		SHROOM3	4q21.1	rs17319721	
		SPATA5L1/ GATM	15q21.1	rs2467853	
		UMOD	16p12.3	rs12917707	
Estimated GFR (cystatin)		JAG1	20p12.1	rs6040055	
		STC1	8p11.2-21	rs1731274	
		UMOD	16p12.3	rs12917707	
		CST3-CST9	20p11.2	rs1303830	
Lupus nephritis	Caucasian	STAT4	2q32.2	rs7574865	Taylor *et al.* 2008[38]
Gout and renal function	Caucasian Black	ABCG2	4q22	rs2231142	Woodward *et al.* 2009[43]
IgA nephropathy	Japanese	PIGR	1q31-41	rs2911002	Obara *et al.* 2003[44]

Although the GWAS approach is unprecedented revolutionary since it permits interrogation of the entire human genome at levels of previously unattainable, it still not

successfully bridges the gap between genotype and phenotype. From the information listed above, we can seldom find them overlapped the results with each other and many such associations failed to be replicated in subsequent studies. The gene *SLC12A3* associated with DN in Japanese population, which is not replicated in Caucasian T2D patients with DN [45]. And the variants in the gene *ELMO1* showed different susceptibility to different populations [46, 47]. It is evident that the statistic powers of some GWA studies are problematic. Besides the sample size, pooling data across ethnic groups in GWAS without proper consideration of the population structure will also raise confounding leading to spurious significant results. Meantime, misclassification of case and control participants can markedly reduce study power and bias study results toward no association.

Candidate Gene Study

This approach mostly focuses on specific candidate genes, which although successful in detecting relatively modest effect of a gene on the certain disease in a certain ethnic group, it has yielded inconsistent and sometimes conflicting results. The concern with this method is that the gene selection is driven solely by previously known biology and therefore is limited in spectrum. And many candidate gene reports were based on a small sample size and minimal coverage (one or a few markers) within or near the gene. Therefore, results are hard to interpret because most studies lack adequate power to detect an association. In spite of those disadvantages, several genes such as *ACE*, *PPARγ*, *SLC2A2*, *ENTPD1*, *ICAM-1*, *ELMO1*, *VEGF*, *TGF-β*, *TNF-α*, *MYH9*, *UMOD* and *ACTN4* have been implicated in diabetes-associated nephropathy. The candidate gene study is still very useful as a replication to demonstrate the findings of GWA and GWS studies.

1.4 CHROMOSOME 3q

Several GWS and linkage analyses show that this chromosomal region is linked with coronary heart disease, T2D, obesity, metabolic syndrome, hypertension, renal function, hypertensive nephropathy, and DN in T1D and T2D [23, 24, 28, 48, 49, 50, 51, 52, 53, 54, 55, 56].

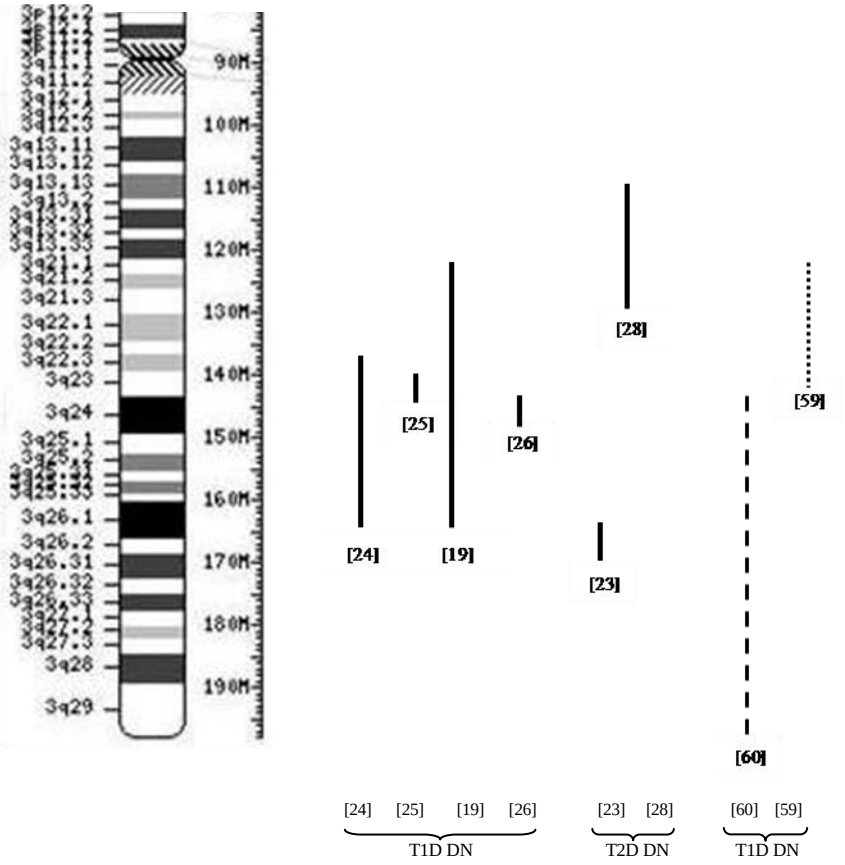
1.4.1 Linkage regions in Chr 3q to diabetic nephropathy

From the summarized two tables of GWS and GWAS together with other published evidence, we can find a recurring signal resides on Chr 3q. It is obvious that this region is not just linked to DN, but also lots of phenotypes related to diabetes and kidney diseases (Figure 3).

The first report about this region is among a cohort of T2D Pima Indians with DN in 1998 [23]. At the same time, another GWS in Caucasians also detected a 20cM region on Chr 3q associated with advanced DN in T1D patients [24]. Several years later, the locus was again confirmed in Russian T1D DN patients [25]. After that, Chr 3q was tautologically reported associated with earliest onset of renal failure [28], renal function phenotypes such as ACR, eGFR, serum creatinine, creatinine clearance, cystatin and

proteinuria [31]. In 2007 and 2008, further evidence for susceptibility gene(s) on Chr 3q was provided by two GWS studies in Finnish and Caucasian T1D DN patients [19, 26]. During the same time, multiple studies mentioned Chr 3q was associated with T2D [49, 50, 51], hypertension [55], coronary heart disease [48], obesity [52, 53], hyperglycemia [57] and hyperuricemia [43], those diseases or traits are closely bound up to DN. Besides, Chr 3q is related to ESRD caused by T1D and T2D, which can be easily find in Table 2. According to the sufficient evidence, we selected Chr 3q as our study region to identify the susceptible genes of DN.

Figure 3. Chromosome loci on 3 q-arm linked to DN



1.4.2 Genes located on locus from Chr 3q22- qter

Chromosome 3 q-arm is a long region with hundreds of genes. We decided to focus just on the locus of 3q22- qter instead of the locus from pericenter to 3q21 for two reasons. Firstly, a hot spot has been revealed on 3q21.3 [58]. Paralogous sequence blocks were found at human 3q21, approximately 4Mb proximal to the evolutionary breakpoint cluster region, which may cause the LOD score peak shift in the published linkage studies. Secondly, two recently published studies indicated some hints for our choice. An association study genotyped 3072 SNPs, chosen from a 26Mb region of Chr 3q22 at 121 to 147Mb (between two microsatellites: D3S1267 and D3S1308, NCBI build37.1), and found a variant in a non-coding region influencing the risk of DN [59]. Another study genotyped 94 SNPs from 14 plausible candidate genes located in Chr 3q23- q29, and detected *SLC2A2*, *KNG1* and *ADIPOQ* with nominal association with DN [60]. Both studies are shown in Figure 3.

Since then, we combined the positional candidate gene approach and appropriate animal models to perform genetic and functional analyses of DN with special focus on Chr 3q. We carefully checked all the genes located in Chr 3q22- qter by Map Viewer NCBI build 36.3. There are totally 431 known coding genes and 138 pseudo or unconfirmed genes in this region. We selected around 40 genes and looked up their properties, confirmed and potential functions from NCBI Entrez Gene database, Ensembl annotation and GeneCard database. Among them, four candidates have been included into my Ph.D study.

2 AIMS

2.1 AIMS

This thesis focuses on identification of the susceptibility and/or resistance genes for DN in Chr 3q with a positional candidate gene approach. The specific aims of each candidate gene study included in this thesis are:

Paper I – Adiponectin (*ADIPOQ*)
To evaluate the association between *ADIPOQ* promoter SNPs and T1D DN, and search for the possible transcriptional factors in the *ADIPOQ* promoter region.

Paper II – MCF.2 cell line derived transforming sequence-like 2 (*MCF2L2*)
To investigate the genetic effects of *MCF2L2* gene on the development of T1D DN, and to study the multi-genetic effects on DN involving *MCF2L2*, *ADIPOQ* and *SOX2* genes.

Paper III – Membrane metallo-endopeptidase (*MME*)
To assess whether the *MME* gene polymorphisms contribute to DN in T1D patients, and to detect the gene expression level in the kidney tissues of a diabetic animal model.

Paper IV – Transient receptor potential channel 1 (*TRPC1*)
To evaluate the genetic and functional roles of *TRPC1* in the development of DN in the patient material of Genetics of Kidneys in Diabetes (GoKinD) and Wake Forest University (WFU).

2.2 CANDIDATE GENE SELECTION

2.2.1 *ADIPOQ* – Chr 3q27

Adiponectin, the most abundant adipokine secreted mainly from adipocytes, plays an important role in the regulation of lipid and glucose metabolism. Circulating adiponectin concentrations are decreased in human with T2D, obesity, hypertension and coronary heart disease [61]. In contrast, plasma adiponectin has been shown to be increased in patients with T1D especially in the presence of DN [62]. The adiponectin levels are thought to be influenced by genetic variants in the *ADIPOQ* gene [63], but the relationship is not fully understood.

The *ADIPOQ* gene, located on chromosome 3q27 spans 15.79kb and contains 3 exons. The promoter of *ADIPOQ* gene consists of 2834bp nucleotides and no TATA box. The promoter variants -11426A/G (rs16861194), -11391G/A (rs17300539) and -11377C/G (rs266729) are the most common and widely studied polymorphisms. They cover an 80bp region, construct as a LD block and are known for their association with promoter activity, circulating adiponectin level, metabolic syndrome, obesity and T2D [63, 64, 65]. But the relationship is inconsistent in different ethnical groups. The variant -11377C/G is described to be associated with T2D in Swedish Caucasians [66]. Together with another variant -11391G/A, the same relationship with T2D are detected with French and German Caucasians [67, 68].While the association between the three promoter variants and diabetes is not replicated in the Japanese, Italian, Finnish T2D populations [69, 70, 71].

When we planned the *ADIPOQ* study, few studies have addressed genetic variants in *ADIPOQ* and association with DN till the end of December 2006. Rudofsky et al. reported the +45T/G (rs2241766) has no association with DN in either T1D or T2D Germany Caucasians [72]. No contribution of variant +276A/C (rs1501299) to DN was found in Japanese T2D patients [73]. Both variants were announced association with T1D but not with DN among Swedish Caucasians [74]. The only reported associated variant is the SNP -11391G/A, which confers the risk susceptibility to T1D DN in Danish population, but not in the French and Finnish populations [60, 74]. In order to evaluate and further analyze the association between *ADIPOQ* promoter SNPs and DN, we carry out a genetic analyses for three promoter SNPs in the *ADIPOQ* gene, including -11426A/G, -11391G/A and -11377C/G, in the patient material of GoKinD collection.

2.2.2 *MCF2L2* – Chr 3q27.1

MCF.2 cell line derived transforming sequence-like 2 belongs to guanine-nucleotide exchange factors (GEFs), which are directly responsible for the activation of Rho-family guanosine triphosphatase (GTPase) in response to diverse extracellular stimuli, and ultimately regulate numerous cellular responses such as proliferation, differentiation and movement [75].

The gene encoding for *MCF2L2* expands around 250kb long and contains 20 exons (NC_000003.11). The protein composed of 1114 amino acids contains 4 conserved

domains: SEC14, PH-like, SPEC and RhoGEF (NP_055893.2). The last domain RhoGEF also called Dbl-homology (DH) domain, which appears when pleckstrin homology (PH) domains invariably occur C-terminal to RhoGEF/DH domains. It has been announced that Dbl-related GEFs represent the largest family of direct activators of Rho GTPases such as *Cdc42*, *Rac* and *Rho* in humans. The interconversion of GTPases between active and inactive stage is regulated by GEFs and GTPase activating proteins (GAPs). GEFs catalyze the release of GDP to allow GTP loading by regulating the nucleotide status of GTPases, while GAPs increase the rate of GTP hydrolysis and terminal the GTPase-mediated signaling processes [76]. The failure of this conversion can have significant consequences and is reflected in the aberrant function of Dbl-family GEFs in some human diseases.

The *MCF2L2* gene is located on chromosome 3q27.1, a region was shown to be linked to DN. Considering the potential function of *MCF2L2* involved in the diabetic complications, the congruence of positional and biological significances led us to evaluate it as a candidate gene for DN.

More recently, *MCF2L2* genetic polymorphisms were reported to be associated with T2D in the Japanese population [50]. We checked the latest information from dbSNP database and found the three reported polymorphisms rs684846, rs35069869 and rs35368790. They are located in 3' untranslated region, intron 15 and intron 10 of *MCF2L2* gene respectively and belong to different haplotype blocks. In the same haplotype block where polymorphism rs35368790 located, we found a non-synonymous polymorphism rs7639705, which is located in exon 10 and causes amino acid change from Leu to Ile at the position 359 of *MCF2L2* cDNA sequence. After carefully checking its validity, rs7639705 was included for genotyping analysis. Therefore, we performed a case-control study to test the genetic association between *MCF2L2* and nephropathy in a well-characterized cohort selected from the GoKinD collection.

2.2.3 *MME* – Chr 3q25.1- q25.2

The *MME* gene encodes human neutral endopeptidase (NEP). NEP also named as neprilysin, enkephalinase, CALLA, CD10 and EC 3.4.24.11 is a transmembrane zinc matalloendopeptidase widely distributed but particularly abundant in the kidney [77]. NEP is present on glomerular epithelium and the brush border of proximal tubules [78], and appears excessive activity induced by hypertension [79], hyperglycemia and hyperlipidemia associated with diabetes [80]. It degrades and inactivates a variety of biologically peptides including bradykinin, enkephalins, substance P, atrial natriuretic peptide (ANP), C-type natriuretic peptide and endothelins etc. [79, 81]. Recent evidence has clearly implicated NEP in the intrarenal renin-angiotensin system (RAS) located glomeruli by metabolizing Ang I to Ang (1-7) and Ang (2-10) predominantly together with aminopeptidase A [82]. Given the evidence that activation of the intrarenal RAS contributes to the pathogenesis of DN [83], and Ang I is the major precursor to Ang II the essential biological active peptide of RAS, NEP may be involved in the intrarenal

RAS homeostasis and exerts a pivotal role in the mechanisms of glomerular injury of progressive kidney diseases.

During the past five years, accumulating evidence has revealed vasopeptidase inhibitor (VPI), an agent that inhibits both NEP and angiotensin converting enzyme (ACE) simultaneously, demonstrated unusual efficacy in preclinical studies for renal and cardiovascular diseases [84, 85, 86, 87, 88, 89, 90]. Superior beneficial effects were also obtained when VPI was used in T1D, T2D and DN animal models [91, 92, 93], which implicated NEP was involved in the pathogenesis of DN through unexplored mechanisms. At the present stage, the VPI is tested in phase IIb/III clinical trials for hypertension and phase II trials for DN. The present study was undertaken to evaluate the association of the *MME* polymorphisms with DN in American-Caucasian T1D patients.

2.2.4 *TRPC1* – Chr 3q22- q24

The transient receptor potential channel 1 gene (*TRPC1*) gene belongs to the super TRPC family. TRPC family includes 7 related members, designated as *TRPC1* through 7. TRPCs are ubiquitously expressed non-selective cation channels, which take part in a wide range of physiological functions, including nerve growth [94, 95], vascular tone [96, 97], permeability of vascular endothelium [98], cell proliferation [99], and mechanosensations [100].

Although *TRPC1* is the first cloned mammalian TRP channel [101, 102] and is expressed almost ubiquitously, its unique physiological function still remains elusive. *TRPC1* is considered as a core component of a mammalian stretch-activated and store-operated calcium channel [103]. In human glomerular mesangial cells, *TRPC1* was detected predominantly expressed in glomeruli and glomerular mesangial cell (MC) both in rat and human [104, 105]. *TRPC1* takes part in the vasoconstrictor-induced mesangial contraction by mediating Ca^{2+} entry, and knockdown of *TRPC1* significantly attenuates the contractile response in rat MCs [106]. Moreover, store-operated Ca^{2+} entry in human MCs reduced significantly by knocking down *TRPC1*, but enhanced by *TRPC1* over-expressing [105].

Recently, *TRPC1* gene expression is described significantly decrease in kidney tissue of Zucker diabetic fatty rats (ZDF) and streptozotocin (STZ)-treated rats compared to normal rats, and *TRPC1* is proposed that as a possible molecular rationale in DN [107]. In order to evaluate the susceptibility of *TRPC1* genetic polymorphisms in the development of diabetes and DN, we have carried out a genetic association study of the *TRPC1* gene in two populations, GoKinD collection and WFU material.

3 MATERIALS AND METHODS

3.1 SUBJECTS

The subject in the present study includes both DN patients and DN animal models.

3.1.1 GoKinD collection

GoKinD collection was supported by the Juvenile Diabetes Research Foundation in collaboration with the Joslin Diabetes Center, George Washington University, and the United States Centers for Diabetes Control and Prevention. It is consisted of 1286 T1D patients (683 female / 603 male) with and without DN, all the enrolled T1D patients were diagnosed T1D before 31 years of age. Treatment with insulin within one year of diagnosis, and had been uninterrupted since then. Among the GoKinD populations, 1177 (91.5%) (622 F/ 555 M) were of European descent, 109 (8.5%) were African Americans (n=42), Hispanic Americans (n=38), Native Indians (n=7), Asian Americans (n=4) and 18 unknown.

T1D subjects with DN (cases) had either persistent proteinuria, defined by a urinary albumin-to-creatinine ratio ≥300 μg/mg in two of the last three measurements taken at least 1 month apart, or ESRD (dialysis or renal transplant). T1D subjects without DN (controls) had T1D for at least 15 years and normoalbuminuria, defined by an albumin-to-creatinine ratio <20 μg/mg in two of the last three measurements taken at least 1 month apart, without ever having been treated with ACE inhibitors or angiotensin receptor blockers. They were not being treated with antihypertensive medication at the time of recruitment into the study. Further information refers to the report by Mueller et al. [108].

Characteristics of the case and control populations are presented in Table 4. Within the case group, a subset of 69.4% (n=461) DN patients had reached ESRD that was being managed by dialysis or renal transplantation, formed T1D with ESRD group. For highlighting the differences between these two subgroups, characteristics of ESRD group were also summarized in Table 4. For another case subgroup with proteinuria (30.5%, n=203) data were not shown.

The average age and diabetic duration were 4 years older and 6 years longer in cases than in controls (P<0.0001), the differences were due partly to the much older age and longer diabetic duration of cases with ESRD as compared to the cases with proteinuria (data not show). As expected the average blood pressure was higher in cases than in controls, consistent with the renal disease phenotype serum creatinine and cystatin levels. The mean BMI and cholesterol were similar between cases and controls. All the phenotypic characteristics were well matched in T1D with DN and with ESRD groups except age and diabetic duration. The subgroup ESRD showed older age and longer diabetic duration at entry than the patients with proteinuria (P<0.05).

3.1.2 WFU material

WFU material is provided by Wake Forest University School of Medicine, composed of 850 African Americans (434 F/ 416 M) from three groups. The first group is 284 T2D-associated ESRD patients; the second group is 284 hypertension associated ESRD patients; and the third group is 282 non-diabetic controls (ND). The characteristics are presented in Table 5. In WFU material, T2D was diagnosed in patients developing diabetes after the age of 35 years and treated at the time of recruitment with oral hypoglycemic agents, insulin, or diet and exercise, where treatment was considered permanent (i.e., excluding steroid-induced diabetes and gestational diabetes).

Medical records were reviewed to verify the etiology of the nephropathy. Impaired renal function was attributed to diabetes in the presence of the following criteria: serum creatinine ≥1.5 mg/dl, diabetes duration for >10 years or presence of proliferative diabetic retinopathy, and/or proteinuria ≥100 mg/dl or 500 mg/24 hr, in the absence of other known causes of renal failure [109].

Primary hypertension associated nephropathy was diagnosed in the presence of hypertension greater than 10 years duration when proteinuria ≤30 mg/dI or 500 mg/24 hr was present. Other detail clinical features in these patients have been described previously [110].

Exclusion criteria included families containing members with renal failure attributes adult polycystic kidney disease, Alport's syndrome (hereditary nephritis) or urologic disease (urinary reflux or surgical nephrectomy), affected relatives less than 18 years old, members unable to provide informed consent or with self-reported race other than African-American.

Table 4. Clinic characteristics of GoKinD collection

	T1D without DN			**T1D with DN**			***T1D with ESRD***		
	All	Female	Male	All	Female	Male	*All*	*Female*	*Male*
Number (Female/Male)	622	370	252	664	313	351	*461*	*227*	*234*
Age (years)	40.0±8.3	39.5±8.6	40.2±8.2	44.4± 6.4	43.0±7.2	44.5±6.4	*44.4± 6.2*	*43.8±6.6*	*45.5±6.0*
DM Duration (years)	26±8	26±7	26±8	31± 8	30±8	32±8	*32± 7*	*32±7*	*33±7*
Creatinine (mg/dL)	0.9±0.2	0.8±0.1	1.0±0.1	2.2±2.0	2.1±2.1	2.3±2.0	*2.4±2.3*	*2.3±2.3*	*2.5±2.3*
Cystatin (mg/L)	0.8±0.1	0.8±0.1	0.8±0.1	2.3±1.8	2.3±1.8	2.4±1.7	*2.6±2.0*	*2.5±2.1*	*2.6±1.9*
BMI (kg/m^2)	26±4	26±5	27±4	26±5	26±6	26±5	*25±5*	*25±6*	*25±5*
HbA1c (%)	7.5±1.1	7.5±1.1	7.4±1.1	7.4±1.9	7.4±2.1	7.4±1.7	*6.9±1.8*	*6.8±1.9*	*6.9±1.7*
Cholesterol (mg/dL)	185±31	189±31	180±31	187±46	190±46	184±46	*181±45*	*185±44*	*178±46*
HDL (mg/dL)	59±16	65±15	51±14	54±18	59±19	49±15	*54±18*	*60±19*	*49±15*
Systolic BP (mm Hg)	118±12	116±12	122±12	132±19	130±20	133±18	*131±20*	*130±21*	*133±19*
Diastolic BP (mm Hg)	71±8	70±7	73±8	74±11	72±11	76±11	*74±11*	*72±11*	*75±11*

Table 5. Clinic characteristics of WFU material

	T2DM-ESRD	**HTN-ESRD**	**ND**
N (Female/ Male)	284 (176/108)	284 (126/158)	282 (132/150)
Age (years)	64±10 (65±11/62±9)	54±15 (56±16/52±14)	50±10 (50±10/49±10)
Age of onset HTN	---	33±12 (31±12/35±11)	---
Age of onset T2DM	43±11 (43±11/43±11)	---	---
Age of onset ESRD	60±10 (62±11/58±10)	48±16 (50±17/47±15)	---
BMI (kg/m2)	29.3±7.3 (29.9±7.8/28.5±6.3)	26.5±7.3 (27.5±8.4/25.8±6.2)	29.3±6.8 (31.2±7.6/27.6±5.5)

3.1.3 db/db mouse

The *db/db* mouse was identified initially in Jackson Labs as an obese mouse in 1966 that was hyperphagic soon on weaning [111]. The diabetic gene (*db*) encodes an inactivating G-to-T point mutation of the leptin receptor, resulting in a shorter intracellular domain of the receptor that is unable to transduce signals [112]. Their phenotype consists of obesity, insulin resistance and diabetes, similar to T2D in humans.

The *db/db* mouse has a long history as an animal model of human DN since it develops progressive renal histologic changes and functional derangements, including loss of renal function, similar to humans with T2D. The key common feature with the human condition is renal hypertrophy, glomerular enlargement, albuminuria, and mesangial matrix expansion [113]. The kidney hypertrophy has been noted in *db/db* mice at the age of 16 wk [114]. The glomerular hypertrophy was found during the early stages of diabetes at 8 wk of age and remained increased at 16 wk of age [115]. Enlarged glomeruli with increased mesangial matrix were consistently noted after the age of 16 wk in *db/db* mice [113]. Also the *db/db* mouse appears to most closely mimic the progressive nature of mesangial matrix expansion seen in human DN.

Given all the characters exhibited by the *db/db* mouse, in Paper III and IV, we used the RNA extracted from kidney tissue of *db/db* mice and its lean littermate as the control, to detect the *TRPC1* gene expression level in this DN animal model at different ages.

3.2 GENOTYPING

There are a lot of genotyping techniques, including PCR-restriction fragment length polymorphism (RFLP), Dynamic allele specific hybridization (DASH), TaqMan allelic discrimination, Bi-directional PCR amplification of specific allele (Bi-PASA), Melting curve analysis of SNPs (McSNP), direct sequencing and mini-sequencing. Each method has its advantage and disadvantage. The ideal SNP genotyping platform should be gel-free, robust, inexpensive, simple assay design, high-throughput capacity, automated genotype calling and accurate and reliable results. Following this idea, two SNP scoring methods, DASH and TaqMan allelic discrimination were used for genotyping in this thesis.

3.2.1 TaqMan allelic discrimination

TaqMan allelic discrimination is a method combining PCR and mutation detection in a single step, by measuring the increase in fluorescence of dye-labeled DNA probes. It provides a rapid and sensitive method for detecting polymorphisms.

This method employs a probe technology that exploits the 5'-3' nuclease activity of Taq Gold DNA Polymerase to allow direct detection of the PCR product by the release of a fluorescent reporter as a result of PCR. In this procedure, two TaqMan probes corresponding to two target alleles are used. Each probe consists of an oligonucleotide with a 5'-reporter dye and a 3'-quencher dye. Carboxy fluorescein FAM and VIC, also tetrachlorofluorescein TET are used as 5'-reporter dye for detecting different allele; the

3'-quencher dye used tetramethylrhodamine (TAMRA). The probe hybridizes to a smaller 20- to 24mer sequence within the PCR product, which includes the SNP. The fluorescent signal given from the reporter is suppressed by the quencher when the probe is intact. Taq DNA polymerase carries out the extension of the primer and replicates the template till the verge of the probe, and the enzyme cleaves the probe with its 5'-exonuclease activity. When the probe is cleaved the reporter dye is separated away from the vicinity of the quencher, resulting in increased fluorescence intensity of the reporter. This process occurs in every cycle and does not interfere with the accumulation of PCR product. The increase in fluorescence is measured, and is a direct consequence of target amplification during PCR. At last, the allelic discrimination plate will be scorning by laser light of Applied Biosystem instrument for detecting the fluorescence signals, with simple FAM/VIC/TET signals corresponding to different homozygous, with mixed signals meaning heterozygous.

3.2.2 Dynamic allele specific hybridization

DASH is a relatively cheap method for scoring SNPs, which is essentially an enhanced form of allele-specific hybridization [116]. It is transferred from the traditional radioactive/chemiluminescent and membrane-based platform to a convenient microtiter plate format that uses a simple duplex-DNA intercalating dye for signal production and a dynamic low-high temperature sweep to capture all phases of probe-target-DNA melting.

Firstly, BLAST Human Sequences program is used to explore whether the target region (100bp around the SNP) is a single-copy genomic sequence. Secondly, assay for two ~22bp primers (one labeled with biotin) and a ~17bp probe (with centered upon the target SNP position) were designed. The primer pair sequences were checked by MFOLD program (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) [117] to avoid they form intra- or intermolecular duplex regions. Meanwhile, PCR amplified fragments are usually designed to be less than 70bp in length.

After the common PCR procedure, PCR products are transferred into a streptavidin-coated plate for immobilization and denatured into single strand. The biotinylated strand will bind to the streptavidin on the well surface, whereas the non-biotinylated strand will be washed out by NaOH solution. Then, the specific probe is added into the well along with a hybridization buffer containing SYBR green. SYBR green is a widely used non-specific double-strands DNA intercalating fluorescent dye. The probe, specific to one allele, will hybridize to the target strand forming a duplex DNA region with intercalating SYBR green.

When scoring the plate in a DASH instrument with increasing temperature, the temperature at which the rate of fluorescence decrease is maximal indicates the melting temperature of the probe-target duplex, and this is directly related to the degree of bp matching between the probe and the target. A melt-curve indicating the melting behavior of the probe-target duplex is available on the screen. The curve peaks at low or high temperature representing the mismatch and match probe-target duplexes, respectively. The two peaks of melt-curve indicate the heterozygous.

3.3 GENE EXPRESSION

3.3.1 TaqMan real-time RT-PCR

The kidney tissues of *db/db* and control mice were collected and quickly submerged in RNA/later solution (Ambion, Austin, Tex., USA). The kidney tissue was disrupted using Mini Beadbeater (Biospec Products, Bartlesville, OK, USA) with 0.5 ml of 2-mm diameter Zirconia beads and further processed with the RNAeasy Mini kit protocol according to the manufacturer (Qiagen, Hilden, Germany). The RNA integrity was assessed by electrophoresis through a 1.2% agarose gel and the RNA concentration was determined by measuring the A_{260}/A_{280} ratio. cDNA transcription was performed by the QuantiTect® Reverse Transcription kit (Qiagen, Valencia, CA) .

Specific gene primers, including the reference gene β-actin primers, were purchased from Applied Biosystems and used following to the manufacturer's protocols in an ABI 7300 real-time PCR system. Gene amplification was performed according to the program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Experiments were carried out in triplicate.

3.3.2 Western blotting

Kidney tissues of *db/db* mice were kept at –80°C until extraction and homogenized in RIPA buffer (150 mmol/l NaCl, 1% Igepal, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mmol/l Tris-HCl, pH 8.8, supplemented with freshly made protease inhibitor cocktail) and centrifuged at 4°C with 20,000 g for 20 min. Protein was quantified by an assay kit (Bio-Rad Laboratories, Hercules, California, USA), electrophoresed (50 µg) with SDS 7.5 % PAGE, transferred to nitrocellulose membrane, and blocked with 10% nonfat milk. Primary antibodies were added at a concentration of 1:1000 for NEP (Santa Cruz Biotechnology, Heidelberg, Germany, sc-46656) and incubated overnight at 4°C with gentle shaking. Membranes were washed 3 times in PBS containing 0.1% Tween 20. The secondary anti-rabbit antibody conjugated to horseradish peroxidase were added at a concentration of 1:3000 and incubated for 1.5 h at room temperature with gentle shaking, after which cells were washed 3 times in PBS containing 0.1% Tween 20. Bound antibody was detected by ECL Western blotting detection system (GE Healthcare, Piscataway, NJ, USA).

3.4 BIOINFORMATICS

Bioinformatics is the application of computer technology to the management of biological information. It has been widely used in genomics and genetics. Over the past few decades rapid developments in genomics, genetics and other molecular research technologies and developments in information technologies have combined to produce a tremendous amount of public database related to molecular biology.

In this thesis, the SNP selection was done mainly based upon the information from NCBI Nucleotide databases (http://www.ncbi.nlm.nih.gov/Database/), International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) and GeneCard (http://www.genecards.org/). We used Human Genome Mapview Blast 35.1 (2006) to

inspect for all the genes on the determinative chromosome locations. Then by Reference Sequence (RefSeq) database we check all the information about the candidate genes. Last, by dbSNP database, together tag marker information from HapMap, polymorphisms are finalized. In paper I, binding proteins and transcription factors were identified with Transcription Element Search System (TESS) (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME).

3.5 STATISTICS

All statistic analyses were performed using PASW Statistic Base 18 (SPSS Inc, Chicago, Illinois, USA) and/or BMDP version 1.1 (BMDP Statistical Software Inc., Los Angeles, CA, USA). A p-value less than 0.05 was considered as significant.

3.5.1 Single marker association analysis

Hardy-Weinberg equilibrium, genotypes frequencies and allele frequencies of different SNPs were compared between cases and controls by using the chi-square test. Amitage's trend test was also performed bases on the genotype results. Towards a further understanding, genetic association analyses with the dominant, recessive and additive models (Cochran-Armitage's trend test) were conducted. The relative risk for association was estimated by calculating odds ratio and 95% confidence intervals. Statistic powers of the samples were calculated with the software of PS PowerandSampleSize Calculations version 3.0 (http://biostat.mc.vanderbilt.edu/PowerSampleSize).

3.5.2 Haplotype and diplotype analysis

For multiple-marker association analyses, the estimation of pair-wise linkage disequilibrium (LD) values, haplotype and diplotype frequencies, also the Haplotype block structure was performed by using Arlequin program version 2.0 (http://lgb.unige.ch/arlequin/) and HaploView program version 4.1. LD between different SNPs was summarized using r^2 and $|D'|$. Haplotype prevalent at >5% were further used for haplotype association analysis.

3.5.3 Genotype and phenotype association analysis

Traits interesting with DN have been included in the quantitative trait analyses. Phenotype comparisons among different genotypes and diplotypes were used ANOVA or Kruskal-Wallis method. Normal distributions were tested with the Kolmogorov-Smirnov test, and the natural logarithm or logarithm with base 10 was applied to normalize the skewed distribution parameters.

3.5.4 Gene-gene interaction analysis

Generalized multifactor dimensionality reduction (GMDR) program (http://www.healthsystem.virginia.edu/internet/addiction-genomics/software/gmdr.cfm) is for detecting gene-gene and gene-environment interactions [118]. GMDR Software is a nonparametric and genetic model-free alternative to linear or logistic regression

program for detection of gene-gene or gene-environmental interactions. This program provides a number of parameters including cross-validation consistency, testing balanced accuracy and empirical P-values to assess each selected interaction. We employed this program to investigate gene-gene interactions in paper III. The GMDR analyses were performed separately in different gender subjects. Logistic regression models were used for confirmation of the data from GMDR analyses.

4 RESULTS

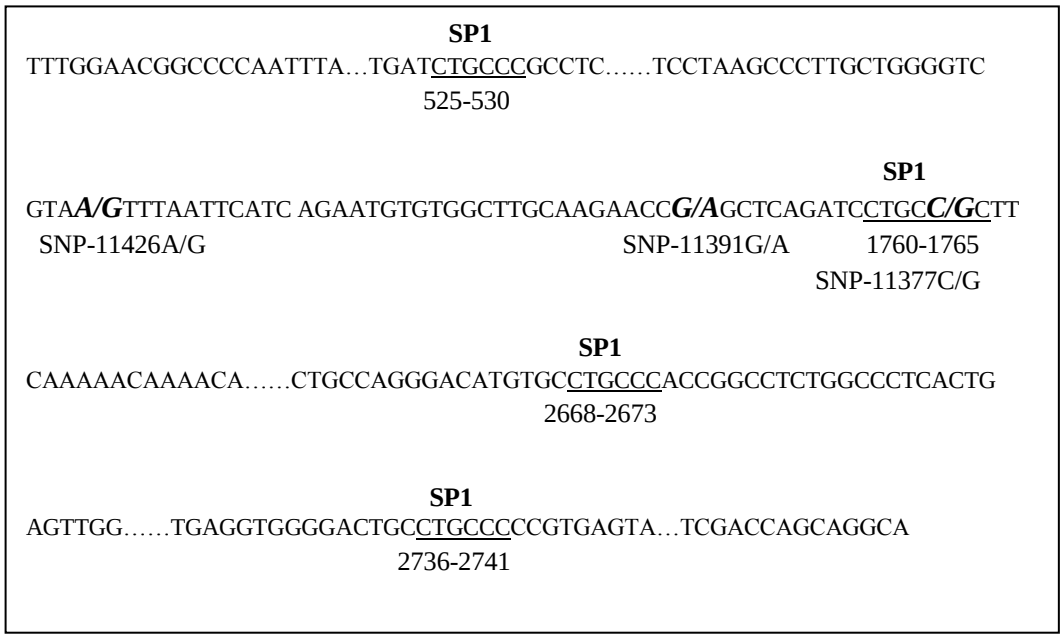
4.1 PAPER I - *ADIPOQ* STUDY

This study mainly evaluated the association between adiponectin promoter SNPs and DN. We also searched for the possible transcriptional factors and their binding sites in the adiponectin promoter in order to predict the possible biological influence of the SNP(s) in the activity of the adiponectin promoter. We performed genetic analyses for three promoter SNPs in the adiponectin gene, including -11377C/G, -11391G/A and -11426A/G, in the patient material of the GoKinD collection. The distributions of genotypes for three SNPs were kept in HWE (P>0.05).

4.1.1 Bioinformatics results

With the TESS program, we identified four binding sites for the transcriptional stimulatory protein (SP1) at the positions of g.525-530, 1760-1765, 2668-2673, and 2736-2741 as indicated in Figure 4. SNP -11377C/G is involved in the sequence of SP1 transcriptional binding site at g.1760-1765. In the Figure 4 below, the partial sequence from *Homo sapiens* adiponectin promoter is represented. Three adiponectin promoter polymorphisms including -11426A/G, -11391G/A, and -11377C/G are shown with bold and italic letters. The binding sites for transcriptional factor SP1 are indicated with underline letters.

Figure 4. The ADIPOQ promoter SNPs and the binding sites for transcriptional factor SP1



4.1.2 Single marker association

The female T1D patients with DN had higher G allele frequency in SNP -11377C/G, comparing with the female patients without DN (27.9% vs 22.3%, P=0.022, OR=1.352, CI 95% 1.044-1.752). No significant difference between the male T1D patients with and without DN was observed. Further analyses showed, the heterozygous, allele G positivity and trend of Cochran Armitage's were significant different between the

female T1D patients with and without DN (*P*=0.015, 0.012 and 0.018, respectively), not in males.

4.1.3 Haplotype analysis

The SNP -11377C/G resides at a LD block with SNP -11391G/A and -11426A/G. So these three SNPs were used to predicted four common haplotypes (>5%) including A-G-C, A-G-G, A-A-C and G-G-C. Among the females, patients with DN had higher frequency of haplotype A-G-G compared to the patients without DN (25.8% vs 19.7%, *P*=0.011, OR=1.417, CI 95% 1.149-1.685). The sequence and frequency of the haplotype in females were shown in Table 6.

Table 6. Common haplotypes of three polymorphisms in females

Haplotypes	T1D with DN	T1D without DN	P-value
A-G-C	0.562	0.612	0.077
A-G-G	0.258	0.197	0.011*
A-A-C	0.108	0.095	0.475
G-G-C	0.051	0.069	0.133

Two haplotype-tagging markers SNP -11391G/A and -11377C/G were used for diplotypes analysis to evaluate the association with DN. Five diplotypes (haplotypic genotypes), including H1/H1, H1/H2, H1/H3, H2/H3 and H2/H2 with over 5% frequency, were formed with three common haplotypes (H1-H3). The Table 7 showed the sequence and the frequency of diplotypes in females. The most common diplotype H1/H1 was significantly associated with the female T1D patients with DN (*P*=0.013, OR=1.513, CI 95% 1.188-1.838).

Table 7. Diplotype frequencies in female T1D patients with and without DN

Diplotypes	T1D with DN	T1D without DN	P-value
H1/H1	0.362	0.462	0.013*
H1/H2	0.373	0.308	0.071
H1/H3	0.125	0.132	0.793
H2/H3	0.068	0.048	0.277
H2/H2	0.053	0.042	0.527

4.2 PAPER II - *MCF2L2* STUDY

In the present study, we have conducted an association study in GoKinD collection. Two SNPs associated with T2D i.e. rs35069869 and rs35368790 as previously reported, and a non-synonymous SNP rs7639705 causing amino acid change from Leu to Ile at the position 359 of *MCF2L2* cDNA sequence were genotyped.

4.2.1 Single marker association

All SNPs were kept in HWE. The SNP rs7639705 (T1165G, Leu359Ile) was found to be significantly associated with DN in female subjects. The G allele frequency in the patients with DN compared to the patients without DN was significantly lower in females (*P*=0.017). The G allele had a dominant effect (*P*=0.005) on DN among females, but not in males (Table 8). The GG genotype carriers among female patients

with DN showed lower but not significant creatinine and cystatin levels compared to the carriers with either TT or TG genotypes.

Table 8. Association between the SNP rs7639705 and DN

	T1D with DN	T1D without DN	P-value		
			Allelic association	Dominant model	Armitage's trend test
G	0.220	0.165	0.017	0.005	0.018
T	0.780	0.835			

4.2.2 Gene-gene interaction

We ever reported SNPs rs266729 in the *ADIPOQ* gene and rs11915160 in the *SOX2* gene are associated with DN in the same cohort of GoKinD collection. Thus, we employed the GMDR program to assess the impact of combinations with the model of *MCF2L2* (rs7639705)-*SOX2* (rs11915160)-*ADIPOQ* (rs266729) in GoKinD female subjects. Table 9 presents the *MCF2L2* polymorphism rs7639705 together with SNPs rs266729 in the promoter of the *ADIPOQ* gene and rs11915160 in UTR-3 of the *SOX2* gene had combined effects on the association with DN in females (P=0.001), but not with either *ADIPOQ* or *SOX2* alone.

Table 9. Gene-gene interaction and impact on DN in female patients

Best combination	Test accuracy (%)	P-value
MCF2L2 (rs7639705)-*SOX2* (rs11915160)-*ADIPOQ* (rs266729)	0.562	0.001
MCF2L2 (rs7639705)-*SOX2* (rs11915160)	0.513	0.623
SOX2 (rs11915160)-*ADIPOQ* (rs266729)	0.545	0.377

4.3 PAPER III - *MME* STUDY

4.3.1 Single marker association

The present study aimed at evaluation of the genetic impact of *MME* gene on the development of DN and ESRD. Eight SNPs in the *MME* gene were genotyped in American Caucasians from GoKinD collection. All the SNPs were kept in HWE. For subgroup analyses, a subset of DN subjects who had reached ESRD (408, 70.6%) constructed T1D with ESRD group.

SNPs rs3796268 and rs3773885 were found to be nominally associated with DN (P=0.044 and P=0.058) and ESRD (P=0.050 and P=0.052) in females, not in males. Both minor alleles of rs3796268-G and rs3773885-T showed higher frequency in the female controls either comparing with female DN or ESRD subjects (Table 10).

Table 10. Allelic associations of two SNPs in female patients

	T1D without DN	T1D with DN	T1D with ESRD	P-value	
				Allele fre.	Dominant model
rs3796268	G 0.345	G 0.291	G 0.286	0.044*	0.043*
				0.050[#]	0.043[#]
rs3773885	T 0.429	T 0.297	T 0.291	0.058*	0.064*
				0.052[#]	0.065[#]

Note: *T1D without DN *vs* T1D with DN; [#]T1D without DN *vs* T1D with ESRD.

4.3.2 Haplotype analysis

SNPs rs3796268 and rs3773885 constructed four haploytpes: A-C, G-T, A-T and G-C. The highest frequency of haplotype *MME*-AC was associated with increased risk of DN and ESRD (P=0.015 and P=0.010) in female subjects. No association of the haplotype A-C with DN and ESRD in male subjects was detected.

Setting A-C haplotype as a risk, all female individuals were divided into three diplotypes dependent on carrying copies of A-C: +/+, +/− and −/− denote two copies, one copy and non-copy of the A-C haplotype, respectively. Individuals with two copies of the A-C risk haplotype were at a significant 1.95-fold increase risk for having DN (P=0.014) and 2.16-fold increase risk for having ESRD in female T1D subjects (P=0.012) (Table 11).

Table 11. Diplotype frequencies of SNP rs3796268 and rs3773885 in female patients

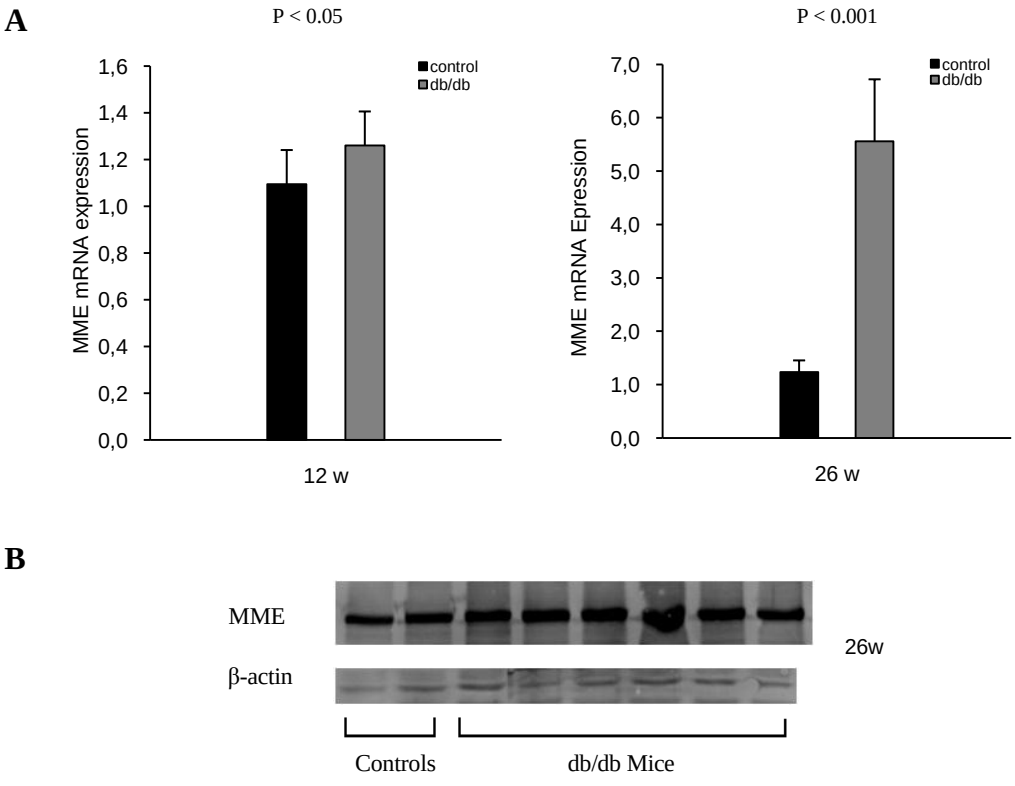
	T1D without DN	T1D with DN	T1D with ESRD	P-value	
+/+	0.351	0.428	0.445	0.014*	0.012[#]
+/−	0.497	0.477	0.466	0.108*	0.125[#]
−/−	0.152	0.095	0.089	Ref.	Ref

Note: *T1D without DN *vs* T1D with DN; [#]T1D without DN *vs* T1D with ESRD.

4.3.3 Gene expression at mRNA and protein levels

The mRNA expression levels in kidney tissues of both in 12-week-old and 26-week-old *db/db* mice were significantly elevated compared with the same age lean littermates (P<0.05)(Figure 5A). The protein level in kidney tissues of 26-week-old db/db mice was higher than the control mice (P<0.05)(Figure 5B).

Figure 5. MME expression at the levels of mRNA (A) and protein (B)



4.4 PAPER IV - *TRPC1* STUDY

4.4.1 Single marker association

A recent study has demonstrated that the *TRPC1* gene expression is decreased in kidney and liver in diabetic ZDF- and STZ-treated rats, also the reduction of the *TRPC1* protein level is detected in kidney of patients with DN [107]. This study mainly evaluated whether the *TRPC1* genetic polymorphisms have influence in the development DN or ESRD. Both the GoKinD collection and the WFU material were included in this study. Seven SNPs in the *TRPC1* gene were genotyped.

Firstly, the analyses were performed in American Caucasians of GoKinD collection. Only the minor allele frequencies of SNP rs7621642 and rs2033912 were of borderline significant difference (P=0.045 and P=0.047, respectively) in GoKinD collection of female T1D patients with versus without DN, but not in males. SNP rs3821647, trended toward a higher frequency in female T1D with DN, compared to those without DN (0.187 vs. 0.151, P=0.096). Then, tests in the entire GoKinD population including were performed. When African Americans of GoKinD collection were combined with American Caucasians, the minor allele frequencies of these three polymorphisms became statistically significant in T1D patients with versus without DN (P=0.003, 0.003 and 0.004, respectively; Pc=0.042, 0.042 and 0.056, respectively, after Bonferroni correction). We further genotyped the three SNPs rs7621642, rs2033912 and rs3821647 in WFU material. We found the genotype distributions of SNPs rs7621642 and rs2033912 were reversed in African Americans, compared to what had been detected in American Caucasians (Table 13). SNP rs3821647 showed different minor allele frequency between American Caucasians and African Americans, which implied the population stratification as a confounding factor interfered the results. No significant association for these three polymorphisms was detected with type 2 diabetes-ESRD or hypertensive-ESRD in African Americans.

Table 12. Minor allele frequencies of TRPC1 polymorphisms in American Caucasians

dbSNP ID	Gender	MA	T1D with DN	T1D without DN	P-value
rs7621642	Female	A	0.217	0.172	0.045
	Male		0.209	0.220	0.661
rs2033912	Female	T	0.217	0.173	0.047
	Male		0.209	0.219	0.694
rs3821647	Female	A	0.187	0.151	0.096
	Male		0.192	0.190	0.946

Table 13. Minor allele frequencies of TRPC1 polymorphisms in American Caucasians

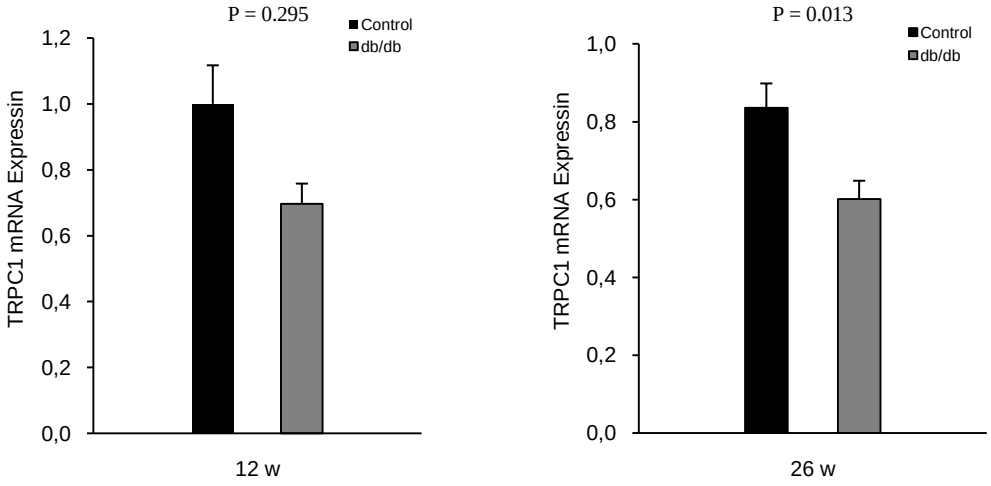
dbSNP rsID	Gender	MA	T2D with ESRD	HTN with ESRD	ND	P-value	P-value
rs7621642	Female	G	0.305	0.312	0.261	0.847[¤]	0.204[#]
	Male		0.306	0.271	0.302	0.382[¤]	0.392[#]
rs2033912	Female	A	0.286	0.256	0.242	0.416[¤]	0.722[#]
	Male		0.280	0.245	0.285	0.365[¤]	0.261[#]
rs3821647	Female	A	0.207	0.258	0.239	0.142[¤]	0.612[#]
	Male		0.259	0.193	0.265	0.070[¤]	0.033[#]

Note: MA means minor allele, [¤] T2D with ESRD vs ND, and [#] T2D with DN vs HTN with ESRD.

4.4.2 Gene expression at mRNA level

TRPC1 gene mRNA expression in kidney was found to be trendily reduced in 12-week and significantly in 26-week-old *db/db* mice, when compared to the same age controls (Figure 6).

Figure 6. TRPC1 gene mRNA expression levels in kidney tissues of db/db mice



5 DISCUSSION

Association of the ADIPOQ polymorphisms with DN

Recent epidemiologic studies support the concept that the SNPs in the *ADIPOQ* gene are associated with T2D and other metabolic disorders in several populations. Serum *ADIPOQ* shows gender-specific prediction effects on kidney disease in T1D patients [119, 120]. In the current study, we investigate three associated promoter SNPs (-11426A/G, -11391G/A, and -11377C/G) within an 80bp region in GoKinD collection.

In single marker analysis, the minor allele G of SNP -11377C/G has association with DN in female T1D patients. Also in the four common haplotypes constructed by the three SNPs, only one haplotype A-G-G contained this minor allele G of SNP -11377C/G showed association with DN in female T1D patients.

We have identified four binding sites of the transcriptional factor SP1 in a partial 300bp *ADIPOQ* promoter region. The SP1 binding activity is enhanced during adipocyte differentiation and had stimulatory effects on the *ADIPOQ* promoter activity from the Barth et al. report [121]. The allele G of SNP -11377C/G alters the sequence of the binding site for SP1 at the position of g.1764, which cause a loss of SP1 binding effect. Because the full promoter of *ADIPOQ* gene has not been identified and there are at least four SP1 binding sites in the whole promoter region, it is not feasible for us to investigate the influence of this binding site on the promoter activity. We estimated the transcriptional activity might become relatively lower but not significantly if the allele G of SNP -11377C/G is transfected into the constructor with the *ADIPOQ* gene promoter sequence. Two recent reports partially confirmed our hypothesis. Bouatia-Naji et al. transfected the G allele of SNP -11377C/G into COS-7 cell line, it showed lower but not significantly transcriptional activity than the allele C [122]. Laumen et al. used the same approach to investigate the three promoter SNPs effects on the promoter activity, together analyzed with circulating adiponectin levels in epidemiologic samples [123]. They found the G allele of SNP -11377C/G showed consistent association with decreased circulating adiponectin levels and promoter activity assays. Since we have not the serum adiponectin data of GoKinD collection, further prospective work is essential to confirm this association.

We found the SNP -11377C/G is associated with DN in GoKinD collection, another SNP -11391G/A was reported to be associated with DN in Danish and British, but not in French and Finnish Caucasians [60, 124]. It has been accounted that in different populations, the associated polymorphisms in the same gene may be different.

Association of the MCF2L2 polymorphisms with DN

The *MCF2L2* genetic polymorphisms were firstly reported to be associated with T2D in Japanese population [50]. Three polymorphisms were mentioned in this report, two are intronic polymorphisms and one resides at 3'- strand sequence but outside of the *MCF2L2* gene. In the present study we included these two intronic SNPs and a non-

synonymous SNP rs7639705, all are tag markers. Genetic analyses showed the SNP rs7639705 minor allele G was associated with decreased risk of DN in females, but not in males. Quantitative trait analyses presented that patients with GG genotype have lower creatinine and cystatin levels compared to the TT and TG genotype carriers, although the p-value was less significant most likely due to the high standard deviation of variables.

Gene-gene interaction analyses

In Papers I and II, *ADIPOQ* SNP -11377C/G and *MCF2L2* SNP rs7639705 are found to be associated with female DN in GoKinD collection. Recently, a *SOX2* gene polymorphism rs11915160 has been found to be associated with female DN in GoKinD collection [125]. Thus, we employed the GMDR program to assess the combination impact of the three polymorphisms in females of GoKinD collection. We found there was no significant interaction when analyzing each of two, but a significant interaction was confirmed among three genes. This significance was also demonstrated by a logistic regression model.

Association of the MME polymorphisms with DN

We have conducted a genetic association and functional study for the *MME* gene in GoKinD collection. We observed a significant increased expression of *MME* gene at both mRNA and protein levels in kidney tissue of *db/db* mice. The study also revealed that two SNPs rs3796268 and rs3773885 were nominally associated with DN and ESRD in females, but not in males. Both minor allele of rs3796268-G and rs3773885-T showed higher frequencies in the female controls compared to female cases of DN and ESRD. The common haplotype of *MME*-AC constructed by the major allele of rs3796268 and rs3773885 was found associated with increased risk of DN and ESRD in females. Also the homozygote diplotype *MME*-AC/AC was associated with increased risk of DN and ESRD. So we presume that the two SNPs are likely to be true risk (major allele) or protective (minor allele) factors because of the consistent haplotype and diplotypes results. In addition, the minor allele rs3773885-T allele was correlated with lower creatinine and cystatin levels in females with DN and ESRD. Thus, the present study provides evidence that *MME* genetic polymorphisms are associated with decreased risk of DN and ESRD in GoKinD collection.

TRPC1 and DN

TRPC1 gene was reported as a candidate gene contributing to DN, since the gene expression at mRNA and protein levels was reduced in both diabetic animal models and DN patients [107]. We have examined the *TRPC1* gene expression in two ages of *db/db* mice, and found the gene expression trendily reduced in 12-week-old and significantly in 26-week-old *db/db* mice. We further performed a genetic study to evaluate the genetic contribution of *TRPC1* gene in the development of DN. The results showed that no significant association of *TRPC1* polymorphisms with DN or ESRD was detected in GoKinD collection and WFU material. Based upon the data from our

study and the recent report [107], we hypothesized that the reduction of *TRPC1* gene expression may be the subsequent defect in the development of diabetic nephropathy.

Gender specificity and protective effects

Several polymorphisms including SNP -11377C/G (*ADIPOQ*), rs7639705 (*MCF2L2*) and rs3796268, rs3773885 (*MME*) are found to be associated with DN. Except SNP -11377C/G, all other SNPs have higher minor allelic frequencies in patients without DN than with DN, which implicates that those SNPs may have protective effects on DN. SNP -11377C/G had higher MAF in patients with DN than without DN, while the minor allele G was identified association with lower circulating adiponectin level in epidemiology studies and reduced promoter activity assays *in vitro* studies [122, 123]. Therefore, we infer that T1D patients without DN with lower G allele frequency are prone to have higher adiponectin level than T1D patients with DN. As an insulin-sensitizing adipokine, adiponectin is thought to have significant anti-inflammatory, anti-atherogenic and cardio-protective properties [126, 127]. Higher adiponectin levels and lower G allele frequency in T1D patients without DN tend to be protective factors. Actually, there are more than one polymorphisms in the *ADIPOQ* gene affected the circulating adiponectin level. SNPs such as -11391G/A and +276A/C in the *ADIPOQ* gene were reported to be associated with adiponectin level in multiple ethnic groups [74, 128, 129]. The relationship between the *ADIPOQ* genetic polymorphisms and its circulating levels, also the corresponding mechanisms, still need further investigation.

All our detected significant results are just in females. DN is a disease with gender specificity. It appears more frequently in men than in women in a ratio of approximately 1.3:1 from the epidemiologic reports [130]. All the associated polymorphisms that we identified tend to have protective effect on female T1D patients. The combined effects and/or interaction among these genes may partly interpret the linkage of chromosome 3q to DN.

Additionally, the sample composition is different between female and male in American Caucasians of GoKinD collection. In females, the case to control ratio is 265 (42.6%) vs 357 (57.3%), while the ratio in males is 313 (56.6%) vs 242 (43.6%). With more controls, the statistic power in females is definitely higher than in males as indicated by PS program (PS Power and Sample Size Calculations, Version 3.0) [131]. While with less controls in males, false positive results may be prone to be found. In the studies including in this thesis, none positive association in males was actually detected.

Chr 3q and the genes for DN

Chr 3q is a region reported associated with DN among different ethnic groups. Our entire findings are relevant to the association with DN, and the results can partly interpret the linkage on Chr 3q. However, no major susceptibility gene for DN in this chromosomal arm has been found yet. There could be two reasons. First, it may exist unidentified major gene(s) in this locus. As we mentioned before, in the region from

Chr 3q22 to the telomere, there are approximate 500 genes including 138 pseudo or unconfirmed genes. Even among the confirmed genes we actually know relatively little about their functions and interactions. Second, there may be no major gene(s) exerting an independence effect on DN in this locus, instead of a number of genes with relevant contributions and combined effects, which can still explain the evidence of linkage to this region.

6 CONCLUSIONS

Paper I – *ADIPOQ* study

We have demonstrated that SNP −11377C/G alters the sequence in one of the SP1 binding sites in the adiponectin promoter region. This polymorphism, together with another promoter SNP −11391G/A are associated with DN in T1D patients among the GoKinD collection.

Paper II – *MCF2L2* study

We found that *MCF2L2* genetic polymorphism rs7639705 was associated with decreased risk for DN, together with *ADIPOQ* and *SOX2* genetic polymorphisms serve as genetic protective factors of DN in females, which may partially explain the linkage with DN in chromosome 3q.

Paper III – *MME* study

We provide the first evidence suggesting the *MME* gene may associate with decreased risk on DN in female T1D patients among the GoKinD collection.

Paper IV – *TRPC1* study

We confirmed that *TRPC1* gene expression is decreased in kidney tissues of *db/db* mice. But *TRPC1* genetic polymorphism may not fundamentally contribute to the development of DN.

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