IDENTIFICATION OF THE SUSCEPTIBILITY GENES IN TYPE 1 DIABETES AND DIABETIC NEPHROPATHY

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马 章

Stockholm 2007
To my family with love
ABSTRACT

Genetic susceptibility plays an important role in the pathogenesis of type 1 diabetes (T1D) and diabetic nephropathy (DN). The present study aims to investigate the association between single nucleotide polymorphisms (SNPs) in candidate genes and DN. Three candidate genes, the intercellular adhesion molecule-1 (ICAM-1), adiponectin (AdipoQ) and neuropeptide Y (NPY), have been selected based upon the approaches: i) from the specific chromosomal regions predicted as important for development of DN by the previous genome wide scan and linkage studies; ii) from the main regulatory steps in the pathways participating in development of diabetic microvascular complications. The studied subjects include 445 Swedish T1D patients with and without DN and 1282 American T1D patients with and without DN selected from the Genetics of Kidneys in Diabetes (GoKinD) study. Genotyping experiments were performed using dynamic allele specific hybridization (DASH). Pyrosequencing and direct sequencing techniques were used for confirmation experiments.

Both ICAM-1 and AdipoQ genes are the strong positional and biological candidates for genetic association study in T1D and DN. We first carried out a genetic association study of the ICAM-1 gene in Swedish subjects and found that SNP rs5498 E469K(A/G) had a high heterozygous index. Frequencies of the allele G in this SNP were decreased gradually from non-diabetic controls, to T1D patients without DN and the patients with DN. Direct sequencing analysis for the subjects with high heterozygous index was performed, but no duplcon in the genomic region around the SNP was found. We further evaluated association between the ICAM-1 genetic polymorphisms and DN with the GoKinD subjects, and found that the allele G in SNP rs5498 E469K(A/G) was significantly associated with the decreased risk susceptibility of DN in female T1D patients. To evaluate the association of AdipoQ genetic polymorphisms with DN in T1D, we have identified 4 binding sites of transcriptional stimulatory protein (SP1) in the AdipoQ putative promoter. The allele G of SNP -11377C/G in the promoter altered the sequence for one of SP1 binding sites. This promoter polymorphism and its common diplotype (haplotypic genotype) constructed with this SNP and -11391G/A are found to be associated with DN in female T1D patients among the GoKinD population. Leu7Pro polymorphism in the NPY gene is found to be associated with DN and coronary heart disease in Finnish women with T1D. We replicated a genetic association study of this polymorphism in both Swedish and GoKinD subjects. The allele C frequency of Leu7Pro polymorphism in Swedish T1D was higher than in the GoKinD population. This polymorphism was found to be significantly associated with DN in Swedish female T1D patients.

The present study provides evidence that ICAM-1, AdipoQ and NPY genetic polymorphisms are associated with DN in T1D. Genetic susceptibility of the polymorphisms may be influenced by many factors, including gender specificity and ethnic stratification.

Key words: Type 1 Diabetes, Diabetic Nephropathy, Single Nucleotide Polymorphism, Genetic Association
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>angiotension-converting enzyme</td>
</tr>
<tr>
<td>ACR</td>
<td>urine albumin/urine creatinine ratio</td>
</tr>
<tr>
<td>AdipoQ</td>
<td>adiponectin</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end-product</td>
</tr>
<tr>
<td>AGT1R</td>
<td>angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>AQP1</td>
<td>aquaporin 1</td>
</tr>
<tr>
<td>AR</td>
<td>aldose reductase</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T-lymphocyte associated 4</td>
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<tr>
<td>DASH</td>
<td>dynamic allele specific hybridization</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
</tr>
<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
<tr>
<td>DN</td>
<td>diabetic nephropathy</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELMO1</td>
<td>engulfment and cell motility 1</td>
</tr>
<tr>
<td>ESRD</td>
<td>end-stage renal disease</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FTO</td>
<td>fat mass and obesity-associated</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GoKinD</td>
<td>the Genetics of Kidneys in Diabetes study</td>
</tr>
<tr>
<td>GPX1</td>
<td>glutathione peroxidase 1</td>
</tr>
<tr>
<td>GWA</td>
<td>genome-wide association</td>
</tr>
<tr>
<td>GWS</td>
<td>genome wide scan</td>
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<tr>
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<td>hemoglobin A1c</td>
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<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HHEX</td>
<td>haematopoietically expressed homeobox</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy-Weinberg equilibrium</td>
</tr>
<tr>
<td>IA-2</td>
<td>insulinoma-associated protein 2</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell autoantigen</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IFIH1</td>
<td>interferon-induced helicase 1</td>
</tr>
<tr>
<td>Ig</td>
<td>immuno-globulin</td>
</tr>
<tr>
<td>IGF1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>INS</td>
<td>insulin</td>
</tr>
<tr>
<td>IL2RA</td>
<td>interleukin 2 receptor alpha</td>
</tr>
<tr>
<td>KCNJ11</td>
<td>potassium inwardly-rectifying channel, subfamily J, member 11</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 TYPE 1 DIABETES

Diabetes mellitus (DM) includes two major types: type 1 and type 2 diabetes. Type 1 diabetes (T1D) patients are characterized by low or absent levels of circulating endogenous insulin and thereby dependent on injected insulin to prevent ketosis and sustain life. T1D is an autoimmune disorder in which the immune system attacks the pancreas, rendering it unable to produce insulin. Auto-antibodies against specific islet cell antigens (ICA, GAD, IA-2 and insulin) are presented in the majority of patients at the onset of T1D. Furthermore, certain human leukocyte antigens (HLA, DR3 and/or DR4) are strongly associated with the disease (Nerup et al., 1974; Palmer et al., 1983; Baekkeskov et al., 1990; Payton et al., 1995; Di et al., 2007). T1D onsets predominantly in youth but can occur at any age. Although the etiology of T1D has not been understood fully, evidence indicates that genetic and environmental factors are involved in the development of this disease (Adeghate et al., 2006).

1.2 DIABETIC MICRO-VASCULAR COMPLICATIONS

Patients with T1D often develop diabetic vascular complications (Figure 1). The diabetic macro-vascular complications include coronary artery, cerebrovascular and peripheral vascular diseases (Bate, Jerums, 2003). The diabetic micro-vascular complications consist of diabetic retinopathy, nephropathy and neuropathy, which are caused by damage to affecting capillaries and small blood vessel in the eyes, kidneys and nerves (Bate, Jerums, 2003).

Figure 1. Diabetic vascular complications

Chronic hyperglycemia is the most important cause for development of micro-vascular complications (Bate, Jerums, 2003). There are several hypotheses about the direct toxic effects of glucose, which causes diabetic micro-vascular complications: activation of protein kinase C (PKC), increased advanced glycation end-product (AGE) formation (Parving, 2001); increased flux through hexosamine pathway (Wild et al., 2004) and...
increased polyol pathway. In particular, hyperglycaemia and increased free fatty acid (FFA) levels result in the production of reaction oxygen species (ROS), such as the superoxide anion, which causes oxidative stress. The generation of oxidative stress results in endothelial dysfunction and a reduction in flow-mediated vasodilation, which may be central to the development of not only micro- but also macro-vascular complications. Hyperglycaemia increases the flux of glucose through the tricarboxylic acid (TCA) cycle, leading to increased levels of the electron donors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2). The increased mitochondrial membrane potential inhibits electron transport at complex III and also extends the half-life of free radical intermediates, which increases the production of superoxide from O2. Central to this activation is the inhibition of glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) by superoxide. This causes an over-utilisation of upstream glycolytic intermediates.

In addition, diabetes and hypertension frequently coexist, and hypertension accelerates the course of micro-vascular complications in the patients with T1D (Schutta, 2007). The development of proteinuria is paralleled in most cases by a gradual rise in systemic blood pressure (SBP).

1.2.1 Diabetic nephropathy

Diabetic nephropathy (DN) was discovered by British physician Clifford Wilson (1906-1997) and Germany-born American physician Paul Kimmelstiel (1900-1970), and description of the disease was published for the first time in 1936. DN, also known as Kimmelstiel-Wilson syndrome and intercapillary glomerulonephritis, is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is characterized by nephrotic syndrome and nodular glomerulosclerosis. Due to longstanding diabetes, the kidney becomes damaged and more protein than normal collects in the urine. As the disease progresses, more of the kidney is destroyed. Over time, the ability of kidney to function starts to decline, which eventually leads to chronic kidney failure.

The cumulative incidence of overt DN in T1D is approximately 20-30% (Marshall, 2003). Diabetes is the leading cause of treated end-stage renal disease (ESRD), accounting for almost half of the new cases each year (Jones et al., 2005). Among European Americans with T1D approximately 30% develop severe DN that leads to ESRD (Krolewski et al., 1996).

1.3 GENETIC STUDIES OF DIABETIC NEPHROPATHY IN TYPE 1 DIABETES

It is widely accepted that the development and progression of DN result from a combination of genetic predisposition, metabolic and hemodynamic abnormalities, and environmental factors (Brownlee, 2001; He, King, 2004; Chow et al., 2005; Jones et al., 2005; Liu, Freedman, 2005; Ng, Krolewski, 2005; Ritz, 2005). There are substantial evidences for a genetic contribution to DN. For instance, in a study of 110 families who
had multiple siblings with T1D, the cumulative risk of DN was 71.5% in those who had a sibling with DN, but just 25.4% for those in which siblings did not show the complications (P<0.001) (Quinn et al., 1996). Epidemiologic studies have shown that DN is strongly clustered in families and that race has a major effect on DN susceptibility and rate of progression (Seaquist et al., 1989; Iyengar et al., 2002; Ng, Krolewski, 2005). Therefore, genetic study may provide useful information for better understanding the pathogenesis of DN.

1.3.1 Genome wide scan and linkage study

Genome wide scan (GWS) and linkage analysis approach has been used to reveal evidence for DN susceptibility loci in chromosomal regions. With this research approach, polymorphic genetic markers for instance micro-satellites with known positions and spaced in smaller intervals along the entire genome are used. The markers distributed across the genome are genotyped in large family pedigrees. Markers that occur more commonly in family members with DN may indicate the presence of a nearby susceptibility loci along the same chromosome. In the recent years, several GWS and linkage analyses have been reported. For instance, chromosomal region 3q is found to link with DN and T1D (Mein et al., 1998; Moczulski et al., 1998; Chistiakov et al., 2004; Osterholm et al., 2007). The linkage peak in this chromosomal region is also observed in T2D with DN (Bowden et al., 2004). Furthermore, two microsatellites D10S558 and D10S1435 in chromosome 10 are found to be significantly associated with DN in T1D (McKnight et al., 2006). These evidences of chromosomal regions linked to DN and T1D provide information for further investigations, including not only fine mapping and positional cloning, but also positional candidate gene single nucleotide polymorphism (SNP) genotyping analyses.

1.3.2 Candidate gene-based association study

Candidate gene-based association studies have been the most common approach employed to identify susceptibility genes for DN. The strategy is the use of either population-based or family-based approaches. Many factors may contribute to variable association studies: multiple hypothesis testing, ethnic stratification, population-specific linkage disequilibrium (LD) between markers and causal variants, inadequate statistical power, publication bias, and gene-gene and gene-environmental interactions. The population-based approach is used to determine whether gene variants are found more or less commonly in patients with DN (cases) than in patients without DN (controls). The family-based approach, such as the transmission-disequilibrium test (TDT), is used to test the frequency of transmission of variants from heterozygous parents to their off-springs in comparison with the expected 50:50 ratio (Spielman et al., 1993). However, TDT study requires analysis of both parents. In the parents of patients with DN, it is difficult to recruit sufficient family trios to provide adequate power (Conway et al., 2006). Using the approach of TDT, Ewens et al. have tested 115 candidate genes in 72 T1D families of European descent. Of the candidates, several genes, including aquaporin 1 (AQP1), B-cell leukemia/lymphoma 2 (BCL2), catalase (CAT), glutathione peroxidase 1 (GPX1), insulin-like growth factor 1 (IGF1), laminin, alpha 4 (LAMA4),
laminin, gamma 1 (LAMC1), mothers against DPP homolog 3 (SMAD3), transforming growth factor, beta receptor II (TGFBR2), transforming growth factor, beta receptor III (TGFBR3), tissue inhibitor of metalloproteinase 3 (TIMP3) and upstream transcription factor 1 (USF1), are found to be associated with DN (Ewens et al., 2005).

A number of genetic association studies have been reported. Aldose reductase (AR) is the first and rate-limiting enzyme in the pathway that catalyses the reduction of glucose to sorbitol. Several studies have indicated that the polymorphisms of a micro-satellite located at 5’ strand of the aldose reductase gene are associated with DN. Further association studies have recently demonstrated that a promoter polymorphism C-106T in this gene confers genetic susceptibility to DN in both T1D and T2D (Moczulski et al., 2000; Neamat-Allah et al., 2001). Angiotensin II type 1 receptor (AGT1R) is expressed in the vascular smooth muscle cells of the afferent and efferent arterioles of the kidney, glomerular mesangial cells, proximal tubule cells of the kidney and adrenal glomerulosa cells. The polymorphism C1166A of the AGT1R gene is strongly associated with renal hemodynamic function in early T1D (Miller et al., 2000). Vascular endothelial growth factor (VEGF) is a potent angiogenic and vascular permeability factor and is implicated in DN. An insertion/deletion C-460 polymorphism in the promoter region of the VEGF gene has been found to be associated with promotion of neovascularization (Ray et al., 2004). The relationship between the angiotension-I-converting enzyme (ACE) insertion/deletion polymorphism and DN in T1D was established (Rudberg et al., 2000; Hadjadj et al., 2001).

1.3.3 Genome wide association study

With the improvement of genotyping technologies and the exponentially growing number of SNPs, case-control genome-wide association (GWA) studies have become a useful tool for identification of the susceptibility genes in diabetes and DN. A recent GWA study on seven diseases, including T1D, has been performed, and the results have demonstrated association between T1D and six chromosomal regions 12q24, 12q13, 16p13, 18p11, 12p13 and 4q27 (The Wellcome Trust Case Control Consortium, 2007). Several genes, including ERBB3 in chromosome 12q13, C12orf30 in 12q24, KIAA0350 in 16p13 and PTPN2 in 18p11, are found to be associated with T1D (Todd et al., 2007). Furthermore, Hakonarson et al. have also performed a GWA study and indicated that the KIAA0350 gene might be involved in the pathogenesis of T1D (Hakonarson et al., 2007). In addition, the solute carrier family 12 (sodium/chloride transporters), member 3 (SLC12A3) and engulfment and cell motility 1 (ELMO1) genes are found to be associated with DN in T2D (Shimazaki et al., 2005; Maeda et al., 2007). Hanson et al. have recently identified that the plasmacytoma variant translocation (PVT1) gene is a susceptibility gene for ESRD in T2D (Hanson et al., 2007). Recent successes in identifying the susceptibility genes in T1D, T2D and DN in T2D suggest that GWA is an useful approach to identify the susceptibility gene for DN in T1D as well.
2 THE PRESENT STUDY

2.1 AIMS

The research project is focused on genetic association study between the candidate genes and DN in T1D. The specific aims are:

- To evaluate the association between SNPs in the intercellular adhesion molecule-1 (ICAM-1) gene and DN in T1D;
- To investigate the genetic influence of adiponectin (AdipoQ) variation in the development of DN and T1D;
- To study the association of neuropeptide Y (NPY) Leu7Pro polymorphism and DN in T1D.

The results may provide useful information for a better understanding pathogenesis of DN in T1D.

2.2 SELECTION OF CANDIDATE GENES

We have selected the candidate genes for our study based upon two approaches:

i) From the specific chromosomal regions predicted as important for development of DN by the previous genome wide scan and linkage studies.

ii) From the main regulatory steps in the pathways participating in the development of endothelial dysfunction and micro-angiopathy in the patients with DN.

2.2.1 Intercellular adhesion molecule-1

ICAM-1 is a 90-kD cell surface glycoprotein of the immuno-globulin (Ig) superfamily involved in the firm attachment of leukocytes to endothelium (Staunton et al., 1988; van, van, 1996). It is normally expressed at low levels on the surface of arterial endothelial cells. But, its expression can be induced by multiple factors, including inflammatory cytokines, reactive oxygen species and shear stress (Koulu et al., 2004; Nordman et al., 2005). Particularly, ICAM-1 expression at both mRNA and protein levels is found to be increased by hyperglycemia (Kado et al., 2001; Omi et al., 2002).

The ICAM-1 gene is located in chromosome 19p13. GWS has predicted that the T1D susceptibility genes may reside in this chromosomal region (Mein et al., 1998). The levels of plasma adhesion molecules are elevated either in T1D or T2D patients with DN and relate to renal dysfunction (Lampeter et al., 1992; Hirata et al., 1998; Clausen et al., 2000; Mohamed-Ali et al., 2001; Guler et al., 2002; Nelson et al., 2005). Vascular cell adhesion molecule-1 (VCAM) is elevated in the setting of moderately decreased glomerular filtration rate (GFR) in T2D patients and may be the link between renal insufficiency and increased risk for cardiovascular events in American population (Lin et al., 2006). Soluble vascular adhesion molecule-1 (sVCAM-1) and intercellular...
adhesion molecule-1 (sICAM-1) are increased in T1D patients with DN (Nelson et al., 2005). Furthermore, it has been proposed that the increased levels of plasma adhesion molecules may be the sign of pre-clinical T1D in children (Toivonen et al., 2004).

The experimental studies using T1D animal models demonstrate that the increased ICAM-1 expression accompanies the progression of T1D (Balasa et al., 2000) and DN (Matsui et al., 1996; Sugimoto et al., 1997). Furthermore, ICAM-1 expression in glomeruli and tubulointerstitium is significantly elevated in the streptozotocin (STZ)-diabetic rats (Qi et al., 2006). Increased mesangial matrix, glomerular hypertrophy, and albuminuria are found to significantly suppressed in diabetic ICAM-1(-/-) mice compared with diabetic ICAM-1 (+/+) mice (Okada et al., 2003). Thus, up-regulation of kidney ICAM-1 appears to be a common response to diabetic renal injury. Therefore, the ICAM-1 gene is a strong positional and biological candidate for genetic association study in T1D and DN.

2.2.2 Adiponectin

Adiponectin is a hormone exclusively secreted by adipose tissue. Serum adiponectin concentrations are decreased in the patients with T2D and in the subjects with obesity (Arita et al., 1999; Statnick et al., 2000). In contrast, serum adiponectin levels are increased in the patients with T1D (Imagawa et al., 2002; Diez, Iglesias, 2003; Lindstrom et al., 2006) and also in T1D patients with micro-angiopathy (Frystyk et al., 2005; Hadjadj et al., 2005) and DN (Saraheimo et al., 2005). The mechanism behind an increase of circulating adiponectin in DN is not fully understood. The recent report indicates that renal insufficiency and tubular injury possibly play a contributory role in increases of serum and urinary adiponectin levels in overt DN (Fujita et al., 2006).

The adiponectin gene is encoded as AdipoQ (adipocyte C1q and collagen domain containing). The alternative names for this gene are GBP28, ACRP30, APM1 and ACDC. The AdipoQ gene is located on chromosome 3q27, within the chromosomal region linked with T2D, obesity and hypertension (Kissebah et al., 2000; Vionnet et al., 2000; Francke et al., 2001; Zacharova et al., 2005), and also with DN in T1D and T2D (Imperatore et al., 1998; Moczulski et al., 1998). Several reports have indicated that the AdipoQ genetic polymorphisms are associated with T2D (Hara et al., 2002; Vasseur et al., 2002; Gu et al., 2004; Vasseur et al., 2005; Schwarz et al., 2006). In order to investigate the susceptibility of the AdipoQ genetic polymorphisms in T1D and DN, we have conducted a genetic association study with Swedish T1D patients with and without DN. A recent study has suggested that the AdipoQ promoter polymorphisms may confer the risk susceptibility to the development of DN in T1D patients in Danish but not in French and Finnish populations (Vionnet et al., 2006). We thus further evaluate the association between the AdipoQ promoter polymorphisms and DN with patient material from the Genetics of Kidneys in Diabetes (GoKinD) study.
2.2.3 Neuropeptide Y

NPY is a 36-amino acid polypeptide that is present in the central and peripheral nervous system, several peripheral organs and plasma (Jamal et al., 1991; Wang et al., 1994; Bennet et al., 1996; Myrsen-Axcrona et al., 1997). NPY has multiple functions and mainly plays a role in the regulation of satiety, ingestive behaviors, energy balance and energy expenditure (Billington et al., 1994; Levine et al., 2004). NPY also stimulates lipoprotein lipase activity in the adipose tissue (Billington et al., 1991) and regulates insulin release (Moltz, McDonald, 1985).

NPY exists at a high concentration in the hypothalamus and increased hypothalamic NPY and noradrenaline overflow may contribute to diabetic hyperphagia (Morris, Pavia, 2004). NPY release is found to be increased from the hypothalamus in STZ-diabetic rats (Gozali et al., 2002; Morris, Pavia, 2004). Furthermore, NPY has been shown to regulate renal blood flow (Bischoff, Michel, 1998). Stimulation of the NPY receptors in the kidney, using active agonists, may decrease GFR, aldosterone concentration and plasma renin activity, but increase sodium excretion in the kidney (Playford et al., 1995). NPY is also a potent vasoconstrictor of renal arteries (Michel et al., 1992) and decreases renal blood flow and glomerular filtration (Echtenkamp, Dandridge, 1989). Interestingly, the Leu7Pro polymorphism in the NPY gene is found to be associated with high blood lipid concentrations and accelerated rate of atherosclerosis as well as diabetic retinopathy. The subjects with Leu7/Pro7 genotype of this polymorphism had 54% higher maximal increases in the plasma GH concentrations compared to the subjects carrying with Leu7/Leu7 genotype (Kallio et al., 2001). NPY may play a role in the pathogenesis of diabetes and DN.
3 SUBJECTS AND METHODS

3.1 SUBJECTS

Patient material in the present study consists of two sets of subjects, i.e. Swedish and the GoKinD populations. T1D patients were diagnosed according to the World Health Organization criteria. The studies were approved by the local ethics committees, and informed consent was obtained from all participates.

3.1.1 Swedish subjects

This set of subjects includes 445 (male 210/ female 235) Swedish T1D patients (Table 1). Most of T1D patients were collected from Department of Endocrinology, Metabolism and Diabetes, Karolinska University Hospital, while 146 T1D patients were collected from Umeå University Hospital. T1D patients had at least 10 years of diabetes duration. All patients had the urinary albumin excretion rate (UAER) measured in at least two consecutive overnight urine samples. The patients with UAER <20 µg/min were considered as being free from DN (n=249). The patients with UAER of 20-200 µg/min and of >200 µg/min in at least 2 out of 3 consecutive overnight samples were considered as microalbuminuria (incipient nephropathy, n=105) and macroalbuminuria (overt nephropathy, n=91. 22 of them were ESRD), respectively. The patients without DN had never been treated with ACE inhibitors when UAER was measured. In addition, 187 non-diabetic healthy individuals, with no family history of diabetes, were also included. All subjects were Swedish Caucasians.

Table 1. Swedish subjects

<table>
<thead>
<tr>
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<th></th>
<th>T1D with DN</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Male</td>
<td>Female</td>
<td>All</td>
</tr>
<tr>
<td>N</td>
<td>249</td>
<td>117</td>
<td>132</td>
<td>196</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44±12</td>
<td>45±12</td>
<td>43±12</td>
<td>46±12</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>30±10</td>
<td>29±10</td>
<td>31±10</td>
<td>29±11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6±3.1</td>
<td>24.9±2.2</td>
<td>24.4±3.6</td>
<td>25.8±3.7</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>127±15</td>
<td>129±14</td>
<td>125±16</td>
<td>138±21</td>
</tr>
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<td>DBP (mmHg)</td>
<td>74±7</td>
<td>75±7</td>
<td>72±7</td>
<td>78±10</td>
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<tr>
<td>HbA1c (%)</td>
<td>7.0±1.1</td>
<td>6.9±1.0</td>
<td>7.2±1.2</td>
<td>7.4±1.4</td>
</tr>
</tbody>
</table>

All data=means±SD.

3.1.2 The GoKinD subjects

This set of subjects was selected from the GoKinD study (Table 2) and collected by the Juvenile Diabetes Research Foundation in collaboration with the Joslin Diabetes Center and George Washington University, and the United States Centers for Diabetes Control and Prevention (Mueller et al., 2006). Totally, 620 T1D patients without DN and 662 patients with DN were included. Among the patients, 1177 (91.8%) were of European descent, while 105 (8.2%) patients were Americans of Black, Asian, Hispanic or Indian descent. T1D patients were diagnosed before 31 years of age. Treatment with insulin was instituted within one year of diagnosis, and has been uninterrupted since then.
patients with DN had T1D for at least 10 years and had persistent proteinuria or ESRD (not due to condition other than diabetes). Persistent proteinuria was defined as 2 out of 3 tests positive for albuminuria (at least 1 month apart), i.e. dipstick (Albustix or Multistix) at least 1+ or Urine Albumin/Urine Creatinine Ratio (ACR) value exceeding 300 µg albumin/mg of urine creatinine. Patients without DN had persistent normoalbuminuria despite duration of T1D for at least 15 years and had never been treated with ACE inhibitors. Persistent normoalbuminuria was defined as at least 2 out of 3 of ACR measurements (at least 1 month apart) in random urine specimens being less than 20 µg albumin/mg of urine creatinine. If 3 ACR measurements were needed, the highest must also be less than 40 µg albumin/mg of urine creatinine. ESRD is recognized as the patients need chronic dialysis or kidney transplant treatment to maintain their life. The onset of ESRD is defined as the date of the first dialysis or kidney transplant, whichever occurred first. Other kidney diseases in cases were excluded (Mueller et al., 2006).

Individuals were excluded from the study if they did not meet the inclusion criteria just described or if any of the following exclusion criteria were met: unable or unwilling to give informed consent; unable to communicate with staff; major psychiatric disorder such as schizophrenia; exclusion in relation to medication: any antihypertensive medication for controls; infectious disease: self-reported HIV positivity and active tuberculosis; pregnant women (although they may be reconsidered 3 months after delivery); other kidney disease due to condition other than diabetes.

Table 2. The GoKinD subjects

<table>
<thead>
<tr>
<th></th>
<th>T1D without DN</th>
<th>T1D with DN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Male</td>
</tr>
<tr>
<td>N (M/F)</td>
<td>620</td>
<td>251</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40±8</td>
<td>40±9</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>26±8</td>
<td>26±8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.1±4.3</td>
<td>26.6±3.7</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118±12</td>
<td>122±12</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>71±8</td>
<td>74±8</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.5±1.1</td>
<td>7.4±1.1</td>
</tr>
</tbody>
</table>

All data=means±SD.

3.2 METHODS

3.2.1 Dynamic allele specific hybridization

Dynamic allele specific hybridization (DASH) is a high throughput genotyping method for scoring SNPs and small insertion/deletions. This method is based upon hybridization with a specific probe. To most readily interpret DASH results, the negative first derivatives can be shown on the computer screen. The sample mismatched to the probe by one base pair produces a single lower temperature peak. The sample matched to the probe produces a single high-temperature peak. The heterozygous sample undergoes two phases of denaturation (one for each allele present)
and therefore produces two peaks in the negative first derivative (Figure 2) (Howell et al., 1999).

**Figure 2. An example from DASH genotyping experiments**

3.2.2 Pyrosequencing

Pyrosequencing is based on the detection of released pyrophosphate (PPI) during synthesis of DNA sequence. The synthesis starts with a nucleic acid polymerization reaction in which inorganic PPI is released as a result of nucleotide incorporation by DNA polymerase. The released PPI is subsequently converted to ATP by ATP sulfurylase, which provides the energy to luciferase to oxidize luciferin and generate light. Because the added nucleotide is known, the sequence of the template can be determined (Figure 3). This method can be used not only for sequencing analysis in short DNA or RNA templates but also for SNP genotyping (Ronaghi et al., 1998).

**Figure 3. Pyrosequencing and SNP genotyping**


3.2.3 Direct sequencing analysis

Segmental duplications (duplicants) with >90% similarity between the copies comprise at least 5% of the human genome, which may cause specific allelic and genotypic diversities such as high heterozygous index in complex diseases (Venter et al., 2001; Shaw, Lupski, 2004). In order to detect the possible duplicants in the sequences that the studied SNPs reside, the direct sequencing analysis with ABI BigDye terminator cycle sequencing kit and protocol was used. The kit and protocol are suitable for performing fluorescence-based cycle sequencing reactions on large single-stranded or double-
stranded DNA templates, and on polymerase chain reaction (PCR) fragments and on large templates.

### 3.2.4 Bioinformatics

Sequences of the studied genes are recorded in the GenBank of NCBI. The studied SNPs are selected from public SNP databases, including dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and HGVbase (http://hgvbase.cgb.ki.se/) according to their types, locations, validation status in HapMap (http://www.hapmap.org/) and information from the previous reports.

For each studied SNP, the surrounding approximately 50 bp sequences in 5’- or 3’-direction were examined for repeats and duplicated sequences (http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker) and blasted in human genome sequences (http://www.ncbi.nlm.nih.gov/blast).

To design DASH assays, the possible structures of PCR products for DASH genotyping experiments were analyzed with mFold (version 3.1; http://www.bioinfo.rpi.edu/applications/mfold/old/dna/). To search for the possible transcriptional binding factors in the sequence of the AdipoQ promoter, Transcription Element Search System (TESS) is used for predicting transcription factor binding sites in DNA sequences (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

### 3.2.5 Hardy-Weinberg equilibrium test

Deviation from the Hardy-Weinberg equilibrium (HWE) for alleles in each SNP was assessed using the appropriate Chi-square distributed statistics.

In the general population the genotype distribution of a SNP should always be in HWE. The law of HWE was founded in 1908, by Godfrey H. Hardy and Wilhelm Weinberg and is basically stating that in a randomly mating population the relative frequencies of heterozygotes and homozygotes are mathematically related and that allele frequencies will remain constant from generation to generation provided that none of the alleles is under positive or negative selection. Several criteria must be fulfilled for a SNP to be in HWE, namely random mating, absence of selection and gene flow, a large enough population, no change of mutation frequency and finally no chromosomal rearrangements.

HWE can easily be tested by using the equation: \((p+q)^2 = p^2 + 2pq + q^2 = 1\), where \(p\) is the allele frequency of allele 1 and \(q\) is the allele frequency of allele 2 and \(2pq\) is the frequency of heterozygotes. The observed allele frequencies are calculated and used to find the expected genotype frequencies. The observed and expected genotype frequencies are compared using the Chi-square test. A \(P\) value that is \(<0.05\), indicates
that a polymorphism is not in HWE, which means that the observed and expected genotype distribution values differ significantly.

If a SNP does not follow the HWE law in the patient group it could be interpreted to strengthen the evidence of association between the SNP and the phenotype because of increased frequency of a certain genotype that is associated with the phenotype. On the other hand, HWE must always be obtained in the control group when performing association studies. One other explanation why a SNP does not follow the HWE law could be complex SNP related sequence variations, a recently discovered form of genome polymorphisms, called multisite variations (MSVs) that usually do not follow the HWE. An MSV can masquerade as a SNP, but may in reality be a duplicon marker that arises from segmental duplications of a sequence (Fredman et al., 2004).

3.2.6 Single marker association analysis

To detect single marker association between cases and controls, two models are tested comparing either allele frequencies in 2×2 contingency tables or genotypes in 3×2 contingency tables. If heterozygous individuals confer significant risk to the carriers with non-risk homozygosity, recessive mode of inheritance is rejected. Additive and dominant modes are considered to be used. If homozygous individuals confer significant risk to heterozygous carriers, additive and dominant modes are not suitable. The recessive mode is then used. Statistical powers are calculated using a software of PowerSampleSize (PS version 2.1.31, http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). Tests for association between genotypes and quantitative traits are performed using Kruskal–Wallis analysis of ranks for traits with non-normal distributions, or alternatively, ANOVA for normally distributed traits. For association estimation, odds ratios (OR) and 95% confidence intervals (CI) are estimated with unconditional logistic regression models between cases and controls. All statistical analyses are performed using the BioMedical Data Program (BMDP) version 1.12 and/or STATISTICA version 7.0.

3.2.7 Linkage disequilibrium and haplotype analysis

SNPs are positioned on the same chromosome can segregate together more often than expected and are then said to be in LD. LD can be calculated and is dependent on allele frequencies as well as of recombination. The two most common measures of LD are Lewontin’s LD parameter D’, which is a measure of recombination, and r^2, which is a measure of correlation. When D’=1 the two SNPs are in total LD, meaning that there is no recombination and when r^2=1 the allele frequencies of the SNPs are equal and there is no recombination meaning that the SNPs are in total LD. The two LD measures do not have to follow each other, i.e. D’=1, but r^2= 0.6 for the same two SNPs means that the SNPs segregate together more often than expected, but the allele frequencies are not equal. LD is an association between polymorphisms at the population level, where alleles in close proximity of each other are often inherited together, since they originate from a common chromosome. A polymorphism can be in LD with another
polymorphism on the same chromosome, which is associated with a trait and thereby act as a marker for the particular disease.

The estimation of pair-wise LD values and haplotype frequencies was performed using Arlequin program version 2.0 (http://lgb.unige.ch/arlequin/). Haplotype prevalence at >5% was further used for haplotype association analysis.
4 RESULTS AND DISCUSSION

4.1 ASSOCIATION STUDIES OF SNPS IN THE ICAM-1 GENE AND DN IN T1D (PAPERS I AND II)

We carried out a genetic association study of the ICAM-1 gene in T1D. A total of 8 SNPs, including these two non-synonymous polymorphisms rs5498 E469K(A/G) and rs1799969 R241G(A/G) in the ICAM-1 gene, were validated with 32 DNA samples from Swedish subjects. Of the studied SNPs, three had allele frequencies at <1% and were therefore excluded from further analyses. Genotyping results indicated that SNPs rs281432 (C/G) and rs5498 E469K(A/G) were significantly associated with T1D. Frequencies of the C allele in SNP rs281432 (C/G) and the A allele in SNP rs5498 E469K(A/G) increased gradually from non-diabetic controls, to T1D patients without DN and T1D patients with DN among Swedish T1D patients. However, no significant association of these two polymorphisms with DN was detected. We then evaluated the association between the ICAM-1 genetic polymorphisms and DN in the GoKinD population. We found that the significant association of heterozygosity and allele G positivity in SNP rs5498 E469K(A/G) with DN in female T1D patients. The allele G of this polymorphism might confers the decreased risk susceptibility to the development of DN.

Rs5498 E469K(A/G) resides in the 5th immunoglobulin-like domain of ICAM-1. This domain may play a role in an immunodominant epitope of B lymphocytes and dendritic cells (Jolting et al., 1994). Furthermore, a recent study has demonstrated that this SNP affects ICAM-1 mRNA splicing pattern and TPA-induced apoptosis (Iwao et al., 2004). Interestingly, the genotype distribution of SNP rs5498 E469K(A/G) was in HWE, and this polymorphism had high heterozygous index, which was not reported previously. By communication with the previous reporters, we found that the phenomenon of high heterozygous index of this SNP existed in all populations studied. Segmental duplications (duplicons) in the human genome may cause specific allelic and genotypic diversities such as high heterozygous index in complex diseases (Venter et al., 2001; Shaw, Lupski, 2004). To ascertain whether SNP rs5498 E469K(A/G) is involved in a duplicon, we have confirmed the genotyping data by using another method called pyrosequencing, and also performed direct sequencing analyses. Results indicated that no duplicon was involved in the genomic sequences around this SNP. The present study thus provide evidence that the heterozygous genotype of SNP rs5498 E469K(A/G) in the ICAM-1 gene may have genetic influence in the pathogenesis of DN and T1D.

4.2 GENETIC ASSOCIATION ANALYSES OF THE ADIPOQ GENE IN T1D AND DN (PAPERS III AND IV)

We first performed validation for 13 SNPs in the AdipoQ gene with Swedish subjects. Of the studied SNPs, 3 non-synonymous polymorphisms in exon 3, including G84R(G/C), G90S(G/A) and Y111H(T/C), had low allele frequencies (<3%). These 3
polymorphisms were excluded for further analysis. We then found that SNPs +45G15G(T/G) and +276(G/T) were in a LD block. These two polymorphisms and their common haplotypes were associated with T1D. Comparison analysis among T1D patients carrying three different genotypes in SNP +276G/T indicated that the GG carriers had lower levels of serum adiponectin. However, no significant association of the AdipoQ genetic polymorphisms with DN was found most likely due to the limitation of sample size.

A recent study demonstrated that an AdipoQ promoter polymorphism -11391G/A was associated with DN in T1D patients among Danish, but not French, Finnish populations (Vionnet et al., 2006). We thus further studied the association between AdipoQ promoter polymorphisms, including -11377C/G, -11391G/A and -11426A/G, and DN with the GoKinD subjects. We identified 4 binding sites of transcriptional stimulatory protein (SP1) in the AdipoQ promoter, and found that the allele G of SNP-11377C/G alters the sequence for one of SP1 binding sites. This polymorphism was significantly associated with DN in female T1D patients. Additionally, two promoter polymorphisms -11377C/G and -11391G/A are located in a LD block and the common diplotype (haplotypic genotype) H1/H1, constructed with these two polymorphisms was found to be significantly associated with DN in females T1D patients.

Genetic variation in the promoter may alter the binding sequences for transcriptional elements and consequently influence gene regulation and expression. It has been demonstrated that SP1 belongs to the SP family of transcriptional factors and is ubiquitously expressed in mammalian cells (Krikun et al., 2000; Li et al., 2004). Barth et al. have previously reported that SP1 binding activity is enhanced during adipocyte differentiation, and has stimulatory effects on the AdipoQ promoter activity (Barth et al., 2002), but number and location of SP1 binding sites are unknown. Bouatia-Naji et al. have recently investigated the functional proprieties of SNP -11377C/G with the transfection approach and electrophoretic mobility shift assays. The data demonstrated that the allele G had lower, but not significantly, transcriptional activity than the “wild-type” allele C (Bouatia-Naji et al., 2006). Taking together, we suggest that the promoter polymorphisms in the AdipoQ gene may have genetic and biological influence related to the development of DN in T1D. However, we still look forward to having the possibility to examine the serum adiponectin levels in T1D patients with or without DN according to the genotypes of the AdipoQ promoter polymorphisms.

4.3 DISTRIBUTION OF NPY LEU7PRO POLYMORPHISM IN T1D PATIENTS IN SWEDISH AND GOKIND POPULATIONS (PAPER V)

NPY Leu7Pro (T1128C) is a non-synonymous polymorphism and the allele C may have an impact on NPY processing on circulating NPY levels, on growth hormone secretion, and on serum cholesterol and triglyceride levels (Karvonen et al., 1998; Kallio et al., 2001; Mattevi et al., 2002; Pihlajamaki et al., 2003; Karvonen et al., 2006). Furthermore, this polymorphism distributes a geographical north to south
gradient of decreasing frequency (Ding, 2003), suggesting that it may be a population-
specific causal variant.

We have genotyped this polymorphism with the subjects from both Swedish and
GoKinD populations. Results indicated that the allele C frequency of NPY Leu7Pro
polymorphism in Swedish T1D was significantly higher than in the GoKinD
population. Interestingly, this polymorphism was found to be associated with DN in
Swedish female T1D patients, but not in the patients from the GoKinD population.
Pettersson-Fernholm et al. reported that the allele C frequency was 7.5% in Finnish
T1D patients, and the allele C had the risk susceptibility for DN and coronary heart
disease (CHD) in Finnish female but not in male patients with T1D (Pettersson-
Fernholm et al., 2004). The allele C frequency in Swedish T1D patients is 6.3%, which
is slightly lower than in the Finnish T1D patients (Pettersson-Fernholm et al., 2004) but
significantly higher than in the American T1D patients of the GoKinD study (4.0%).
The results are consistent with other observations that frequency of the allele C was
highest in Finnish, second high in Swedish (Karvonen et al., 1998; Ding, 2003; Ding et
al., 2005; Nordman et al., 2005) but low in the GoKinD and other populations (Makino
et al., 2001; Ding, 2003).
5 CONCLUSIONS

- SNP rs5498(E469K) in the ICAM-1 gene, with a high heterozygous index, is found to be associated with DN in female T1D patients among the GoKinD population. Although frequencies of the allele A in this polymorphism is found to be increased gradually from non-diabetic controls, to T1D patients without DN and T1D patients with DN in Swedish population, no statistically significant association with DN is detected.

- We have identified four SP1 binding sites in the promoter region of the AdipoQ gene. The promoter SNP -11377C/G is found to be associated with DN among female T1D patients of the GoKinD study. This polymorphism may have a role in the regulation of AdipoQ promoter transcription activity. Additionally, SNPs +45G15G(T/G) and +276(G/T) in the AdipoQ gene are found to be associated with T1D in Swedish Caucasians.

- Frequency of the allele C in NPY Leu7Pro polymorphism is higher in Swedish T1D patients than in American GoKinD subjects. This polymorphism is found to be associated with DN in Swedish female T1D patients.
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7 REFERENCES


