Identifying genes for diabetic nephropathy—current difficulties and future directions

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Keywords: candidate gene analysis; diabetic nephropathy; genetic susceptibility; genome-wide screens; single nucleotide polymorphism

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease in the Western world [1], placing considerable demands on health care resources and causing significant morbidity and mortality for individuals with diabetes. Improvements in glycaemic and blood pressure control, together with the introduction of inhibitors of the renin–angiotensin system, have delayed the development and progression of nephropathy [2]. However, a substantial proportion of diabetic patients still develop nephropathy despite apparently good glycaemic control [3]. This suggests that additional, as yet unidentified, pathogenic mechanisms are likely to exist and that novel alternative therapeutic strategies could significantly retard progression to nephropathy in susceptible diabetic individuals.

Evidence for a genetic predisposition to diabetic nephropathy

There is growing evidence that genetic background determines the risk of nephropathy in patients with diabetes. Familial clustering of nephropathy is well-documented [4–6], and significant concordance, both for the degree of proteinuria [7] and for the severity of the glomerular lesions [8] has been observed within families. Furthermore, the likelihood of developing nephropathy is greater in diabetic patients who have a parental history of hypertension or cardiovascular disease, implicating genes that regulate blood pressure and cardiovascular risk [9,10]. Further support for genetic susceptibility to nephropathy is derived from the natural history of the disease. If nephropathy were solely due to the accumulating glycaemic burden, its prevalence would rise inexorably over time until the majority of diabetic patients developed renal disease. In contrast, the peak incidence of nephropathy occurs between 15 and 20 years after the onset of type 1 diabetes, beyond which it declines rapidly, so that the cumulative incidence of renal disease is less than 30% [11]. It therefore appears that a subset of patients with diabetes is destined to develop nephropathy, and these patients usually have evidence of clinical nephropathy within 20 years of their diabetic diagnosis.

Advances in molecular medicine offer promise that the genetic basis of complex diseases such as diabetic nephropathy may be unravelled. This would allow intensive multifactorial treatment to be targeted towards susceptible individuals and, perhaps more importantly, identification of novel therapeutic targets.
Furthermore, genetic factors may determine therapeutic response; for example, polymorphisms in the angiotensin converting enzyme (ACE) and angiotensin-II type 1 receptor genes may influence the progression of nephropathy in patients treated with ACE inhibitors [12].

The complexity of common disease

No gene variant has, as yet, been conclusively shown to influence predisposition to nephropathy. Meta-analyses can help in rationalizing the results from several conflicting studies, however, these analyses may still have inherent problems such as including individual studies that employ widely different phenotype criteria. Nevertheless, a recently published meta-analysis, incorporating data from 14,727 diabetic patients, provided evidence that the ACE insertion (I)/deletion (D) polymorphism influences the development of diabetic nephropathy in both type 1 and type 2 diabetes. There was a statistically significant protective role for the II genotype especially in Asians with type 2 diabetes [13]. However, even if this analysis is correct, the ACE I/D polymorphism only accounts for a small fraction of the total attributable genetic risk of nephropathy. The identification of additional nephropathy susceptibility gene variants represents an enormous challenge, and there are major limitations in the methodologies employed to date. Hence, it is not surprising that the causative gene variants remain elusive.

It is estimated that the human genome contains approximately 25,000 genes and more than 10 million common (occurring in more than 1% of the population) variations, known as single nucleotide polymorphisms (SNPs). Unlike monogenic disease, the risk of complex disorders such as diabetic nephropathy depends on the effect of many genetic variants acting additively or synergistically with each other and with environmental factors. In contrast to the apparent all-or-nothing effect of monogenic disorders, the genetic variants demonstrate incomplete penetrance, that is, they confer variable, but comparatively modest (generally much <2-fold) risk of susceptibility to or protection from disease. The search for multiple variants, each with a small individual phenotypic effect, will require additional strategies to those used successfully in monogenic disorders.

Candidate gene studies

Candidate gene-based association studies have been the most common approach employed to identify susceptibility genes for diabetic nephropathy. Candidate genes have frequently been assessed by determining whether gene variants are found more or less commonly in patients with diabetic nephropathy (cases) than in patients who exhibit no evidence of nephropathy despite having had diabetes for many years (controls). Genes are selected to test for association with diabetic nephropathy based on our current understanding of the pathogenesis of the disease (e.g. the genes coding for ACE, angiotensin-II receptor, and aldose reductase). Nevertheless, examining individual genes is laborious and unsystematic, as genes are prioritized on what is essentially a 'best guess' basis. Furthermore, studies have often focused on a single polymorphism or a few polymorphisms within an individual candidate gene, selected for ease of genotyping or because they are postulated to affect gene function. Hence some candidate genes may have been discounted prematurely without a thorough assessment of all their genetic variation.

Case-control studies are susceptible to a variety of potential methodological flaws. First, rigorous criteria must be employed to correctly assign phenotype. For example, in studying the genetic predisposition to diabetic nephropathy, employing the following criteria should minimize misclassification of subjects. All patients should be diagnosed with type 1 diabetes before 31 years of age. Those with nephropathy must have had diabetes for at least 10 years prior to the onset of persistent proteinuria (>0.5 g/24 h), associated hypertension and later progressive renal failure in the absence of clinical, serological or radiological evidence of non-diabetic renal disease [14,15]. In contrast, controls must have had type 1 diabetes for at least 15 years, with no microalbuminuria on repeated testing, and not be in receipt of antihypertensive medication, which may mask underlying microalbuminuria. Given the evidence that spontaneous regression of microalbuminuria can occur in type 1 diabetes it is important that microalbuminuric patients are not assigned a case phenotype [16].

Sufficient number of cases and controls must be recruited in order to minimize the risk of identifying false-positive associations that are due to chance alone (type 1 errors) or, conversely, of failing to detect a true association between a variant and a disease (false negatives or type 2 errors). Lack of statistical power is a problem common to many genetic analyses, although case-control studies are relatively more powerful for detecting disease susceptibility variants than cohort studies.

False positives may also occur due to population stratification resulting from ethnic admixture, the presence of two or more populations with different genetic backgrounds [17]. If, for example, a particular ethnic group is over-represented in the cases compared with controls, then polymorphisms that occur more frequently in this ethnic group may lead to spurious results. This problem may be minimized by examining homogenous populations such as the Pima Indians or by employing cases and controls that are matched for ethnicity, and other potential confounding variables such as age and sex. Alternatively, statistical methods can be employed to identify and adjust for ethnic admixture. Markers that are informative regarding ancestry can be used to match cases and controls for ethnicity, or to make an appropriate adjustment in the
threshold of \( P \)-value required for significance [18]. Another strategy is the use of family-based approaches, such as the transmission-disequilibrium test (TDT), in which the frequency of transmission of variants from heterozygous parents to their offspring is compared with the expected 50:50 ratio [19]. However, this approach is less powerful than case-control studies, and it requires analysis of both parents [20]. Given the increased risk of premature death, largely due to cardiovascular disease, in parents of patients with diabetic nephropathy, it is difficult to recruit sufficient family trios to provide adequate power.

**Linkage studies**

An alternative strategy, genome-wide linkage analysis, has been very successful in monogenic disorders. In this approach, markers distributed across the genome are genotyped in large family pedigrees. Markers that occur more commonly in family members with nephropathy may indicate the presence of a nearby susceptibility variant travelling with the marker along the same chromosome. As large pedigrees in which many members exhibit diabetic nephropathy are difficult to ascertain, an alternative is to examine the observed versus the expected rate of marker sharing by diabetic sib-pairs who are either concordant or discordant for nephropathy. A major advantage of this approach over candidate gene analysis is that it can detect chromosomal regions that harbour genes that were not previously implicated in the pathogenesis of nephropathy.

Although this approach has proved successful in identifying genomic regions containing susceptibility variants in both type 1 diabetes [21] and inflammatory bowel disease [22], in general it has been less effective than for monogenic disorders. This is largely because it is less powerful in detecting variants with modest effect on phenotype and therefore prohibitively large family pedigrees or numbers of sib-pairs are required [20]. For example, several thousand affected sib-pairs would be required to attain adequate power to identify gene variants conferring a genotype relative risk of two at a minor allele frequency of 0.05. This is in contrast to association studies, which can provide adequate statistical power under the same parameters using approximately 1000 cases and 1000 controls, even when adopting a stringent significance level (5 \( \times 10^{-8} \)) [17]. Only two linkage analyses have been performed in diabetic nephropathy to date, and these have arguably been inadequately powered or employed a highly selected population, e.g. Pima Indians [23,24].

**Recent advances**

Several recent advances have improved the quality of genetic studies. An important prerequisite is the establishment of large multi-centre collections such as the Diabetes UK Warren 3 collection [25], the FinnDiane study [26], the Juvenile Diabetes Research Foundation Genetics of Kidneys in Diabetes (GoKinD) study [27] and the Family Investigation of Nephropathy and Diabetes (FIND) initiative [28]. International collaborations involving the use of such collections will improve the statistical power of association studies in diabetic nephropathy beyond that obtainable in even the largest individual centre.

The identification of several million SNPs as part of the human genome project and the development of high-throughput, low-cost genotyping technologies will significantly reduce the time and cost required for a comprehensive analysis of candidate gene variants. Additional improvements in efficiency have been generated from the concept of haplotype-tag SNPs (htSNPs) [29]. SNPs located in close proximity along the same chromosome are in linkage disequilibrium, that is, they tend to be inherited together within a given population as a single unit or haplotype, due to the low probability of recombinination between them (Table 1). Hence for genotyping purposes many SNPs will be redundant and all the common variation along that haplotype may be captured by genotyping a much smaller proportion of variants, the htSNPs.

Gene expression profiling has been used to identify genes that are up-regulated or down-regulated within cells under varying conditions. This approach is increasingly being utilized to dissect the molecular basis of disease, thereby generating large numbers of new candidate disease-susceptibility genes. An example pertinent to diabetic nephropathy is that over 200 genes are differentially expressed in mesangial cells cultured in high compared with normal extracellular glucose conditions [30]. The results from different strategies may be analysed together to rationalize selection of an attractive

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candidate gene. For example, caldesmon, a gene up-regulated in mesangial cells in high extracellular glucose [30], is located on chromosome 7q35 in a region previously linked to susceptibility to diabetic nephropathy in two independent genome-wide screens [23,24]. We identified that a polymorphism in the promoter region of the caldesmon gene is associated with type 1 diabetic nephropathy in the Irish population [14]. This association has been replicated in an independent population [31], and warrants further analysis.

Future directions

There is a strong argument for directing future resources towards a high-density genome-wide SNP-based association screen in diabetic nephropathy, employing large numbers of cases and controls. Although genome-wide screens will be exceptionally expensive, by adopting a systematic approach they are likely to be more cost-effective per true positive identified than candidate gene analysis. The availability of three major resources has made such screens increasingly feasible: high-throughput, low-cost genotyping technologies, a sufficiently dense SNP marker set and DNA collections such as FinnDiane, Warren 3, GoKinD and FIND. However, in view of the expense involved, it is imperative that due consideration is given to study design in advance.

Careful selection of SNP markers may improve cost-effectiveness. Focusing on variants located in the coding regions of genes, rather than including variants distributed randomly across the genome would reduce the number of SNP markers required to 30,000–60,000. This, however, assumes that coding variants are more likely to influence disease than intronic variants. Although true for monogenic disorders, this may not be the case for complex disease in which more subtle changes may be important, such as altered gene expression due to variation in key regulatory regions. An alternative approach is to employ tag SNPs and this will be facilitated by the recent completion of phase one of the HapMap, which identified patterns of linkage disequilibrium in four ethnic groups and determined the tag SNPs required for such a screen. Preliminary data has shown that 75–90% of all SNPs will be redundant as they are in close correlation with another SNP lying along the same chromosome, thereby reducing the total number of markers required by a factor of ten [32]. While this approach will capture most of the common (>5% minor allele frequency) variation in the genome, it will not detect association between rare variants and common disease.

 Genome-wide screens should only be attempted if they are sufficiently powered to detect gene variants that confer at least a moderate risk of nephropathy. For instance, approximately 500 cases and 500 controls would be required to provide 80% power to detect an allelic odds ratio of two at a minor allele frequency of 0.1 and at an appropriate level of significance for a genome-wide screen [33]. However, many nephropathy susceptibility variants will confer a much lower magnitude of risk, necessitating larger patient collections. For example, approximately 2000 cases and 2000 controls are required to detect an allelic odds ratio of 1.5 using the same parameters as aforementioned. In addition, the number of patients required increases exponentially as the minor allele frequency decreases. This has major implications if rare variants, with minor allele frequencies much <5%, are responsible for the majority of genetic predisposition to nephropathy. However, the relative contribution of rare vs common variants to disease susceptibility is, as yet, uncertain [33]. Hence, it can be seen that in any genome-wide study there will be a trade-off between efficient genotyping and the ability to detect gene variants with relatively modest effects on disease susceptibility and with lower minor allele frequencies.

Due to the large number of SNPs genotyped, genome-wide screens will identify many potential disease-susceptibility variants, most of which will be associated with the disorder by chance (for example, in a screen employing 100,000 SNPs, one would expect 5000 SNPs to be associated by chance alone at the 5% confidence level). The risk of false positives may be minimized by adjusting for the number of SNPs genotyped using the Bonferroni factor. In the above example, adoption of a significance threshold of $P = 5 \times 10^{-7}$ for individual SNPs would result in a genome-wide false positive rate of 1 in 20, the accepted level of confidence for an individual genotype. However, as many of the SNPs tested will be in linkage disequilibrium, each test performed will not be truly independent, therefore this correction is likely to be too rigorous.

Consideration should be given to strategies that minimize genotyping costs while maintaining adequate statistical power such as the staged design illustrated in Figure 1. In the first stage, the full complement of SNPs markers is examined in a subset of cases and controls. The majority of SNPs tested would not be associated with the disease being assessed (e.g. diabetic nephropathy) and would not be examined in subsequent stages of the study design [34]. The threshold of significance for passing markers in the first screen would presumably be set low (e.g. a liberal $P$-value of $<0.01$), so that it maintained sufficient power to detect loci with a relatively modest effect on disease despite employing a fraction of the total number of samples available. The ability to identify susceptibility variants in this initial screen could be enhanced by including only extreme phenotypes: cases with onset of nephropathy after a relatively short duration of diabetes or despite good glycaemic control; and conversely controls who have not developed nephropathy despite a long duration of diabetes or poor glycaemic control. The majority of SNPs showing statistically significant association with the disease of interest in the initial screen are then reassessed in a second stage, employing a larger number of cases and controls. A joint analysis using combined stages 1 and 2 data is then performed for these SNPs thereby improving cost efficiency without sacrificing much of the statistical power [35,36].
In a screen employing a two-stage strategy, over 80,000 SNP markers were genotyped in a Japanese population with type 2 diabetes, to identify two potential nephropathy susceptibility genes: a sodium-chloride co-transporter known to be mutated in Gitelman syndrome [37] and the ELMO1 gene, which is up-regulated in a high glucose environment and promotes accumulation of extracellular matrix proteins [38]. These interesting findings have yet to be replicated in other populations, but if validated they could represent attractive novel therapeutic targets.

**Conclusion**

While the early promise of the human genome project has yet to be realized, technological advances have permitted dissection of the molecular basis of disease and made genome-wide screens with high-density SNP markers feasible. In order to capitalize fully on these advances, multi-centre precisely phenotyped patient collections will be required. Hence, by combining the expertise of geneticists and clinicians, there is real hope that the genetic basis of diabetic nephropathy and other complex renal diseases may be unravelled offering new opportunities for screening and therapeutic intervention.

**Acknowledgements.** BRC was supported by the Northern Ireland Kidney Research Fund and the Juvenile Diabetes Research Foundation.

**Conflict of interest statement.** None declared.