Original Article

Parallel Genotyping of 10,204 Single Nucleotide Polymorphisms to Screen for Susceptible Genes for IgA Nephropathy

Keng Thye Woo,[1]FRACP, Yeow Kok Lau,[1]PhD, Kok Seng Wong,[1]FRCP, Yi Zhao,[2]PhD, Choong Meng Chan,[1]FRCP

Abstract

Introduction: IgA nephritis (IgAN) is the most common glomerulonephritis worldwide. We aim to genotype SNPs (single nucleotide polymorphisms) genomewide in patients with IgAN to search for genetic clues to its aetiology. **Materials and Methods:** Genotyping for 10,204 SNPs genomewide was done with the Gene Chip Human Mapping 10K Microarray (Affymetrix). Twenty-eight patients with IgAN and 30 normal subjects were screened and analysed for differences in genotype frequency, allele frequency and heterozygosity reduction. **Results:** Among the most significantly associated SNPs, 48 SNPs were found mapping directly to the intron of 42 genes that localised in 13 somatic chromosomes and chromosome X. Genotype distribution of these SNPs did not deviate from the Hardy-Weinberg equilibrium in normal subjects. The most significantly associated gene, glial cells missing homolog 1 (GCM, χ^2 =13.05, P = 0.000) is a transcription factor mapped to 6p12.2. GCM1 reported decreased in placenta of patients with pre-eclampsia. The second gene, Tenascin-R (TNR, χ^2 = 9.85, P = 0.002) is a glycoprotein and extra-cellular matrix component mapped to 1q25.1. Tenascin-R was associated with motor coordination impairment and enhanced anxiety profile in deficient mice. Interestingly, Triadin (TRDN, χ^2 = 9.16, P = 0.01) is an integral membrane protein mapped to 6q22.31 within the IgAN1 locus. Triadin was shown to participate in cardiac myocyte arrhythemia. However, there is no published study of these genes in IgAN. **Conclusion:** Forty-two associated genes (particularly GCM1, TNR and TRDN) are identified as possible susceptibility or marker genes for IgAN. Knowledge of their mesangial expression and binding capacity for IgA-containing complexes may help elucidate the pathogenesis of IgAN.

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Introduction

Worldwide, IgA nephropathy (IgAN) is now recognised as the most common form of primary glomerulonephritis.[1,2] It is an important cause of chronic kidney disease and up to 30% to 40% of patients progress to end-stage renal failure within 20 years after diagnosis.[3] Its causes remain unknown and treatment is symptomatic.[4] The lesion is characterised by the predominant deposition of IgA in the mesangium and para-mesangial regions. These deposits may be immune-complexes with a wide range of antigens. They may be aggregates of abnormal IgA1 with aberrant galactosylation in the hinged-region.[5] Such depositions conceivably activated the renal cells to express various cytokines and growth factors.[6] The results are mesangial cell proliferation and expansion of the mesangial matrix,

both characteristic histological features of the disease. Damage to the glomerular structure can lead to leakage of serum proteins and red blood cells giving rise to proteinuria and haematuria which are frequently observed in the urine of patients with IgAN. The proteinuria, if heavy and left unchecked, will lead to further damage in the tubules and progression to end-stage renal failure.[7,8]

A locus for familial IgAN called "IgAN1" on chromosome 6q22-23 had been described in man[9] and in mice.[10] The European IgAN Consortium in a genome-wide scanning of 22 multiplex families identified 2 regions with the strongest evidence of linkage, 4q26-31 and 17q12-22.[11] However, no identification of any responsible gene was made. Nevertheless, there is great potential in genetic studies that might reveal new insights in the aetiology and

[1] Renal Medicine Department, Singapore General Hospital, Singapore
[2] Clinical Research Department, Singapore General Hospital, Singapore
Address for Correspondence: Dr Keng Thye Woo, Department of Renal Medicine, Singapore General Hospital, Outram Road, Singapore 169608.
Email: woo.keng.thye@sgh.com.sg

mechanism of pathogenesis. New data in this area may suggest more specific approaches in seeking preventive measures and better treatment for IgAN. The GeneChip Human Mapping 10K Xba 142 Array genotypes 10,204 single nucleotide polymorphisms (SNPs) on a single array allows rapid, accurate, cost-effective and whole-genome scan for susceptibility genes in the genetic study of diseases. Using this chip, we had screened 30 patients with biopsy proven IgAN and 30 normal subjects for differences in their genetic composition.

Materials and Methods

Clinical Samples

For this study, 30 patients with biopsy-proven IgAN and 30 healthy normal subjects were enlisted. To eliminate any difference in ethnic groups, only Chinese were selected. Prior ethical approval had been obtained from the hospital research ethics committee before blood sampling for this study. Patient's consent was also obtained at the time when each peripheral blood sample (2 mL EDTA-Blood) was taken.

Choice of Methodology

GeneChip system from Affymetrix was the first of its kind to be launched in the market.

The GeneChip® Human Mapping Assay is a well-established product from a series of GeneChip systems. It is a powerful tool for genome-wide SNPs screening and provides a widespread in terms of genomic coverage, giving researchers the ability to detect SNPs on a genome-wide basis. It was the best available at the time the research was performed, costing about $1000 Singapore dollars per patient, still relatively expensive, but able to detect more than 10,000 SNPs in a patient's sample. This was about 2 years ago.

The GeneChip® Human Mapping Assay is easy to use and cost much less compared to other products in the market. In addition, the amount of DNA required for the test is much less compared to other similar products. Other systems now available and much more efficient but more costly are: SNP genotyping and CNV Analysis (Affymetrix) for genome-wide human SNP array 6.0 and 5.0, mapping for 500,000 array set and 100,000 array set. Yet others are: Targeted Genotyping like Universal 70,000 tag array, 25,000 tag array, 10,000 tag array, 5000 tag array and 3000 tag array. The targeted ones cost less compared to the genome-wide ones.

Another important reason for the choice of the GeneChip® Human Mapping Assay is the pre-existing Affymetrix GeneChip detection systems already in place in the Singapore General Hospital, including the GeneChip hybridisation oven, fluidics station and scanner.

Methodology

10K GeneChip® Mapping Array Genotyping

Genomic DNA was extracted using standard procedures. Whole-genome scan was performed with the Affymetrix GeneChip Human Mapping 10 K Xba 142 Array.

In short, 250 ng of genomic DNA was digested with 10 units of Xba I (New England Biolabs, Beverly, MA) for 2 hours at 37°C. Adaptor Xba (P/N 900410, Affymetrix, Santa Clara, CA) was then ligated onto the digested ends with T4 DNA Ligase for 2 hours at 16°C. After dilution with water, samples were subjected to PCR using primers specific to the adaptor sequence (P/N 900409, Affymetrix) with the following amplification parameters: 95°C for 3 minutes initial denaturation, 95°C for 20 seconds, 59°C for 15 seconds, 72°C for 15 seconds for a total of 35 cycles, followed by 72°C for 7 minutes final extension. PCR products were then purified and fragmented using 0.24 units of DNase I at 37°C for 30 minutes. The fragmented DNA was then end-labelled with biotin using 100 units of terminal deoxynucleotidyl transferase at 37°C for 2 hours. Labelled DNA was then hybridised onto the 10K Mapping Array at 48°C for 16 to 18 hours at 60 rpm by hybridisation oven.

The hybridised array was then washed, stained with streptavidin phycoerythrin and biotinylated antibody on fluidics station. After washing and staining, the arrays were scanned with Affymetrix 3000 scanner according to the manufacturer's instructions.

The data from the scanning were saved with CAB file and analysed with GDAS and GDOS software to generate genotype call for each of the SNP probe on the array.

The values from SNP Call, signal detection, alleles call frequency and 4 QC performance probe (AFFX-5Q-123, AFFX-5Q-456, AFFX-5Q-789 and AFFX-5Q-ABC) from samples CEL data were tested for array QC performance control. For all of the sample data collected, the value of SNP Call was higher than 90%, and the average value was among 96% to 99%. The percentage of signal detection was around 99%.

Using GDAS analysis, SNP call from 10 K SNP probe was obtained for each array.

Statistics

Three analysis methods were used to identify the SNPs that are significantly different in frequency between patients and normal subjects. Chi-square tests are applied to all SNPs for difference in genotype frequency distributions and allele frequencies. The fixation index, F_{ST} measures overall reduction in heterozygosity among the patient and normal subpopulations. Microsoft Excel is excellent for handling genotype-call data for the 10,204 SNPs. Data patients and normal subjects were imported into Excel files for processing

and statistical analysis. SPSS for Windows was used to confirm chi-square calculations done in Excel. SPSS was also used to perform Odds Ratio analysis and paired-t test for allele and heterozygote frequency comparison between our data and Affymetrix data for Asians.

Allele and Genotype Counts

Genotype and allele counts were first established by inserting conditional functions in the first row for the first SNP. The functions were then replicated with instant results down the column for the 10,204 SNPs.

Chi-square Test for Difference in Genotype Frequency Distributions

Counts of the 3 genotypes in patients were compared to corresponding counts in normal subjects. The chi-square statistic was calculated with the formulas for fit of class to sample given in page 50 of Statistics at Square One.[12] Formulas were inserted into cells in the first row to automate calculation for the first SNP. The formulas were then replicated down the columns for all the remaining SNPs with instant results. Sorting by chi-square values in descending order, the top 100 SNPs with the most significant differences between patient and normal groups were identified for further analysis. Accuracy of chi-square calculation for these SNPs were verified by re-calculation in SPSS for Windows.

Chi-square Test for Difference in Allele Frequencies

Counts of the 2 alleles in patients were compared with corresponding counts in normal subjects. The special form of chi-square test for a 2 x 2 contingency table was given in Statistics at Square One.[12] The procedure was same as described above for comparing genotype frequency distributions.

Heterozygosity Reduction

The fixation index, $F_{ST} (=\{H_T + H_S\}/H_T)$ measures overall reduction in heterozygosity. It is a measure of genetic difference among subpopulations.[13] H_S is the expected heterozygosities in subpopulations (patients, normals) given by $(H_{expIgAN} \times N_{IgAN} + H_{expNormal} \times N_{Normal})/(N_{IgAN} + N_{Normal})$ where $H_{exp} = 1- (p^2 + q^2)$. H_T (given by $1-[p^2 + q^2]$) is the expected heterozygosities for overall total population (patients and normals combined). $F_{ST} > 0.05$ suggests significant genetic differentiation. Accuracy of calculation in Excel was confirmed with published data and results.[14]

Hardy-Weinberg Equilibrium

Genotype distributions in normal subjects were tested for deviation from Hardy-Weinberg equilibrium to confirm homogeneity of the population under study and proper

genotyping procedure. The Hardy-Weinberg (HW) principle states: gene and genotype frequencies remain constant between generations in an infinitely large, random mating population. Accordingly, the expected frequencies of genotypes (E_{AA}, E_{Aa}, E_{aa}) are given by p2, 2pq and q2. The allele proportions p and q are calculated from observed genotype frequencies ($P = (2O_{AA} + O_{Aa})/2N, q = (2O_{aa} + O_{Aa})/2N$, N is the total number of subjects studied). Departure from HW equilibrium is examined by the chi-square test for goodness of fit between observed frequencies and calculated expected frequencies. Probability of deviation was looked up in a chi-square distribution table at 1 degree of freedom for 2 alleles.[15]

Correlation Analysis to Estimate Linkage between Alleles of 2 SNPs

Alleles of 2 SNPs in random association are said to be in linkage equilibrium. Conversely, alleles that are inherited together are said to be in linkage disequilibrium (LD). From genotypic data, it is not possible to determine exact values for LD. In studies of LD, gametic frequencies of alleles at two loci $P_{11}, P_{12}, P_{21}, P_{22}$ can only be estimated using complex statistical procedures. However an impression of the degree of linkage between alleles at 2 loci may be gained by a simple correlation test. With the A and B alleles taking the value of 1 and 2 respectively, the sum values of 2, 3 and 4 may be assigned to the AA homozygote, AB heterozygote and BB homozygote respectively at each locus. Correlation r = 1 or -1 indicates absolute linkage.

Selection of 48 SNPs and 42 Associated Genes

From an initial number of 10,204 SNPs tested, a pool or 300 SNPs were derived by choosing those with the most significant statistical differences between the patients with IgA nephritis and the normal subjects (controls). These 300 were further reduced to 173 when those that were called by more than one analysis were excluded. At <80% genotype call rate, 12 more SNPs were excluded. Of the remaining 161 SNPs left, annotation data revealed that only 48 SNPs were mapped directly to the intron of 42 genes.

Results

Genotype data were successfully obtained from 28 of the 30 IgAN patients and from all 30 normal subjects for further analysis. Patients and normal subjects were compared and analysed for differences in genotype distribution, allele frequency and level of heterozygosity reduction. Among all the 10,204 SNPs tested, each analysis identified 100 SNPs with the most significant differences. From the pooled 300 SNPs, the unique number of SNPs was reduced to 173 as many were called by more than one analysis. At <80% genotype call rate, 12 more SNPs were excluded. Of the

remaining 161 SNPs, annotation data (from Affymetrix. com) revealed only 48 that mapped directly to the intron of specific genes. There was no SNP mapping to an exon. The 48 SNPs were tabulated in Table 1 with their genotype counts and results of all the 3 analyses. Allele frequency chi-square values were consistent with F_{ST} values. However genotype frequency chi-square values are not consistently in accordance with both the former values. Hence the chi-square values for allele frequency were favoured as a measure of significance of association for ranking genes in descending order. The genotype distribution in all 48 SNPs did not deviate from the Hardy Weinberg equilibrium in the normal subjects, confirming the genetic homogeneity and proper genotyping procedure of this study. The lowest allele frequency is 6% in the total study cohort (a variant is a SNP when the frequency is 1% or more in the population). The allele and heterozygote frequency of these SNPs in our normal Chinese subjects were similar to the population frequency data for Asians that Affymetrix reported in their validation studies [paired-t test: Allele frequency, 0.48+0.21 (observed) vs 0.48+0.24 (Affymetrix.), n = 48, P =0.89; Heterozygote frequency, 0.37+0.18 (observed) vs 0.41+0.10 (Affymetrix.), n = 48, P = 0.09].

Annotation data were extracted from the Affymetrix database. Table 2 shows the 48 most significantly associated with SNPs, each mapped to specific chromosome, cytoband, physical position and the gene identified at the intron locus. Six SNP-pairs were mapped to the same gene. The 2 SNPs of each of the 6 SNP-pairs are very close, only 36 to 668 base-pair apart, hence recombinant events between SNPs of each pairs are unlikely. In accordance, correlation analysis of genotype distribution gave values of r=1 or -1 only, demonstrating that 2 members of each SNP-pair were in absolute linkage. This was observed in both the patient and normal groups. The observation also attested to the reliability and reproducibility of the 10K Mapping procedure of Affymetrix. The 42 genes are located in chromosomes 1 (7 loci), 2 (3 loci), 3 (5 loci), 5 (1 locus), 6 (4 loci), 7 (1 locus), 9 (1 locus), 10 (4 loci), 11 (4 loci), 14 (4 loci), 16 (2 loci), 18 (2 loci), 20 (2 loci) and X (2 loci). In total, associated genes were localised in 13 of the 22 somatic chromosomes and in the sex chromosome at 42 loci.

With the accession number of identified genes, the National Center for Biotechnology Information (NCBI) website was visited to search the Nucleotide database for information on the 42 gene products. While 8 gene products were hypothetical proteins, the majority of gene products (18) were component of integral to membrane. Three were parts of cyto-skeleton, 1 was component of extra-cellular matrix and remaining products were extra-cellular or intracellular components. The majority of these products had binding functions to metal ions, protein, nucleotide,

DNA, histones, ATP, electron carrier and many other life functions. In addition, some are receptors and others had enzyme activities. The biological processes involved were wide-ranging and included transcription regulation, morphogenesis, signal transduction, metabolism, cell cycle progression, cell proliferation, cell-cell and cell-matrix interaction, neurogenesis and other cellular activities.

Clinical relevance for the 42 genes was obtained from literature search in the PUBMED database at the NCBI website. In chi-square analysis of allele frequencies, the most significantly associated gene was Glial cells missing homolog 1 (Drosophila) (GCM1, χ^2 = 13.05, P = 0.000), a transcription factor mapped to chromosome 6p12.2. The second gene was Tenascin-R (TNR, χ^2 = 9.85, P = 0.002), a glycoprotein component of extracellular matrix mapped to chromosome 1q25.1. By chi-square analysis of genotype frequencies, the most significantly associated gene was a hypothetical gene (ENST00000359835, χ^2 = 21.45, P = 0.000) mapped to chromosome 10q21.3 and the second was GCM1 (χ^2 =13.95, P = 0.001). Among the others, 15 genes had association with various cancer and 7 more were for hypothetical proteins. Several genes were known causes of specific diseases: gene 5 had been linked to non-specific mental retardation in males; gene 21 was the cause of Duchenne Muscular dystrophy; gene 6 was found responsible for autosomal recessive cerebellar ataxia; and gene 38 was associated with uric acid nephrolithiasis. The rest of the genes were involved in normal cell activities such as excitory synaptic transmission, inflammation control, membrane trafficking and protein sorting, modulation of gene expression and regulating the release of neurotransmitters.

The GCM1 and TNR genes reside separately on chromosomes 6 and 1, respectively, and are randomly associated in inheritance. The combined effect of GCM1 and TNR polymorphism on risk of IgAN was examined. Odds ratio (OR) analysis of individual genotype showed that GCM1-B allele increased the risk for IgAN (GCM1-AB, OR = 18.8, P = 0.001) whereas the TNR-B allele is protective from IgAN (TNR-AB, OR = 0.25, P = 0.040). In combination, the GCM1 and TNR polymorphisms did not change the Odds Ratios very much (GCM1-AB:TNR-AA, OR = 16.1, P = 0.002; GCM1-AA:TNR-AB, OR = 0.13, P = 0.011). Of 9 possible combinations, 2 have not occurred and 4 have very low counts.

Discussion

In both patients and normal subjects, absolute linkage between members in all 6 SNP-pairs that mapped to the same gene confirmed reproducibility of genotyping with the GeneChip Mapping 10K Arrays. The low overall error rate of 0.1% had been reported for this GeneChip.[16] The

normal population showed Hardy-Weinberg equilibrium in genotype distribution in all the 48 identified SNPs. Normals also showed allele and heterozygote frequencies that were similar with the Affymetrix frequency data for Asians in all the associated SNPs. These observations showed that the Affymetrix microarray procedure was robust and our study population was homogenous.

There are few genetic studies that search for susceptibility genes in IgAN using high-throughput SNP technologies. A locus for familial IgAN had been described with strong evidence of linkage to "IgAN1" on chromosome 6q22-23.[9] Two other loci were reported at 4q26-31 and 17q12-22.[11] However, none of these reports identified the gene responsible. In a large Lebanese family with 38 members,[17] no evidence was found of linkage to the loci, 2q36, 4q22-31 and 6q22-23. In our study, 6 SNPs were mapped to chromosome 6. The SNP-pair (Affymetrix Prob Set-ID: SNP_A -1510472 / SNP_A-1519262) mapped to Triadin (TRDN) at 6q22.31 which is within the IgAN1 region of 6q22-23. There were 3 SNPs mapping to chromosome 2 but none to the region of 2q36. No associated SNP mapped to chromosomes 4 and 17. Thus besides the IgAN1 locus, association with other reported loci had not been detected here. However, we had only screened 10,204 SNPs or about 0.3% of the estimated 3 million SNPs in the whole human genome of 3 billion base pairs. Therefore it is highly likely that the chromosome regions of these known loci may not be covered by the SNPs included in the GeneChip Human Mapping 10K Array.

Detected by all 3 analyses, the most significantly associated gene identified is glial cells missing homolog 1 (GCM1, a neural transcription factor in Drosophila) which is expected to regulate gliogenesis. However in mice, GCM1 is placenta-specific and is necessary for placental development. Its absence causes abnormal placental labyrinth formation that resulted in embryo death.[18] In human beings, decreased placental GCM1 expression was found in pre-eclampsia and may contribute to the aetiology.[19] A complication of pregnancy, pre-eclampsia shared common features with the IgAN lesion. Both are believed to be multisystem disorders with the presence of proteinuria and elevated blood pressure. GCM1 has not been investigated in IgAN. Tenascin is a large glycoprotein component of the extracellular matrix. It increases rapidly after inflammation or injury. For this reason, Masaki et al[20] investigated and reported that Tenascin protein expression may be an indicator of chronicity and Tenascin mRNA expression may be an indicator of disease activity in IgAN. However in this early study, it was not specified which member of the protein family was involved. Also detected by all analyses, the second most significantly associated gene is Tenascin-R, a family member known to express exclusively in the central nervous system. Its deficiency in mice caused motor coordination impairment and consequently an enhanced anxiety profile in elevated plus maze test.[21] Specifically, family member Tenascin-R has not been investigated in IgAN. Triadin (TRDN) is detected by genotype analysis only, ($\chi^2 = 9.16$, $P = 0.02$) and is 31st on the list of associated genes. It is an integral membrane protein with a role in cardiac myocyte arrhythmia.[22] Though mapped to the IgAN1 locus, whether it is the gene responsible or just a marker of gene close-by remains to be investigated.

Examination of the combined effects of GCM1 and TNR gene polymorphisms showed no great synergistic effects on the strength of association. This is may be because the 2 polymorphisms are on separate chromosomes and are randomly linked in inheritance. This observation seems to support the hypothesis that IgAN is a complex disease probably with pathology involving the interactions of many genes[23] and environmental factors such as infections and dietary antigens.[24,25] With significant association demonstrated (at significant level $P < 0.05$), all 42 identified genes are legitimate targets for further study in IgAN. A unique feature of IgAN is the mesangial binding and deposition of IgA immune-complexes or abberant IgA molecules with defect in O-glycosylation. In this respect, it is noted that many associated genes code for proteins are integral of membranes, cytoskeleton or extra-cellular matrix, many with protein-binding properties. These genes have not been studied in IgAN. It would be of interest to study their expression in the mesangium and to test their binding capacity for IgA-containing complexes. Nevertheless, priority should go to GCM1 and TNR, the top 2 genes with the highest significant values from all 3 analyses. GCM1 and TNR may have better chances of success in being established as genetic markers of disease or having a role in the aetiology. At the rate of 2 per 10,204 SNPs, approximately 600 more target genes may be identified from the estimated 3 million SNPs in the human genome for further study. Affymetrix already have a 500K GeneChip in the market for genotyping 500,000 SNPs in one microarray. Computerised analysis technique is available for examining hundreds of proteins and their network of interactions.[26,27] All the tools needed are now at hand to search for genetic clues to the causes of IgAN. In conclusion, 42 associated genes (particularly GCM1, TNR and TRDN) are identified as possible susceptibility genes or as marker genes. Knowledge of their mesangial expression and binding capacity for IgA-containing complexes may help to elucidate the pathology of IgAN.

Conclusion

At the end of our search, we found among the patients with IgA nephritis 48 SNPs which mapped directly to the

intron of 42 genes which were significantly different from normal healthy controls. This offers an avenue for further investigation of the 42 genes which may be associated with IgA nephritis. Future studies may show that some of these associated genes may play a role in the pathogenesis of IgA nephritis, the most common form of glomerulonephritis of unknown etiology with an elusive cure. This is a pilot study on a small group of patients. What is needed is a larger cohort of patients with much funding to study a wider array of genes. A large consortium may have to be engaged for this.

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