DNA hypermethylation and DNA hypomethylation is present at different loci in chronic kidney disease

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Genetic risk factors for chronic kidney disease (CKD) are being identified through international collaborations. By comparison, epigenetic risk factors for CKD have only recently been considered using population-based approaches. DNA methylation is a major epigenetic modification that is associated with complex diseases, so we investigated methylome-wide loci for association with CKD. A total of 485,577 unique features were evaluated in 255 individuals with CKD (cases) and 152 individuals without evidence of renal disease (controls). Following stringent quality control, raw data were quantile normalized and β values calculated to reflect the methylation status at each site. The difference in methylation status was evaluated between cases and controls with resultant P values adjusted for multiple testing. Genes with significantly increased and decreased levels of DNA methylation were considered for biological relevance by functional enrichment analysis using KEGG pathways in Partek Genomics Suite. Twenty-three genes, where more than one CpG per loci was identified with $P_{adjusted} < 10^{-8}$, demonstrated significant methylation changes associated with CKD and additional support for these associated loci was sought from published literature. Strong biological candidates for CKD that showed statistically significant differential methylation include *CUX1*, *ELMO1*, *FKBP5*, *INHBA-AS1*, *PTPRN2*, and *PRKAG2* genes; several genes are differentially methylated in kidney tissue and RNA-seq supports a functional role for differential methylation in *ELMO1* and *PRKAG2* genes. This study reports the largest, most comprehensive, genome-wide quantitative evaluation of DNA methylation for association with CKD. Evidence confirming methylation sites influence development of CKD would stimulate research to identify epigenetic therapies that might be clinically useful for CKD.

Introduction

Chronic kidney disease (CKD) with multifactorial etiology leading to end-stage renal disease (ESRD) is a significant public health concern with increasing numbers of individuals affected worldwide.[1-3] CKD is defined as an estimated glomerular filtration rate (eGFR) <60 mL/min/1.73m^2.[4] ESRD (also known as stage 5 CKD), is defined as an eGFR <15 mL/min/1.73m^2, and is the most severe form of CKD; many individuals with ESRD require renal replacement therapy, such as dialysis or kidney transplantation. In 2010, 50 965 adult UK patients received renal replacement therapy reflecting a UK ESRD prevalence of 832 per million population and a 63% increase in renal replacement therapy population over the past decade.[5] Currently, more than half of UK healthcare costs for CKD are spent on renal replacement therapy.[6] CKD leads to reduced quality of life for affected individuals and is associated with an increased risk of premature death.[7]

There is an urgent need to identify biological markers that help identify individuals who are at higher risk of developing CKD so that targeted therapies can be used to delay the onset or progression of CKD. An inherited predisposition to CKD exists, and research to date has focused on common genetic variants in large cohorts of individuals.[8-10] Nevertheless, currently identified genetic markers account for a limited component of the heritability for this disorder. More integrated strategies that explore the interaction between an individual's genome and environmental stimuli are required to determine inherited risks for CKD.[11-13] It is hypothesized that the genetic background interacts with multiple potential modifying stimuli including intrauterine environment, nutrition, toxins, pathogens, and drugs, to influence the risk of development and progression of CKD.[14] While it is possible that rare genetic variants, copy number variation, or mitochondrial mutations account for some of the "missing heritability," it is also plausible that epigenetic phenomena may be critical determinants of CKD.[15,16]

Emerging evidence for epigenetic phenomena has revolutionized investigations of heritable influences on disease and, complementary to GWAS, it is now cost-effective to perform population-based studies of the epigenome (EWAS).[17-19] Epigenetic changes have been associated with CKD[15,16,20-22] and the development of novel genetic-epigenetic risk profiles are key translational medicine priorities for targeted therapies (**Fig. 1**).[15,23,24] Drugs that directly target the epigenome are already in clinical use for the treatment of cancer and, potentially, such drugs may have efficacy in the treatment of CKD.[25]

Methylation is a key epigenetic feature of DNA that plays an important role in chromosomal integrity and the regulation of gene expression with different methylation profiles now being associated with many complex diseases.[26] DNA methyltransferases are required for the process of DNA methylation and sequence variants in these genes have been associated with diabetic kidney disease.[27] Preliminary research evidence provides support for the importance of differential methylation in CKD.[28-30] We describe a relatively large-scale case-control study that aimed to quantitatively identify changes in DNA methylation with single site resolution associated with CKD.

Results

Samples

Methylation status was determined for 485 577 unique sites in a total of 407 individuals (cases with CKD, n = 255; controls with no evidence of renal disease n = 152). There were no significant differences for age (36.2 ± 12.0 vs. 36.0 ± 11.8 y) or gender [132 (52%) vs. 76 (50%) males] between case and control groups, respectively, with slightly more males present in the case group. Additional clinical characteristics are provided in **Table S1**. The 113 individuals with type 1 diabetes had at least 15 y of type 1 diabetes with no evidence of kidney disease (controls) and were compared with 113 individuals with persistent proteinuria and type 1 diabetes (cases) using the same inclusion criteria as our recent meta-analysis of GWAS.[10]

Quality control

Following stringent quality control and analysis, significantly associated differences in methylation status were observed between 255 individuals with CKD compared with 152 control individuals with no evidence of renal disease. Greater than 99% concordance was observed between duplicate samples ($r^2 > 0.98$ for each of the eight pairs of samples), and experimentally defined genders matched each individual submitted for analysis based on Y-chromosome specific loci.

DNA analysis

Statistically significant association was observed for 399 unique genes in top-ranked markers with $P < 10^{-8}$. Focusing on top-ranked clusters of associated CpG sites revealed 23 genes, where more than one CpG per gene was identified with $P_{adjusted} < 10^{-8}$ (**Table 1**; **Table S2**). Top ranked genes are involved in various diseases, regulation, signaling, and apoptotic pathways (**Table S3**), and several genes are strong biological candidates for kidney disease including *CUX1*, *ELMO1*, *FKBP5*, *INHBA-AS1*, *PTPRN2*, and *PRKAG2* (**Fig. S1**).
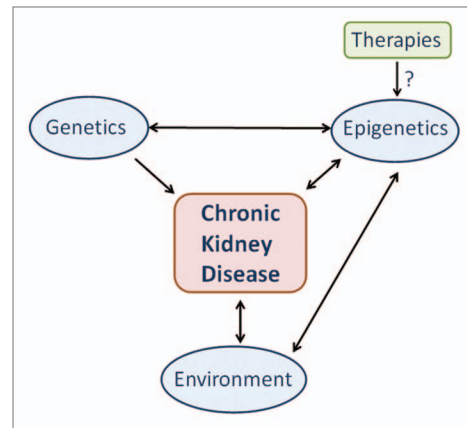
Figure 1. A role for epigenetics for chronic kidney disease.

Subgroup analysis of cases and controls without diabetes revealed significant association with the same top-ranked loci as in the original combined analyses. Enrichment based on gene ontology terms revealed 326 processes with $P < 0.05$ for gene ontology (GO) enrichment when considering CpG sites where $P < 10^{-8}$ for association with CKD; this decreased to 80 when restricted to genes with more than one top-ranked CpG site. Partek pathway analysis identified only three pathways with $P < 0.05$: mucin type O-Glycan biosynthesis ($P = 0.02$), DNA replication ($P = 0.02$), and tryptophan metabolism ($P = 0.03$) (**Tables S4–6**). Minimal interactions have been reported between top-ranked genes (**Fig. S2**).

SNPs were identified within the vicinity of CpG sites for key genes (**Fig. 2**). Fine mapping by genomic and bisulfite treated DNA sequencing confirmed methylation at target sites and also identified two SNPs (rs12673766, *PRKAG2*, minor allele frequency in our population (9% rather than the 4% reported for CEU population in dbSNP and *ELMO1* at 4% minor allele frequency); dbSNP is not presently accepting new SNP submissions so we have included an image of these changes as **Figure S3**. *INHBA-AS1* was not fine-mapped as neither Sanger sequencing (Life Technologies), nor Sequenom Epityper (www.epidesigner.com) primers were successfully designed for bisulfite treated DNA. Examining ENCODE data by means of a regional plot in UCSC's genome browser for *CUX1*, *ELMO1*, *FKBP5*, *INHBA-AS1*, *PTPRN2*, and *PRKAG2* genes revealed that clusters of CpG sites in PRKAG2 overlapped with a DNaseI hypersensitivity cluster (indicating a gene regulatory region) with *ELMO1* and *PTPRN2* also having differentially methylated CpG sites in potentially functional sites.

RNA analysis

Gene expression, based on total RNA extracted from whole blood, was evaluated by RNA-seq in two cases with CKD stage 5 compared with two controls with no evidence of kidney disease. An average of 71% loading was achieved for chip loading (~70 million reads) with 97% successful enrichment, 24% polyclonal reads for ion sphere particles, and a mean read length of 123 bp. Twenty to thirty percent polyclonal reads are expected and these were excluded from further analysis. Samples were

Table 1. Top-ranked 23 genes showing most significant differences in methylation status between case and control groups

Symbol	Description	Number of top ranked CpGs and features	Potential role	Top-ranked CpG	Cytogenetic location	*P* value (delta β)	Function and processes by gene ontology
ANKRD11	ankyrin repeat domain 11	Three; 5′UTR, north and south shelves	Encoded protein inhibits ligand-dependent activation of transcription. Mutations associated with syndromes, including renal dysfunction.	cg26957471	16q24.3	3.67E-12 (0.03)	Development and homeostasis
BANP	BTG3 associated nuclear protein	Three; 5′UTR, CpG island, body and north shelf	Encoded protein binds to matrix attachment regions	cg07018090	16q24	1.70E-10 (-0.36)	DNA binding, cell cycle, chromatin modification, transcription
BRD2	bromodomain containing 2	Three; genic	Located within the MHC region, this gene encodes a transcriptional regulator that associates with transcription complexes and with acetylated chromatin during mitosis, and it selectively binds to the acetylated lysine-12 residue of histone H4	cg01716683	6p21.3	4.68E-10 (0.09)	Chromatin binding and modification, transcription, nucleosome assembly
CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2	Two; enhancer	Not evident	cg01704698	16q24.1	1.53E-09 (0.2)	Extracellular matrix organization, heparin binding
CUX1	cut-like homeobox 1	Two; body, south shore, Promoter associated	Involved with kidney development and dysfunction. May regulate gene expression, morphogenesis, and differentiation, and it may also play a role in the cell cycle progression	cg02858808	7q22.1	3.67E-09 (0.05)	Chromatin binding, DNA binding, differentiation, transcription
DCUN1D2	DCN1, defective in cullin neddylation 1, domain containing 2 (S. cerevisiae)	Two; genic	Upregulated in animal model of transplantation following administration of a methyltransferase inhibitor	cg02128308	13q34	1.11E-09 (0.06)	Induces cell-growth arrest
ELMO1	engulfment and cell motility 1	Two; Enhancer	Encodes a member of the engulfment and cell motility protein family and has been associated with diabetic kidney disease	cg08044454	7p14.1	2.74E-09 (-0.2)	Protein binding, actin cytoskeleton organization, protein signal transduction, cellular component movement and apoptotic process

Delta β is positive for increased methylation in cases and negative for decreased methylation between case and control groups.

Table 1. Top-ranked 23 genes showing most significant differences in methylation status between case and control groups (continued)

Symbol	Description	Number of top ranked CpGs and features	Potential role	Top-ranked CpG	Cytogenetic location	*P* value (delta β)	Function and processes by gene ontology
EXOC3	exocyst complex component 3	Two; body, island	Interacts with the actin cytoskeletal remodeling and vesicle transport machinery	cg17500228	5p15.33	1.57E-14 (0.3)	Protein binding, exocytosis, protein transport
FKBP5	FK506 binding protein 5	Three; 5′UTR, TSS1500, north and south shores, enhancer, promoter associated	Role in immunoregulation and basic cellular processes involving protein folding and trafficking. Mediates calcineurin inhibition	cg00130530	6p21.31	3.33E-10 (-0.09)	Heat shock protein binding, FK506 binding, protein folding, isomerization
GALNT2	UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetylgalacto saminyltransferase 2 (GalNAc-T2)	Two; Enhancer, Promoter Associated	Member of the GalNAc-transferases family and associated with cholesterol	cg23843953	1q41-q42	1.10E-10 (0.08)	Glycan processing, cellular metabolism, protein modification, immunoglobulin biosynthetic process
IFLTD1	intermediate filament tail domain containing 1	Two; genic	Not evident	cg13134916	12p12.1	2.27E-10 (0.2)	Cell proliferation
INHBA-AS1	INHBA antisense RNA 1	Two; body, south shore, enhancer	Long non-coding antisense RNA potentially involved in kidney damage and transplant rejection	cg26478599	7p14.1	2.90E-11 (0.3)	
JARID2	jumonji, AT rich interactive domain 2	Three; enhancer	DNA-binding protein that functions as a transcriptional repressor. Associated with blood pressure and cholesterol	cg12230983	6p24-p23	8.47E-18 (0.1)	DNA binding, chromatin binding, development, cell proliferation, histone methylation, transcription, differentiation
MCM2	minichromosome maintenance complex component 2	Two; body, island, enhancer	Highly conserved mini-chromosome maintenance proteins (MCM) that are involved in the initiation of eukaryotic genome replication. Prognositic marker for kidney cancer	cg13296238	3q21	5.03E-11 (0.1)	ATP binding, DNA binding, replication, histone binding, cell cycle
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Two; enhancer	Component of an important energy-sensing enzyme that monitors cellular energy status and functions by inactivating key enzymes involved in regulating de novo biosynthesis of fatty acid and cholesterol. Associated with CKD	cg10370262	7q36.1	3.67E-09 (0.08)	ATP binding, protein kinase activity, metabolic processes, insulin signaling,

Delta β is positive for increased methylation in cases and negative for decreased methylation between case and control groups.

Table 1. Top-ranked 23 genes showing most significant differences in methylation status between case and control groups (continued)

Symbol	Description	Number of top ranked CpGs and features	Potential role	Top-ranked CpG	Cytogenetic location	*P* value (delta β)	Function and processes by gene ontology
PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	Two; body, north shore	Major autoantigen associated with insulin-dependent diabetes mellitus. Associated with CKD	cg24701780	7q36	8.87E-10 (0.02)	Transmembrane receptor protein tyrosine phosphatase activity, GTPase activity, protein dephosphorylation
SLC6A16	solute carrier family 6, member 16	Two; TSS200, island	Not evident	cg00041047	19q13.33	8.33E-11 (-0.2)	Neurotransmitter transport and activity
STK24	serine/threonine kinase 24	Two; enhancer	Not evident	cg07804470	13q31.2-q32.3	1.50E-10 (0.2)	ATP binding, metal ion binding, protein binding, protein kinase activity, apoptosis, regulation of cell migration, phosphorylation, signal transduction
SYNPO2	synaptopodin 2	Two; enhancer	Not evident	cg13541713	4q26	7.53E-11 (0.2)	14–3-3 protein binding, actin binding
TP53BP2	tumor protein p53 binding protein, 2	Two; Enhancer, Promoter Associated	Regulates apoptosis and cell growth through interactions with other regulatory molecules	cg03992323	1q41	2.10E-09 (0.2)	NFkB binding, SH3 domain binding, SH3/SH2 adaptor activity, protein binding, apoptosis, cell cycle, development, signal transduction
VPS52	vacuolar protein sorting 52 homolog (S. cerevisiae)	Three; body, south shelf and shore	Involved in vesicle trafficking	cg26987613	6p21.3	1.50E-14 (0.1)	Protein binding, protein transport
WDR46	WD repeat domain 46	Two; body, north shore	Not evident	cg16458752	6p21.3	8.13E-10 (0.05)	Molecular function, biological process
ZFP36L1	zinc finger protein 36, C3H type-like 1	Three; body, island, promoter associated	Putative nuclear transcription factor most likely functions in regulating the response to growth factors. Congenital anomalies of the kidney and urinary tract gene	cg08169020	14q22-q24	5.23E-10 (-0.3)	DNA, RNA, transcription factor, and protein binding, differentiation, gene expression, metabolic processes, regulation of mRNA stability, transcription and translation, vasculogenesis

Delta β is positive for increased methylation in cases and negative for decreased methylation between case and control groups.

identified based on their IonXpressRNA barcode and individually analyzed for *CUX1*, *ELMO1*, *FKBP5*, *INHBA-AS1*, *PTPRN2*, and *PRKAG2* genes. None of the key genes demonstrated significant changes in gene expression. However, a non-significant decrease in gene expression was observed for *PRKAG2* and a small increase in expression for the *ELMO1* gene; of interest, these results do correlate with those expected based on the DNA methylation results. In silico comparisons revealed additional supporting data and some conflicting results (**Fig. S4**) depending on the source tissue and phenotypes compared for each population; none of this publicly available data examines a CKD population similar to the one investigated in this study.

Several of the loci highlighted in this study were recently identified as differentially methylated genes in kidney tissue [*ELMO1* (*P* = 0.001), *PRKAG2* (*P* = 0.003), *CUX1* (*P* = 0.001), and *PTPRN2* (*P* = 0.005)] comparing 12 individuals with CKD to 14 healthy controls. *CUX1* (*P* = 0.000 000 01) and *PTPRN2* (*P* = 0.000 000 008) genes were also identified in the same paper evaluating methylation profiles between individuals with and without diabetic kidney disease.[31]

Discussion

Epigenetic modification provides a dynamic link between each individual's genetic background and relevant environmental
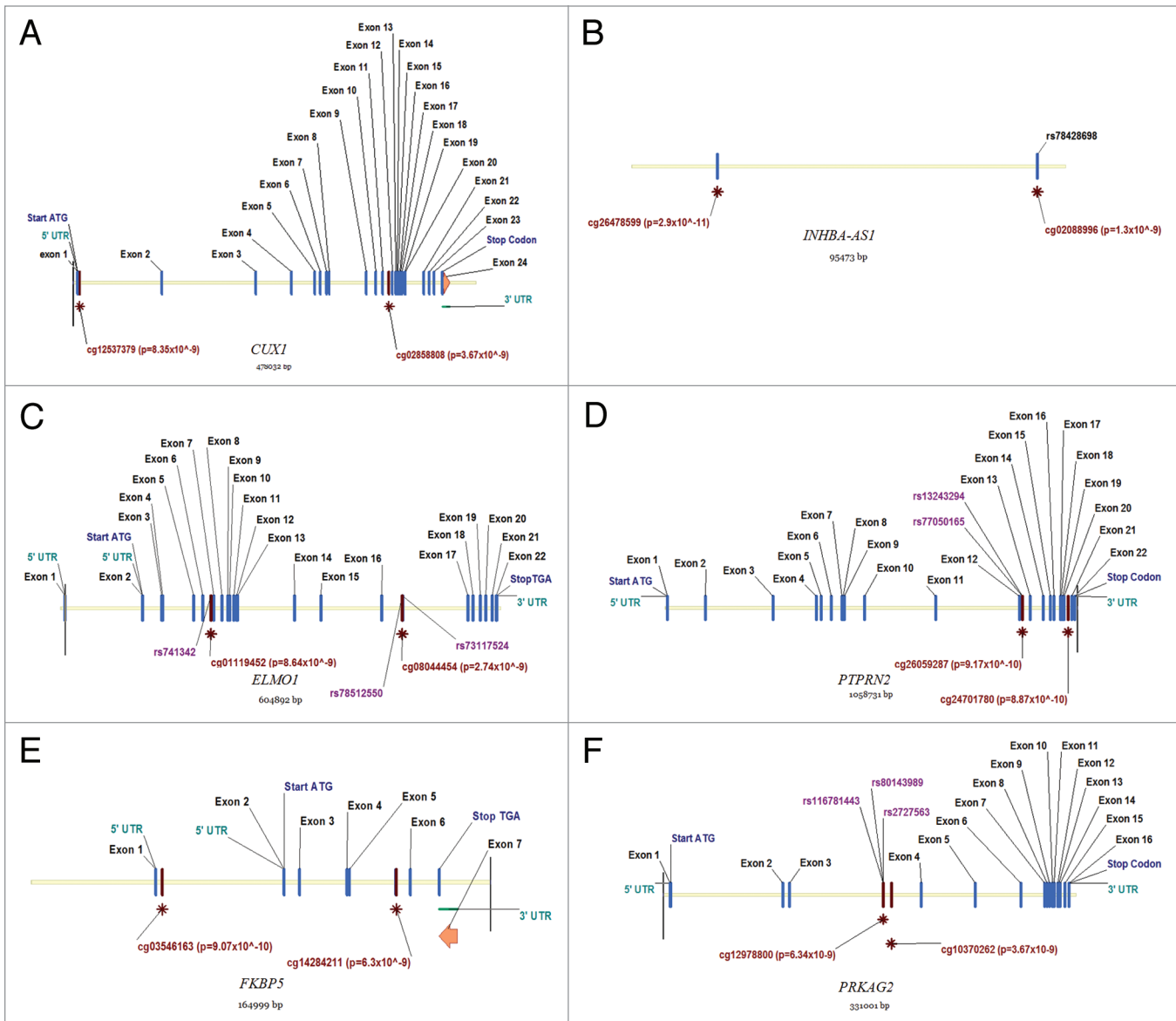
Figure 2. Location of key features for genes that are strong biological candidates for kidney disease including *CUX1*, *ELMO1*, *FKBP5*, *INHBA-AS1*, *PTPRN2*, and *PRKAG2*.

exposures. The results described above suggest that adequately powered genome-wide studies of the methylome are both cost-effective and necessary to improve our understanding of CKD. In particular, this novel data suggests that differential methylation in key genes is associated with chronic kidney disease. When not adjusted for age, gender or cell heterogeneity, the majority of differentially methylated sites were hypomethylated. This is not unsurprising as hypomethylation is associated with aging, in particular aging-associated changes in the relative subpopulations of white blood cell types, and age-related disorders.[32-34] It is interesting to note that Reinius and colleagues[35] observed that methylation in the promoter-related CpG islands tends to be similar among all white blood cell types so that measurements in whole blood is a reflection of true methylation status across subpopulations at those loci; we observed only 10% of differentially methylated loci in

CpG islands (**Fig. S5**). Following adjustment for age and gender, as well as in silico adjustment for the proportions of white blood cell types using Houseman's approach,[36] many of the hypomethylated regions were not sustained. Hypermethylation of genes has been reported in individuals with ESRD[37] and is associated with increased mortality in CKD patients.[38] Consistent with these reports we also observed increased DNA methylation in CKD patients (cases) compared with persons without renal disease (controls). However, it must be highlighted that whole-genome methylation is dependent on the method of experimental analysis and that gene-specific methylation depends on the function of the gene under investigation. For example, anticipated methylation differences were observed between case and control groups for pro and anti-inflammatory markers traditionally associated with CKD, but these were not top-ranked in the association tests.

Top-ranked genes have been reported with roles in disease, regulation, signaling, and apoptotic pathways, however formal Partek pathway analysis identified only three pathways with $P < 0.05$; top-ranked was mucin type O-glycan biosynthesis ($P = 0.02$) and it is of note that mucin-type O-glycosylation is important for kidney development with abnormalities associated with renal dysfunction, abnormal glomeruli, proximal tubules, and renal podocytes.[39-41] Enrichment based on gene ontology also highlighted O-glycosylation with the most significant results being for biological processes protein O-linked glycosylation via serine ($P = 0.002$, GO ID = 18242) and protein O-linked glycosylation via threonine ($P = 0.002$, GO ID = 18243). Analysis of the interaction profile did not reveal anything of note. Of particular interest are the following genes associated with CKD: *CUX1*, *ELMO1*, *FKBP5*, *INHBA-AS1*, *PTPRN2*, and *PRKAG2*. None of the key genes demonstrated significant changes in gene expression based on RNA derived from whole blood, but this is not unexpected as only two case and two control individuals were compared. Nevertheless, it is encouraging that this limited comparison did reveal small changes in gene expression that correlate with our reported DNA methylation status in cases and controls for several genes, including *ELMO1* and *PRKAG2* in particular. The distribution of methylation in cases and controls for *ELMO1* and *PRKAG2* is presented in **Figure S6**.

Comparison with publicly available microarray data sets for gene expression in Nephromine showed conflicting results, although it should be noted that significant differences were observed for *PTPRN2, CUX1*, and *FKBP5* genes in a diabetes cohort comparing 22 microdissected human renal glomerular and tubule samples from healthy patients and from patients with CKD (<60 mL/min/1.73 m^2). A recently published paper[31] supports association of several of the loci highlighted by our blood-based analysis as *ELMO1*, *PRKAG2*, *CUX1*, and *PTPRN2* genes are differentially methylated in kidney tissue comparing individuals with CKD compared with healthy controls.

CUX1 is a member of the homeodomain family of DNA binding proteins that acts as a transcription factor.[42] *CUX1* is important for kidney development with abnormal expression of *CUX1* implicated in kidney disease, particularly polycystic kidney disease.[42-44]

ELMO1 encodes a member of the engulfment and cell motility protein family and has been associated with diabetic kidney disease.[45,46] Decreased methylation was observed in the CKD case group relative to controls and blood-derived gene expression data suggests this may contribute to kidney damage by increasing *ELMO1* expression potentially leading to abnormal regulation of the extracellular matrix.[47] This decrease in methylation was supported by a higher level of expression for the *ELMO1* gene based on RNA-seq results. Increased ELMO1 production may also facilitate more interactions with COX2, thus promoting COX2 activity leading to extracellular matrix accumulation.[48]

FKBP5 is involved in regulation of the stress hormones and decreased methylation at this *FKBP5* locus has been linked to increased stress-dependent gene transcription.[49] Decreased methylation was observed in this study, suggesting that FKBP5 may be involved with the development and progression of CKD, as well as the previously reported influence on response to immunosuppressive medication and rejection of kidney transplants.[50]

INHBA-AS1 encodes a long non-coding antisense RNA potentially involved in kidney damage and transplant rejection.[51,52] INHBA acts as a hormone and is involved in growth / differentiation, nephrotoxicity,[53] and expression is upregulated in response to immunosuppressive drugs.[54]

PRKAG2 is located on chromosome 7 and is an important regulator of metabolic functions. Rare genetic variants have been linked to enlarged kidneys and the association of a *PRKAG2* SNP (rs7805747, $P = 4.2 \times 10^{-12}$) with CKD was highlighted by Kottgen and colleagues; the CKDGen consortium performed a meta-analysis of genome-wide association data revealing *PRKAG2* as one of 13 new loci affecting renal function.[55,56]

PTPRN2 is an autoantigen associated with type 1 diabetes mellitus[57,58] and variation in the *PTPRN2* gene has been associated with CKD in Japanese individuals with hypertension and diabetes mellitus.[59] SNP rs13243294 in this gene may affect the methylation of cg26059287, but genotypes at this position did not account for the significant difference in methylation in a UK population.

Key strengths of this study include the use of Illumina's 450K BeadChip, which enables cost-effective, high throughput epigenome-wide association studies examining 485764 sites distributed across all chromosomes based on selected input from 22 methylation experts across the world.[19] Included are unique markers that cover 99% of RefSeq genes with an average of 17 CpG sites per gene region distributed across the promoter, 5′ untranslated region, first exon, gene body, and 3′ untranslated region. This array also includes dedicated content for CpG sites outside CpG islands and microRNA promoter regions.

Similar to our GWAS investigations,[10] careful attention was paid to quality control measures for this relatively large-scale epigenetic typing effort. Stringent quality control, consideration of available covariates and strong significance values were employed to minimize false positive associations. Previous methylation studies have shown that <200 individuals are sufficient to identify associations with common, complex diseases[60-64] and our data supports that proof-of-concept rationale for CKD. This data suggests that larger epigenome-wide association studies of carefully phenotyped individuals may reveal further insights into the biological mechanisms, clinically relevant biomarkers and potential therapeutic options for CKD.

A potential limitation of this study is that blood-derived DNA was used rather than DNA obtained from affected and unaffected kidneys. Laser captured, micro-dissected kidney tissue would allow direct evaluation of methylation chances in relevant tissues; however, this material is not readily available for human population-based studies. Interestingly, increased DNA hypermethylation of *RASAL1* appears to influence fibrosis with a model protected from kidney fibrosis following inhibition of DNA methylation.[65,66] Kidney biopsies are invasive procedures with attendant surgical risks to the patient, provide a minimal amount of material for analysis, and are not performed routinely unless clinically necessary in the UK. Furthermore, renal tissue within a biopsy sample is heterogeneous consisting of multiple

different cell types that make up the microscopic anatomy of the glomerulus, tubule, interstitium, and renal microcirculation. Therefore, the primary aim of our study was to identify biomarkers from a readily accessible source (peripheral blood leucocytes) that may have functional relevance in a routine clinical setting. Differential methylation profiles using blood-derived DNA have previously identified risk factors and biomarkers associated with complex phenotypes.[22,28,67-70] A recently published paper[31] supports association of several of the loci highlighted by our blood-derived analysis by confirming differentially methylated sites in 2/3 of our top-ranked genes *ELMO1*, *PRKAG2*, *CUX1*, and *PTPRN2* genes in kidney tissue (tubular epithelial cells) comparing 12 individuals with CKD to 14 healthy controls. This is a particularly intriguing discovery as it shows the majority of markers identified from peripheral blood were also reflected in kidney specific analysis for CKD.

Recently we identified changes in DNA methylation that were significantly associated with ESRD in our carefully phenotyped, longitudinal renal transplant recipient-donor cohort revealing several biologically relevant pathways under epigenetic control.[71] Further large-scale, collaborative endeavors are necessary to characterize genetic and epigenetic risk factors that influence renal function. Meta-analyses are challenging for studies of the methylome due to cross-platform differences and a lack of standardized reporting for methylation studies. Nonetheless, accumulating evidence suggests that DNA methylation differences are associated with complex disease and aggregated results from cross-center studies are revealing promising biomarkers.[72,73] The approach described in this manuscript carefully considered tissue type and cell heterogeneity, employed cost-effective 450K analysis for discovery at genome-wide significance, followed by verification and validation using an alternative approach to minimize experimental artifacts, and explored biological relevance alongside expression data for top-ranked markers. Following our identification of associated methylation changes with CKD, replication in additional cohorts is required to confirm these variants and ideally prospective studies would be performed to resolve the methylation influences of cause or effect.

Significant challenges exist in identifying individuals at risk of CKD and optimally managing those diagnosed with CKD in a cost-effective manner.[74] Recent studies suggest a complex relationship between genetic variation, DNA methylation and genes expression and it is likely that a model incorporating all of these factors may provide much better biomarkers for CKD. The identification of a genetic-epigenetic profile that can develop understanding of biological mechanisms, lead to earlier diagnosis, and offer improved clinical evaluation would not only provide significant health benefits for affected individuals, but would also have major utility for the efficient use of healthcare resources.

Methods

Samples

All recruited individuals provided written, informed consent and this study was approved by the Office of Research Ethics Northern Ireland. All individuals were from the United Kingdom with White ancestry. Individuals with concurrent disease such as HIV, hepatitis, tuberculosis, lupus or cystic disease were excluded from this study. Cases and controls were carefully selected for age and gender with multivariate regression analysis performed using these covariates for top-ranked loci. DNA was extracted using the salting out method; all samples were treated in a consistent manner, normalized using PicoGreen quantitation, and frozen in multiple aliquots to minimize potential damage from freeze-thaw cycles.

Methylation on 450K

Blood-derived DNA was accurately quantitated using PicoGreen®, normalized, and bisulfite treated using the EZ-96 DNA Methylation-Gold™ Kit (Zymo Research) with case and control samples randomly distributed across arrays. The Infinium Human Methylation 450K BeadChip[19,75] (Illumina Inc.) was employed according to manufacturer's instructions. Raw data were adjusted for dye bias and quantile normalized with data derived from sites using Infinium I or Infinium II assay chemistry considered separately. This high-throughput platform enables quantitative evaluation of methylation levels with single nucleotide resolution, generating a methylation score per individual (a β value ranging from 0 for unmethylated to 1 representing complete methylation) for each CpG site. Standard quality control included evaluation of bisulfite conversion efficiency, staining, hybridization, target removal, extension, dye specificity, and 600 integral negative controls, as well as assessing outliers for gender mismatches, non-White ethnicity and experimental batch effects. All sites with detection $P \geq 0.05$ for individual sample's β value were set to "missing," as were samples where more than 10% of probes did not generate useful data. Chromosomes X and Y were excluded from association analyses, as were all markers that were ambiguously mapped to the human genome. Normalization of raw methylation data and case-control comparisons for the 450K BeadChip were independently performed using arrayQuality-Metrics,[76] methylumi (http://tinyurl.com/methylumi), Limma,[77] GenomeStudio's methylation module v1.9 (Illumina) software and customized R scripts; probes were excluded if they failed QC following normalization in any software using standard parameters. Software was used following the developer's instructions and standard parameters. Formal comparison of normalization methods was not performed, but only those top-ranked sites that were present in independent analyses employing multiple approaches were taken forward for further investigation; we have observed previously that artifacts from the normalization and ranking process (i.e., these are not true methylation changes that can be validated by bisulfite sequencing or Sequenom EpiTyper analysis) are minimized when only those top-ranked loci common to multiple approaches are included. Significance values for differences in methylation levels between groups were adjusted for multiple testing using the Benjamini and Hochberg approach for all probes that passed quality control filters and were included in the association testing for CKD.[78] Loci were prioritized where more than one CpG site per gene was identified in top-ranked markers.

In silico analysis

Support for top-ranked genes was sought using published literature and open-access data sources. PubMed (www.ncbi.nlm.

nih.gov/pubmed), Scirus *(*www.scirus.com), Web of Knowledge (www.webofknowledge.com), American Society of Nephrology meeting abstracts (2009–2012), the Renal Association UK abstracts (2009–2012), and Nephromine (www.nephromine.org) were searched using "kidney" or "renal" together with the gene names of primary interest to determine published links. Interactions between top-ranked genes were visualized using STRING 9.05 interaction network.[79] NCBI BioSystems Database[80] was searched to investigate which pathways were affiliated with top-ranked genes. Illumina's manifest file was used to filter for known SNPs and dbSNP build 37 was searched for SNPs that may interfere with probe binding. Genotypes for SNP rs13243294 were compared with methylation data for cg26059287 in the *PTPRN2* gene from a separate GWAS-EWAS study of 437 UK individuals with type 1 diabetes (McKnight et al., unpublished data/submitted). VectorNTI software (Life Technologies) was employed to visualize the location of features for genes (**Fig. 2**) with probe sequences, target CpG sites and local SNPs highlighted (**Fig. S2**). Gene set enrichment and pathway analysis was conducted using Partek Genomics Suite 6.6.

Nephromine™ (Compendia Bioscience™, part of Life Technologies™ www.nephromine.org) was used for analysis and visualization of in silico gene expression data sets.

Fine mapping

Primers were designed to fine map the regions immediately surrounding top-ranked CpG sites in *CUX1*, *ELMO1*, *FKBP5*, *INHBA-AS1*, *PTPRN2*, and *PRKAG2* genes (**Fig. S7**; **Table S**7). Forty-six samples (23 cases and 23 controls) were bidirectionally analyzed for SNPs using Sanger sequencing on a 3730 genetic analyzer (Life Technologies) as this provides 95% power to detect all polymorphisms with greater than 5% minor allele frequency.[81] Methylation was confirmed using bisulfite treated DNA compared with genomic DNA for the same individuals.

RNA-Seq

To evaluate the relationship between gene expression and methylation status for *CUX1*, *ELMO1*, *FKBP5*, *INHBA-AS1*, *PTPRN2*, and *PRKAG2* genes, blood was collected within 5 min in both EDTA and PAX gene tubes and processed for DNA and RNA extraction for four individuals; two cases with CKD and two controls with no evidence of kidney disease. RNA was extracted from 2.5 ml of whole blood using the PAXgene RNA System following manufacturer's instructions. RNA was selectively depleted for rRNA using the *RiboMinus™ Eukaryote System v2 (Life Technologies*) and libraries were prepared according to the Ion Total RNA-Seq Kit v2 for Whole Transcriptome Libraries standard protocol, with samples barcoded using RNA-Seq Library Barcodes 1–4 (Life Technologies). Reference spike-in controls from the External RNA Controls Consortium (ERCC) were included for quality control purposes. Prepared libraries were diluted to 12 pM using TE 0.1 and enriched using a One-Touch Two machine and automated enrichment system with the Ion PI™ Template OT2 200 Kit version two. Enriched products were prepared for sequencing using the PI™ Sequencing 200 Kit version two and run on a Proton using version two of the P1 chip (Life Technologies). Data was analyzed using Torrent Suite v3.6, Partek Flow and key genes examined using the template workflow in Partek Genomics Suite 6.6.

Additional data are available from the authors or at www.qub.ac.uk/neph-res/CORGI.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/27161

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