Cytosine methylation predicts renal function decline in American Indians

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Diabetic nephropathy accounts for most of the excess mortality in individuals with diabetes, but the molecular mechanisms by which nephropathy develops are largely unknown. Here we tested cytosine methylation levels at 397,063 genomic CpG sites for association with decline in the estimated glomerular filtration rate (eGFR) over a six year period in 181 diabetic Pima Indians. Methylation levels at 77 sites showed significant association with eGFR decline after correction for multiple comparisons. A model including methylation level at two probes (cg25799291 and cg22253401) improved prediction of eGFR decline in addition to baseline eGFR and the albumin to creatinine ratio with the percent of variance explained significantly improving from 23.1% to 42.2%. Cg22253401 was also significantly associated with eGFR decline in a case-control study derived from the Chronic Renal Insufficiency Cohort. Probes at which methylation significantly associated with eGFR decline were localized to gene regulatory regions and enriched for genes with metabolic functions and apoptosis. Three of the 77 probes that were associated with eGFR decline in blood samples showed directionally consistent and significant association with fibrosis in microdissected human kidney tissue, after correction for multiple comparisons. Thus, cytosine methylation levels may provide biomarkers of disease progression in diabetic nephropathy and epigenetic variations contribute to the development of diabetic kidney disease.

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Nephropathy is a serious complication of diabetes mellitus that often leads to end-stage renal disease (ESRD). Diabetic nephropathy is the most common cause of ESRD in the United States and most developed nations.[1,2] Virtually all of the excess mortality experienced by diabetic individuals relative to those without diabetes occurs in those with nephropathy.[3] Treatment of hyperglycemia and blood pressure can reduce the risk of nephropathy to some extent,[4–7] but the molecular mechanisms by which diabetic kidney disease develops remain unknown. Familial aggregation of diabetic nephropathy indicates that genetic factors may be involved in disease pathogenesis.[8–10] Although a few well-replicated single nucleotide variants associated with the disease were recently identified,[11,12] most of the heritability remains unexplained. Epigenetic variations, such as in DNA methylation, may play a role in disease susceptibility and may explain part of the heritability of complex traits.[13,14] DNA methylation mostly occurs at the cytosine base in cytosine-phosphate-guanine (CpG) sequences. Most of the genome has a low CpG content, and cytosines in low CpG regions are generally methylated. Most promoter regions, in contrast, have high CpG content (CpG islands), and methylation of CpG islands is variable. Methylation of CpG islands plays a key role in regulating gene expression as methylation levels can interfere with transcription factor binding. Methylation changes that occur in gene regulatory regions, such as promoters and enhancers, are likely to be functional.[15]

Several lines of evidence suggest that epigenetic factors may contribute to the risk of diabetic nephropathy. Epigenetic mechanisms have been implicated in the "metabolic memory" by which reduction in the risk of diabetic complications induced by intensive treatment of glycemia remains reduced after the treatment period is over.[16] Studies in cellular and animal models have shown that hyperglycemia can induce persistent histone modification changes, which could influence pathways related to diabetic kidney disease,[17–20] but few studies have been performed in patients to validate these results. Ko et al.[21] identified significant cytosine methylation differences in tubule cells of kidneys obtained from patients with chronic kidney disease compared with those from healthy individuals. Kidney-specific cytosine methylation changes were enriched in regulatory regions and

correlated with expression of profibrotic genes, indicating that epigenetic changes are likely to play a role in disease development.

Several studies have identified particular CpG sites, measured in peripheral blood, that are differentially methylated between individuals with and without diabetic nephropathy.[22–24] Apart from a small study in the Chronic Renal Insufficiency Cohort (CRIC),[24] these studies have been cross-sectional, and because cytosine methylation levels can change over time, it is uncertain whether the differences in methylation preceded onset of diabetic nephropathy or whether they developed as a consequence of nephropathy. Longitudinal studies are required to determine the extent to which cytosine methylation levels are associated with the risk of the development of diabetic kidney disease. The identification of CpG sites at which methylation levels are predictive of diabetic nephropathy may not only provide markers that can be useful indicators of disease prognosis, but may also yield important clues about disease pathogenesis. In this study, we analyzed the extent to which DNA methylation, measured in the peripheral blood in diabetic Pima Indians with chronic kidney disease, is associated with development of subsequent ESRD and subsequent changes in renal function, as measured by estimated glomerular filtration rate (eGFR). The Pima Indians are an American Indian population with a high prevalence of type 2 diabetes and a high risk of diabetic ESRD;[25,26] >90% of the ESRD which occurs in this population is attributable to diabetes.[26]

RESULTS

Cytosine methylation levels and association with baseline eGFR

We conducted a nested case-control study[27–29] to assess the association of methylation levels with the development of ESRD in individuals with diabetes and established chronic kidney disease (albumin:creatinine ratio [ACR] ≥300 mg/g or eGFR <60 ml/min per 1.73 m^2) at baseline. The study involved 181 Pima Indians, selected from a longitudinal study.[25] Baseline characteristics for participants are shown in Table 1. In keeping with the matched case-control design, there were no significant differences between those in whom ESRD developed and in those in whom did not in sex, duration of diabetes, or age.

Cytosine methylation was measured in baseline peripheral blood samples with the Infinium HumanMethylation450 Beadchip (Illumina, San Diego, CA), resulting in methylation level measures at 397,063 CpG sites. We performed an epigenomewide association analysis between cytosine methylation levels and baseline eGFR. Results are shown in Figure 1a. The relationship between effect size and the *P* value for association is shown in Figure 1b. After adjustment for multiple comparisons, none of the sites achieved genomewide significance (false discovery rate [FDR] <0.05), but the association with eGFR at several sites approached significance. The strongest association between methylation levels and eGFR levels was with cg13525276 (on chromosome 14) in the gene body of *TSHR* ($P = 3.87 \times 10^{-7}$, FDR = 0.119). Figure 1c shows the relationship of methylation level at the top probe with the eGFR. The top 20 sites with the strongest evidence of association of methylation with the baseline eGFR are shown in Table 2.

We also performed analyses without adjustment for cell type. The order of the top 20 associated sites changed modestly, but methylation differences at several sites remained strongly associated, and 1 site in *CRBN* achieved an FDR <0.05 (cg00294109, $P = 1.03 \times 10^{-7}$, FDR = 0.041, Supplementary Table S1). This suggests that results are largely due to cell-type autonomous methylation changes rather than cell-type composition-induced methylation variation.

Top eGFR-associated regions are localized to gene regulatory regions of metabolic genes

Cytosine methylation levels of gene regulatory regions, promoters, or enhancers are more likely to be functional compared with those that lie outside of regulatory regions. To expand the analysis, we used all 1435 probes whose

Table 1 | Characteristics of participants in methylation studies

Characteristic	All	Case	Control	*P* value
N	181	80	101	
Age (yr)	53.5 ± 12.0	52.9 ± 10.0	54.0 ± 13.4	0.53
Sex	66 male, 112 female	32 male, 48 female	37 male, 64 female	0.65
Duration (yr)	18.1 ±7.1	18.3 ± 13.2	17.9 ± 7.7	0.71
SBP (mm Hg)	140.3 ± 20l8	145.1 ± 19.7	136.6 ± 20.9	0.0055
DBP (mm Hg)	80.9 ± 11.3	83.3 ± 12.3	79.0 ± 10.1	0.011
Mean BP (mm Hg)	100.7 ± 12.6	103.9 ± 13.2	98.2 ± 11.4	0.0028
HbA1c (%)	10.0 ± 2.2	10.3 ± 2.1	9.7 ± 2.2	0.069
ACR (mg/g)	985.7	2026.3	542.2	8.2E-10
eGFR (ml/min per 1.73 m^2)	78.1 ± 31.0	69.8 ± 33.8	84.7 ± 27.0	0.0015
Serum creatinine (mg/dl)	1.11 ± 0.68	1.34 ± 0.90	0.92 ± 0.34	0.00015
Body mass index (kg/m^2)	31.6 ± 6.6	31.4 ± 6.8	31.7 ± 6.5	0.75
Bisulfite conversion efficiency	0.94 ± 0.01	0.94 ± 0.01	0.94 ± 0.01	0.93

ACR, albumin:creatinine ratio; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; mBP, mean blood pressure; SBP, systolic blood pressure; SCr, serum creatinine.
Data are mean ± SD, except for *N* and sex, which are reported as counts, and ACR, which is reported as the median. *P* values are for the comparison between cases and controls and were calculated by *t* tests, except for sex (Fisher exact test) and ACR (Wilcoxon rank sum test).