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Epigenome-Wide Association (DNA Methylation) Study of Sex Differences in Normal Human Kidney

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Abstract

Studies have identified epigenetic sex differences in several human tissues and have implicated epigenetic factors in the regulation of tissue-specific expression. Studies have also shown that women and men respond differently to various drugs, thereby influencing the pharmacokinetics, pharmacodynamics, adverse reactions, efficacy, and safety of a drug. Using Illumina Human Methylation450 BeadChip kit, we investigated the influence of sex on DNA methylation patterns in normal human kidneys (16 females and 15 males). We then related the methylome to mRNA expression levels in kidney structure/function and Drug Metabolizing Enzyme and Transporter (DMET) genes (32 females and 59 males). Our findings indicate that 429 methylated sites on autosomal chromosomes had significant sex-specific differences in the normal human kidney. Methylated sites in/near regions associated with DMET genes or with genes involved in renal structure/function and disease were identified for subsequent analysis. Validation of 2 DMETs genes (POR and ABCA3) and 2 renal structure/function/disease genes (LAMA5 and PLAT) exhibited significant sex-specific differences in mRNA expression. Our results highlight site-specific sexual dimorphisms (epigenetic-based) in normal human kidney. Importantly, we provide a reference methylome for normal human kidney, which may be utilized to improve our understanding of renal disease and assessing the overall safety and effectiveness of a drug in the kidney.

Keywords: DNA methylation; Kidney; Sex differences; DMETs; Kidney injury

Abbreviations:

ADR: Adverse Drug Reaction; DMET(s): Drug Metabolizing Enzyme and Transporter(s); EWAS: Epigenome-Wide Association Study; PC: Principal Component; KEGG: Keto Encyclopedia of Gene and Genomes; IPA: Ingenuity Pathway Analysis; ECM: Extracellular Matrix; FDA: Food and Drug Administration

Introduction

There has been a wealth of clinical data demonstrating sex differences in drug responses and efficacy [1-6]. Around 6-7% of new drug applications that include a sex analysis component have shown statistically significant differences in pharmacokinetic profiles as well as toxicokinetic activities, adverse drug reactions, and drug efficacy and safety [7]. In addition, according to analyses of the Food and Drug Administration's (FDA) adverse events reporting database, women have been found to experience more Adverse Drug Reactions (ADRs) than men [8]. This has led to many drugs being withdrawn from the market. However, the underlying mechanisms for these sex differences are not well understood.

One of the key organs involved in drug response is the kidney. The kidney performs a number of functions that include the excretion of the parent drug, xenobiotic agents, and their metabolites, the removal of metabolic waste products, maintenance of blood pH and osmolarity, release of hormones that regulate blood pressure, and production of an active form of vitamin D that promotes strong, healthy bones [9-12]. Kidney toxicity is one of the leading causes of pharmaceutical development failure [13]. Typically, 7% of drug agents are removed from preclinical studies due to nephrotoxicity [13]. Additionally, it is believed that drug-induced nephrotoxicity is responsible for 30-50% of all drugs failing phase III clinical trials [14]. The prevalence of nephrotoxicity might be explained by differences in the expression of drug metabolizing enzymes and transporters (DMETs) [15]. In the kidney, DMETs play a critical role in the biotransformation process of pharmaceuticals, substances, and xenobiotics [16]. Drug metabolism is typically divided into three phases. Phase I DMETS are involved in the modification reactions by oxidation (e.g. cytochromes P450), reduction (e.g. aldo-keto reductases), and hydrolysis (e.g. epoxide hydrolases) of the drug [17]. Phase II DMETs typically consist of conjugate reactions catalyzed by transferase enzymes such as uridine 5'-diphospho-glucuronosyltransferase (UDP-Glucuronosyltransferase (UGT)) and Glutathione S-Transferase (GST), which make the drug agents or xenobiotics water soluble and more excretable [16]. Phase III DMETs entail the uptake and excretion process of the drug and its metabolites by drug transporters. Drug transporters are divided into two categories: Efflux transporters (ATP-binding cassette (ABC) superfamilies) and uptake transporters (Solute Carrier (SLC) superfamilies) [18]. DMETs have been identified as key elements in the

pharmacokinetic and toxicokinetic process of a variety of drugs and have significant influence on a drug's overall effect [19-22].

In the kidney, a number of these biological factors can be regulated by epigenetic mechanisms, which, if altered, can affect the kidney's response to drugs or increase susceptibility to kidney disease [23]. Epigenetic regulation in drug metabolizing and transporting tissues can be a critical determinant in how individuals respond to drugs [24]. These epigenetic modifications can lead to alterations in kidney function influencing drug absorption, metabolism, and pharmacokinetics in humans and promote inter-individual variability in drug efficacy, safety, and adverse drug reactions. One of the major mechanisms of epigenetic modifications, DNA methylation, is known to play an important role in tissue-specific gene expression, X-chromosome inactivation, chromosomal stability, genomic imprinting, and mammalian development [25-28]. In mammalian cells, DNA methylation most frequently occurs when a methyl group is transferred to the 5'-carbon position of cytosine in CpG dinucleotide region [29]. DNA methylation is known to play a significant role in transcriptional activity depending on where in the genome it occurs [30-32]. It is generally recognized that DNA methylation of gene promoters can lead to gene silencing [33]. Additionally, recent findings suggest that DNA methylation of the first exon may be linked to transcriptional silencing and decreased gene expression [31]. Furthermore, several studies have indicated that tissue-specific DNA methylation occurs mainly in the CpG shores, not in the CpG island [32,34]. Yet, other studies have shown that there was a positive correlation between DNA methylation and gene expression when DNA methylation occurred in the gene body [30,35-37].

Previous epigenome-wide studies demonstrated sex-specific DNA methylation differences in specific genes of several human tissues and cell types such as liver, heart, blood, pancreatic islets, brain, and saliva [38-44]. Hall et al. analyzed the impact of insulin secretion in human pancreatic islets and found that epigenetic changes were associated with sex-based differences in insulin secretion [39]. Liu et al. concluded that females tend to have higher levels of DNA methylation on the X–chromosomes and the autosomal chromosomes in saliva cells [45]. In contrast, other studies revealed that sex differences in DNA methylation on the autosomal chromosomes had no or minimal effects [38,42-44].

Although a number of genome-wide expression studies have been performed to evaluate and understand kidney function in animal models and humans [46-48], this is the first to investigate epigenome-wide methylation in normal human kidney. Thus, the aim of this study was to perform an Epigenome-Wide Association Study (EWAS) in normal human kidney tissue obtained from a cohort of 15 male and 16 female donors and to elucidate sex-specific differences from the genome-wide DNA methylation profiles. The study design presents an opportunity to evaluate sex-specific epigenetic variations and identify underlying patterns in the normal human kidney tissue. We also investigated the association between DNA methylation and the mRNA expression of DMET genes and genes associated with renal disease/function/structure in normal kidney tissue from 59 males and 32 females. This study provides a critical baseline for exploring sex-based differences in DNA methylation variation and patterns in renal disease and drug response, thus contributing to the improvement of the efficacy and safety of drugs for the public.

Materials and Methods

Tissue samples

Normal (non-diseased) human kidney samples from 91 donors were obtained from the US Cooperative Human Tissue Networks (CHTN) (Table 1).

Experiment	Phenotype	Males	Females	p-values
Illumina Methylation Array	n	15	16	-
	Age (years)	54 ± 18	52 ± 18	0.792
	Ethnicity (AA/EA)	(7/8)	(8/8)	0.493
mRNA Expression	n	59	32	-
	Age (years)	60 ± 16	57 ± 19	0.355
	Ethnicity (AA/EA)	(11/48)	(11/21)	0.0949
AA: African American, EA: European American. Age data are represented as mean ± standard deviation				

Table 1: Characteristics of normal (non-diseased) human donors stratified by sex.

The renal tissues were obtained from patients with no stated kidney disease; only tissues confirmed as non-diseased by pathological analysis performed by CHTN were utilized [49]. Kidney tissues from 16 females and 15 males were used in our analysis of DNA methylation profiling. All 91 kidney tissues were used for qRT-PCR analysis. This project was reviewed by the National Center for Toxicological Research (NCTR) and FDA's Research Involving Human Subject Committee (RIHSC) and received an exempt status.

DNA and RNA extraction

DNA and RNA were extracted from normal human kidney using QIAamp DNA Mini Kit and using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Nucleic acid purity and concentrations were determined using NanoDrop Spectrophotometer ND1000 (Thermofisher, Grand Island, NY). All DNA samples had A260/A280 ratios of 1.8 to 2.0, whereas the A260/A280 ratios of the RNA were 1.9 to 2.1. The integrity and quality of the RNA were assessed using Biorad Experion Automated Electrophoresis Station (Hercules, CA) and samples with RNA integrity number (RIN) values between 9 and 10 were utilized.

DNA methylation data pre-processing and analysis of differentially methylated probes/sites

Epigenome-wide DNA methylation analysis of normal human kidney was performed using the Illumina Infinium Human Methylation450 BeadChip kit. (San Diego, CA). Triplicate genomic DNA (500 ng) for each sample was bisulfite converted using Zymo Research EZ DNA Methylation Kit (Irvine, CA) following the alternate incubation conditions specific for Illumina Infinium Methylation Assay according to the manufacturer's protocol. The total amount of bisulfite converted DNA was used to analyze DNA methylation with Infinium Human Methylation450 BeadChip according to the manufacturer's protocol. The bead chips were imaged using the Illumina iScan. The Illumina Infinium Human Methylation450

BeadChip contains 485,577 probes with probes targeting sites in the promoter region 5′UTR, first exon, gene body, and 3′UTR including CpG islands, island shores, and island shelves (sites flanking island shores), CpG sites outside of CpG islands, and miRNA promoter regions among others. Methylation levels are quantified by beta (β) values derived from the ratio of intensities between methylated and unmethylated alleles; β-values for each probe range from 0 (unmethylated) to 1 (completely methylated).

Data preprocessing steps to filter data based on quality thresholds for Illumina detection p-values were carried out using the wateRmelon package in R [50]. Samples with at least 1% of CpG probes with a detection p-value >0.05 were filtered out. Additionally, probes were removed if at least 1% of samples had a detection p-value >0.05 or if at least 5% of samples had a bead count <3. Based on these criteria, all samples met the quality control threshold; however, approximately 2600 probes were filtered from analysis. Methylation data were normalized using quantile normalization to correct for between-array technical biases. Subsequently, Beta-Mixture Quantile (BMIQ) normalization was used to correct for within-array variations resulting from Infinium I/II probe biases [51]. No chip or row effects were detected by principal component analysis. Beta values were highly correlated (r>0.99) across technical replicates. In general, all three replicates clustered together in hierarchical cluster analysis. However, two particular biological samples, which demonstrated a minimal degree of separation between a single replicate and the other two replicates, were excluded from analysis. Methylation data were averaged across replicate samples for the remaining samples. The resulting 482,954 probes for the 31 biological samples were converted to M-values via the logit transformation [43]. Approximately, 10,000+cross-reactive probes [52] and probes within 2 nucleotides of a SNP with minor allele frequency greater than 0.1 were filtered from analysis. Finally, to identify sex-based differences in DNA methylation, only probes located on autosomes were retained for further analysis. The final dataset consisted of 429,304 autosomal probes.

We conducted Principal Component (PC) analysis to assess the effect of known clinical variables (i.e., ethnic group, age, and sex) on the variation in genome-wide DNA methylation. The first PC (PC1) accounted for 98.5% of the variance in genome-wide methylation sites. PC1 was not significantly associated with ethnic group (African American/European American) (p=0.865), age (p=0.919), or sex (male/female) (p=0.686). Thus, differential methylation analysis of each probe was assessed by evaluating the influence of sex on DNA methylation, after adjusting for ethnic group, age, and PC1, which may reflect unknown or unmeasured biological variability. P-values were adjusted for multiple comparisons testing using the Bonferroni correction. Probes with a Bonferroni-adjusted p-value <0.05 were deemed statistically significant.

The R-project version 3.3.1 (http://www.r-project.org) was used for all statistical computing. The Gene Expression Omnibus accession number for the methylation data is GSE79100.

Distribution of significant CpG sites

The frequency distribution of significant sex-based CpG sites was compared to the frequency distribution of all CpG sites. CpGs were categorized in two ways: CpG island content/neighbouring context and functional genomic annotation. CpG island content/neighbouring context was categorized as North shelf or shore, South shore or shelf, CpG Island, and open sea. Functional genomic annotation was categorized as TSS1500, the region 200 to 1500 nucleotide upstream of the Transcription Start Site (TSS); TSS200, the 200 nucleotides immediately upstream of the TSS; 5' Untranslated Region (UTR); first exon; gene body, 3'UTR; or intergenic region (Supplemental Figure 1).

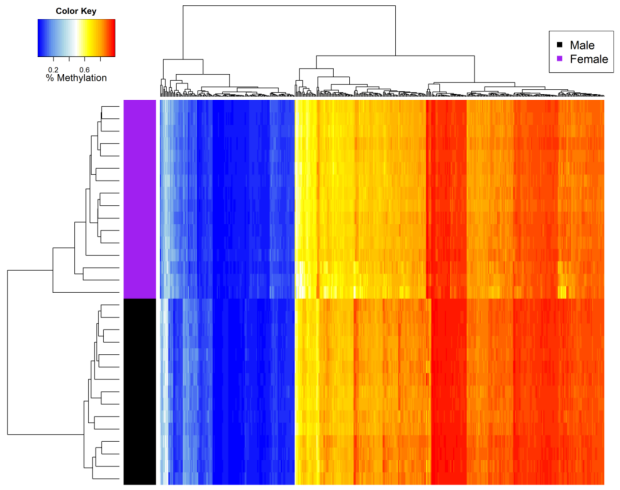

Figure 1: Heatmap of the 429 differentially methylated sites between males and females in normal human kidney. Normal human kidneys are depicted by sex (males and females are indicated in black and purple, respectively). Beta values from the 429 significantly methylated CpGs are used for hierarchical clustering. Methylation levels are depicted from lowest (blue) to highest (red) expression.

A chi-square test (GraphPad Prism 7.0, La Jolla, CA) was performed to determine whether the frequency distribution of significant CpGs differed from that of all CpGs. When the chi-square test was found to be significant, post-hoc testing was performed using residual analysis [53]. Standardized residuals with an absolute value greater than 3 were used to identify the source of the significant result.

Biological relevance of differentially methylated sites

We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to identify biological processes associated with significant sex-based CpGs. KEGG pathway analysis was carried out using the missMethyl R package [54], which accounts for the varying numbers of CpG sites associated with each gene when computing the probability of a gene being selected. KEGG pathways with a false discovery rate <0.05 (based on the Benjamini-Hochberg method [55]) were considered significant.

To assess the biological function of the 316 genes associated with the 429 differentially methylated sites, we used the Ingenuity Pathway Analysis software (IPA, Qiagen Bioinformatics, Redwood City, CA). IPA analysis was performed by limiting the knowledge database to kidney-related biological functions.

Complementary DNA and qRT-PCR

Complementary DNA (cDNA) was synthesized from 0.4 μg of high quality RNA (RIN>8) using the Advantage RT-for-PCR Kit according to the manufacturer's protocol (Clontech Laboratories Inc., Mountain View, CA). The resulting cDNA was amplified using qRT-PCR: SYBR green assay (SYBR Green PCR mastermix, Applied Biosystems,

Thermofisher, Grand Island, NY) or multiplex assay (multiplex power mix solution, BioRad, Hercules, CA). The list of primers included GAPDH (Sigma-Aldrich: St. Louis, MO), RPS13, PPIA, POR, ABCA3, LAMA5, AND PLAT (Integrated DNA Technologies: Coralville, Iowa) (Additional File 1). All samples were assayed in triplicate using a BioRad CFX96 C1000 system (BioRad, Hercules, CA). Sex-based differences in mRNA expression were analyzed using the ΔCT values (with GAPDH, RPS13, and PPIA as the endogenous control genes). PC1 and PC2 accounted for 83.3% of the variation in mRNA expression. PC1 was significantly associated with sex; however, PC2 was not significantly associated with age, ethnic group, or sex. Thus, sex-based differential expression analysis was assessed with a linear regression model, adjusting for ethnic group, age, and PC2. P-values were adjusted for multiple comparisons testing using the FDR approach. FDR-adjusted p-values <0.05 were deemed statistically significant. Fold-change was determined using 2-ΔΔCT calculations [56]. All data analysis was performed using R (version 3.3.2).

Results

Genome-wide DNA methylation analysis between male and female non-diseased human kidney samples

We obtained methylation data from normal (non-diseased) human kidney from 31 subjects (15 males and 16 females) using the Illumina Infinium HumanMethylation450 BeadChip. Summary characteristics of the covariates for the kidney donors are presented in Table 1. There was no significant difference in mean age (p=0.792) or in the proportion of African Americans (p=0.849) between the two sex groups. A total of 429,856 were included in the study. We assessed differential methylation between males and females at each of these sites using a linear regression model. Overall, 429 CpG sites showed significant sex-based differences at a Bonferroni-adjusted p-value <5% (Additional Files 2 and 3).

Following DNA methylation analysis, the 429 CpG sites (corresponding to 316 genes) that were differentially methylated between males and females were further analyzed. A heatmap of the significant sex-based CpG sites shows that the samples separate by sex (Figure 1). Furthermore, a volcano plot of the methylation differences between male and female samples indicates that, in general, males demonstrated higher methylation levels compared to females (Figure 2A). Of the 429 CpG sites with significant sex-based differences, 339 sites had higher methylation in males; these sites were associated with 232 unique genes (Additional File 2). Likewise, the 90 CpG sites with higher methylation in females were associated with 81 genes (Additional File 3). The distribution of deviation in male and female methylation levels for significant sites where females or males had higher methylation is presented in Figure 2B and Figure 2C, respectively. Three genes, FUT4, LBX1, and FLJ41350, exhibited higher DNA methylation in both males and females at different methylated sites. We assessed the average degree of methylation for the 429 significant sex-based sites; there was no difference in average methylation between males and females (p=0.224) (Figure 2D).

Furthermore, average DNA methylation levels were not significantly differentiated between males and females when stratified in relation to CpG island content/neighboring context and functional genomic region (Figure 3A and 3B).

Additional, frequency distributions of the 429 significant sites (in comparison to all CpG sites) were summarized by CpG island content/neighbouring context and functional genomic annotation (Figures 3C and 3D, respectively). When classifying sites by CpG island content/neighbouring context, the chi-square test (p=0.004) followed by post-hoc residual analysis indicated that the significant sex-specific sites were enriched in the S shelf region and less enriched in the S shore region.

Biological relevance of significant sex-specific methylated sites in normal (non-diseased) human kidney

The 429 differentially methylated sites with significant sex-based differences were involved in several biological pathways (Table 2).

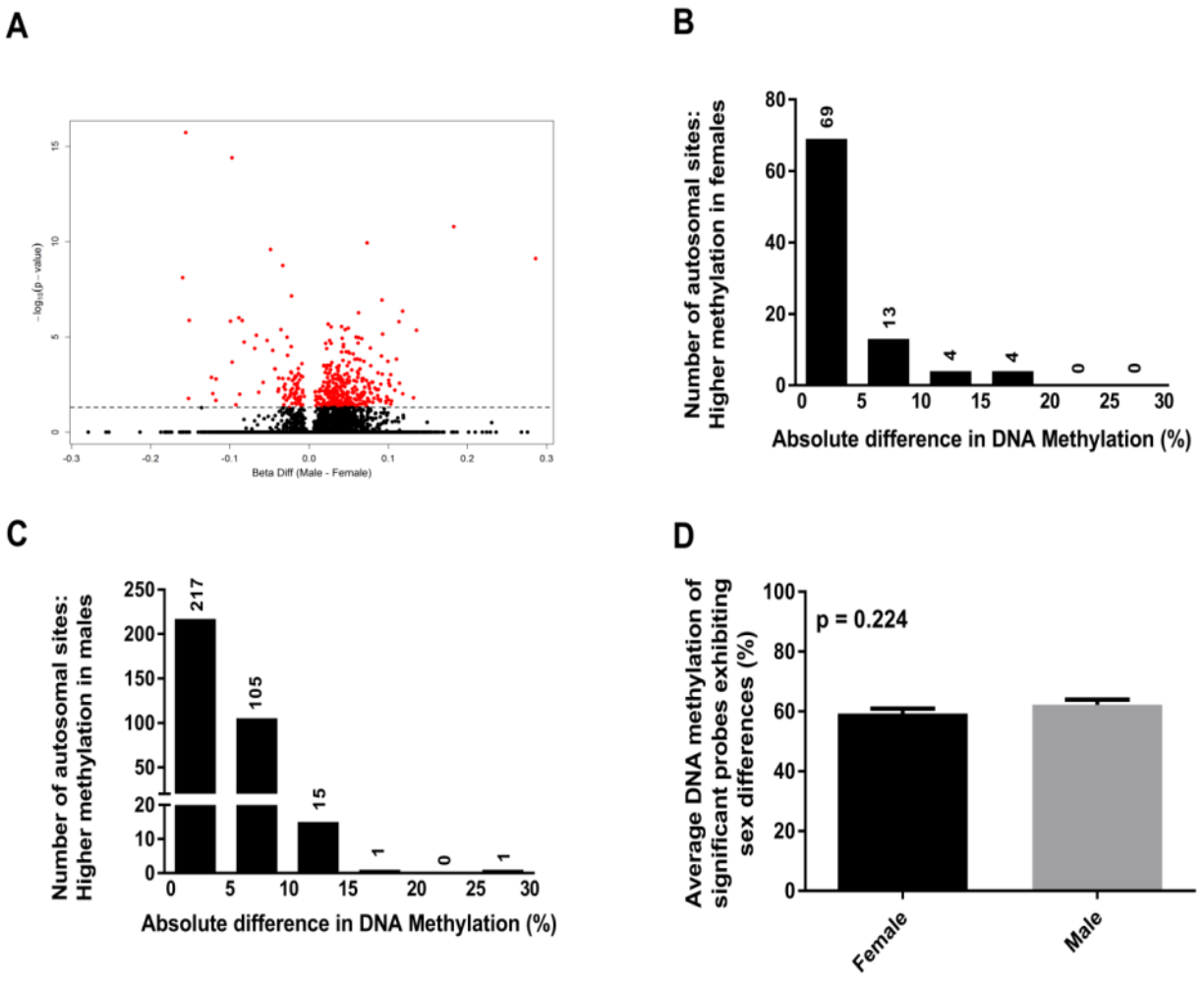

Figure 2: DNA methylation patterns of human normal (non-diseased) kidney samples from female and male donors. Epigenome-wide association study of normal (non-diseased) human kidney using Infinium HumanMethylation450 BeadChip array. Data excludes CpG sites on the sex chromosomes. A) Volcano plot of the 429,856 analyzed CpGs. The difference between male and female beta values is plotted on the x-axis and log10 transformed p-value is presented on the y-axis. Sites with significant sex-based differences are colored in red. Frequency distribution of deviation between male and female DNA methylation levels (%) for significant sites with, B) higher methylation levels in females (n=90) and C) higher methylation levels in males (n=339). D) Average DNA methylation (%) for males and females for the 429 significant sex-based CpGs. Data are presented as mean ± SEM.

Cell cycle regulation reached the highest level of significance (FDR-adjusted $P=3.4 \times 10^{-8}$). We performed Ingenuity Pathway Analysis (IPA) analysis to determine that the 316 genes associated with significant CpGs were involved in several kidney-related biological functions (Table 3).

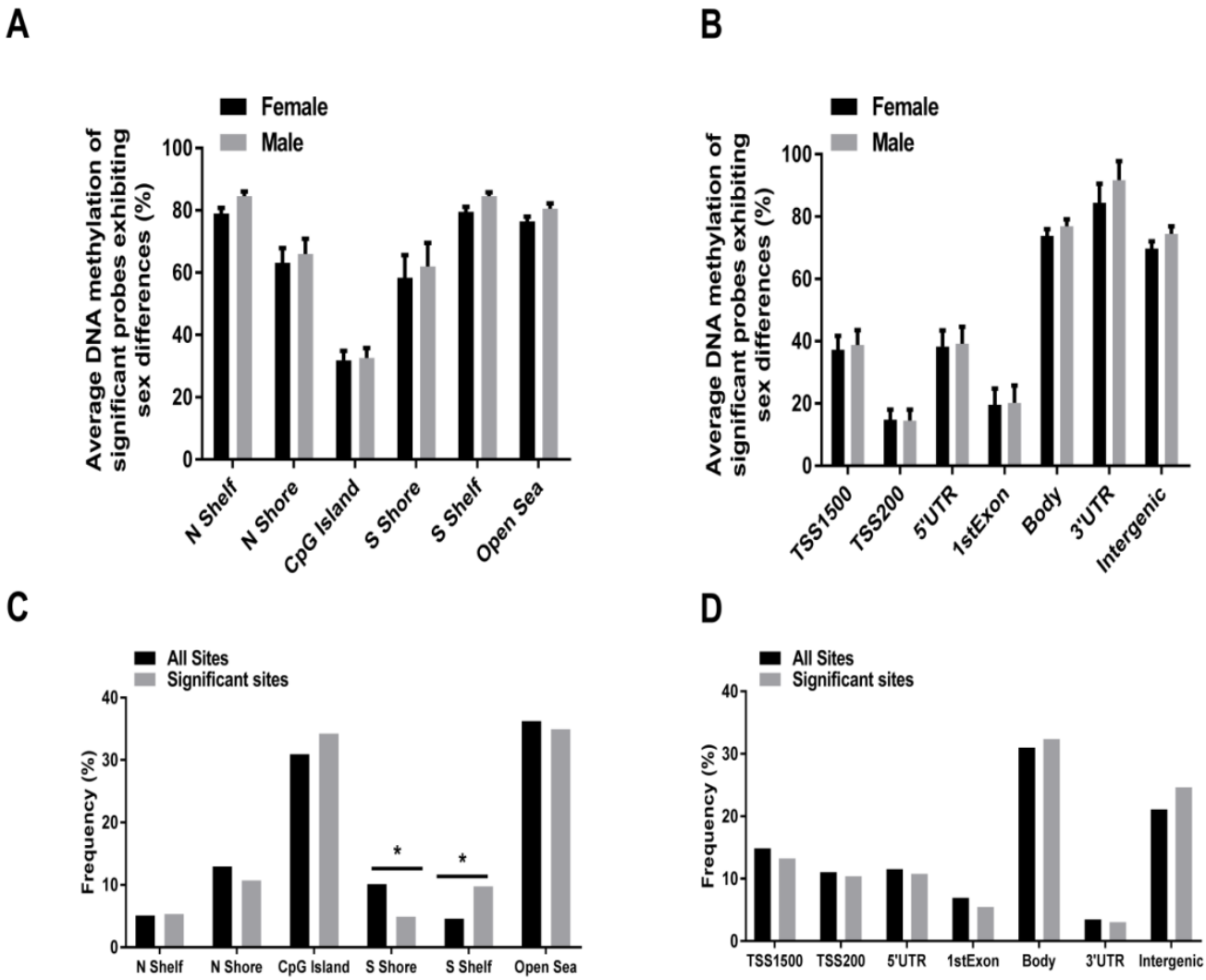

Figure 3: Average DNA methylation (%) and frequency distribution of the 429 significant sex-specific methylated sites. Average DNA methylation (%) between males and females for the 429 significant sex-based CpGs when categorized in relation to A) CpG island content/neighbouring context classes and B) functional genome annotation. Data are presented as mean ± SEM. The frequency distribution of all CpG sites compared to significant CpG sites when sites are classified by C) CpG island content/neighbouring context (chi square test was significant, p=0.004) and D) functional genome annotation.

Pathway	N	DE	FDR
Cell cycle	121	10	3.40E-08
Pathways in cancer	386	13	1.29E-05
Circadian rhythm	30	5	2.89E-05
Proteoglycans in cancer	200	9	7.14E-05
Metabolic pathways	1186	17	7.14E-05
Transcriptional misregulation in cancer	165	7	1.01E-03

Viral carcinogenesis	193	7	1.01E-03
Calcium signaling pathway	172	7	1.01E-03
TGF-beta signaling pathway	83	5	1.07E-03
Oocyte meiosis	111	5	3.30E-03
Peroxisome	81	4	4.31E-03
HTLV-I infection	250	7	4.79E-03
Ubiquitin mediated proteolysis	129	5	4.79E-03
Non-small cell lung cancer	55	4	7.88E-03
AMPK signaling pathway	120	5	9.42E-03
Adrenergic signaling in cardiomyocytes	144	5	1.39E-02
Wnt signaling pathway	138	5	1.49E-02
Small cell lung cancer	82	4	1.49E-02
Th17 cell differentiation	102	4	1.62E-02
Lysosome	118	4	2.02E-02
Ribosome biogenesis in eukaryotes	69	3	2.26E-02
Melanogenesis	101	4	2.26E-02
Inflammatory bowel disease (IBD)	61	3	2.26E-02
Vascular smooth muscle contraction	119	4	2.42E-02
Circadian entrainment	95	4	2.43E-02
Hepatitis B	131	4	4.34E-02
Focal adhesion	193	5	4.35E-02
Basal cell carcinoma	55	3	4.35E-02
Glycine, serine and threonine metabolism	35	2	4.51E-02
MicroRNAs in cancer	279	5	4.51E-02
Huntington's disease	179	4	4.76E-02

Table 2: KEGG pathway analysis of the 429 significant sex-based methylated sites.

	Category	Diseases or Functions Annotation	P-value	Molecules
Disease and Biofunction	Cellular Function and Maintenance, Molecular Transport	Flux of K+	0	NR3C1,NR3C2
	Cellular Function and Maintenance, Molecular Transport	Flux of Na+	0	NR3C1,NR3C2
	Cellular Function and Maintenance	Ion homeostasis of cells	0.01	ADRB2,NR3C1,NR3C2
	Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response, Tissue Development	Accumulation of M1 macrophages	0.02	PLAT
	Renal and Urological System, Development and Function	Adhesion of glomerular membrane	0.02	LAMA5
	Cell Death and Survival	Adhesion of glomerular basement membrane	0.02	PLAT
	Tissue Morphology	Apoptosis of M1 macrophages	0.02	PLAT

	Embryonic Development, Organismal Development, Tissue Development	Functional integrity of basement membrane	0.02	LZTS2
	Organ Development	Outgrowth of metanephric bud	0.02	PLAT
	Developmental Disorder, Organ Morphology, Organismal Development, Organismal Injury and Abnormalities, Renal and Urological Disease, Renal and Urological System Development and Function	Recovery of kidney	0.02	LZTS2
	Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	Renal duplication	0.02	FLT1
	Tissue Morphology	Reorganization of cytoskeleton	0.02	PLAT
	Drug Metabolism	Structural integrity of basement membrane	0.02	POR
	Molecular Transport	Toxicity of acetaminophen	0.02	NR3C2
	Molecular Transport	Transport of K+	0.02	SLC14A2
	Cellular Movement, Organismal Injury and Abnormalities, Renal and Urological Disease, Tissue Morphology	Breakdown of glomerular basement membrane	0.04	LAMA5
	Cardiovascular Disease, Organ Morphology, Organismal Injury and Abnormalities, Renal and Urological Disease	Distension of glomerular capillary	0.04	LAMA5
	Cancer, Organismal Injury and Abnormalities, Renal and Urological Disease	Renal cancer	0.04	ABCA3,AHRR,ARID1B, CCNA1,CDH, 23,DROSHA,EPHB2,IF T74,KDM2B, PCDH15,PRDM4,PTC H1,SERF2,SM AD2,TBC1D1,UBE3C
Toxicity Function	Renal Degradation	Breakdown of glomerular basement membrane	0.04	LAMA5
	Glomerular Injury	Distension of glomerular capillary	0.04	LAMA5

Table 3: IPA analysis of the 316 genes (associated with the 429 significant methylated sites) in relation to kidney toxicity, disease, and bio-function.

Disease, bio-function, and toxicity function analysis identified several functional categories in the kidney, which can be grouped into three main categories: renal disease/injury (LAMA5, LZTS2,ABCA3, AHRR, ARID1B, CCNA1, CDH23, DROSHA, EPHB2, IFT74, KDM2B, PCDH15, PRDM4, PTCH1, SERF2, SMAD2, TBC1D1, UBE3C, and PLAT); cellular function/maintenance/structure (NR3C1, NR3C2, ADRB2, PLAT, LAMA5, LZTS2, and FLT1); and metabolism/transport (POR, NR3C2, and SLC14A2) (Table 3).

qRT-PCR analysis of DMETs and structure/function genes associated with significant sex-specific methylated sites

Since the kidney is one of the major organs involved in drug metabolism and transporting, we identified sites associated with DMETs for subsequent analysis. A total of 10 significant sex-specific sites associated with DMET genes were identified (Table 4).

Of those 10, 2 had higher DNA methylation in females; they were associated with genes POR and SLC44A3 (Table 4). DNA methylation levels in males exceeded DNA methylation levels in females for the other 8 CpGs; these sites were associated with DMET genes ABCA3, SLC13A2, ALDH1L2, CYP51A1, SLC14A2, SLC25A17, SLC43A2, and SLC37A1 (Table 4).

Furthermore, since methylation may regulate gene transcription, we examined whether two selected DMET genes (ABCA3 and POR) had significant sex differences in mRNA expression in normal human kidney. These DMET genes were selected based on IPA analysis, which indicated that POR was involved in the drug metabolism-toxicity of acetaminophen and that ABCA3 was involved in renal disease/injury. Additionally, changes in the kidney structure may influence its ability to metabolize and transport drug substrates. Hence, structure/function genes PLAT and LAMA5 were also of interest due to their role in renal disease/structure/function. Based on qRT-PCR analysis, POR (FDR-adjusted p=0.0091), ABCA3 (FDR-adjusted p=0.014), LAMA5 (FDR-adjusted p=0.0091), and PLAT (FDR-adjusted p=0.0068) had significant differences in mRNA expression levels in males and females (Figure 4 and Table 5).

POR demonstrated higher mRNA expression in males, which corresponds to lower methylation in males (Figure 4 and Table 5). ABCA3, LAMA5, and PLAT demonstrated significantly higher mRNA expression in females (and hence lower methylation in females; Figure 4 and Table 5). The results of qRT-PCR mirrored our results from DNA methylation analysis.

Probe/Site	Female Mean	Female SD	Male Mean	Male SD	Bonferroni-Adj p-value	Functional genome annotation	CpG island content/neighbor	Gene Name
cg09632163	0.909	0.004	0.929	0.007	3.15E-04	Body	Island	ABCA3
cg10701168	0.911	0.008	0.936	0.009	2.69E-03	TSS1500	Open Sea	SLC13A2
cg20955458	0.866	0.029	0.915	0.01	8.06E-03	Body	N_Shelf	ALDH1L2
cg05435065	0.025	0.004	0.017	0.003	8.47E-03	TSS1500	Island	POR
cg10655371	0.71	0.039	0.789	0.018	9.03E-03	Body	Open Sea	CYP51A1
cg04314247	0.127	0.012	0.095	0.011	1.57E-02	5'UTR;1stExon;Body	Island	SLC44A3
cg22064129	0.803	0.017	0.855	0.018	1.61E-02	TSS1500	Open Sea	SLC14A2
cg26548134	0.738	0.03	0.813	0.022	1.96E-02	Body	Island	SLC25A17
cg10754697	0.87	0.016	0.905	0.009	3.54E-02	Body	Open Sea	SLC43A2
cg01879556	0.857	0.023	0.906	0.015	3.78E-02	5'UTR	N_Shore	SLC37A1

Table 4: List of DMET genes associated with the significant sex-based methylated sites.

DNA methylation					qRT-PCR			
Gene Name	Female	Male	Functional	CpG island	Avg	Avg	Fold Change	FDR
Probe/Site	Mean	Mean	genome	content/	female	male	(Female:Male)	adjusted
	± SD	± SD	annotation	neighbor	± SEM	± SEM		p-value
ABCA3	0.909 ±	0.929 ±	Body	Island	9.45 ±	11.7 ±	4.69 : 1.00	0.014
cg09632163	0.004	0.007			0.932	0.528		
PLAT	0.322 ±	0.424 ±	Body	Open Sea	6.58 ±	9.40 ±	7.05: 1.00	0.0068
cg06931905	0.041	0.034			0.889	0.565		
LAMA5	0.797 ±	0.855 ±	Body	S_Shelf	9.12 ±	11.6 ±	5.45: 1.00	0.0091
cg12382846	0.03	0.024			0.821	0.497		
Gene Name	Female	Male	Functional	CpG island	Avg	Avg	Fold Change	FDR
Probe/Site	Mean	Mean ±	genome	content/	female	male	(Female:Male)	adjusted
	± SD	SD	annotation	neighbor	± SEM	± SEM		p-value
POR	0.025 ±	0.017 ±	TSS1500	Island	11.6 ±	8.54 ±	0.117: 1.00	0.0091
cg05435065	0.004	0.003			0.787	0.597		

Table 5: DNA methylation levels, gene expression ΔCT values and mRNA expression fold change of selected genes involved in drug metabolism, drug transport, and renal disease/structure function in the kidney.

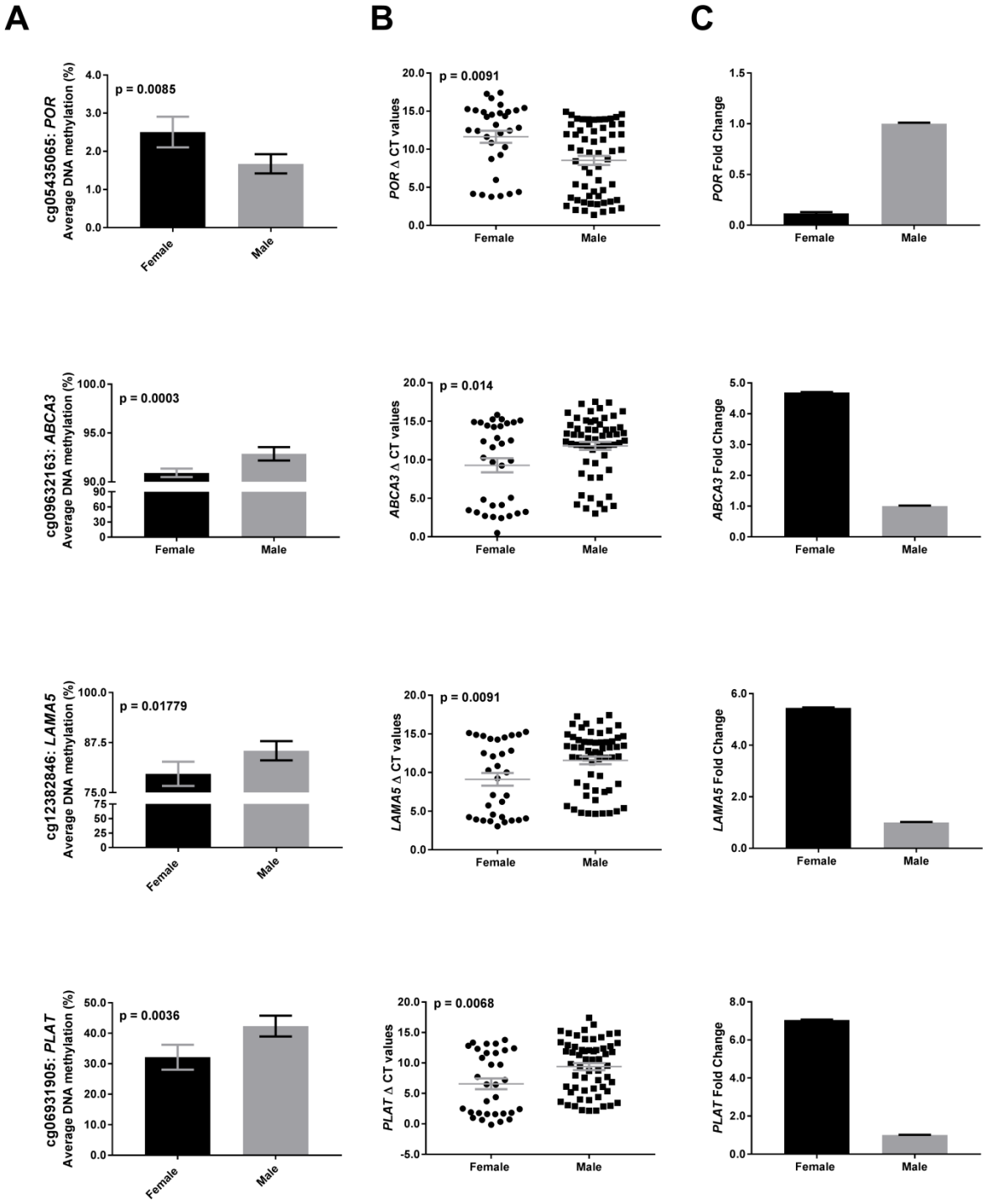

Figure 4: Impact of sex on DNA methylation and mRNA expression in human normal (non-diseased) kidney for selected kidney DMETs and cellular function/structure genes. Sex-based results of ABCA3, POR, LAMA5, And PLAT for A) DNA methylation beta values (data are presented as mean ± SD), B) ΔCT values (ΔCT=CTgene target–CT endogenous controls), and C) mRNA expression fold change using 2-ΔΔCT (ΔΔCT was determined using avg. ΔCT values and avg. ΔCT of males utilized as the reference group).

Discussion

The kidney is the most important excretory organ in the body. It is responsible for excreting soluble metabolic waste products and pharmaceutical drugs, maintaining normal biological functions, and secreting hormones and enzymes [9-12,57]. Numerous global gene expression studies involving diseased kidney have identified sex differences in several functions performed by the kidney, such as glomerular filtration, tubular secretion, and tubular reabsorption [6,58]. In addition, studies have demonstrated that genes involved in the excretory function of the kidney may be regulated through epigenetic mechanisms [59]. Epigenetic deregulation has also been suggested as a mechanism for drug response [60]; however, little is known about the epigenome of normal human organs involved in drug metabolism and transport such as the kidney. Therefore, our study, the first to examine sex-based differences in genome-wide DNA methylation in normal human kidneys, is critical to better understanding sex differences in normal kidney responses and function.

In this study, we analysed CpG sites on autosomal chromosomes of normal human kidney samples to evaluate sex-based differences in DNA methylation. Site-specific methylation analysis identified 429 sites (associated with 316 genes) with significant sex-based differences. There were no sex differences in average methylation, no matter how the sites were categorized e.g. functional genomic annotation or CpG island content/neighbouring context. Of the 429 significantly methylated sites, 79.0% were higher methylated in males. In addition, when compared to the distribution of all CpGs, significant sex-specific sites were enriched in the S shelf region and less enriched in the S shore region. This finding agrees with earlier studies which indicate that tissue-specific DNA methylation does not occur mainly in the CpG island region [32,34]. Our work suggests that kidney-specific DNA methylation may occur in the S shore and S shelf regions.

Based on pathway analysis of the 429 differentially methylated sites, we identified 31 significant biological pathways, several of which were associated with cancer. The pathway reaching the highest level of significance was cell cycle regulation. Cell cycle regulation under normal condition is essential for maintaining homeostasis, where cells in the kidney undergo low or slow turnover [61,62]. However, renal disease or injury can promote cell proliferation, cell death, or hypertrophy through activation of cycle cell cycle regulation [63]. Through IPA analysis of the 316 genes associated with the 429 methylated sites, we identified several genes of interest that may be involved in renal disease, injury, cellular function/maintenance/structure, and drug metabolism and transport. Drug-induced nephrotoxicity is an increasing public health concern not only in the United States but worldwide [64] and is one of the leading causes of failure in the drug development and approval process. This, in part, may be due to the variance in expression of DMETs and cellular/structural integrity in the kidney. Similar to the liver, the kidney contains DMETs. The DMETs in the kidney enhance the excretion of drugs through the urine [49]. Due to the critical role of DMETs in metabolizing and transporting therapeutic drugs, variations in the expression and activity of DMETs can lead to significant inter-individual differences in the renal structure and disposition of chemical compounds including Absorption, Distribution, Metabolism, and Excretion (ADME) of pharmaceutical products [65]. The observed within- and between-subjects (individuals) variance may be caused by epigenetic regulation. Studies have identified several drug metabolism and transport genes under epigenetic regulation; it is believed that 90% of epigenetic regulation in these genes involves DNA methylation [17]. Thus, our work also focused on profiling methylated sites associated with DMET genes and genes involved in renal disease/structure.

In this study, we identified 10 DMET genes: POR, SLC44A3, ABCA3, SLC13A2, ALDH1L2, CYP51A1, SLC14A2, SLC25A17, SLC43A2, and SLC37A1, and several genes involved in renal disease, injury and cellular structure/function: ABCA3, LAMA5, LZTS2, AHRR, ARID1B, CCNA1, CDH23, DROSHA, EPHB2, IFT74, KDM2B, PCDH15, PRDM4, PTCH1, SERF2, SMAD2, TBC1D1, UBE3C, NR3C1, NR3C2, ADRB2, PLAT, and FLT1. Two DMETs (POR and ABCA3) and 2 genes involved in renal disease/structure (LAMA5 and PLAT) were selected for validation through qRT-PCR analysis and evaluated based on sex. Sex difference is known to characterize the predisposition or expression of many diseases. Sexual disparities in response to acute and chronic renal disease have been identified for many years. Animal studies have demonstrated that males are more susceptible to renal injury compared to females [66], and females have a significantly higher ratio of survival when exposed to profound renal ischemia [67]. In the United States (as of 2014), chronic kidney disease is known to affect approximately 30 million adults, where males exhibit a higher prevalence and progression of renal disease, and is associated with a higher incidence of End Stage Renal Disease (ESRD) [68]. There has also been a wealth of clinical data demonstrating sex differences in drug responses and efficacy [1-6]. In this study, 3 of the 4 genes selected for validation, ABCA3, LAMA5 and PLAT, exhibited lower mRNA expression levels in males (higher methylation in their corresponding differentially methylated sites). Huls et al. hypothesized that ABCA3 plays a role in lipid homeostasis in the kidney as a phospholipid transporter [69]. The restructuring of membrane phospholipids may be an important function in the renewal process of damage tissue, such as the kidney [70]. Hence, DNA methylation of members of the ABCA family, for example ABCA3, may limit the availability of phospholipids needed in the regeneration of an injured kidney. Members of the laminin family of protein have been shown to be essential for normal kidney development, where experiments in mice have shown that LAMA5 is crucial for normal glomerular development and function in the kidney [71,72]. Therefore, DNA methylation of LAMA5 may lead to lower LAMA5 expression, and thus prevent the kidney from performing its function and recovering from renal injury. The role of PLAT in the kidney depends on it pathological function. As a serine protease, PLAT, plays a fundamental role in regulating Extracellular Matrix (ECM) degradation and accumulation in the kidney. Conventionally, PLAT is beneficial in the pathogenesis of renal interstitial fibrotic lesions, where elevated levels of PLAT leads to increased degradation of the accumulation and deposition of ECM associated with renal interstitial fibrotic lesions [73,74]. Accumulation and deposition of ECM associated with renal interstitial fibrotic lesions are part of the common cascade of actions leading to chronic kidney disease [75,76]. However, as a cytokine, PLAT may be damaging in promoting the development of kidney fibrosis [77]. In its role as a serine protease, DNA methylation of sites associated with PLAT may lead to PLAT gene silencing. Thus, under conditions leading to the formation of interstitial fibrotic lesions males may be more prone to chronic disease mediated by renal interstitial fibrotic lesions. The fourth gene selected for validation, POR, exhibited lower mRNA expression (higher methylation in its corresponding differentially methylated site) in females. Chronic and excessive ingestion of acetaminophen has been linked to renal toxicity or failure [78]. Renal toxicity due to acetaminophen has been, in part, attributed to the members of

cytochrome P450 oxidase enzymes, such as POR, in the kidney [79]. POR plays a role in metabolism of acetaminophen into a toxic metabolite [80]. Excess of toxic acetaminophen metabolite in the kidney mediates renal toxicity [80]. Hence, higher gene expression levels for POR (corresponding to lower DNA methylation) in males may lead to a higher level of acetaminophen toxic metabolites, and thus greater risk in kidney failure upon chronic or excessive ingestion of acetaminophen. This study reiterates previous findings that document factors contributing to higher prevalence and progression of renal disease in males. The data suggests that DNA methylation pattern of genes associated with renal injury, cellular structure/function, and drug metabolism may account in part for the higher prevalence in kidney disease/toxicity, and the lower survival or recovery ratios in males. Overall, this study allowed us to elucidate the epigenetic patterns in the kidney and to demonstrate the role of epigenetics in the regulation of genes involved in drug metabolism/excretion, cellular function/structure, and disease in the human kidney. This work is of paramount importance to understanding normal renal function. A more complete understanding of what constitutes a relatively normal epigenome, and the degree to which the epigenome varies based on sex has the potential to dramatically improve the success of studies related to epigenetic alterations in the human kidney.

Conclusion

This study identified methylated sites on autosomal chromosomes in human normal (non-diseased) kidney with sex-based differences. We also demonstrated that the pattern of epigenetic expression in significantly methylated sites in/near regions associated with DMET genes and involved in renal disease and cellular function/structure in the normal human kidney agreed well with mRNA expression patterns. The data supports the importance of DNA methylation as a source of sex-specific biases in drug response and renal disease/structure and thus increases our appreciation for the dynamic nature of the kidney epigenome. Most importantly, we provide a reference methylome for normal human kidney, which may be utilized to improve our understanding of renal disease and the overall safety and effectiveness of a drug's effect on the kidney.

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Authors' Contributions

The project was designed and planned by SJ, BLC, BGK, TN, SH, and BW. DNA and RNA extractions were performed by SJ, BLC, and GH. Methylation array and qRT-PCR studies were performed by SJ and BGK. The methylation raw data analysis, qRT-PCR statistical analyses, and pathway analysis were performed by NIG. Post-methylation data analyses and data interpretation were performed by SJ. The paper was written by SJ, NIG, and BLC. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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