Association Analysis of the Ephrin-B2 Gene in African-Americans with End-Stage Renal Disease

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Key Words

African-Americans • End-stage renal disease • Ephrin • Genetics • Hypertensive nephrosclerosis

Abstract

Background: Genome scans in African-Americans with end-stage renal disease (ESRD) identified linkage on chromosome 13q33 in the region containing the ephrin-B2 ligand (*EFNB2*) genes. Interactions between the ephrin-B2 receptor and ephrin-B2 ligand play essential roles in renal angiogenesis, blood vessel maturation, and kidney disease. **Methods:** The *EFNB2* gene was evaluated as a positional candidate for non-diabetic and diabetic ESRD susceptibility in 1,071 unrelated African-American subjects; 316 with non-diabetic etiologies of ESRD, 394 with type 2 diabetes-associated ESRD and 361 healthy controls. Single nucleotide polymorphism (SNP) genotyping was performed on the Sequenom Mass Array System. Statistical analyses were computed using Dandelion version 1.26, Snpaddmix version 1.4 and Haploview version 3.32. **Results:** Twenty-eight HapMap tag SNPs were genotyped spanning the 39 kilobases (kb) of the *EFNB2* coding region, with average spacing of 1.43 kb. Analysis of 710 ESRD patient samples and 361 controls provided no evi-

dence of single SNP associations in either diabetic or non-diabetic ESRD; although nominal evidence of association with all-cause ESRD was observed with a two SNP ($p = 0.022$) and three SNP ($p = 0.023$) haplotype, both containing SNPs rs7490924 and rs2391335 in intron 1. **Conclusions:** Although an attractive positional candidate gene, polymorphisms in the *EFNB2* gene do not appear to contribute in a substantial way to non-diabetic, diabetic or all-cause ESRD susceptibility in African-Americans. Additional genes within the chromosome 13q33 linkage interval are likely contributors to African-American non-diabetic ESRD.

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Introduction

Familial aggregation of end-stage renal disease (ESRD) is observed worldwide [1] and is independent from the prevalence of diabetes and hypertension within families [2]. African-American men and women demonstrate the strongest familial aggregation of ESRD, with a 2.5- to 3-fold higher risk when compared to European-Americans [3]. These factors strongly implicate the presence of renal failure susceptibility genes.

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Three genome scans have been published in African-American families enriched for multiple members with ESRD. These reports focused on diabetic, non-diabetic (hypertensive nephrosclerosis and glomerular diseases) and all-cause etiologies of ESRD [4–6]. Significant evidence for linkage to ESRD was detected on chromosome 13q33 in both the non-diabetic and all-cause ESRD genome scans. Thus, a renal failure susceptibility gene(s) may reside under this linkage peak.

The ephrin-B2 (*EFNB2*) gene is located on 13q33 and contains 5 exons spanning 45 kb. Splicing of these exons resulted in a 4,317-bp transcript encoding a 333 amino acid protein. The ephrin-B2 ligand is expressed in the developing nephron and interactions between ephrin-B2 receptor tyrosine kinases and transmembrane-type ephrin-B2 ligands appear to play an important role in vasculogenesis in the developing embryo [7, 8]. Ephrin-B2 also induces migration of adult endothelial cells and promotes angiogenesis via the phosphatidylinositol-3 (PI3) kinase pathway [9]. In related studies, ephrin-B1 was recently shown to co-localize with CD2-associated protein (CD2AP) and nephrin at the podocyte slit diaphragm and plays an important role in maintaining barrier function at the slit diaphragm [10] and ephrin B4 receptor kinase transgenic mice develop glomerulopathy, manifested by fused afferent and efferent arterioles bypassing the glomeruli (aglomerular vascular shunts) [11].

Diseases of small intrarenal arterioles and arteries have been implicated in the development of arteriolar nephrosclerosis (hypertensive renal disease). Hypertensive ESRD is more often diagnosed in African-Americans compared to European-Americans, with a 4-fold higher incidence rate nationally and a twenty-fold higher incidence rate in the southeastern US [12]. The small blood vessel injury observed in arteriolar nephrosclerosis fails to correlate with systemic blood pressure [13, 14], suggesting the presence of an innate renal blood vessel disease that secondarily leads to systemic hypertension with resultant renal failure. In order to determine whether *EFNB2*, a positional and functional candidate gene for nephropathy susceptibility was involved in the pathogenesis of kidney failure, we performed an association analysis of the *EFNB2* gene in African-Americans with ESRD.

Methods

Participants

Self-reported African-Americans born in North Carolina, South Carolina, Georgia, Virginia or Tennessee formed the study population. Peripheral blood specimens for DNA extraction were

collected from unrelated, prevalent patients performing hemodialysis in Winston-Salem, Greensboro and Hickory, North Carolina. Medical records were reviewed by a single investigator (B. I.F.). Non-diabetic subjects with ESRD attributed to hypertension on the CMS 2728 form and with hypertension preceding initiation of renal replacement therapy and hypertensive target organ damage (retinopathy or left ventricular hypertrophy), or with chronic glomerulonephritis (proteinuria detected on dipstick or quantitated urine specimen) were diagnosed as having non-diabetic ESRD. Type 2 diabetic ESRD was diagnosed in subjects whose diabetes developed after the age of 34 years, with ≥ 5 years diabetes duration prior to dialysis onset, in the presence of diabetic retinopathy and/or proteinuria exceeding 500 mg/24 h. Cases with polycystic kidney disease, hereditary nephritis, urologic diseases and surgical nephrectomy were excluded.

Healthy, unrelated non-diabetic African-Americans who were born in one of the above five states served as controls. Control subjects denied a personal or family history (in first-degree relatives) of diabetes or kidney disease (kidney disease defined as kidney failure, dialysis treatments or kidney transplantation). The study was approved by the Institutional Review Board at the Wake Forest University School of Medicine and met the criteria outlined in the Declaration of Helsinki. All participants provided written informed consent. DNA from these participants was extracted using Puregene (Gentra, Minneapolis, Minn., USA). Table 1 contains demographic data from study participants. Thirty-nine unrelated European-American (EA) controls were recruited, as described for the African-American controls, and DNA was obtained from 44 Yoruba Nigerians from the National Institute of General Medicine Sciences (NIGMS) Human Variation Collection (Coriell Repositories, Camden, N.J., USA).

SNP Selection and Genotyping

Twenty-eight haplotype tagging SNPs were chosen for genotyping based on linkage disequilibrium (LD) information from the HapMap Yoruba in Ibadan Nigerian dataset to maximally capture ($r^2 > 0.80$) common variants (minor allele frequency $> 5\%$). Selected SNPs were: rs9520087, rs3742160, rs4399422, rs9301140, rs9301141, rs9514540, rs11069646, rs9587148, rs7983579, rs7983611, rs9514543, rs8002701, rs8001826, rs7327929, rs11840214, rs9520090, rs9520091, rs9520092, rs7320751, rs4772795, rs9514545, rs7490924, rs2391335, rs2391336, rs2391337, rs2391339, rs8000078 and rs9520094. All SNPs were intronic, with the exception of rs9520087 which was located in the noncoding portion of exon 5. Figure 1 depicts the *EFNB2* gene and the relative location of each SNP. These SNPs spanned the entire *EFNB2* gene at an average density of 1.43 kb with inter-SNP distances ranging from 22 bp to 6.5 kb. SNP genotyping was performed on a Sequenom Mass Array Genotyping System (Sequenom, San Diego, Calif., USA).

Genotyping for Admixture Analysis

Seventy diallelic Admixture Informative Markers (AIMs) were genotyped to determine whether population substructure biased our conclusions [15]. The 44 YRI, 39 EA controls, 361 African-American controls, and 730 African-American ESRD samples were genotyped using Illumina Inc's Custom Genotyping Services (San Diego, Calif., USA) or using the Sequenom Mass Array (San Diego, Calif., USA).

Fig. 1. Genomic map and LD plot of the ephrin-B2 gene. The labeled shaded regions are exons, numbered 1–5. The nonshaded region represents the 3'UTR. The ruler represents the relative location of SNPs in kilobases within the 39-kb region of EFNB2. The SNP numbers and their locations (relative to each other) are indicated at the top. The LD block structure was calculated using the solid spine method in Haploview.

Table 1. Demographic data in African-American participants: mean (median) $\pm$ SD, where applicable

	Controls (n = 361)	T2DM ESRD (n = 394)	Non-DM ESRD (n = 316)
Female, %	44.8	61.6	42.4
Age at recruitment, years	50.1 (49) $\pm$ 10.1	64 (64) $\pm$ 10.1	53.4 (53) $\pm$ 14.8
BMI at recruitment	29.4 (28.9) $\pm$ 6.7	29.2 (28.2) $\pm$ 7.3	26.9 (24.9) $\pm$ 7.6
Maximum reported BMI	31.5 (30.3) $\pm$ 7.7	36.3 (34.9) $\pm$ 9.6	32.9 (30.5) $\pm$ 9.5
Age at T2DM onset, years	N/A	42.7 (40) $\pm$ 11.9	N/A
Age at ESRD onset, years	N/A	60.3 (60) $\pm$ 10.1	47.6 (47) $\pm$ 15.6
Predialysis diabetes duration, years	N/A	17.6 (17) $\pm$ 10.7	N/A

T2DM ESRD = Type 2 diabetes mellitus-associated ESRD; non-DM ESRD = nondiabetic ESRD (hypertensive nephrosclerosis and chronic glomerular diseases).

Table 2. Association analysis for *EFNB2* SNPs and ESRD

SNP	Alleles (minor/ major)	MAF T2DM-ESRD (n = 394)	MAF Non-DM ESRD (n = 316)	MAF all-cause ESRD (n = 710)	MAF controls (n = 361)	p value (all-cause ESRD vs. controls)
rs9520087	G/A	0.412	0.399	0.406	0.410	0.824
rs3742160 ^a	A/T	0.393	0.367	0.382	0.360	0.3329
rs4399422 ^b	A/G			N/A	N/A	
rs9301140	C/G	0.483	0.469	0.477	0.438	0.083
rs9301141	G/C	0.273	0.279	0.276	0.270	0.7589
rs9514540	A/T	0.342	0.336	0.340	0.316	0.2688
rs11069646	A/G	0.339	0.343	0.340	0.357	0.4978
rs9587148 ^a	G/A	0.475	0.487	0.482	0.482	0.997
rs7983579	T/G	0.460	0.465	0.462	0.456	0.8311
rs7983611 ^b	A/G			N/A	N/A	
rs9514543 ^a	C/T	0.454	0.425	0.441	0.450	0.6764
rs8002701	T/C	0.483	0.487	0.485	0.482	0.1547
rs8001826	C/G	0.280	0.288	0.284	0.272	0.5536
rs7327929	G/A	0.332	0.333	0.332	0.332	0.9807
rs11840214 ^a	G/A	0.403	0.441	0.420	0.422	0.9604
rs9520090	G/C	0.251	0.254	0.252	0.258	0.7298
rs9520091	G/T	0.389	0.409	0.398	0.401	0.7263
rs9520092	T/C	0.350	0.347	0.348	0.347	0.9673
rs7320751	C/G	0.258	0.249	0.253	0.272	0.3521
rs4772795	G/A	0.312	0.307	0.310	0.320	0.6255
rs9514545	C/T	0.253	0.245	0.250	0.270	0.2963
rs7490924	G/A	0.414	0.417	0.415	0.394	0.3648
rs2391335	G/T	0.308	0.302	0.304	0.346	0.056
rs2391336	G/A	0.381	0.390	0.384	0.404	0.3044
rs2391337	G/A	0.259	0.244	0.252	0.237	0.4257
rs2391339	G/A	0.335	0.327	0.330	0.358	0.214
rs8000078	T/G	0.348	0.351	0.350	0.346	0.8871
rs9520094	G/T	0.258	0.233	0.247	0.231	0.4182

MAF = Minor allele frequency; N/A = not applied.

^a Deviated from Hardy-Weinberg equilibrium in both cases and controls; ^b <95% genotyping efficiency.

A power analysis was performed to determine the odds ratio (OR) detectable with 710 ESRD cases and 361 controls, assuming a prevalence of 2.5%. Computations were completed using QUANTO (version 1.1.1) for $\alpha = 0.05$ and $\alpha = 0.001$, over a range of minor allele frequencies and genetic models (i.e. dominant, additive and recessive).

Statistical Analyses

Statistical analyses to test for Hardy-Weinberg equilibrium (HWE) were determined by calculating a χ^2 statistic and corresponding p value. Pair-wise LD and single SNP allelic associations were performed using Haploview 3.32 [16]. Dandelion was used to perform haplotypic association analysis [17] and Snpadmix was used to calculate haplotypic association adjusted for African ancestry proportions as reported in Keene et al. [15].

Results

Using Tagger [18], the 28 SNPs that were analyzed captured 94% of the known variation at the *EFNB2* locus with an $r^2 > 0.80$ (mean $r^2 = 0.96$). In the case and control populations, four SNPs failed to meet HWE; rs3742160 ($p = 0.003$ controls, $p = 0.038$ cases), rs9587148 ($p = 0.0001$ controls, $p = 0.0001$ cases), rs9514543 ($p = 0.002$ controls, $p = 0.006$ cases) and rs11840214 ($p = 0.022$ controls, $p = 0.0018$ cases). Two additional SNPs did not conform to HWE, one in the case population rs8002701 ($p = 0.021$) and one in the control population rs8000078 ($p = 0.041$). Two SNPs (rs4399422 and rs7983611) were removed from further analysis when they failed to meet genotyping efficiency (<95% genotyping success rate). Genotyping

Table 3. Haplotypic association analysis for all-cause ESRD

Haplo-type	Frequency cases	Frequency controls	p value	Z statistic	OR	Empirical p value*
rs7490924, rs2391335						0.022
AG	0.078	0.085	0.721	-0.36	0.92	
AT	0.507	0.521	0.663	-0.44	0.94	
GG	0.228	0.261	0.238	-0.84	0.88	
GT	0.187	0.133	0.029	2.18	1.48	
rs9514545, rs7490924, rs2391335						0.023
CAG	0.002	0.005	0.367	-0.90	0.38	
CAT	0.122	0.152	0.166	-1.38	0.77	
CGG	0.016	0.027	0.209	-1.26	0.57	
CGT	0.110	0.086	0.219	1.23	1.31	
TAG	0.078	0.080	0.890	-0.14	0.96	
TAT	0.384	0.368	0.616	0.50	1.07	
TGG	0.211	0.234	0.392	-0.86	0.87	
TGT	0.079	0.048	0.064	1.85	1.67	
* Ancestry-adjusted; please see text for details.						

success rates for the *EFNB2* SNPs were 96.1–99.7% for both the cases and controls, 208 duplicate samples genotyped had a 100% concordance rate, while the AIM SNPs were genotyped at a rate of 93.3–97.4%.

Using the solid spine block definition in Haploview, SNPs successfully genotyped at the *EFNB2* locus were contained in eight haplotype blocks of two or more SNPs, with a mean size of 1.57 kb (0.05–4.15 kb) capturing 20/26 (76.9%) of the markers. Block structure is depicted in figure 1, where the absolute value of the inter-SNP D' from the control population is shown. A similar LD structure was observed in the case population (data not shown).

Table 2 summarizes allele frequencies and the results of the single SNP allelic association analysis for the African-American ESRD cases and control subjects. Initial analysis did not reveal any single SNP allelic associations with ESRD, however, SNP rs2391335 trended towards association ($p = 0.056$). This result remained unchanged when adjusted for admixture ($p = 0.057$).

Following single SNP analysis, haplotypic analysis was performed with Dandelion [17] using 2- and 3-marker moving windows for all-cause ESRD. Nominal evidence of association in a 2- and 3-marker haplotype, both containing the SNPs rs7490924 and rs2391335 in intron 1, was observed. Table 3 describes the association analysis of the significant 2- and 3-SNP haplotypes. The frequency for cases and controls, p value, Z score and OR of each haplotype is presented with the empiric p value for

all haplotypes constructed within the interval. Significance within this interval does not appear to be the result of a rare genotype since the significantly associated GT haplotype driving both 2-SNP ($p = 0.029$) and 3-SNP (haplotype TGT, $p = 0.064$) (table 3), haplotypic associations is present at a frequency of 18% in cases and 13% in controls. The positive Z statistic of 2.18 and an OR of 1.48 suggest the GT haplotype is a risk haplotype, i.e. associated with increased prevalence of disease.

FRAPPE (Frequentist Estimation of individual ancestry proportion) was used to calculate the proportion of African ancestry in our case and control population [19]. AIMS genotyped in our population revealed a mean African ancestry proportion of 0.792 (SD = 0.110) in controls and 0.807 (SD = 0.108) in ESRD cases. The individual African ancestry proportion data was used as a covariate in the program Snpadmix [Langefeld and Stiebert, unpubl.] to calculate haplotype analysis. The p values seen in the original analyses did not change significantly, further proving that admixture was not a factor. The p values for the 2-marker ($p = 0.022$) and 3-marker ($p = 0.023$) haplotypes remained significant after adjustment for ancestry.

A power analysis was computed to examine the magnitude of the OR plausibly detectable (table 4). For a SNP with a minor allele frequency of 0.20 and with 0.50 power, 710 ESRD cases and 361 controls can detect an OR = 1.21 and OR = 1.44 for $\alpha = 0.05$ and $\alpha = 0.001$, respectively. These estimates are consistent with published ORs for complex genetic traits.

Discussion

Factors other than high blood pressure per se contribute to the development of arteriolar nephrosclerosis or 'hypertension-associated end-stage renal disease'. Two renal biopsy studies demonstrated that the degree of systemic hypertension did not correlate with arterio- or arteriolo-nephrosclerosis [13, 14]. Epidemiologic analyses strongly support this conclusion [20]. In addition, strict blood pressure control fails to halt the progression of hypertensive nephropathy in African-Americans, while it appears to do so in Caucasians [21, 22]. The ephrin-B2 ligand (*EFNB2*) gene is an attractive candidate gene for susceptibility to hypertensive renal failure (e.g. non-diabetic ESRD), since polymorphisms in this gene could lead to arterio-nephrosclerosis. Disease in small arterioles could result in high systemic blood pressures and progressive kidney failure. However, we

Table 4. Odds ratio detectable under varying genetic models and minor allele frequencies (q) for 710 ESRD cases and 361 controls, assuming disease prevalence 0.025

Type I error	Power	Odds ratio detectable (OR > 1)								
		dominant			additive			recessive		
		q = 0.45	q = 0.3	q = 0.2	q = 0.45	q = 0.3	q = 0.2	q = 0.45	q = 0.3	q = 0.2
0.05	0.50	1.33	1.29	1.30	1.20	1.21	1.24	1.36	1.51	1.78
	0.70	1.44	1.38	1.39	1.26	1.28	1.32	1.46	1.68	2.05
	0.80	1.50	1.44	1.45	1.30	1.32	1.36	1.54	1.78	2.22
	0.90	1.62	1.52	1.54	1.35	1.38	1.43	1.64	1.94	2.47
0.001	0.50	1.63	1.54	1.54	1.35	1.38	1.44	1.65	1.95	2.50
	0.70	1.76	1.65	1.66	1.42	1.45	1.52	1.78	2.14	2.83
	0.80	1.86	1.72	1.72	1.46	1.49	1.57	1.86	2.27	3.05
	0.90	2.00	1.82	1.82	1.52	1.56	1.64	1.98	2.45	3.37

did not observe compelling evidence that polymorphisms in the *EFBN2* gene on chromosome 13q33 were associated with nondiabetic, diabetic or all-cause ESRD in this cohort of African-American subjects with advanced nephropathy, though haplotype analysis suggests a possible genetic link. Extension of this study to include additional study samples or other populations is likely worthwhile.

Ephs and ephrins are membrane-bound proteins that function as receptor-ligand pairs. Angiogenesis, the process of sprouting branches from the immature vascular plexus, differential growth of endothelial cells and recruitment of pericytes and smooth muscle cells, are dependent on the effects of vascular endothelial growth factor (*VEGF*), angiopoietins and ephrins [23]. Ephrin-B2 ligand and ephrin-B2 receptor appear to trigger the development of angioblasts into mature endothelial cells that are committed to either an artery or vein lineage [24]. In addition, ephrin-B1 expression has been detected along the glomerular capillary loop, where it is restricted to the glomerular slit diaphragm [10]. Ephrin-B1, nephrin and CD2-associated protein all co-localize at the slit diaphragm and there is extensive evidence supporting ephrin-B1 as playing an important role in maintaining slit diaphragm barrier function [10]. Abnormalities in this barrier lead to proteinuria, a risk factor for progressive renal injury. As a primary defect in small intrarenal arterioles may underlie hypertensive nephrosclerosis in African-Americans, polymorphisms in the *EFBN2* gene under the 13q linkage peak could have accounted for development of this disease. However, thorough analysis of this gene using 28 SNPs at best revealed limited evidence

for such an association. This leaves open the likelihood that another gene or genes in this region contribute to the evidence of linkage.

We conclude that polymorphisms in the *EFBN2* gene on chromosome 13q33 were not strongly associated with ESRD as defined by the chromosome 13 linkage peak observed in African-American families enriched for members with severe nephropathy, although two haplotypes in *EFBN2* were nominally associated with all-cause ESRD. Additional gene(s) in this region likely contribute to ESRD susceptibility and efforts to identify these genes should receive high priority. Detection of the genes underlying non-diabetic or hypertension-associated ESRD, such as chromogranin A [25], may allow us to determine why select individuals with high blood pressure ultimately develop ESRD, as well as identify novel pathways for intervention to prevent hypertension-associated renal failure.

Acknowledgements

This work was supported in part by NIH grants RO1 DK 070942 (BIF), RO1 DK066358 (M.M.S.), and RO1 DK53591 (D.W.B.). The authors report no conflicts of interest in this work. The authors are indebted to Drs. James Deterding, David Harvey, Richard Paul, William Scott Moore, Todd Greenwood and their colleagues, without whom this work would not have been possible, and acknowledge the successful recruiting efforts of Joyce Byers, Carrie Smith, Mitzie Spainhour, and Sharon Warren.

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