Vitamin D Receptor Gene Polymorphisms and Environment Influencing the Impact on Survival in Hemodialysis Patients

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Keywords. hemodialysis, environment, vitamin D receptor gene, gene polymorphisms, survival

Introduction. The vitamin D-receptor axis is involved in multiple physiological functions and altered states such as hypertension, mineral metabolism disorders, and inflammation. These disturbances are major risk factors for progression to end-stage kidney disease and cardiovascular disease. In addition, changes in internal systemic environment could be influencing the impact of survival in patients with kidney disease. This study aimed to evaluate the impact of vitamin D receptor (*VDR*) polymorphisms on hemodialysis patients' survival.

Material and Methods. A total of 122 hemodialysis patients and 120 healthy controls were compared for *VDR* gene polymorphism. Markers for full coverage in the *VDR* gene were selected and genotyped. The hemodialysis patients were followed until death event, which was considered the primary endpoint for the survival analysis.

Results. Two tag SNPs (rs10875695 and rs11168293) showed significant differences between the hemodialysis and healthy patients. In survival analysis, the CC genotype for rs2248098, compared to the TT genotype, was associated with a worse mortality rate. After adjustments for age, sex, diabetes mellitus, and cardiovascular disease, the genotype CC (rs2248098) was associated with a higher risk of mortality in a multivariable analysis.

Conclusions. Polymorphisms specific to patients with kidney disease could be influencing different conditions associated with mortality. Thus, these genetic markers, rs2248098 for example, would act in a specific time in the history of kidney disease and would bring different results of patient survival outcomes.

IJKD 2018;12:223-31
www.ijkd.org

Original Paper

INTRODUCTION

Patients with end-stage kidney disease treated with hemodialysis present a number of complications and comorbidities that are responsible for the high mortality rates, primarily related to cardiovascular disease. Traditional risk factors do not fully explain the higher mortality rates among these patients.[1] In addition, genetic risk factors, particularly those involved in the control of inflammatory response and bone metabolism, have shown relevance in this population.[2] Vitamin D, and its receptor (VDR), classically play an essential role in a large spectrum of biological actions not only related to mineral metabolism, but also in cell proliferation and immunomodulation.[3] Moreover, cardiomyocytes, vascular endothelial, and smooth

muscle cells express VDR and respond to the active product of vitamin D.[4] Human VDR is a product of a single gene located on 12th chromosome in 12q12-14 position, and consists of 11 exons, spanning 63,495 bp.[5] It has been demonstrated that mutations in the *VDR* gene significantly alter its subcellular distribution and that subtle variations in expression or function of VDR may contribute to major differences in the regulation of other target genes.[6] The *VDR* gene is polymorphic and presents hundreds of polymorphisms,[7] which could make the entire physical gene coverage unviable. However, with the use of bioinformatic genetic tools, such as those presented in the extinct HapMap Project (https://www.genome.gov/10001688/international-hapmap-project), the coverage of all polymorphisms becomes feasible. These tools include formulas considering the high linkage disequilibrium, providing a small number of single nucleotide polymorphisms (SNPs), which can capture the entire genomic region named target SNPs (tag SNPs).

The *VDR* gene is a candidate locus for susceptibility to different diseases, such as bone mineral disorder,[8] osteoporosis,[9] periodontal disease,[10] breast cancer,[11] cardiovascular calcification,[12] and coronary artery disease.[13] Another important point is that patients with kidney disease present an intense modification in the systemic parameters throughout the progression of their disease. It is known that the environment ultimately contributes to a certain weight in genotype expression. Thus, progression through the stages of renal disease could be contributing along with genetic polymorphisms.

The hypothesis of this study was that polymorphisms in the *VDR* gene could be more related to susceptibility to end-stage kidney disease and hemodialysis patient outcomes. To approach that, we aimed to compare the prevalence of polymorphism, covering the whole *VDR* gene, between end-stage kidney disease patients and controls to investigate the association of *VDR* SNPs in this population. Our second objective was to evaluate whether polymorphisms in *VDR* were determinants of survival in hemodialysis patients.

MATERIALS AND METHODS

Study Population

The first part of this work comprised a cross-sectional study with 242 unrelated male and female patients with a mean age of 44.8 years (range, 20 to 77 years) selected from the Pro-Renal Foundation and the Pontifical Catholic University of Paraná in Curitiba. Participants completed personal and medical history questionnaires following a protocol approved by an Institutional Review Board and signed a consent form (approved by the Ethics Committee in Research at Pontifical Catholic University of Paraná, CAAE 25141813.4.0000.0020). Details from the original study design can be accessed in a preliminary study performed with this cohort.[10]

The sample was divided into 2 groups of 122 hemodialysis patients and a healthy control group with 120 individuals without chronic kidney disease (CKD). Laboratory measurements were obtained from routine on dialysis clinics for the hemodialysis patients. All individuals without CKD presented a glomerular filtration rate greater than 90 mL/min estimated according to the Modification of Diet in Renal Disease formula.[14] After finishing the inclusion phase of the participants, the lack of matching in our sample was verified, mainly due to the characteristics related to the variables age and sex.

DNA Collection and Purification

Buccal epithelial cells for all of the participants were obtained by a mouthwash with 3% glucose.[15] DNA was extracted using proteinase K, ammonium acetate (10 M), and precipitated with isopropanol. After that, it was suspended with Tris 10mM and ethylenediaminetetraacetic acid 1mM.[16]

Marker Selection and Genotyping

Markers for coverage (target polymorphism, tag SNP) of the *VDR* gene locus were selected according available information in the International HapMap Project website (https://www.genome.gov/10001688/international-hapmap-project). Searches for the following criteria were applied: release 24/phase 2_Nov08, minor allele frequency of 5%, multimarker option, $r^2 = 0.8$ (80%) for linkage disequilibrium in Yoruba population. After applying the above criteria, 40 tag SNPs were selected by the program: rs100875695, rs11168266, rs11168267, rs11168268, rs11168287, rs11168288, rs11168293, rs115740065, rs1157400700, rs115741100, rs11574114, rs11574138, rs1230080082, rs12314197, rs12717991,

rs15400339, rs15444100, rs21894800, rs2239179, rs2239182, rs2239185, rs22480098, rs22542100, rs25250044, rs2853563, rs2853564, rs37829005, rs3819545, rs3858733, rs43340089, rs47600648, rs7136534, rs730050032, rs731236, rs7963776, rs7975128, rs7975232, rs79793600, rs886441, and rs987849.

The tag SNPs selected were genotyped by polarized fluorescence (Taqman probe-based methodology) using the ABI 7500 platform. The genotype (allelic discrimination) of each sample was carried out by determining the intensity of the fluorescence emitted. Using a fluorescent specific probe, an increase of the fluorescence signal indicates homozygosity for one of the alleles. Increasing two-color fluorescence is indicative of the presence of heterozygosity. Tag SNPs used in the study met the following quality control criteria: overall call rate greater than 94% and Hardy-Weinberg equilibrium among controls.

Each tag SNP was assessed for genotypic modes of transmission (additive, dominant and recessive models). For a SNP with a major allele "*a*" and a minor allele "*b*", the dominant model tests the hypothesis that the collective genotypes "*bb*" and "*ab*" are associated with an increased or decreased risk compared with the genotype "*aa*".

Survival Analysis

The second part of the study took place after the initial collection period of hemodialysis patients. All patients with kidney disease were prospectively followed in the dialysis clinic until the primary endpoint (mortality from any cause), end of study (censored), or lost to follow-up. After the follow-up, all causes of death were recorded through an active search of the medical records. Details of survival for these patients can be accessed in a preliminary study performed with this cohort.[17]

Statistical Analysis

The genotypic frequencies were compared by univariate analysis using the chi-square test or the Pearson test for the dominant model, and logistic regression for the additive model. The strength of association was expressed by odds ratio (OR). The Kaplan-Meier curves were used to visualize the survival of patients according to the genetic variables. The log-rank test was used to assess the difference between the curves. A logistic regression model was used in multivariate analyses and variables of significant clinical importance (genetic polymorphisms, for example) or with *P* values less than .20 in the univariate analysis were included. In this analysis, only the final models that showing significant *P* values for all variables included were considered. Analyses were performed the SPSS software (Statistical Package for the Social Sciences, version 13.0, SPSS Inc, Chicago, IL, USA). The Hardy-Weinberg equilibrium and the linkage disequilibrium estimations were identified using the Haploview software, version 4.2.

RESULTS

Baseline characteristics of the study population are shown on Table 1. Healthy controls (n = 120; 49.6%) compared with the hemodialysis group (n = 122; 50.4%) were younger (39.0 ± 9.5 years versus 49.7 ± 12.8 years; *P* = .001). There were fewer females in the hemodialysis group (n = 41; 33.6%) than in the controls (n = 82; 68.3%) and a higher prevalence of diabetes mellitus (21.3% versus 1.7%) and cardiovascular disease (22.1% versus 1.7%).

Serum markers among the hemodialysis patients

Table 1. Baseline Characteristics of the Study Population

Characteristic	Hemodialysis (n = 122)	Control (n = 120)	Odds Ratio (95% Confidence Interval)	*P*
Age, y	49.7 ± 12.8	39.9 ± 9.5	…	.001
Sex				
Male	81 (66.4)	38 (31.7)		
Female	41 (33.6)	82 (68.3)	1.75 (0.75 to 4.10)	.001
Diabetes mellitus	26 (21.3)	2 (1.7)	0.63 (0.00 to 0.27)	.001
Cardiovascular disease	27 (22.1)	2 (1.7)	0.60 (0.01 to 0.26)	.001
Anemia	41 (33.6)	3 (2.5)	0.51 (0.02 to 0.17)	.001
Hypertension	89 (73.0)	2 (1.7)	0.01 (0.00 to 0.23)	.001
Smoking	28 (23.0)	8 (6.7)	0.24 (0.10 to 0.55)	.001

*Values are mean ± standard deviation or frequency (percentage).

were as follows: serum calcium, 9.0 ± 0.7 mg/dL (range, 7.8 mg/dL to 11.8 mg/dL); serum phosphorus, 5.9 ± 1.3 mg/dL (range, 3.5 mg/dL to 9.4 mg/dL); calcium-phosphorus product, 51.2 ± 12.5 mg^2/dL^2 (range, 24.8 mg^2/dL^2 to 88.6 mg^2/dL^2); serum potassium, 5.3 ± 0.6 mg/dL (range, 3.6 mg/dL to 7.4 mg/dL); serum alkaline phosphatase, 129 ± 118 IU/L (range, 49 IU/L to 824 IU/L); and serum albumin, 3.7 ± 0.3 mg/dL (range, 3.0 mg/dL to 4.5 mg/dL).

Tables 2 and 3 show results of genotyping for tag SNPs in the hemodialysis and healthy controls. All polymorphisms were assessed for genotypic modes of transmission (additive, dominant and recessive models). The rs10875695 and rs11168293 presented, in relation to their genotypes, significant difference between the hemodialysis patients and the controls.

Figure 1 illustrates all 40 tag SNPs in the *VDR* gene and the values found for linkage disequilibrium between polymorphisms. All allele frequencies in the control group were in the Hardy-Weinberg equilibrium (data not shown).

The survival analysis showed there were 27.3% death events in the hemodialysis population during a mean follow-up time of 40 ± 15 months. Cardiovascular disease (acute myocardial infarction) was the leading cause of mortality (53%), followed by infectious complications (22%). Older age (P = .001), male sex (P = .007), and the presence of diabetes mellitus (P = .006), and cardiovascular disease (P < .001) were associated with increased mortality risk (data not shown in the Figure).

Figure 2 illustrates the survival curves for only 5 tag SNPs. Present worse association with mortality the CC genotype for rs2248098, AA genotype for rs7963776, TT genotype for rs7975232, CC genotype for rs10875695, and GT genotype for rs11168293. The best result for mortality analysis was to rs2248098, showing that the genotype. CC for this SNP was associate with worse survival compared to TT.

Still considering the survival analysis, the authors would like to show other polymorphisms that were not significant within the statistical concept. However, it seems important to present them because the outcome involving these tag SNPs are significant from the renal patient's point of view. The GG genotype (for rs7963776 and rs7975232) obtained the best survival curves compared to the other possible genotypes within this genetic marker (P = .09 and P = .09).

After the genotypes and survival analysis, logistic regression models were performed. Table 4 shows that even after adjustments for age, sex, diabetes mellitus, cardiovascular disease, rs2248098 (CC genotype), rs7963776 (AA genotype), and rs7975232

Table 2. Univariable Genotypic Analysis of Target Single Nucleotide Polymorphisms (Tag SNPs) in Vitamin D Receptor (*VDR*) Gene in Addictive Model*

Tag SNPs DbSNP ID†	Variation [1/2]‡	Group	Homozygous 1	Heterozygous	Homozygous 2	*P*
			CC	**CA**	**AA**	
rs10875695	[C/A]	Control	55 (46.6)	51 (43.2)	12 (10.2)	
		Hemodialysis	76 (62.3)	38 (31.1)	8 (6.6)	.049
			GG	**GT**	**TT**	
rs11168293	[G/T]	Control	56 (47.1)	56 (47.1)	7 (5.9)	
		Hemodialysis	62 (50.8)	42 (34.4)	18 (14.8)	.03
			CC	**CT**	**TT**	
rs11574070	[C/T]	Control	115 (95.8)	2 (17)	3 (2.5)	
		Hemodialysis	119 (97.5)	3 (2.5)	0 (0.0)	.20
			AA	**CA**	**CC**	
rs2189480	[A/C]	Control	61 (51.3)	44 (37.0)	14 (11.8)	
		Hemodialysis	47 (38.5)	62 (80.8)	13 (10.7)	.09
			GG	**GA**	**AA**	
rs4334089	[G/A]	Control	58 (48.3)	50 (41.7)	12 (10.0)	
		Hemodialysis	74 (60.7)	38 (31.2)	10 (8.2)	.15
			AA	**AG**	**GG**	
rs731236	[A/G]	Control	41 (34.2)	68 (56.7)	11 (9.2)	
		Hemodialysis	51 (41.8)	55 (45.1)	16 (13.1)	.19

*Values are frequency (percentage). Only variables with *P* values less than .20 in the univarieble model are shown in this table.
†SNP identifier based on NCBI dbSNP
‡The first allele is designated as the major allele and the second allele is designated as the minor allele.

Table 3. Univariable Genotypic Analysis of Target Single Nucleotide Polymorphisms (Tag SNPs) in Vitamin D Receptor (*VDR*) Gene in Dominant or Recessive Model*

Tag SNPs dbSNP ID†	Alelles‡	Groups	Genotypes	Genotypes	*P*
			AA + CA	**CC**	
rs10875695 (Dom A)	[C/A]	Control	63 (53.4)	55 (46.6)	
		Hemodialysis	46 (37.7)	76 (62.3)	.02
			GG + AG	**AA**	
rs11168287 (Dom G)	[A/G]	Control	73 (64.6)	40 (35.4)	
		Hemodialysis	90 (74.4)	31 (25.6)	.12
			GG + GT	**TT**	
rs11168293 (Rec G)	[G/T]	Control	112 (94.1)	7 (5.9)	
		Hemodialysis	104 (85.2)	18 (14.8)	.03
			CC + CT	**TT**	
rs11574070 (Rec T)	[T/C]	Control	117 (97.5)	3 (2.5)	
		Hemodialysis	122 (100.0)	0 (0.0)	.12
			AA + CA	**CC**	
rs2189480 (Rec A)	[A/C]	Control	58 (48.7)	61 (51.3)	
		Hemodialysis	75 (61.5)	47 (38.5)	.05
			TT + CT	**CC**	
rs2254210 (Rec C)	[C/T]	Control	57 (47.9)	62 (52.1)	
		Hemodialysis	70 (57.4)	52 (42.6)	.16
			CC + CG	**GG**	
rs3782905 (Dom C)	[G/C]	Control	76 (63.3)	44 (36.7)	
		Hemodialysis	66 (54.1)	56 (45.9)	.15
			CC + CT	**TT**	
rs3819545 (Rec T)	[T/C]	Control	61 (52.6)	55 (47.4)	
		Hemodialysis	76 (62.3)	46 (37.7)	.15
			AA + GA	**GG**	
rs4334089 (Dom A)	[G/A]	Control	62 (51.7)	58 (48.3)	
		Hemodialysis	48 (39.3)	74 (60.7)	.07
			TT + CT	**CC**	
rs7136534 (Dom T)	[C/T]	Control	50 (42.7)	67 (57.3)	
		Hemodialysis	40 (32.8)	82 (67.2)	.14
			GG + AG	**AA**	
rs7979360 (Rec G)	[G/A]	Control	21 (17.5)	99 (82.5)	
		Hemodialysis	13 (10.7)	109 (89.3)	.14

*Values are frequency (percentage). Only variables with *P* values less than .20 in the univarieble model are shown in this table. Dom indicates dominant model and Rec, recessive model.
†SNP identifier based on NCBI dbSNP
‡The first allele is designated as the major allele and the second allele is designated as the minor allele.

(TT genotype) were associated with a higher risk of mortality.

DISCUSSION

This study proposed a theory about the influence on kidney patient survival that a change in the internal environment of these patients, probably caused by the progression of the disease, could influence the action of genetic polymorphisms. This important hypothesis, already considered in other studies, reflect the relationship between genetics and the environment. Renal patients undergo metabolic modifications that would generate different types of environment throughout the stages of CKD. For this reason, genetics would not have a linear development in their actions. To attempt to help in the understanding of this conceptual approach we would have to recall the terminology known as the *antagonist gene*. This concept addresses whether at any given time there be a consistent environmental condition in which the gene would be influencing a type of result and from a point of inflection, such as the internal environment change, this result could change.

A large number of studies have identified several polymorphisms in the *VDR* gene, and VDR activation is involved in multiple physiological functions and in disease states. This modified

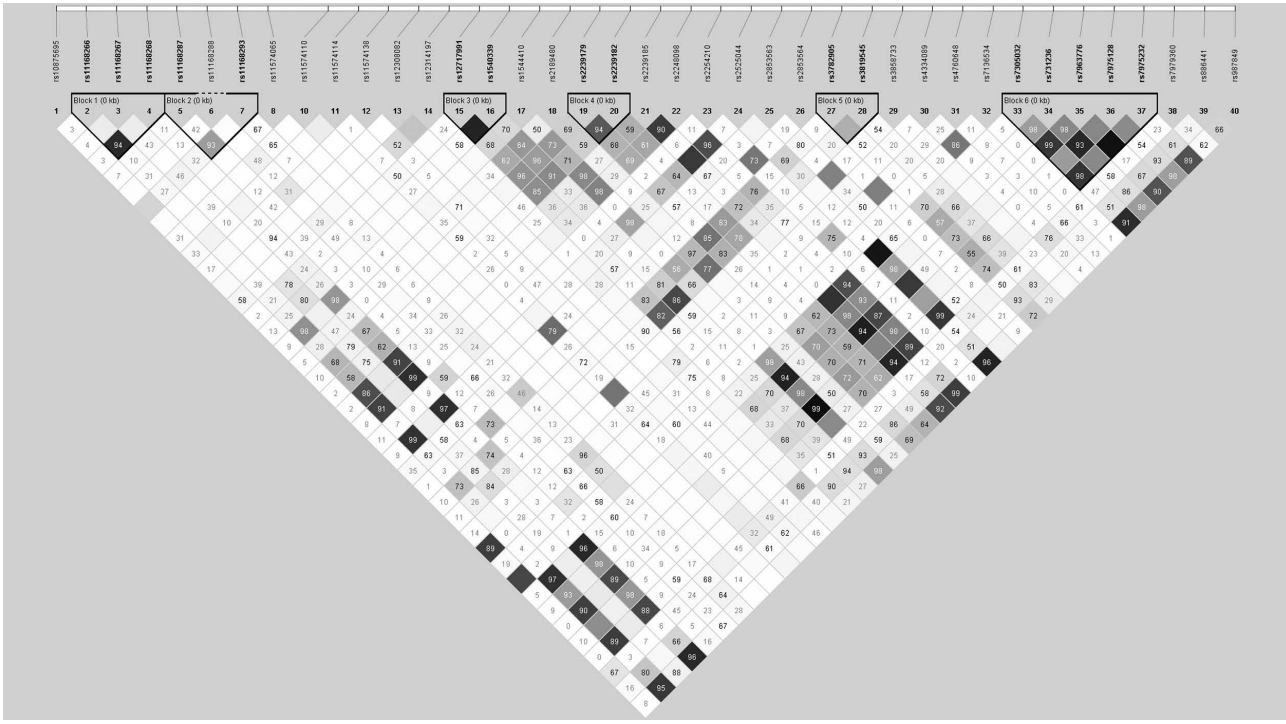

Figure 1. A representation measure of linkage disequilibrium among all polymorphic sites in vitamin D receptor (*VDR*) gene.

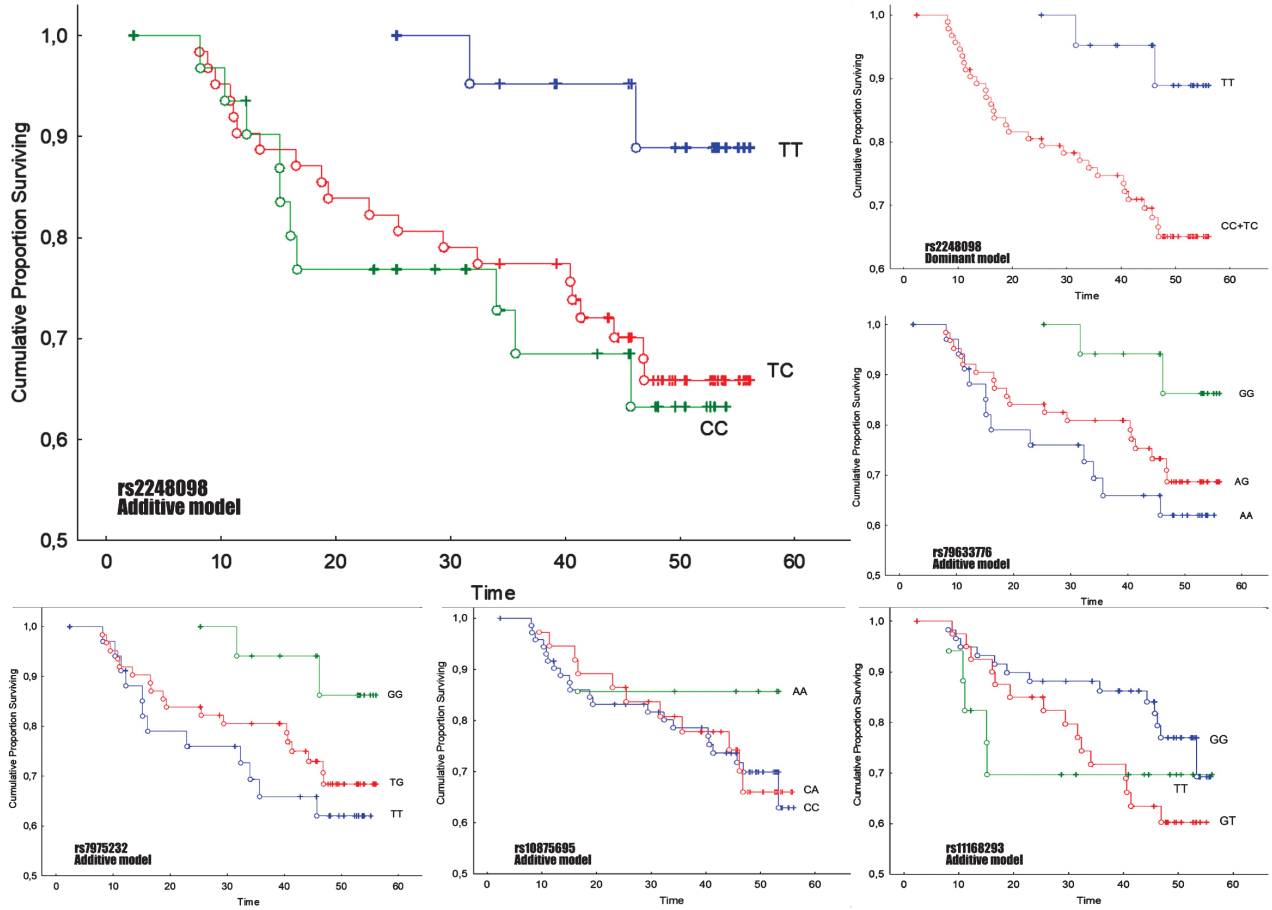

Figure 2. Kaplan-Meier survival analysis to visualize the survival of patients patients according to the genetic variables.

Table 4. Models Made to Multivariate Analysis for Relevant Variables and Target Single Nucleotide Polymorphisms (Tag SNPs) in Vitamin D Receptor (*VDR*) Gene*

Variables in Model	*P*	Coeficient	Hazard Ratio
Model 1			
Sex	.001	1.40	4.06
Age	.002	0.05	1.06
Diabetes mellitus	.045	0.84	2.33
Cardiovascular disease	.04	0.88	2.41
rs2248098 in addictive model (CC)*	.02	1.90	6.69
Model 2			
Sex	.001	1.38	3.99
Age	.002	0.05	1.06
Diabetes mellitus	.03	0.91	2.48
Cardiovascular disease	.047	0.84	2.31
rs7963776 in addictive model (AA)*	.03	1.72	5.58
Model 3			
Sex	.001	1.38	3.98
Age	.002	0.05	1.05
Diabetes	.03	0.91	2.47
Cardiovascular disease	.047	0.84	2.31
rs7975232 in addictive model (TT)*	.03	1.72	5.57

*Genotype (combined or alone) who presented the worst curve

state could transform the internal environment in these patients. Today, new genetic analysis tools allow for the identification of broad markers of genetic variations covering whole genes, but the influence of these polymorphisms on the VDR protein function is largely unknown.[18] The use of tagging SNP methodology for the *VDR* gene was used to search the genetic relationship between, for example, diabetes mellitus,[19] Parkinson disease,[20] and breast cancer.[21] In this study, this methodology was used to help understand the role of genetics action involving the *VDR* gene in a specific group of kidney patients.

After univariable analysis involving hemodialysis patients and healthy controls, the rs10875695 showed significant difference (Table 2 and 3), and the A allele was associated as a protective allele for CKD, because it was with higher prevalence in hemodialysis group. The T allele plays a protection role in hemodialysis group for rs11168293 when comparing the hemodialysis group with controls. Genetic susceptibility is an important determinant of the onset and progression of CKD and its complications, and different studies have identified novel susceptibility loci to reduce kidney function. Moreover, nontraditional risk factors such as oxidative stress, inflammation, and immune processes may be important contributors to the pathogenesis and progression to renal disease. Thus, the interaction between genes and environmental risk factors will undoubtedly play an additional role. In this context, the two tag SNPs (rs10875695 and rs11168293) present alleles with protective characteristics which probably influenced the progression of kidney disease. The relationship of the internal environment in patients with kidney disease with these genetic markers could be producing a specific and resultant condition so that the patient could progress through renal disease to the later stages, rather than being lost by early death. For the patients, the presence of these alleles in the *VDR* gene were probably responsible, obviously together with other genes, to influence the progression of CKD and protect against early death. In the literature, these tag SNPs were studied before with other diseases (diabetes mellitus and colorectal cancer), and these results already indicate the relevance of these previous associations that somehow repeat themselves but in another population.[19,22]

Still considering the univariable analysis, 3 others tag SNPs (rs2248098, rs7963776, and rs7975232) did not present significance between the hemodialysis group and the healthy controls (Table 2 and 3); however, we can attribute the same aspects of protection within patients with kidney disease when the frequency of their genotypes were analyzed only inside of CKD population. Access to these polymorphisms becomes important in the survival analysis to follow.

In a second part of our study, after performing this first round of analyses involving hemodialysis patients and healthy controls, survival analysis was implemented only in patients with kidney disease. The main result of the survival versus tag SNP was to rs2248098 in dominant and additive models, respectively (P = .02 and P = .05). The TT genotype had a positive impact on patient survival presenting protection aspect when compared with combined CC+TC and CC genotypes. Similar analysis can be made with other four tag SNPs show in Figure 2.

Despite this significance attributed to rs2248098 in survival analysis, we would like to reinforce is that the environment again, although presenting

differently in patients with kidney disease, probably continues to influence along with genetics the important outcome now in relation to mortality. Thus, the genetic markers presented in the univariate analysis will also, even without significance, be responsible for influencing the better or worse survival rate.

The classic publication by Marco and colleagues in 2001 showed the influence that *Bsm*I polymorphism in the *VDR* gene had survival in hemodialysis patients.[23] In addition to raising the possibility of other SNPs in linkage disequilibrium with this specific polymorphism. Other studies hypothesized the association of genetic markers in *VDR* gene with events, such as cardiac problems and cancer, that would lead to worse survival, and this approach show the importance of this gene.[24,25]

Especially for rs2248098, there are 2 articles that have found some kind of association. In 2013, Azevedo and colleagues[26] found an association with systemic lupus erythematosus. This disease is an autoimmune disorder with heterogeneous clinical manifestations and rs2248098 was found associated with immunological alterations. On the other hand, the same marker, in recent study by Cavalcanti and coworkers,[27] showed no association in a Brazilian population with rheumatoid arthritis.

Finally, several models of logistic regression were designed to better understand the relationship between nongenetic and genetic variables. After multivariable analysis, the final models are shown in Table 4. Interestingly, the point at which these models have always maintained a variable (sex) not related to the main comorbidities presented by patients with kidney disease. Together with gender other risk variables like age, diabetes mellitus, and cardiovascular disease were included. These variables are similar in results of several other studies involving renal disease and are markedly regarded as important comorbidities. Interestingly, in addition to these traditional variables, the rs2248098 (CC genotype), rs7963776 (AA genotype), and rs7975232 (TT genotype) merge as important genetic markers.

Figure 1 shows that rs4334089 presented a strong linkage disequilibrium with rs10875695 and with rs11168293. Thus, this rs4334089 could represent these two other markers. Associated markers analyzed by linkage disequilibrium suggested the existence of many single association signals in all loci. This analysis serves to give us an idea of the behavior within our group when considering the factor known as a linkage disequilibrium. Values inside boxes represent linkage disequilibrium measured using the r^2 parameter and the intensity of shading is proportional to r^2. So, for an upcoming study using a sample from southern Brazil we could be more economical since boxes with values greater than 80 and with grayer colors indicate that any of the used SNPs could be used. This analysis would allow greater savings of time, money and effort.

The present study had limitations that need to be clearly discussed. The first involves the analysis between serum metabolic variables that may have a bias, since at the time of formation of our control group, there was no possibility of obtaining the same data set. The second limitation was the small sample size, and to compensate for this factor, we continued improving our sample after this report. Another problem is the nonmatching of the sample, especially considering the variables age and sex. The latter limitation could occur due to the chosen type of genetic study. The classical approach that uses only 1 candidate gene presents its limitations because we may be underestimating the action of other unacknowledged genes.

CONCLUSIONS

Polymorphisms specific to patients with kidney disease (T allele in rs2248098 for protection, for example) could be producing conditions for some patients to evolve differently during CKD. Thus, these markers (environment and genetic) would act at specific times in the history of kidney disease and would bring different results in outcome history of CKD patients.

ACKNOWLEDGMENTS

This study was supported by Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Paraná (PPSUS CP08 2009) and CNPq (Pós-doutorado Júnior – PDJ: 150725/2010-8).

CONFLICT OF INTEREST

None declared.

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Received December 2017
Revised March 2018
Accepted March 2018