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Plasma Haptoglobin and Group-specific Component Phenotypic Variants Increase the Risk of Chronic Kidney Disease

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ABSTRACT The researchers studied the correlation of Haptoglobin (HP) and Group-specific Component (GC) plasma protein biomarkers in chronic kidney disease (CKD) patients and non-CKD controls using discontinuous-polyacrylamide gel electrophoresis. The results showed a significant difference between the CKD and control clusters of 2-2 variants for HP (p=0.0036) and GC (p= 0.0033). The current research indicates 2-2 phenotype is an independent risk determinant for CKD, while risk analysis reports odds value >1 for both protein polymorphisms signifying an effective risk towards CKD. The multifactor dimensional reduction analysis also detailed HP and GC markers have a strengthening and stronger association and are causative for CKD development. Collectively, the findings signify a potential risk of plasma HP and GC polymorphisms being susceptible to CKD and their key role in the deterioration of kidney functions.

INTRODUCTION

Chronic kidney disease (CKD) is an intricate disease caused by multiple environmental and genetic factors. CKD at times is allied with co-morbid conditions, namely diabetes, hypertension (HTN), and cardiovascular diseases (CVD). CKD is classed into five stages based on the estimated glomerular filtration rate (Levey et al. 2002). A taut interplay of genetic predisposition and environmental factors describes the disease onset and progression rate. Nevertheless, knowing the functional correlation that bridge the gap from the genetic risk loci to disease phenotype is one of the main challenge (Keller et al. 2012). Specific allelic polymorphisms influence the consequences of CKD besides acute kidney injury, glomerulonephritis, end-stage kidney disease (ESKD), and renal transplantation indeed. Generally, kidney function reduces with age but, among healthy subjects this reduction or deteriorate is not similar but exhibits a much personal variation (Dayon and Kussmann 2013). The rate at which kidney function is decreased or lost explains a high degree of inter-individual dissimilarity even among persons with an identical underlying cause of kidney injury (Lindeman et al. 1985).

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Over the past decades, data of plasma proteins are used for diagnosing and giving pathophysiological information on various infections that have improved noticeably. By difference, the detection of novel biomarkers of human plasma is substantially challenging, and the presence of genetically determined polymorphisms of plasma proteins has led to several investigations correlating genetic markers and human diseases (McClellan and Flanders 2003).

Haptoglobin is an alpha-2 globulin acute phase reactant that binds to the free hemoglobin (HB) of blood at the site of globin. The main purpose of HP is it protects against heme and iron-direct oxidative damage to the kidney and vascular system. The HP gene is located on the long (q) arm of chromosome 16 (16q22.2). The molecular position according to Homo sapiens annotation genomic coordinates (GRCh38) on chromosome 16 is within 72,054,504 to 72,061,055 base pairs (6,552 bp in length). The gene is characterized into three major phenotypes: HP 1-1, HP 2-1, and HP 2-2 controlled by two autosomal codominant alleles: $HP^{*}1$ and $HP^{*}2$ in humans. The key function of HP is to hunt for circulating HB freed by hemolysis or typical RBC turnover (Quaye 2008). The gene HP is expressed in the kidney and inducible via inflammatory cytokines with interleukin 6 (Sadrzadeh and Bozorgmehr 2004). The HP-HB complex can stop renal tubular injury by inhibiting the hemoglobin escaping through the glomerulus and proximal tubules, and circumventing body iron loss (Allison

and Rees 1957; Laurell and Nyman 1957). Physiologically, HP functions as an anti-inflammatory, antioxidative agent, and angiogenic factor (Levy et al. 2012). Moreover, HP upholds the growth of new blood vessels and differentiation and the explosion of vascular endothelium.

Group-specific component (GC) is a serum α_2-globulin positioned on the long (q) arm of chromosome 4 with the exact cytogenetic location be 4q13.3. According to human annotation GRCh38, the gene is of 63,828 base pair length (Chr 4:71, 741,692 to 71,805,519). The protein is structurally indistinguishable to Vitamin-D Binding Protein (VDBP) (Bouillon et al. 1976). GC is the serum transport protein of vitamin D and its metabolites. It circulates in the serum as an apoprotein, which is in the form of a free ligand. The discrepancies in comparative electrophoretic mobility disclose the classification of individual sera into three common phenotypes: GC 1-1 fast migrating component, GC 2-2 slow migrating and the third is characterized by the prevalence of both components, GC 2-1. GC*1 and GC*2, the two autosomal co-dominant alleles regulate the common phenotypes (Hirshfeld et al. 1960; Cleve and Bearn 1961; Reinskou and Mohr 1962).

The exact incidence and frequency of CKD disease is not evidently known owing to paucity of renal registries (Dash and Agarwal 2006; Prabahar et al. 2008). A high prevalence was reported in Srikakulam district of Coastal Andhra Pradesh (Reddy and Gunasekar 2013). Further, the frequency distribution of HP and GC alleles alter geographically and there are limited genetic association studies relating to CKD. With this hypothesis, researchers intended at determining the correlation of HP and GC polymorphisms in the development of CKD and the impact of these polymorphic variants on disease progression. The study noticed to be significantly associated ($p<0.01$) with 2-2 phenotypic variant of HP and GC in North Coastal Andhra Pradesh CKD group.

Objective

The present research sought to evaluate the risk association of HP and GC in chronic kidney disease and their correlation with disease progression in different phases of CKD.

MATERIAL AND METHODS

Subjects

The present investigation is a part of the Ph.D. work entitled, "Unraveling the genetics of chronic kidney disease: susceptible genes in the people of North Coastal Andhra Pradesh" instigated in 2013 by the corresponding author in the department of Human Genetics department, Andhra University, Andhra Pradesh for which 300 CKD cases and 300 age and sex-matched unrelated healthy controls were enrolled during 2015-2017. The patients were recruited from the nephrology department of King George Hospital from Visakhapatnam, India. Epidemiological data and clinical information have been collected from each subject along with 5 ml of intravenous blood samples, which were drawn by an expertise laboratory technician. Blood samples were collected after acquiring informed written consensus before their enrolment in the study. The work was ethically approved by the Institutional ethical committee at Andhra University (Reference number is IEC No: 2) and at the local government hospital, King George Hospital (Reference number: 1/18-10-2016), Visakhapatnam, Andhra Pradesh, India. Inclusion criteria for the subjects were: (i) both the genders (males and females), (ii) Age ranging from 20 to 80 years, (iii) Samples diagnosed by disease symptoms and categorized into stages based on the eGFR (that is, stage I to stage V) are included. Due to the limited number of cases with initial stages (that is, I, II, and III), these subjects were pooled into a single cluster naming it as Stage I-III. Patients with eGFR 15-29 ml/ min / 1.73m2, were grouped as the advanced stage (Stage IV) and, patients with eGFR <15 ml/min/1.73m2 were classed as end-stage (Stage V).

Main Reagents

The gels were prepared by mixing 2 volumes of solution A (28% Acrylamide and 0.75% Bis-acrylamide), equal volumes of solution B (TEMED- tetramethylenediamine) and solution C (Glycine and Tris(hydroxymethy l) aminomethane), and 4 Volumes of solution D (APS-Ammonium persulphate) for HP system (that is, 2:1:1:4), whereas for GC system, the solution was mixed in the ratio 1:2:2:4, that is, Solution A (Tris, TEMED), Solution B (30% Acrylamide, 0.8% Bis-acrylamide), Solution C (APS)

and Solution D (Distilled water).The staining solutions used were 0.5% O-dianisidine and 1.25% glacial acetic acid for the HP system and 2% Amido black, methanol, 30% glacial acetic acid for the GC system.

Methodology

Blood collected in EDTA containers was centrifuged at 2,000 rpm for 15-20 min to separate plasma. The plasma samples were extracted and tested utilizing the discontinuous-polyacrylamide gel electrophoresis methodology as described in the study of Ornstein and Davis (1964). For the preparation of test samples, 5 µl of fresh hemolysate is added to 30 µl of plasma prior to incubation at 37ºC for 30 min for the formation of a haptoglobin-hemoglobin complex. To this 2-3 drops of 40 percent sucrose solution containing 5 mg of bromophenol blue was added. After polymerization of gels, the sample (30 µl) is loaded to the gel tubes inserted in the buffer tank. The electrophoresis was carried out with a constant supply of 2.5 mA per tube until the tracking dye (Bromophenol blue) has migrated to the other end of the gel tube. Following staining, the gels were visualized under a white illuminator to ascertain phenotypic variants based on the banding pattern.

Statistical Analysis

Quantitative data were expressed in numbers and percentage frequency. Phenotyping comparisons were analyzed by the Chi-square test using Graphpad Prism (Version 8.4.3). Hardy Weinberg Equilibrium (HWE) and the allelic frequency for maximum likelihood are calculated (Balakrishnan 1989). The power of association between the protein markers and CKD risk was estimated by odds ratio (ORs) and 95% confidence intervals (CIs). To overcome the small sample size Bonferroni correction is performed.

Multifactor Dimensionality Reduction (MDR) analysis to study gene-gene and gene-environment interactions among HP, and GC markers was conducted (Ritchie et al. 2003; Moore 2004). Based on the cross-validation and maximum testing precision the best model with the possible grouping of the polymorphisms was considered. After identifying the best combination of factors, the genotypes are categorized into high and low-risk multifactor levels based on the threshold ratio (that is by taking the proportion of the number of affected to unaffected subjects). The results are determined as high risk if the proportion exceeds one and low risk if the value reported is below 1.

RESULTS

Patients Characteristics

CKD patients screened for proteins association reported males (71.7%) preponderant over females (28.3%) and the mean age of the study group was 49.17± 13.71 for both males and females. The peak age between 41-50 years resulted in active risk towards the disease with the highest proportion, yet age group 51-60 reported risk development. The percentage of smokers, alcoholics, and chewers were found to be 44.3 percent, 43 percent, and 33.3 percent respectively in the present study. Patients with CKD are associated with co-morbid conditions such as diabetes (3.6%), HTN (43.3%), Anemia (24.7), CVD (2.3%) and others have more than one disease conditions, like 19.7 percent were reported to have both diabetes and HTN, 2.7 percent had CVD and HTN and 3.7 percent observed to have CVD, diabetes and HTN. The same is detailed in Table 1.

Table 1: Frequency distribution of CKD individuals

Characters	*Frequency distribution (n=300)*	*Percentage (%)*
Male vs Female	215/85	71.7/28.3
Age (years) Mean±SD	49.17±13.71	NA
Smoking	132	44.3
Chewing (*Gutka*)[a]	99	33.3
Alcohol	129	43.0
Self-reported diabetes	11	3.6
Self-reported hypertension	130	43.3
Self reported anemia	74	24.7
Diabetes and hypertension	59	19.7
Self reported cardiac diseases	07	2.3
Hypertension and cardiac	08	2.7
Co-morbid condition of HTN, CVD and diabetes	11	3.7

Note: [a]*Gutka*= Gutka also called as betel quid is a chewing tobacco prepared by crushing betel nut, tobacco, paraffin wax, catechu, slaked lime and sweet flavors.

Phenotypic and Allelic Frequency Association of HP and GC Markers in the CKD and Non-CKD Subsets

The outcome of phenotypic and allelic frequency distribution for the two subsets are furnished in Table 2. The findings revealed significant correla-

Table 2: Distribution of HP and GC phenotypes and allele frequencies in the study groups

System	*Phenotype*	*Patients (n=300)*	*Controls (n=300)*	*OR (95%CI)*	*p-value*	*Test for heterogeneity*
HP	1-1	08 (2.7%)	12 (4%)	0.657 (0.275-1.540)	0.4951	9.025**
	2-1	52 (17.3%)	80 (26.7%)	0.576 (0.388-0.856)	0.0078**	(0.01> p>0.001)
	2-2	240 (80%)	208 (69.3%)	1.769 (1.214-2.575)	0.0036**	
	Allele 1	29 (11.3%)	52 (17.3%)	-	-	
	Allele 2	261 (88.7%)	248 (82.7%)	0.529 (0.322-0.850)	0.0136*	
GC	1-1	95 (32%)	117 (39%)	0.724 (0.515-1.015)	0.0729	9.848**
	2-1	147 (41%)	151 (50%)	0.948 (0.688-1.305)	0.7440	(0.01 > p>0.001)
	2-2	58 (27%)	32 (11%)	2.007 (1.260-3.196)	0.0033*	
	Allele 1	168 (56%)	192 (64%)	-	-	
	Allele 2	132 (44%)	108 (36%)	0.715 (0.517-1.000)	0.0553*	

Note: HP= Haptoglobin; GC= Group specific component; OR= Odds ratio; CI= Class interval. All relationships are statistically significant for Chi-square test (*p<0.05; **p<0.01; ***p<0.001)

tion for HP polymorphism with CKD ($\chi^2 = 9.025$; df = 2; 0.01>p>0.001). The frequencies distribution in CKD patient's versus controls was 2.7 percent vs 4 percent for homozygous 1-1 phenotype, 17 percent vs 27 percent for heterozygous 2-1 phenotype, and 80 percent vs 69 percent for homozygous 2-2 phenotype respectively. The groups showed a significant difference for 2-1 (OR= 0.576; 95% CI = 0.38-0.85; p=0.0078) and 2-2 (OR= 1.769; 95% 1.21-2.57; p=0.0036) phenotype for the two groups. Allelic frequency in CKD cases were11.3 percent and 88.7 percent for HP*1 and HP*2 respectively. Likewise, 17.3 percent and 82.7 percent was observed in non-CKD individuals, and both groups did differ significantly (p= 0.0136). While GC 1-1 and 2-1 phenotype frequencies was higher in controls (39% and 50%) than in patients (32% and 41%), and the groups did not exhibit any significant association. A significant association was reported in the frequency distribution of 2-2 phenotype between CKD and non-CKD subsets (p = 0.043; OR = 1.769; 95% CI = 1.214-2.575). The GC allele frequency for alleles 1 and 2 is noticed to be 56 percent and 44 percent in CKD cases, and 64 percent and 36 percent in non-CKD groups respectively. Allele distribution did differ significantly (p = 0.0553) and the Chi-square test for heterogeneity reported to be highly significant ($\chi^2 = 9.848$; df = 2; 0.01 >p>0.001) when viewed for the two subsets.

Stage Wise Distribution of HP and GC Phenotypes in CKD Patients

Although there was no significant association of HP ($\chi^2 = 2.395$; df= 4; p-value = 0.663) and GC ($\chi^2 = 0.631$ and p-value = 0.959) phenotype and allelic frequencies with CKD stages (Stage I-III, Stage IV and Stage V), the study resulted in increased number through initial stages to end-stage indicating progression of the disease as a consequence of the HP and GC biomarkers. Stage V CKD patients showed predominance over other stages with the two protein markers indicating progress of disease (Table 3).

Risk Association Analysis

Table 4 shows the findings of heterogeneity, pooled Odds ratios (OR) and 95% class interval (CI). Though all phenotype comparisons of HP and GC polymorphisms showed risk for CKD with OR higher than 1, only heterozygote (OR=1.77, 95% CI= 0.3793-0.836, $\chi^2 = 8.198$, $p_c = 0.012$) and dominant models (2-2+2-1 vs 1-1: OR=1.76, 95% CI= 0.3887-0.8220, $\chi^2 = 9{,}023$; $p_c = 0.006$) for HP phenotype reported significance. Similarly, homozygote (OR=2.23, 95% CI= 0.2692-0.7456, p= 0.001), co-dominant (OR=1.86, 95% CI= 0.3298-0.874, p= 0.011) and recessive (OR=2.01, 95% CI= 0.117-0.986, p= 0.048) genetic models of GC phenotype were observed to be significant and is susceptible to CKD.

Test for Multifactor Dimension Reduction

The two proteins HP and GC that might affect disease development are analyzed for intergroup interaction using MDR software are interpreted and detailed in Table 5. The researchers found that the cross-validation is maximal (10/10) for the two-factor model (HP and GC), and the testing accura-

Table 3: Phenotypic distribution of HP and GC in different stages of CKD

	Phenotype	*Stages of CKD* [a]			*Test for heterogeneity*
		Stage I-III	*Stage IV*	*Stage V*	
HP	1-1	1	3	4	2.395
	2-1	15	11	26	0.7>p>0.05
	2-2	56	71	113	
	Allele 1	11	10	12	
	Allele 2	89	90	88	
GC	1-1	24	37	44	0.631
	2-1	37	43	66	1.0>p>0.05
	2-2	11	15	33	
	Allele 1	59	57	54	
	Allele 2	41	43	48	

Note: All relationships are statistically significant for Chi-square test ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$); [a] gives different stages of CKD patients involved in the study divided basing the eGFR value.

Table 4: Risk association of HP and GC polymorphisms among CKD individuals and controls groups

Polymorphism	*Genotype model*	*Disease and control groups*					
		OR	*95%CI*	*χ^2 value*	*p-value*	*Z-Value*	*$p_c$value*[a]
HP	2-2 vs 1-1	1.73	0.6941-4.3156	1.416	0.234	1.177	0.702
	2-2 vs 2-1	1.77	1.1954-2.6362	8.198	0.004^{**}	2.844	0.012^{*}
	2-2+2-1 vs 1-1	1.52	0.6126-3.7756	9.023	0.366	0.904	1.098
	2-2 vs 2-1+1-1	1.76	1.2166-2.5731	0.828	0.002^{**}	2.986	0.006^{**}
	2 vs 1	1.62	0.2819-1.3687	1.412	0.224	1.181	0.672
GC	2-2 vs 1-1	2.23	1.3412-3.7153	9.743	0.002^{**}	3.089	0.006^{**}
	2-2 vs 2-1	1.86	1.1432-3.0321	6.338	0.012^{*}	2.498	0.036^{*}
	2-2+2-1 vs 1-1	1.38	0.9858-1.9309	8.837	0.061	1.876	0.181
	2-2 vs 2-1+1-1	2.01	1.2604-3.1965	4.98	0.003^{**}	2.935	0.006^{**}
	2 vs 1	1.39	0.7915-2.4651	1.33	0.248	1.153	0.744

Note: HP= Haptoglobin; GC= Group specific component; OR= Odds ratio; CI= Class interval. All relationships are statistically significant for Chi-square test ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$); [a] p_c : values after Bonferroni's corrections.

Table 5: MDR analysis of the genetic factor

Number of loci	*Polymorphism model*	*Testing accuracy*	*CVC*	*Prediction error (%)*
1	HP	0.5460	10/10	45.4^{**}
2	HP, GC	0.5281	10/10	47.19^{*}

Note.- HP= Haptoglobin; GC= Group specific component; CVC= Cross-validation Consistency. All relationships are statistically significant for Chi-square test ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$)

cy is shown to be 0.5281 which is lower than that of the single factor model (HP). The two-locus phenotype combinations associated with high and low risk along with the corresponding distribution of cases and controls for each multilocus phenotype combination are analyzed taking into consideration the ratio of case-control. It is reported to be a risk if the predetermined threshold reaches or exceeds 1 (that is a ratio of total cases and total controls), and considered low risk if the number is below 1. In the two-locus model, the combination of HP 1-1 and GC 1-1, HP 2-2 and GC 2-1, and HP 2-2 and GC 2-2 showed a high risk for CKD (Table 6). Further, based on the entropy values of single attributes (I(A:C) HP=0.0109 and GC=0.0067) and pairwise attributes (I(A:B:C) for GC and HP was 0.0059) the markers were regarded as causative and risk for CKD development.

Table 6: Distribution of high-risk and low-risk genotypes in the best two-locus model

Pattern	*Multilocus-genotype combinations*		*Number of cases*	*Controls*	*Case/Control ratio*[a]	*Association with CKD*
	HP	*GC*				
1	1-1	1-1	2	2	1	High risk
2	1-1	2-1	3	5	0.6	Low risk
3	1-1	2-2	3	5	0.6	Low risk
4	2-1	1-1	18	33	0.5455	Low risk
5	2-1	2-1	26	32	0.8125	Low risk
6	2-1	2-2	8	15	0.5333	Low risk
7	2-2	1-1	75	82	0.916	Low risk
8	2-2	2-1	118	82	1.439	High risk
9	2-2	2-2	47	44	1.0682	High risk

Note: HP= Haptoglobin; GC= Group specific component; CKD= Chronic Kidney Disease.
[a] Ratio of number of cases to controls of phenotype combination. The value above 1 indicates high risk and below to 1 reported as low risk to develop the disease.

DISCUSSION

The link between HP and GC polymorphisms and some principal causes for CKD, such as hypertension and diabetic nephropathy, is well known. Yet, clarification on the relation between the two markers and the occurrence of CKD is unclear. Concentrating on this issue, the study was undertaken to investigate phenotype distribution in CKD. Findings of the current study show a significantly higher frequency of HP 2-2 phenotype and overexpression of HP^2 allele in CKD patients. Results of this study were comparable to earlier studies (Chen et al. 2011; Mogadam et al. 2013; Armaly et al. 2015) that reported significant risk of HP 2-2 in CKD patients. The pathological mechanism by which the 2-2 phenotype results in CKD might be either weaker antioxidant capacity or due to other traditional factors such as hypertension or diabetes as found in the studies. HP 2-2 shows weaker antioxidant activity and HB binding ability as a result, oxidants and free radicals accumulate produced from the free haem-iron, at a higher incidence than HP 1-1 phenotype. The increase of such oxidation derivatives could direct to renal failure (Chen et al. 2011). The variation in relationship with HP phenotypes and ethnic population discloses diverse results. Studies among Irish (Conway et al. 2007) and Egyptian subjects (Bessa et al. 2007) signified that HP 2-2 phenotype confers susceptibility to kidney disease. A contemporary study in IgA nephropathy and CKD patients revealed the prevalence of HP 1-1 phenotype in the Israel population which is in contrast to this study (Habbashe et al. 2020). In converse, studies with Japanese (Koda et al. 2002), North American (Moczulski et al. 2001), and Brazilian subjects (Wobeto et al. 2009) showed no association with the HP protein marker. In the year 2013, Strandhave and his colleagues reported significance occurrence of 2-1 phenotype in a cohort study among CKD with cardiovascular disease (Strandhave et al. 2013). These different outcomes might be due to the difference in the allele distribution in different geographical regions or populations, sample size variation or study design as well as the baseline individuality of the enrolled disease respondents.

Data on genetic variation of the group-specific component with chronic renal infection are limited; hence the researchers have designed the study to investigate its correlation with the disease. Study findings revealed significant differences with the GC 2-2 phenotype which is similar to the Caucasian study (Speeckaert et al. 2008). The structural difference of GC phenotype lies in amino-acid sequence and in glycosylation position where GC^*1F and GC^*2 differ by a single amino acid modification replacing threonine versus lysine. Whereas, GC^*1S and GC^*2 differ at two positions exactly at 416 and 420. Glycosylated status of GC^*1 is with galactose and sialic acid whereas GC^*2 has galactose as a sole. The variation in the glycosylation model causes increased metabolism that lower GC concentration in individuals with 2-2 phenotype (Speeckaert et al. 2006).The findings of this study reported increased expression of a GC^*2 allele in

stage I to stage V CKD patients, however, the risk of mortality is not studied.

A few limitations of this case-control study are to be considered. First, the sample number is restricted to a few regions, hence it is not viable to give a strict conclusion about the correlation. Finally, the number of subjects of initial stages was relatively less, so it was hard to distinguish the correlation in the early stages, consequently stages I, II, and III were pooled to a single platform.

CONCLUSION

The researchers observed the association of HP and GC with the chronic kidney disease cluster suggesting of the markers being a potential risk factor in developing the disease. The phenotype 2-2 variant was observed to be associated with CKD. However, factors such as diverse geographical areas, distinctive distribution of alleles, numerous genetic variations, and the different environments that interact with genomes result in relatively variable phenotypes, which might explain the debatable results.

RECOMMENDATIONS

Though the study resulted in a significant association of HP and GC phenotypes in overall CKD subjects, the modest size of the samples for CKD stages specific cohorts did not yield any significant interactions. Hence, one should await explicitly designed large-scale CKD specific cohort studies for definite conclusions to be drawn on the problems such as the progression of the disease, and to replicate on the leads provided by this study, to establish the role that CKD and relevant genetic variants are significantly associated for the onset, development and progression.

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