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Association of Uromodulin rs13333226 and Angiotensinogen rs699 genes variants with essential hypertension in Arab Iraqis of Babylon province.

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ABSTRACT

Hypertension represent a serious health issue for both developed and developing countries. According to The world health organization (WHO) surveys, 40% of Iraqi adults are suffering from hypertension. Apart from the rare monogenic form of hypertension, the majority of hypertension cases believed to be a multifactorial disorder, caused by an interaction between environmental and genetic factors. The missense variant rs699 of angiotensinogen(AGT) gene and the promoter variant rs13333226 of Uromodulin (UMOD) gene , were found to be associated with essential hypertension in several studied populations. This is the first such study in Iraq, aimed to evaluate the association of those variant with essential hypertension, by conducted a case-control association study, with enrolling individuals belong to Arab ancestry from Babylon province/ Iraq. The study enrolled 100 cases of well diagnosed essential hypertensive patients and a 70 controls of carefully selected normotensive individuals . For genotyping we designed and optimized a 'polymerase chain reaction- restriction fragment length polymorphisms (PCR-RFLP)' and 'polymerase chain reaction amplification of multiple specific alleles (PAMSA)' methods, each sample was genotyped by the two methods to ensure precise genotyping. Concerning AGT rs699, the results showed that there was no significant allelic or genotypic association recorded , except that the patients group was significantly deviated from Hardy-Weinberg equilibrium exact test (P=0.023). The same association results were achieved when the samples were segregated into males and females. And the deviation from Hardy-Weinberg equilibrium was recorded in male group patients only (P= 0.011). Concerning UMOD rs13333226, the results showed that, allele A conferring an additional risk for essential hypertension with odd ratio 1.75 (C.I.1.03-2.96) (P =0.035), while the G allele represent a protective allele conferring a lesser susceptibility for hypertension (OR = 0.57, C.I. 0.33-0.96). The genotypic association under deferent inheritance models, showed that the A allele inherited as a recessive risk allele, the GG+AG to AA odd ratio equal to 2.17 (C.I. 1.14-4.11)(P = 0.022). when the samples segregated into males and females , the association significance was lost among females , while it was radically increased among the males , recorded an allele A odd ratio equal to 3.28 (C.I. 1.43-7.50)(P = 0.0035) and followed the same inheritance pattern , the GG+AG to AA odd ratio equal to 4.58 (C.I. 1.69-12.42)(P= 0.0029). We concluded that the rs13333226 variant of UMOD gene represent a genetic factor which can modulate the susceptibility to develop essential hypertension in males from Arab ancestry of Babylon province.

Keywords: Essential Hypertension, Angiotensinogen, Uromodulin, rs13333226, rs699 , PCR-RFLP, PAMSA .

Abbreviations: AGT: angiotensinogen gene, DALYS: Disability Adjusted Life Years, EH: essential hypertension, PAMSA: polymerase chain reaction amplification of multiple specific alleles, PCR: polymerase chain reaction, PCR-RFLP: polymerase chain reaction- restriction fragment length polymorphisms, TAL: thick ascending limb of Henle, THP: Tamm-Horsfall protein, UMOD: uromodulin gene, WHO: world health organization.

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INTRODUCTION

Hypertension, is a condition of high or up normal blood pressure, in which the blood vessels have persisted high pressure. WHO estimated the mortality due to raised blood pressure during 2010 in more than seven million deaths, this consisting about 12.8% of all annual deaths, furthermore hypertension consist about 3.7% of the total global DALYS (WHO, 2010).

According to hypertension etiology, it is classified into : A) Secondary Hypertension: which can be due to preexisting pathological conditions such as , arteriosclerosis , kidney diseases ,adrenal glands disorders and thyroid glands disorders . and B) Primary or Essential Hypertension (EH) : this type representing up to 95% of the diagnosed hypertension cases , and includes all hypertension cases that do not have an explained etiology or specific origin (Padma and Padma, 2012).

Nowadays hypertension accepted to be a multifactorial condition and can be due to an interaction of several to many genes with small effect for each gene separately . a simple search in 'HuGe navigator®' (Yu et al.,2008) showing the implication of several hundred genes with EH, but the implication of each gene usually could not be replicated in all studies.

Accumulated evidences have been concluded by many studies in different populations, demonstrated the significant association of the AGT gene's (which is one of Renin-angiotensin-aldosterone system RAAS) polymorphisms with EH . among several hundred validated variants in the locus of AGT gene (Ensembl database), the studies identified A number of associated SNPs which including; T174M (rs4762), G-217A (rs5049) and the mostly studied and confirmed variant the M235T (rs669) ,which represent a missense SNP lead to missense change in the mature angiotensin protein by substitute 235 methionine with threonine (Kunz *et al*.,1997; Sethi *et al.,2003;* Fang *et al*.,2010; Srivastava *et al.,*2012 ; Dhanachandra *et al*.,2014).

Since 1950 the researchers identified a muco-protein abundant in urine , they call this protein as Tamm-Horsfall protein (THP) due to the first discovery by Tamm and Horsfall (1950). after decades from THP discovery Muchmore and Decher (1985) study and characterize a urinary glyco-protein they called it Uromodulin (UMOD) . The two proteins UMOD and THP approved to be as the same protein and demonstrated that this protein is expressed exclusively in kidney cells (Pennica *et al.,*1987*)* . nowadays the terms THP and UMOD are exchangeable to refer to the same protein.

The certain role of Uromodulin proteins in blood pressure regulation is under investigation, but It has recently been shown that UMOD protein can modulate the Na+-K+-2Cl− cotransporter (NKCC2) activity and NaCl reabsorption in TAL cells (Mutig *et al.,*2011) . Furthermore, it is known that UMOD protein can bind several cytokines, including tumor necrosis factor-α (TNF-α), via its epidermal growth factor (EGF) domains , TNF-α is produced by TAL and acts in an autocrine manner to down regulate NKCC2A expression, lowering NaCl reabsorption at this site (Hao *et al,.*2011) .

Until the 2010, there was no confirmed association (independently from nephropathic effect) of UMOD gene with hypertension , but a team of researchers from Glasgo university directed a large genome-wide association research, they identified the rs13333226 variant on Uromodulin gene as quantitative blood pressure locus . The minor G allele of rs13333226 is significantly reduced the risk for hypertension and also reduced urinary excretion of uromodulin protein , and associated with better renal function (Padmanabhan *et al.,*2010). Furthermore , transgenic mice experiment found that, UMOD gene risk variants were increased the expression of UMOD protein both in vivo and in vitro. the Uromodulin overexpression mice showed a development of salt-sensitive hypertension and the presence of age-dependent renal lesions (Trudu *et al.,*2013).

This is the first study carried out in Iraq , aimed to investigate the genetic association of UMOD rs13333226 and AGT rs699 variants with essential hypertension . furthermore we aimed to optimize and validate a PCR-RFLP and PAMSA genotyping methods for those variant , which will be suitable for further studies, especially for low and moderate Budget laboratories .

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MATERIALS AND METHODS

The project proposal and sampling method were approved by the research ethics Committee of Babylon Health Directorate according to the directorate Administrative order no.1056 , date :26/3/2014 . also the project achieve the permission of research ethics in Marjan Medical City at the date 7/4/2014 . Samples were collected during the period 10/4/2014 to 15/1/2015 , from the visitors of consult clinic in Merjan medical city , A written Agreement was signed by every participant after his/her understanding of the project aim and tests that would be performed .

All the samples were collected from individuals belong to Arab ancestry (according to their testimony) and lived in Babylon province/ Iraq . The participant's age, sex, alcohol consumption, the age of hypertension onset, family history, etc. were recorded according to the participant testimony . while the participant's weight and height were measured by electronic balance and measuring tape respectively.

Five milliliters of venous blood was obtained from overnight fasted individuals by 5ml disposable syringe (without tourniquet) . Two milliliters of the blood was drained into EDTA tube and mixed gently , the blood in EDTA tube stored at 4C° and used for DNA extraction during the next 5 days . The rest 3ml of blood was drained into gel plain tube for serum preparation, which would be used in biochemical tests during the next 6 hours . The measurements of serum glucose , urea and creatinine, were carried out by the use of 'cobas c111®' device (Roche® , Germany) by using the manufacturer reagents and instructions . while The serum calcium was measured by employing the 'calcium CPC method' kit (Biolabo SA® , France).The DNA was extracted from the venous blood by Wizard® Genomic DNA Purification Kit (Promega. USA) by following the manufacturer protocol.

The DNA quantity and quality was measured by nano-drop (biodrop , UK) , employing the scanning ability of diode array from 200 to 320 Nm wave length , the absorbance profile then processed and analyzed to determine the DNA quantity and quality by calculating the 260/280 and 260/230 ratios . if a sample showed 260/280 ratio lesser than 1.8 and /or 260/230 ratio lesser than 2 , it would be re-extracted .The molecular weight and the integrity of extracted DNA was determined by agarose gel electrophoresis, the electrophoresis was carried out according to (Samboork and Russell ,2001).

To ensure that we would include essential hypertensive patient only in the further genetic study, we omitted all patients that had abnormal biochemical tests values , BMI >30Kg/m^2 , smokers, alcohol consumers , who have any illness which can effect blood pressure, who did not has a diagnosed hypertension from at least 1 year and those who did not have a family history of hypertension , the filtered patient group included 42 males and 58 females with age mean 48.7±9.9 . on the other hand the selected control should be, had a normal blood pressure , aged more than 50 years , did not have a family history of hypertension and did not have any illness that can effect on blood pressure , the filtered control group included 36 males and 34 females with age mean 58.6±7 .

PCR-RFLP analysis design :

The flanked sequence for each SNP was retrieved from dbSNP (http://www.ncbi.nlm.nih.gov/projects /SNP/snp) according to human genome assembly GRCH.p2. The selection of the suitable restriction enzyme was performed by the aid of WatCut® online software (http://watcut.uwaterloo.ca/template) , we selected the Hpy99I and EcoRv restriction enzymes to be used in rs699 and rs13333226 genotyping respectively . The primers were designed by the aid of NCBI-primer BLAST online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK _LOC=BlastHome) , at the same time the produced primers was checked for specificity for their target sequences by performing the BLAST against the human genome . The primer ability to form secondary structure was checked by the aid of Oligo Calc online software (Kibbe,2007) (http://www.basic.northwestern.edu/biotools/ oligocalc.html) . The amplicon of each primers pair was retrieving by the aid of "MFEprimer-2.0" online software (Qu *et al.,*2012) , by performing insilico PCR for the primers pair against the human genome . Each amplicon sequence retrieved by the former step was subjected to in-silico digestion by the selected restriction enzyme, by the aid of "Sequence Manipulation Suite" online software (http://www.bioinformatics.org/sms2/rest_digest.html), the designed primers pairs would be rejected if the amplicon showed unaccepted digestion manner .

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Primers design for *PAMSA* genotyping method:

The primers for PAMSA genotyping method were designed according to Gaudet *et al.*(2009) , briefly Allele specific primers and a primer for the flanked region were designed by the aid of 'Batch primer3' online software (http://probes.pw.usda.gov/batchprimer3/) . A mismatch nucleotide was interred in the one of 5 nucleotides at the 3' end of each allele specific primer . then For each allele specific primers set a mismatch tails of 5 and 15 nucleotides was added to each allele primer respectively . The specificity , secondary structure formation , dimer formation and other primers criteria was checked as described earlier . table (1) list the employed primers for both genotyping methods .

Table (1) : the designed primers sets for rs699 and rs13333226 genotyping by PCR-RFLP and PAMSA methods (the mutated nucleotide marked by square brackets , the lowercase letters refer to the added mismatch tails).

SNP	Genotyping method	Primer name	Sequence (5' to 3')	Amplicon size bp
rs699	PCR-RFLP	forward	AAGACTGGCTGCTCCC[T]GA	99
		Backward	AGAGCCAGCAGAGAGGTTTG	
	PAMSA	Allele G specific	ttactactggacaatAGACTGGCTGCTCC[T]TGAC	101
		Allele A specific	aatatAGACTGGCTGCTCCCTG[C]T	91
		Backward	GAGGTTTGCCTTACCTTGGA	--
rs13333226	PCR-RFLP	forward	GTGTTGTCATCTCCTCAGGATTA	96
		Backward	CTGTTTGGGAAGAGGAGTC[A]ATA	
	PAMSA	Allele G specific	ttagtagtagacagtGAGGTAGCACAGCTGTAG[T]G	125
		Allele A specific	aatatGAGGTAGCACAGCTGTA[T]GA	115
		Backward	AATGCTCAGAACTGGTGAGT	--

PCR conditions:

We followed the routine optimization method steps , by applying a gradient annealing temperature , different cycle number , different reaction gradients, etc. . until the achievement of sufficient and specific product for the target template.

For genotyping rs699 by PCR-RFLP ; the optimized PCR reaction mixture contain, 7.5 µl of 2X GoTaq® Green Master Mix (promiga , USA) , 20Ng of genomic DNA ,1 µl of each primer (10 pmol/ µl , bioneer , Korea) and the total volume completed to 15 µl by molecular grade water (promiga , USA).

For genotyping rs699 by PAMSA ; the optimized PCR reaction mixture contain, 6.25 µl of 2X GoTaq® Green Master Mix (promiga , USA) , 30Ng of genomic DNA ,0.38 µl of each primer (10 pmol/ µl , bioneer , Korea) and the total volume completed to 12.5 µl by molecular grade water (promiga , USA).

For genotyping rs13333226 by PCR-RFLP ; the optimized PCR reaction mixture contain, 5 µl of 2X GoTaq® Green Master Mix (promiga , USA) , 20Ng of genomic DNA ,0.125 µl of each primer (10 pmol/ µl , bioneer , Korea) and the total volume completed to 10 µl by molecular grade water (promiga , USA) .

For genotyping rs13333226 by PAMSA ; the optimized PCR reaction mixture contain, 7.5 µl of 2X GoTaq® Green Master Mix (promiga , USA) , 20Ng of genomic DNA ,0.5 µl of each primer (10 pmol/ µl , bioneer , Korea) , 0.5µl of DMSO (Himedia , India) and the total volume completed to 15 µl by molecular grade water (promiga , USA). The optimized PCR thermo-cycling conditions For genotyping rs699 by PCR-RFLP were : initial denaturation 94C˚ for 4 min. followed by 40 cycles of (94 C˚ for 60 sec. ,54 C˚ for 35 sec. and 72 C˚ for 30 sec.) then final elongation 72C˚ for 4 min.

The optimized PCR thermo-cycling conditions For genotyping rs699 by PAMSA were : initial denaturation 95C˚ for 10 min. followed by 5 cycles of (95 C˚ for 60 sec. ,63 C˚ for 30 sec. and 72 C˚ for 15 sec.) , 5 cycles of (95 C˚ for 60 sec. ,61 C˚ for 30 sec. and 72 C˚ for 15 sec.), 27 cycles of (95 C˚ for 60 sec. ,59 C˚ for 30 sec. and 72 C˚ for 15 sec.) then final elongation 72 C˚ for 4 min.

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The optimized PCR thermo-cycling conditions For genotyping rs13333226 by PCR-RFLP were : initial denaturation 94C˚ for 4 min. followed by 38 cycles of (95 C˚ for 40 sec. ,59 C˚ for 35 sec. and 72 C˚ for 30 sec.) then final elongation 72C˚ for 4 min. The optimized PCR thermo-cycling conditions For genotyping rs13333226 by PAMSA were : initial denaturation 94C˚ for 10 min. followed by 5 cycles of (94 C˚ for 50 sec. ,58 C˚ for 30 sec. and 72 C˚ for 30 sec.), 35 cycles of (94 C˚ for 50 sec. ,55 C˚ for 30 sec. and 72 C˚ for 30 sec.) then final elongation 72 C˚ for 4 min.

For rs699 PCR-RFLP genotyping : The PCR-product was digested with Hpy99I restriction enzyme , this enzyme will cut the product only if the C allele of rs699 present , in this case it will split the 99bp product to two polynucleotide chains with a molecular weight of 21bp and 78bp , otherwise if the allele T of rs699 present the PCR product will not splinted and existing as intact 99bp.

For rs13333226 PCR-RFLP genotyping : The PCR-product was digested with EcoRV restriction enzyme , this enzyme will cut the product only if the G allele of rs13333226 present , in this case it will split the 96bp product to two polynucleotide chains with a molecular weight of 22bp and 74bp , otherwise if the allele A of rs13333226 present the PCR product will not splinted and existing as intact 96bp. The PCR product length , the specificity of the PCR reaction and restricted PCR product were checked by non-denaturing polyacrylamide gel electrophoresis , the electrophoresis was carried out according to (Sambrook and Russell ,2001) , figures (1) and (2).

Statistical analysis : the genetic association statistics were carried out by the aid of SNPStats® online software (Sole *et al.,*2006) (http://bioinfo.iconcologia.net/en/SNPStats_web), except that genotypes association which was carried out by Fisher's exact test according to (Agresti,1992).

RESULTS AND DISCUSSION

Genotyping methods accuracy:

The result of rs699 genotyping by PAMSA method showed that this method represent a good alternate for PCR-RFLP method , among the 170 sample that genotyped by both method there were only two samples that gave a different genotype results , GG to AG and AG to GG by PAMSA and PCR-RFLP methods respectively , this represent 99.4% matching between the two genotyping methods. A better results were achieved for rs13333226 . among the 170 sample that genotyped by both method there was only one samples that gave a different genotype results , AG by PAMSA and AA by PCR-RFLP methods , this represent 99.7% matching between the two methods .

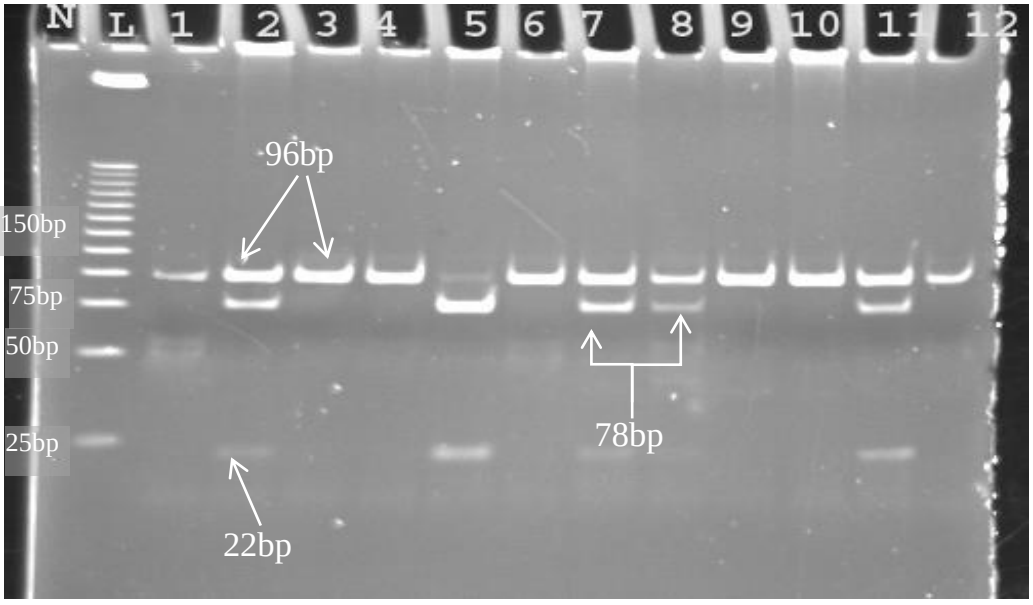

Figure (1) : PCR-RFLP of rs13333226 (Lane N = negative control ;lane L= 25bp step ladder ;lanes 5 = GG genotype ; lanes 2,7,8,11= AG genotype : other lanes = AA genotype).

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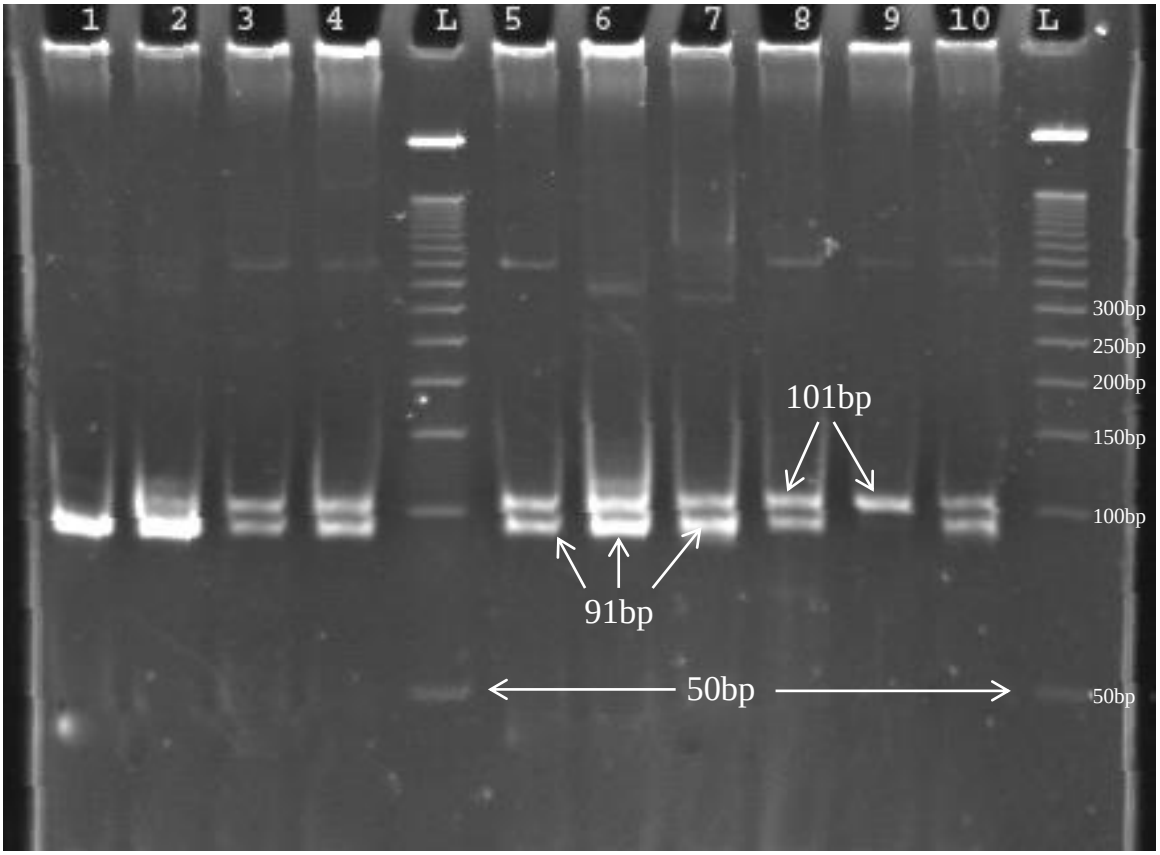

Figure (2): genotyping of rs699 by PAMSA method . lanes: L= 50 bp step ladder; 1&2= AA genotype ; 9 = GG genotype ; other = AG genotype .

Association of AGT rs699 with essential hypertension:

The results showed that there was no significant allele frequency difference between patients and control groups . the segregation of the samples for males and females gave the same insignificant association results as listed in table (2).

Table(2): alleles percentages , allelic odd ratio and allelic difference of rs699

Samples	Allele	Patient	Control	Allelic odd ratio (95% CI)	p-value[a]
All	G	0.58	0.59	0.947 (0.609-1.473)	0.80
	A	0.42	0.41	1.056 (0.679-1.643)	
Males	G	0.58	0.59	0.977 (0.534-1.787)	0.94
	A	0.42	0.41	1.023 (0.559-1.871)	
Females	G	0.57	0.59	0.929 (0.465-1.856)	0.83
	A	0.43	0.41	1.077 (0.539-2.152)	

a:Pearson's goodness-of-fit chi-square (degree of freedom = 1)

The results of the exact test for the deviation from Hardy-Weinberg equilibrium (table: 3) revealed that the control group genotypes frequencies follow the Hardy-Weinberg equilibrium, while a significant deviation was recorded in the distribution of patients group genotypes (p value =0.023). the segregation of samples for males and females revealed that the females follow the distribution of Hardy-Weinberg equilibrium in both patients and control groups , while the patients group in males deviated significantly from Hardy-Weinberg equilibrium .

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Table (3): rs699 genotype percentages and the P-values of exact test for the deviation from Hardy-Weinberg (HW) equilibrium .

Samples	Group	Genotype			H.W. P-value
		AA	GG	AG	
All	patient	27%	61%	12%	**0.023**
	control	31%	56%	13%	0.32
Males	patient	24%	69%	7%	**0.011**
	control	26.6%	64.4%	9%	0.062
Females	Patients	29.3%	55.2%	15.5%	0.43
	control	39.1%	39.1%	21.8%	0.4

We further tested the association of each genotype with the EH and under different models of inheritance , the result showed that there was no significant association between any genotype and EH under any of studies inheritance model , the same results was achieved when the samples segregated for male and females, as in table (4).

Table (4) : association of rs699 genotypes with essential hypertension under different models of inheritance

Model	Samples	Genotype	Patients	Control	OR (95% CI)	P-value[a]
Codominant	All	G/G	27%	30.9%	1.00	0.80
		A/G	61%	55.9%	0.80 (0.40-1.61)	
		A/A	12%	13.2%	0.96 (0.34-2.78)	
	Males	G/G	23.8	26.7	1.00	0.94
		A/G	69	64.4	0.83 (0.31-2.23)	
		A/A	7.1	8.9	1.11 (0.20-6.18)	
	Females	G/G	29.3	39.1	1.00	0.41
		A/G	55.2	39.1	0.53 (0.18-1.59)	
		A/A	15.5	21.7	1.05 (0.27-4.09)	
Dominant	All	G/G	27%	30.9%	1.00	0.6
		A/G-A/A	73%	69.1%	0.83 (0.42-1.63)	
	Males	G/G	23.8	26.7	1.00	0.80
		A/G-A/A	76.2	73.3	0.86 (0.33-2.27)	
	Females	G/G	29.3	39.1	1.00	0.44
		A/G-A/A	70.7	60.9	0.64 (0.23-1.77)	
Recessive	All	G/G-A/G	88%	86.8	1.00	1.00
		A/A	12%	13.2%	1.12 (0.44-2.82)	
	Males	G/G-A/G	92.9	91.1	1.00	0.76
		A/A	7.1	8.9	1.27 (0.27-6.03)	
	Females	G/G-A/G	84.5	78.3	1.00	0.52
		A/A	15.5	21.7	1.51 (0.45-5.12)	
Overdominant	All	G/G-A/A	39%	44.1%	1.00	0.52
		A/G	61%	55.9%	0.81 (0.43-1.51)	
	Males	G/G-A/A	30.9	35.6	1.00	0.82
		A/G	69	64.4	0.81 (0.33-1.99)	
	Females	G/G-A/A	44.8	60.9	1.00	0.22
		A/G	55.2	39.1	0.52 (0.20-1.40)	

a: two tailed p-value of Fisher's Exact Test

For several decades ago, the researchers observed a significant plasma angiotensinogen level increment in hypertensive individual in both sexes and in different age intervals , furthermore the studies also revealed a positive correlation between plasma angiotensinogen levels and blood pressure (Gould and green ,1971 ; Gardes et al.,1982 ; menard et al.,1991) . this finding made the angiotensinogen gene represented candidate gene which thought to be involved directly in the onset and development of hypertension . For this aim, researchers employing several strategies and methods to address the clear physiological role of angiotensinogen (AGT) gene in blood pressure regulation . Kim et al.(1995) designed an experiment based on transgenic mice which mutated to carry a duplication or knockout AGT gene , they found that the AGT gene expression level have a significant impact on the regulation of blood pressure . while other researchers teams

such as Tomita *et al.(*1995) and Tang *et al.*(1999) , employed antisense technology to suppress the AGT gene expression, by designed a specific oligo-nucleotides that complementary to AGT mRNA , which lead to dramatic reduction in this gene expression , the experiments revealed that the treatment with AGT antisense oligo-nucleotides lead to a significant decrease in the blood pressure of spontaneously hypertensive rate , for relatively long term duration ranged from few days to several weeks .

Our results agreed with several other studies carried out in different populations , and disagreed with many other studies which found a significant relationship between rs699 and EH . The implication of rs699 in essential hypertension was investigated by many studies, which employed both association and linkage strategies and in different populations , most of these studies revealed a significant relationship between rs699 and essential hypertension.

May be the earliest clear association between rs699 with EH was recorded in 1992 in Caucasians population when Jeunemaitr *et al.*(1992) observed an increased 235T (rs699) allele frequency in hypertensive individuals compared to the controls group . furthermore Dr Jeunemaitre headed another researchers team (Jeunemaitre *et al.*,1997) to answer the question of , "does the M235T predispose the development of EH by itself or it act as a marker which has a high degree of linkage disequilibrium with another locus that act as EH susceptibility locus? ". for this purpose, they enrolled samples from two populations (Japanese and French) , their results showed that M235T was associated with EH in both two studied populations and there is a di-allelic SNP at -6 position had a complete linkage with M235T ,however they did not find any other polymorphism in AGT gene had a significant association with EH .

Another significant association was also found in Romanian patients between rs699 and EH but the study include a small sample size (38 patients and 21 normal control) (Procopciuc *et al.,*2002) .

Further study was based on families linkage strategy enrolled 126 Chines families which included 434 siblings found a significant over-transmission in rs699 and EH (p =0.036), the significance was increased when a haplotype include rs699 A and rs4762 A alleles was tested for linkage with EH (p = 0.004) (Fang *et al.,*2010) .

The association also tested by A large meta study conducted by Ji *et al.*(2010) analyzed 31 studies which investigate the association of rs699 with EH in Han Chinese population , the study found a significant association for rs699 with EH (OR = 1.54 ,95%CI= 1.16-2.03 , P =0.002) , furthermore the association was held the significant values in all inheritance models .

Concerning Arabic population , Al-najai *et al.* (2013) studied the association of several SNPs in Saudi native population , their results showed a significant association of rs699 with EH (p =0.0001) , also they demonstrated that the rs699 G allele (235T) conferring a risk susceptibility to EH for carrier individuals (OR :1.19 ,95% C.I. :1.08-1.13) ,the study also concluded that AGT polymorphisms were independently conferring a risk for various cardiovascular traits .

On the other hand several other studies failed to found a significant relationship between rs699 and EH , for example : Basak *et al.*(2008) studied the association of T175M and M235T in AGT gene (rs4762 and rs699 respectively) enrolling a Turkish subjects from Trakya region , their results showed that there were no significant association of both SNPs with EH . furthermore Yang *et al.*(2015) study the association of eight RAAS system SNPs with EH , they found that no single SNP a achieve significant association alone , but after analyzing of SNP-SNP interaction by multifactor dimensionality reduction (MDR) they found that the SNPs of AGT gene combined with angiotensin converting enzyme gene insertion/deletion (ACE I/D) can achieve a significant association with EH .

After this mini-review for rs699- EH association studies , we can postulate a theoretical explanation which may be rationalize the fluctuated results of rs699-EH association studies , by the following reasons :

1- The rs699 may represent an EH trait loci , but it is conditional , i.e. it need a specific environmental conditions to express EH, and as a consequences the rs699-EH association is varied from population to other due to the environmental diversity .
2- The rs699 may represent a blood pressure quantitative trait locus (QTL) , which participate in a small to moderate elevation in blood pressure , in this case the onset of hypertension will depend on the

presence of other blood pressure QTLs , which together collaborated to make a significant increment in blood pressure .

3- The rs699 do not effect blood pressure by itself , but it could be in linkage disequilibrium (LD) with another locus or loci which in turn confer the susceptibility for EH or act as blood pressure QTL , in this case the rs699-EH association will depend directly on the degree of (LD) between the rs699 and the other effective locus . it is well demonstrated that the LD between relatively apart two loci on the genome usually varied among populations , this argument was well explained in previous studies (Reich *et al.,* 2001 ; Teo and Sim ,2010) . and clearly noticed by HapMap project results .

Association of Uromodulin rs13333226 with essential hypertension:

The results showed that there was a significant allele frequency differences between patients and control group . allele 'A' represent the risk allele with odd ratio of 1.75 while allele G represent the protective allele with odd ratio of 0.57 . the segregation of samples for males and females revealed that the association significance increased in male , while it was lost in females, table (5).

Table(5): Alleles percentages , allelic odd ratio and allelic difference of rs13333226

Samples	Allele	Patient	Control	Allelic odd ratio (95% CI)	p-value[a]
All	G	17%	26%	**0.57 (0.337-0.965)**	**0.0352**
	A	83%	74%	**1.754 (1.036-2.969)**	
Males	G	11%	28%	**0.305 (0.133-0.697)**	**0.0035**
	A	89%	72%	**3.283 (1.436-7.506)**	
Females	G	22%	23%	0.924 (0.413-2.068)	0.85
	A	78%	77%	1.082 (0.484-2.422)	

a: Pearson's goodness-of-fit chi-square (degree of freedom = 1)

The results of the exact test for the deviation from Hardy-Weinberg equilibrium, revealed that the control group genotype frequency follow the Hardy-Weinberg equilibrium, while a significant deviation was recorded in the distribution of patients group genotype (p value = 0.034). when the samples segregated into males and females , the male patients keep the significant deviation while there was no deviation recorded in females group, table (6).

Table (6): rs13333226 genotypes percentages and the P-values of exact test for the deviation from Hardy-Weinberg (HW) equilibrium .

Samples	Group	AA	AG	GG	P-value
All	Patients	72%	22%	6%	**0.034**
	control	54%	39%	7%	1
Males	Patients	83%	12%	5%	**0.05**
	control	52%	39%	9%	0.73
Females	patients	64%	29%	7%	0.43
	control	58%	38%	4%	1

We further tested the association of each genotypes with essential hypertension and under different models of inheritance, the result showed that the A allele represent a risk factor for essential hypertension and inherited as recessive risk factor , the AA genotype has a related odd ratio compared to (AG+GG) of 2.17 (C.I.95%=1.14-4.11) , while G allele represent a dominate protective genetic factor conferring lesser essential hypertension susceptibility for the carrier individuals . when the samples segregated into male and females , the association significance was increased among the males, while there was no significant association recorded among the females, table (6).

The difference in hypertension prevalence between males and females was noted by several studies , but the specific cause that lead to gender dependent penetrance of UMOD rs13333226 is out the scope of this article , in general , previous studies concluded that sex hormone have a significant effect on blood pressure regulation and hypertension development (Reckelhoff, 2001; Zaidan et al. 2014)

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Table (7) : association of rs13333226 genotypes with essential hypertension under different models of inheritance

Model	Samples	Genotype	Patients	Control	OR (95% CI)	P-value[a]
Codominant	All	A/A	72%	54.3%	1.00	**0.046**
		A/G	22%	38.6%	**2.33 (1.17-4.62)**	
		G/G	6%	7.1%	1.58 (0.45-5.51)	
	Males	A/A	83.3%	52.2%	1.00	**0.0046**
		A/G	11.9%	39.1%	**5.25 (1.72-16.07)**	
		G/G	4.8%	8.7%	2.92 (0.49-17.21)	
	Females	A/A	63.8%	58.3%	1.00	0.79
		A/G	29.3%	37.5%	1.40 (0.51-3.86)	
		G/G	6.9%	4.2%	0.66 (0.07-6.43)	
Dominant	All	A/A	72%	54.3%	1.00	**0.022**
		A/G-G/G	28%	45.7%	**2.17 (1.14-4.11)**	
	Males	A/A	83.3%	52.2%	1.00	**0.0029**
		A/G-G/G	16.7%	47.8%	**4.58 (1.69-12.42)**	
	Females	A/A	63.8%	58.3%	1.00	0.80
		A/G-G/G	36.2%	41.7%	1.26 (0.48-3.33)	
Recessive	All	A/A-A/G	94%	92.9%	1.00	1.00
		G/G	6%	7.1%	1.21 (0.35-4.12)	
	Males	A/A-A/G	95.2%	91.3%	1.00	0.68
		G/G	4.8%	8.7%	1.90 (0.33-10.98)	
	Females	A/A-A/G	93.1%	95.8%	1.00	1.00
		G/G	6.9%	4.2%	0.59 (0.06-5.54)	
Overdominant	All	A/A-G/G	78%	61.4%	1.00	**0.025**
		A/G	22%	38.6%	**2.23 (1.13-4.37)**	
	Males	A/A-G/G	88.1%	60.9%	1.00	**0.0068**
		A/G	11.9%	39.1%	**4.76 (1.57-14.37)**	
	Females	A/A-G/G	70.7%	62.5%	1.00	0.60
		A/G	17 (29.3%)	9 (37.5%)	1.45 (0.53-3.94)	

a: two tailed p-value of Fisher's Exact Test

The certain role of Uromodulin proteins in blood pressure regulation is under investigation but It has recently been shown that UMOD can modulate The Na+-K+-2Cl− cotransporter (NKCC2) activity and NaCl reabsorption in the thick ascending limb of the loop of Henle (TAL) cells (Mutig *et al.,*2011) . Furthermore, it is known that UMOD can bind several cytokines, including tumor necrosis factor-α (TNF-α), via its epidermal growth factor (EGF) domains , TNF-α is produced by TAL and acts in an autocrine manner to down regulate NKCC2A expression, lowering NaCl reabsoprtion at this site (Hao *et al,.*2011) . furthermore, A transgenic mice experiment conducted by Trudu *et al.* (2013) found that UMOD gene risk variants increased the expression of UMOD gene both in vivo and in vitro. the Uromodulin overexpression mice showed a development of salt-sensitive hypertension and a presence of age-dependent renal lesions

The Rs13333226 (HGVS names 16:g.20354332A>G) is a SNP located in the promoter region of UMOD gene , the ancestral G allele of this SNP was found to be associated with low level of uromodulin protein execration in urine (Padmanabhan *et al.,*2010) .

Before 2010 there were a few studies that referred for the implication of UMOD gene promoter polymorphisms with EH , Iwai *et al.* (2006) conducted a study to investigate the association of UMOD gene promoter polymorphism rs6497476 (-744) in Japanese population , the study found that the minor allele of this variant was associated with lower risk for EH , but the association did not achieve the statistical significance. also another study found that the C allele of rs4293393 and the T allele of rs12917707 (which both located in the promoter region of UMOD gene) both alleles associated with increased estimated glomerular filtration rate (eGFR) ,and conferring a resistance against chronic kidney diseases (CKD) , furthermore they found that these polymorphisms were associated with decreased level of urinary uromodulin protein excretion (Kottgen *et al.,*2009 ; 2010).

Our results is partially agreed with the discoverer study of Padmanabhan *et al.* (2010) , which directed a large genome-wide association study by recruited 1,621 cases and 1,699 controls then they performed follow up analyses in 16,541 controls and 19,845 cases, they found that The rs13333226 minor G allele is significantly reduced the risk for hypertension and also reduced urinary excretion of uromodulin protein , and associated with better renal function. Furthermore the same team employed 13,446 subjects and measure their glomerular filtration rate (eGFR) , the results showed that there was independently associated between rs13333226 and hypertension in both eGFR non-adjusted and adjusted calculations , they also found that the rs13333226 G allele associated with lower excretion of urinary uromodulin , furthermore they concluded that this variants can effect in blood pressure by an effect on sodium homeostasis, and suggested the UMOD locus as a potential antihypertensive target to reduce cardiovascular risk of hypertension.

On the other hand Han *et al.*(2012) found that the rs13333226 G allele was associated with elevated diastolic blood pressure comparing to the individual who carry the AA genotype ($p = 0.035$) , the study included 1000 individuals from chimes population .

This contradictory findings lead us to suggest that the rs13333226 do not represent functional polymorphism which effect directly the expression rate of UMOD gene promoter but instead it could have a linkage disequilibrium with another variant or haplotype that has the direct effect on the promoter activity . this suggestion is agreed with the finding of Graham (2013) , he found that the rs13333226 has a linkage disequilibrium with another variant called rs4997081 (also occur in the UMOD promoter region) which may be the functional variant that effect on UMOD promoter activity , he demonstrated an experimental and computational clues suggested that the genotype of rs4997081 implicated in the binding of TEAP2A (transcriptional factor activating enhancer protein 2 alpha) which in turn lead to the change in the UMOD promoter activity , then the uromodulin protein introduce an alteration on sodium reabsorption by activate TNFα signaling .

Another evidence supporting our suggestion come from the HapMap project results , it is clearly showing that the region of UMOD promoter has a significant diversity from population to another , and as a consequences the linkage disequilibrium between any two markers will be differ among the different populations .

CONCLUSION

We concluded that the rs13333226 variant of UMOD gene represent a genetic factor which can modulate the susceptibility to develop essential hypertension in males from Arab ancestry of Babylon province.

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