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(Genetic Studies for Leptin in Hypertensive Patients in Thi-Qar Governorate/ Iraq)

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***Abstract*— The current study showed a high frequency of AA genotype compared to the AC and CC genotypes in patients and control group. In the control group, 71.11% had AA genotype, 22.22% were heterozygote and had AC genotype and 6.67% had CC genotype. However, 53.33% of patients had AA genotype, 40.00% had AC genotype and 6.67% had CC genotype.**

Odd ratio increased by 2.40 times in AC compared to AA genotype (wild type) as a reference with a significant difference between patients and control group (OR 2.40, 95% CI 0.66 – 4.51). While, there was no significant differences between CC genotype and AA genotype in patients and control group (OR 1.33, 95% CI 0.24 – 7.19).

Keywords*— *Leptin , Hypertensive , gene

I. INTRODUCTION

Several studies suggest that leptin is involved in the pathogenesis of arterial hypertension in humans. It was first observed significantly higher plasma leptin in patients with essential hypertension, even in those with normal body weight.)Agata J., et al., 1997).

Many subsequent studies reported a significant positive correlation between plasma leptin and blood pressure, independent of body weight, both in normotensive and in hypertensive subjects (Kazumi T et al., 1999), (Narkiewicz K et al., 1999), (Schutte R et al., 2005). In addition, humans with inherited leptin deficiency are normotensive despite massive obesity (Ozata M et al., 1999). The results of some studies are discrepant, for example demonstrating significantly higher leptin only in males (Schorr U et al., 1998) or only in females (Sheu WH et al., 1999), (Kokot F et al., 1999) with essential hypertension; these differences could be attributed to various populations studied. Interestingly, it has been observed that leptin may be elevated in hypertensive subjects only at night (Ficek J et al., 2002) whereas, in most studies, blood for analysis is obtained in the morning. Recently, it was demonstrated that plasma leptin is elevated in lean normotensive subjects with a high normal blood pressure in comparison to those with optimal blood pressure (Papadopoulos DP et al., 2005). Makris et al. (Makris TK et al., 1999) observed higher leptin levels in healthy offspring of hypertensive patients and suggested that genetically determined hyperleptinemia may precede and contribute to the development of hypertension rather than being secondary to increased blood pressure. That hypertension per se is not responsible for elevating leptin secretion, which is further supported by normal or even reduced plasma leptin in patients with primary aldosteronism (Torpy DJ et al., 1999).

Leptin may also be involved in the pathogenesis of specific forms of secondary hypertension. In particular, because leptin is vigorously metabolized in the kidney, its concentration is markedly elevated in renal failure and thus could contribute to hypertension in these patients (Kokot F et al., 1998). Many studies have demonstrated increased plasma leptin in pregnant women with pre-eclampsia in comparison to normal pregnancy (Ramsay JE et al., 2004). Interestingly, hyperleptinemia may precede the development of hypertension during pregnancy (Anim-Nyame N et al., 2000). Finally, plasma leptin is higher in patients with obstructive sleep apnea than in healthy controls with similar body weight, suggesting that hyperleptinemia may contribute to increased sympathetic drive and hypertension associated with this syndrome(Phillips BG et al., 2000). Nevertheless, because clinical studies are based almost exclusively on correlation data, one cannot definitely conclude if leptin plays a causal role in human hypertension .

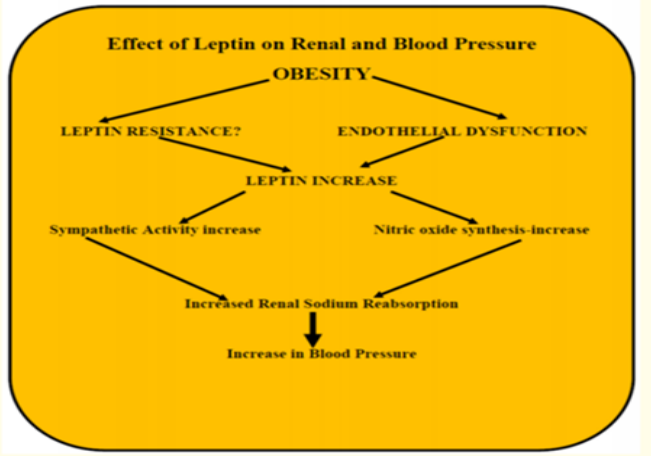

Figure 1: Leptin (LPT) and secondary cause of hypertension.

A significant relationship between the peripheral expression of the ob gene producing leptin and systolic blood pressure was

found in women and non-hypertensive men. In view of the multiple functions of leptin a causal relationship is postulated and potential mechanisms may involve modulatory effects of leptin on neuropeptide Y, angiotensinogen gene expression, the modulation of the autonomous nervous system, or effects on the pituitary adrenal axis. Direct relationships between both plasma renin activity and aldosterone levels and leptin support the potential importance of the relationship between leptin and blood pressure. (Paolo M et al., 1998)

II. Materials and Methods

A. Primers

PCR primers for AT1R gene were designed by (Jinmin et al., 2013)in table (1). These primers were provided from (Macrogen company, Korea) as following tables:

Table (1): The RFLP PCR primers with their sequence and amplicon size:

Primer	Sequence	Amplicon
AT1R gene	GAGATTGCATTTCTGTCAGT	350bp
	ATAATGTAAGCTCATCCACC	

B. Design of Study

This study is conducted at AL-Hussein Teaching Hospital in Thi-Qar/Iraq.

The present study was started with (90) cases (patients and healthy):

1-Control group: Includes (45) persons in healthy, ages (75-45).

2-Patient group: Includes (45) persons with high blood pressure, ages (45-75).

C. Methods

1) Genomic DNA Extraction

Genomic DNA from blood samples were extracted by using gSYAN DNA extraction kit (Frozen Blood) Geneaid. USA, and done according to company instructions as following steps:

A 200 µl of frozen blood was transferred to sterile 1.5ml microcentrifuge tube, and then added 30 µl of proteinase K and mixed by vortex. And incubated at 60℃ for 5 minutes, after that, 200 µl of GSB lysis buffer was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 60℃ for 5 minutes, and inverted every 3 minutes through incubation periods, 200 µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously, DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tube containing the flow.through were discarded and placed the column in a new 2 ml collection tube, 400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube, 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube, all the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix, the dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre.heated elution buffer were added to the center of the column matrix, the tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

2) Genomic DNA estimation

The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA), a dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system, the sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of blood genomic DNA was added to measurement.

3) RFLP-PCR Technique

RFLP-PCR technique was performed for genotyping and detection A1166C gene polymorphisms of AT1R gene in hypertension patients and in healthy control blood samples. This method was carried out according to described by (Jinmin et al., 2013) as following steps:

4) PCR master mix preparation

PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions:

DNA template 5-50ng (5µl) ,Forward primer (10pmol) (1µl) , Reveres primer (10pmol) (1µl), PCR water (13µl).

After that, these PCR master mix component that mentioned in table above placed in standard AccuPower PCR PreMix Kit that contains all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2,stabilizer, and loading dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (Mygene. Korea).

PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

1.5% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 ℃ for 15 minutes, after that, left to cool 50℃, then 3µL of ethidium bromide stain were added into agarose gel solution, agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 5µl of PCR product were added in to each comb well and 10ul of (100bp Ladder) in First well, the gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour, the 350bp PCR products were visualized by using UV transilluminator.

5) RFLP-PCR mix preparation

RFLP-PCR mix was for A1166C gene polymorphisms of AT1R gene was prepared by using Dde restriction enzyme (New England Biolabs. UK) and this master mix done independent according to company instructions .

RFLP-PCR master mix:

PCR product (15µl), Dde Restriction enzyme buffer (10X 2 µl), Dde (5 unit) (0.5 µl), Free nuclease water (2.5µl)

After that, this master mix placed in Exispin vortex centrifuge at 3000rpm for 2 minutes, then transferred into incubation at 37°C for overnight. After that, RFLP-PCR product was analysis by 2.5% agarose gel electrophoresis methods that mention in PCR product analysis. The (C/A) heterozygote, the product digested by restriction enzyme into 350bp, 210bp, and 140bp band. The (CC) wild type homozygote, the product undigested by restriction enzyme and still 350bp band. The (AA) mutant type homozygote that show digested by restriction enzyme into 210bp and 140bp band.

Statistical analysis

The differences in genotype and allele frequencies between patients and controls were analyzed by the odd ratio (OR) test. P values less than 0.05 were considered significant.

III. RESULTS AND DISCUSSION

Genotype

The current study showed a high frequency of AA genotype compared to the AC and CC genotypes in patients and control group. In the control group, 71.11% had AA genotype, 22.22% were heterozygote and had AC genotype and 6.67% had CC genotype. However, 53.33% of patients had AA genotype, 40.00% had AC genotype and 6.67% had CC genotype.

Odd ratio increased by 2.40 times in AC compared to AA genotype (wild type) as a reference with a significant difference between patient and control group (OR 2.40, 95% CI 0.66 – 4.51). While, there was no significant differences between CC genotype and AA genotype in patients and control group (OR 1.33, 95% CI 0.24 – 7.19).

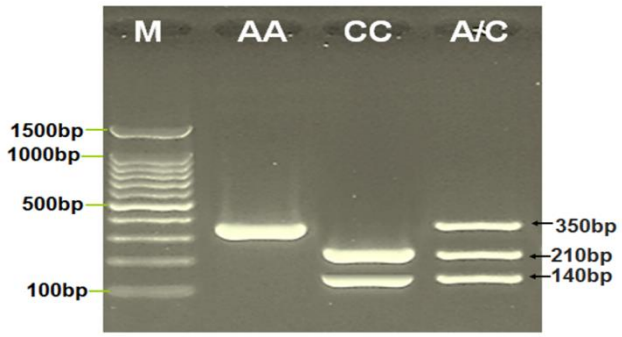

Figure 2: Agarose gel electrophoresis image that show the RFLP-PCR product analysis of AT1R gene A1166C polymorphism by using Dde restriction enzyme. Where M: marker (1500-50bp), lane (AA) wild type homozygote product undigested by restriction enzyme at 350bp bands, lane (C/C) mutant type homozygote, the product digested by restriction enzyme into 210bp and 140bp bands, and lane (A/C) heterozygote, the product digested by restriction enzyme into 350bp,, 210bp, and 140bp bands.

Extensive analysis in spontaneously hypertensive rat had revealed an elevated level of AT1R gene expression in the brainstem (Raizada et al.,1993), as well as a positive correlation between AT1R gene expression and systolic BP (Reja et al.,2006). Such and similar findings from animal model as well as human studies (Chandra et al.,2014;Ceolotto et al.,2011), supports a pivotal role of AT1R gene in BP homeostasis. Several lines of experimental clinical evidence also have alluded a strong evidence that development of hypertension is harboring the AT1R gene. Despite many positive findings of association studies, linkage and sib-pair linkage analysis in clearly linking rs5186 as one of the apt cause of hypertension, overwhelmingly negative results from preceding molecular genetic studies of humans also have appeared across various populations and ethnic groups. We therefore considered it worthwhile to unravel the functional significance of AT1R A1166C polymorphism with respect to disease and indeed found a significant interaction with the disease phenotype. These differences might validate that the AT1R A1166C polymorphism is an important genetic determinant of hypertension. Conspicuously, subgroup analysis by gender or by age did not change this pattern of results. Deepak et al(2018) reported the frequency of 1166 A/A genotype among cases was found to be 29.46% and CC genotype frequency as 25.89%, which was higher and statically significant in regard to that in the control group (p: 0.83). Albeit the genotype distribution resulted in a higher frequency of the C allele in the cases than controls (p: 0.0031;), testing genetic equilibrium between the observed and expected genotypes using Hardy–Weinberg equilibrium showed AT1R gene variants were confirming to the law. C allele of AT1R gene might confer a high risk of developing hypertension, as exemplified by an increased frequency of the rs5186 C allele in hypertensive individuals and its association with hypertension was highly compounded when C allele was present in homozygous state i.e. there was a statistically significant increase in odds of hypertension with the AT1R C/C genotype. This reasserts that the AT1R gene C/C variant is associated with higher susceptibility to hypertension and is substantiated by intervention studies demonstrating greater benefits & physiological response by wild type of rs5186 AT1R gene polymorphism compare to mutant type to pharmacological maneuvers(Brugts et al.,2011). Therefore, the hypothesis purposed by researchers that polymorphisms associated with increased RAAS activity may predispose to hypertension, as in AT1R C allele, which is associated with greater responses to angiotensin II at lower concentrations(Spiering et al.,2000), might all be more prev-alent within a hypertensive population is likely to be true for the studied population. A1166C variant is a nonfunctional mutation(Bonnardeaux et al.,1994), but has been associated with higher antiotensin II type 1 receptor responsiveness(Van et al.,2000), defects in messenger RNA (mRNA) processing, receptor expression(Thekkumkara and Linas,2003) or may well affect the function of regulatory elements(Atkinson and Halfon,2014), which could mechanistically contribute to its effects. Thus, it plays a pivotal role in the genomic context and may influence the expression levels, even being located within the 3′ untranslated regions of the gene. So, although, it do not lead to amino acid substitutions, positive associations observed in this study may be the result of linkage disequilibrium with another polymorphism or with an unidentified functional mutation in the AT1R gene or in another closely linked gene possibly located in regulatory regions and is supported by a study(Sethupathy et al.,2007), that demonstrated that there is miR155 (from chromosome 21,), which down-regulates the expression of the 1166A allele but not of the 1166C. As a result 1166C allele is associated with hypertension just because miR155 cannot negatively control the expression levels of AT1R. Nonetheless, the present study provided only the statistical evidences of the association between the A1166C allele of AT1R with EHT at the gene level and do not imply causation, mechanism or the functionality of the variants. It remains possible that we have not necessarily identified the pathobiological genetic variant but potentially only a variant in the gene in linkage disequilibrium with the causal pathobiological variant. Hence, our data must be interpreted within the context of its precincts for any potential clinical implications. There are also other potential limitations. Firstly, hypertension is a polygenic disease, to which different physiologic pathways contribute. Therefore, a dichotomous classification—hypertension and normotension, may not be sensitive enough to detect genetic predispositions to individual pathways, especially when the gene under study can

exert minor effects on hypertension status itself. Secondly, the number of cases for association study analysis is somewhat insufficient i.e. study involves a small sample size and may not be representative of the entire patient population. Nevertheless, still results of our study are particularly relevant, as our trial fulfils the criteria of a good genetic association study suggested by Hattersley et al. (2005). Finally, we did not make any adjustments for dietary factors (salts, electrolytes) and physical activity in the covariate list of regression analysis, which seem to affect BP variance. In spite of these limitations, we believe that AT1R gene A1166C SNP can be useful for outcome predictions during diagnostic processes and can be helpful in finding new treatment strategies.

Many studies showed an association between essential hypertension and the AC variants. One study found that the frequency of the C allele was elevated significantly (0.36) in a group of 206 Caucasian hypertensive individuals with essential hypertension compared to a frequency of 0.28 in 298 non-hypertensive individuals (Bonnardeaux et al.,1994). Wang et al. (1997) found that the C allele was significantly more frequent (0.40 versus 0.29) in hypertensive subjects with strong family history of essential hypertension compared to non-hypertensive individuals. Kainulainen et al. (1999) found that the A1166C variant was significantly more frequent among hypertensive individuals compared to their non-hypertensive individuals in the Finnish population. Szombathy et al. (1998) suggested that this variant is involved in a severe resistant form of systolic hypertension in old and overweight hypertensive individuals and in diastolic hypertension in overweight hypertensive individuals. Mehri et al. (2012) reported that the CC genotype was significantly associated with hypertension. However, Benetos et al. (1995) observed no difference in blood pressure level according to genotype for never-treated hypertensive individuals. A 1166 C SNP among all study subjects with BMI less than versus greater than or equal to 30 kg/m C polymorphism was not associated with systolic or diastolic blood pressure. Coto et al. (2010) also reported that the frequency of AT1R C-allele carriers did not differ between hypertensives with left ventricular hypertrophy and controls. Assali et al. (2010) found no significant difference in genotype and allele frequencies between hypertensive and non-hypertensive patients. Ramu et al. (2011) concluded that there was no significant association between AT1R A C gene polymorphism and hypertension. In the contrary, Castellano et al. (1996) and Schmidt et al. (1997) showed that blood pressures were lower in carriers of the C allele than they were in those with AA genotype.

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