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Relation of neuropeptide Y gene expression and genotyping with hypertension in chronic kidney disease

Eman A.E. Badr[a,*], Abd El-Aleem Hassan Abd El-Aleem[b], Samah EL-Ghlban[c], Asmaa AH. Swelm[c], Mahmoud Emara[d]

[a] Medical Biochemistry and Molecular Biology Department. Faculty of Medicine, Menoufia University, Shebin Elkom City, Menoufia governorate, Egypt
[b] Department of Organic Chemistry, Faculty of Science, Menoufia University, Egypt
[c] Department of Biochemistry, Faculty of Science, Menoufia University, Egypt
[d] Internal Medicine Department. Faculty of Medicine, Menoufia University, Shebin Elkom City, Menoufia governorate, Egypt

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ABSTRACT

Objectives: The prognosis of high-risk patients might be greatly ameliorated using genetic predisposition risk factors. Sympathetic activity and innate immunity related to neuropeptide Y function may be related to dyslipidemia and atherosclerosis. The aim of this study is to detect the correlation between Neuropeptide Y (NPY) SNP rs16147 and its gene expression in chronic kidney disease with and without hypertension.

Methods: This study carried out on 150 subjects who were divided into 3 main groups group (I) 50 CKD patients with hypertension, group (II) 50 CKD patients without hypertension and group (III) 50 healthy individuals. Carotid intima media thickness (CIMT) was measured by Ultrasound. Kidney function test and lipid profile were performed. Genotyping and gene expression of neuropeptide Y (NPY) were performed using real time PCR.

Results: There was a significant increase in number and percentage of CC genotype and C allele of NPY SNP distribution in CKD patients with and without hypertension when compared to controls. A significant association was found between CC genotype and C allele and the risk of CKD with hypertension with odd ratio 3.26 and 1.77, respectively. There is a significant positive correlation between NPY gene expression level and CIMT among chronic kidney disease patients with highest level of TC, LDLc and CIMT among CC genotype of NPY gene.

Conclusion: A significant association was found between CC genotype and C allele of NPY at rs16147 with increase NPY gene expression and risk of developing hypertension in CKD.

1. Introduction

Chronic Kidney Diseases (CKDs) is progressive and irreversible in nature leading to End stage renal disease (ESRD) over period of few months to years relying on the nature of the causal kidney disease [1].

It remains to be a worldwide public health problem due to its high incidence, major effect on patients, high cost to society, poor public awareness [2].

A sympathetic activity pointer like inflammatory phenomena and heart rate play an vital role as risk factors in ageing chronic kidney disease (CKD) patients [3,4].

Neuropeptide Y (NPY) is a sympathetic neurotransmitter which very expressed in sympathetic neurons, enteric neurons and several brain pathways [5].

Also, NPY has significant effects in inflammation and innate immunity [6. 7], NPY participates in the regulation of several physiological processes, together with energy balance, feeding,vasoconstriction, and anxiety, all of which are intermediated through diverse NPY G-protein-coupled receptors [8,9], *NPY* gene is sited on chromosome 7 and is nearby 8 kb in length with four exons separated by three introns [10]. The expression of NPY in the human brain is associated to polymorphisms in the NPY gene, and change in NPY expression [11].

Single nucleotide polymorphisms (SNP) are genetic variations of one nucleotide and these variants could have functional implications [12].

The chief genetic variant described in this gene is rs16147 (−399) and it is positioned inside the promoter region upstream of the NPY gene [13].

The aim of the work is to detect the correlation between Neuropeptide Y (NPY) SNP rs16147 and its gene expression in chronic

* Corresponding author. Professor of Medical Biochemistry and Molecular Biology, Faculty of Medicine-Menoufia University, 32511, Egypt.
E-mail address: ebadr2014@gmail.com (E.A.E. Badr).

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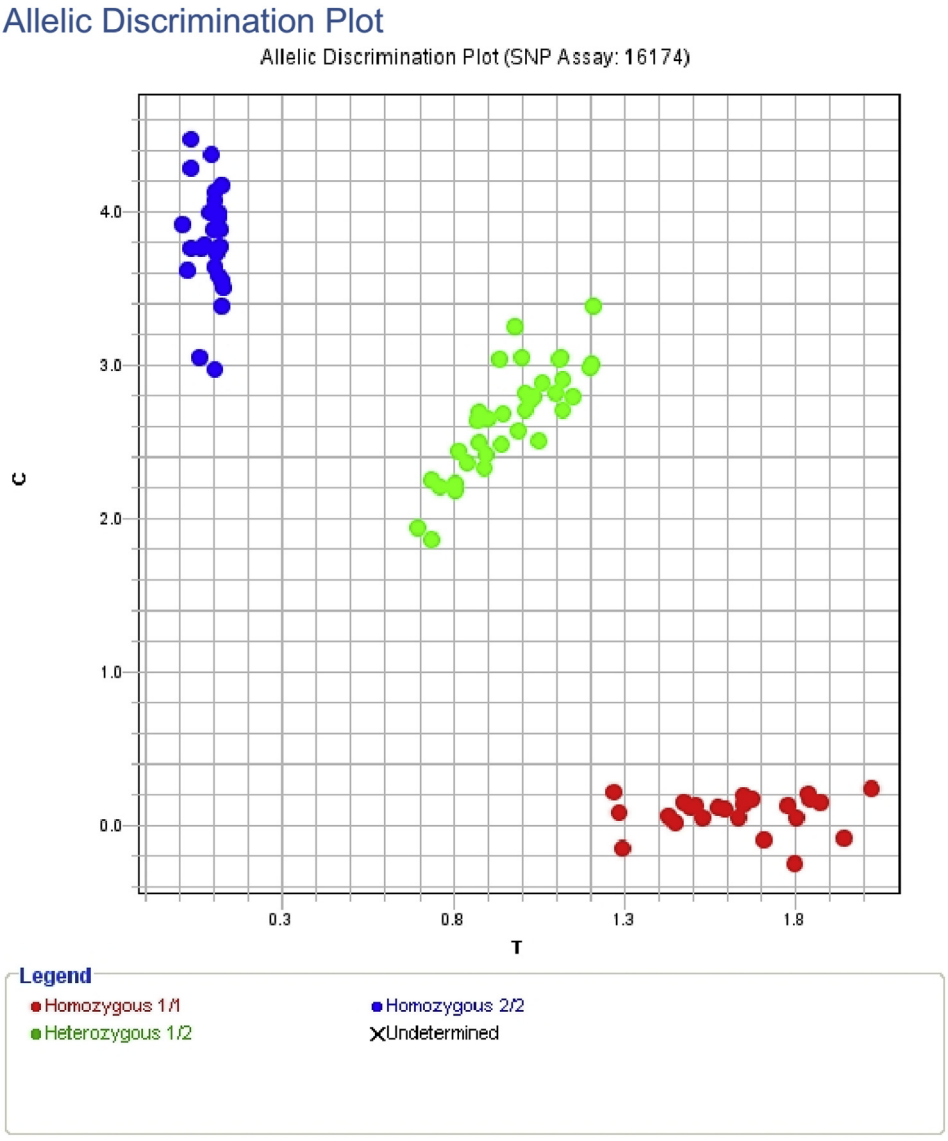


Fig. 1. a: Allelic discrimination plot of rs16147 (−399 T/C) SNP of NPY. b. Amplification plot and melting curve of NPY gene expression.

kidney disease.

2. Subjects and methods

This study was carried out in Biochemistry department, Faculty of Science, Menoufia University, Medical Biochemistry and Molecular Biology, and Nephrology Unit of Internal Medicine Departments, Faculty of Medicine, Menoufia University. During the period from March 2018 to November 2018 the study included a 150 subject who were divided into 3 main groups: **Group I:** Included 50 chronic kidney disease patients with hypertension. **Group II:** Included 50 chronic kidney disease patients without hypertension. The diagnosis of chronic kidney diseases can be ascertained by the presence of albuminuria, defined as albumin-to-creatinine ratio > 30 mg/g in two of three spot urine specimens. GFR can be estimated from calibrated serum creatinine and estimating equations, such as the Modification of Diet in Renal Disease (MDRD). **(Levey et al., 2005)**.

Group III: Included 50 ages and sex matched healthy individuals as a control group.

Patients with diabetes mellitus, cardiovascular disease and end-stage renal disease were excluded from the study. Written informed consent was obtained from all subjects participated in this study. The protocol of study was approved by the ethics committee of medical research of Faculty of Medicine- Menoufia University.

Carotid intima media thickness (CIMT) measurements by Ultrasound was made at the department of diagnostic radiology, Menoufia University Hospital using high-resolution B-mode ultrasonography 2–5 MHz wide band convex, linear array transducer (Philips, HD11XE ultrasound system,USA).

2.1. Blood samples

After overnight fasting [7], ml of venous blood were collectedby venipuncture of the cubital vein and divided as follow: 2 ml of blood were divided into EDTA containing tubes for DNA and RNA extraction. -The remaining blood were divided in plain vacutainer tubes, left 15 min for coagulation, then centrifuged at 3000 rpm for 10 min then the serum was separated into several aliquots for measurement of liver function tests and renal function tests, lipid profile. -'1 ml of blood was collected into Sodium fluoride containing tube for fasting blood glucose for exclusion of diabetic patients.

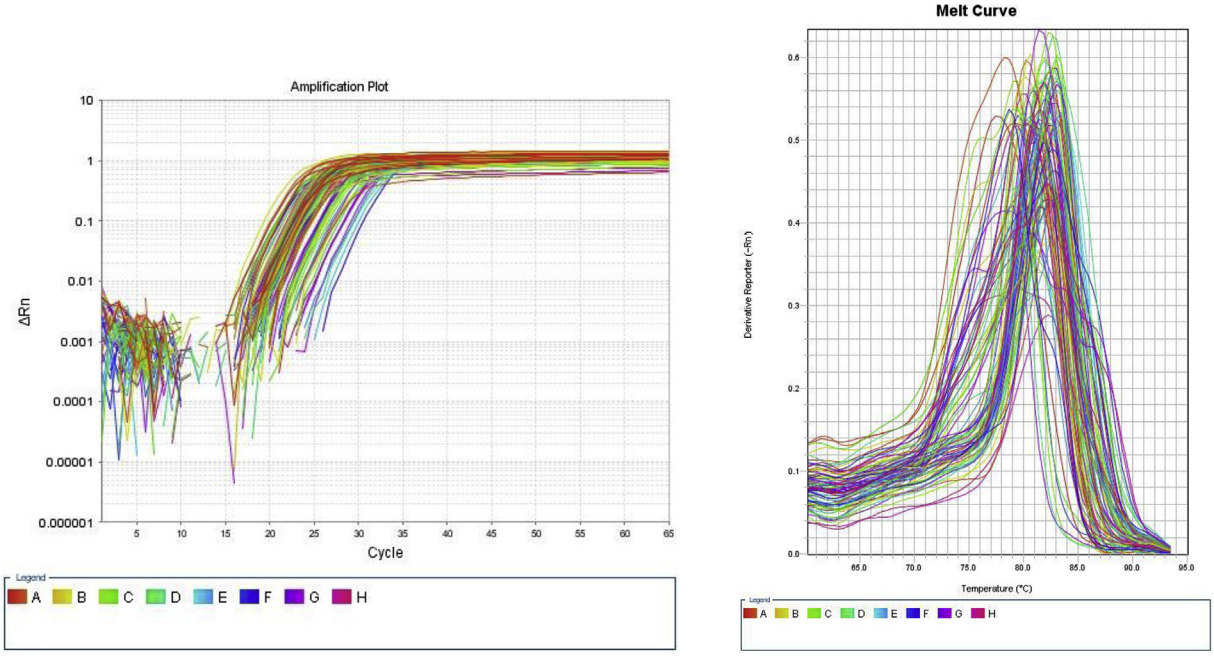
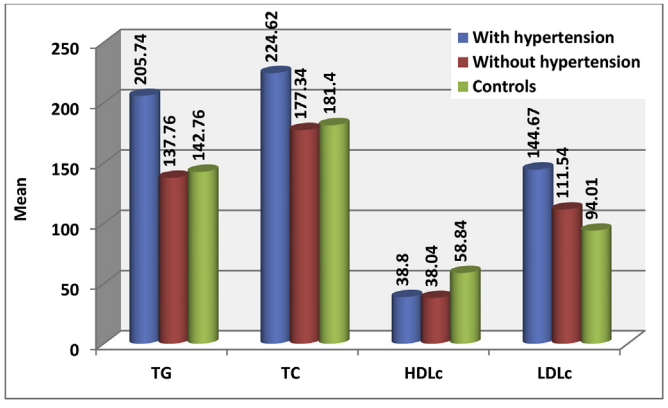


Fig. 1. (*continued*)

Table 1
Distribution of lipid profile and CMIT among the studied groups.

Lipid profile & CMIT	Groups			Test of sig	Post hoc value
	With hypertension (I) (N = 50)	Without hypertension (II) '(N = 50)	Controls (III) (N = 50)		
	Mean ± SD	Mean ± SD	Mean ± SD		
TG	205.74 ± 50.16	142.76 ± 23.63	137.76 ± 9.34	**F = 68.08** **P < 0.001***	< 0.001*(I vs. II-I vs. III) II vs. III = 0.722
TC	224.62 ± 35.93	181.40 ± 26.76	177.34 ± 15.22	**F = 45.99** **P < 0.001***	< 0.001*(I vs. II-I vs. III) II vs. III = 0.738
HDLc	38.80 ± 5.07	38.04 ± 5.04	58.84 ± 5.59	**F = 253.21** **P < 0.001***	I vs. II = 0.749 < 0.001*(I vs. III II vs. III)
LDLc	144.67 ± 41.92	111.54 ± 15.53	94.01 ± 23.94	**F = 38.80** **P < 0.001***	< 0.001*(I vs. II I vs. III) II vs. III = 0.003*
CIMT	0.95 ± 0.14	0.75 ± 0.06	0.65 ± 0.03	**F = 118.95** **P < 0.001***	< 0.001*(I vs. II- I vs. III- II vs. III)

*significant.



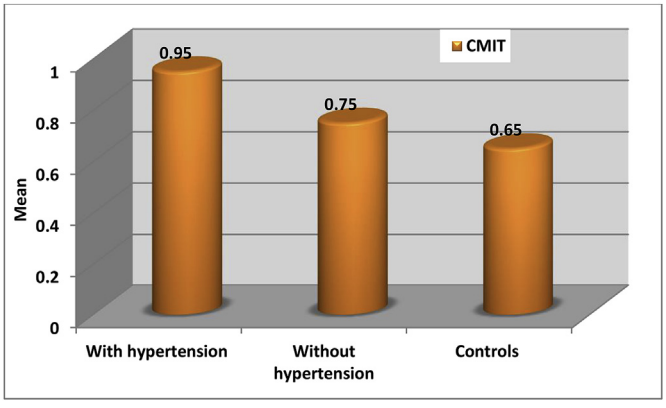


Fig. 2. a: distribution of lipid profile among the studied groups. b: distribution of CIMT among the studied groups.

Fig. 2. (*continued*)

Table 2
NPY SNP and NPY gene expression of the studied groups.

	Groups						Test of sig	P-value
	With hypertension (I) (N = 50)		Without hypertension (II) (N = 50)		Controls (III) (N = 50)			
	no	%	no	%	no	%		
NPY gene expression Mean ± SD	128.98 ± 46.60		53.80 ± 19.84		3.46 ± 2.89		Kruskal-Wallis = 124.04 P < 0.001[a]	< 0.001[a](I vs. II- I vs. III- II vs. III)
NPY SNP							χ^2	
TT	7	14.0	12	24.0	27	54.0	χ_1 = 4.41	0.110
TC	24	48.0	28	56.0	18	36.0	χ_2 = 20.78	< 0.001[a]
CC	19	38.0	10	20.0	5	10.0	χ_3 = 9.61	0.008[a]
NPY allele							χ^2	
T	38	38.0	52	52.0	72	72.0	χ_1 = 3.96	0.046[a]
C	62	62.0	48	48.0	28	28.0	χ_2 = 23.35	< 0.001[a]
							χ_3 = 8.49	0.003[a]

[a] Significant.

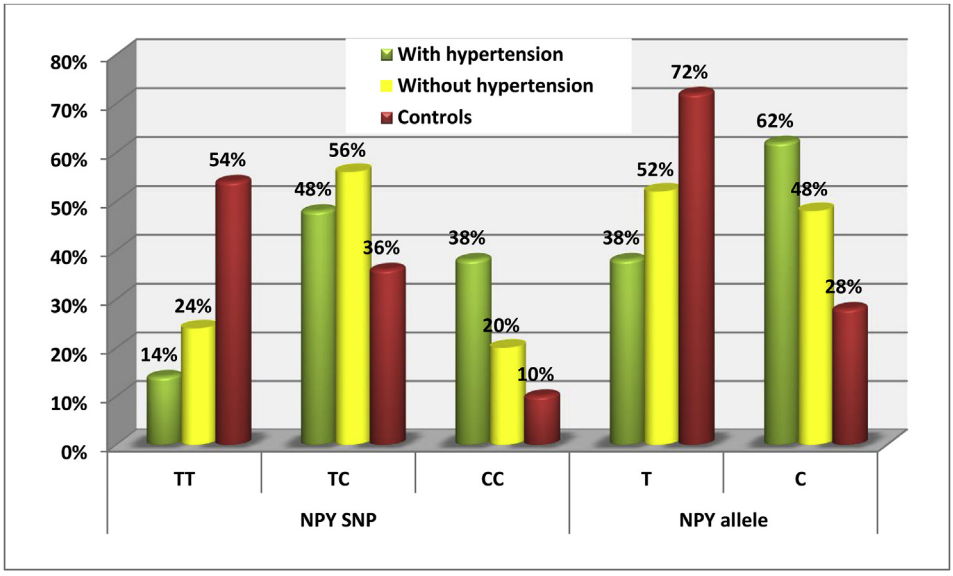


Fig. 3. NPY SNP frequency and alleles of the studied groups.

Table 3
NPY SNP of the studied with and without hypertension groups.

	Groups				Test of sig	P value	OR (CI 95%)
	With hypertension (N = 50)		Without hypertension (N = 50)				
	no	%	no	%			
NPY SNP							
TT	7	14.0	12	24.0			RF
TC	24	48.0	28	56.0	FE = 0.49	0.592	1.47 (0.50–4.33)
CC	19	38.0	10	20.0	χ^2 = 3.80	0.051	3.26 (0.97–10.88)
NPY allele							
T	38	38.0	52	52.0			RF
C	62	62.0	48	48.0	χ^2 = 3.96	0.046*	1.77 (1.01–3.10)

*significant.

3. Assay

Measurement of lipid profile (low density lipoproteins cholesterol (LDLc), high density lipoproteins cholesterol (HDLc), total cholesterol (TC), and triacylglycerol (TG) was done using standard enzymatic colorimetric kits (Spinreact diagnostics kit, Spain) and renal function tests (urea and creatinine) was determined using standard enzymatic colorimetric kits (DIAMOND diagnostics kits, Germany). Determination of GFR by MDRD formula = Estimated GFR (ml/min/1.73 m^2) = 186.3 x (serum creatinine) $^{-1.154}$ x Age $^{-0.203}$ x (0.742 if female) [14].

4. SNP assay of rs16147 (−399 T/C of NPY) by real time PCR

DNA was extracted from from frozen EDTA treated blood sample using Gene JET™Whole Blood Genomic DNA Purification Mini Kit (THERMO SCIENTIFIC, EU/Lithuania) according to the manufacturer's instructions. The samples were analyzed by TaqMan probes rs16147 (−399 T/C of NPY) which were labeled with VIC and FAM fluorescent dyes, respectively, with the probe sequence as follows: GCTTCCTACT CCGGCACCCAGTGGG **[C/T]** TGGTAGTCCTGTTGGCAGGAGACAA.

In a total reaction volume of 20 μL for real-time PCR contains 10 μL of TaqMan Genotyping Master Mix, 1.25 μL of 20 × SNP assay mixture containing both primers and probes, nuclease-free water, and template

Table 4
Correlation between NPY gene expression level and some parameters in studied groups.

Parameters	NPY gene expression level					
	With hypertension (I)		Without hypertension (II)		Controls (III)	
	r	P value	r	P value	r	P value
Urea	0.007	0.962	0.057	0.696	0.016	0.913
Creatinine	−0.141	0.330	−0.037	0.796	−0.197	0.171
GFR	0.105	0.467	0.015	0.915	−0.044	0.760
AST	0.136	0.348	−0.012	0.932	−0.002	0.989
ALT	0.102	0.480	−0.005	0.972	0.142	0.325
TG	0.070	0.631	−0.148	0.305	0.233	0.104
TC	0.143	0.322	0.149	0.303	−0.214	0.136
HDLc	0.277	0.052	−0.171	0.234	0.060	0.677
LDLc	0.098	0.497	0.183	0.198	−0.323	0.022*
CIMT	0.898	< 0.001*	0.890	< 0.001*	−0.131	0.363

*significant.

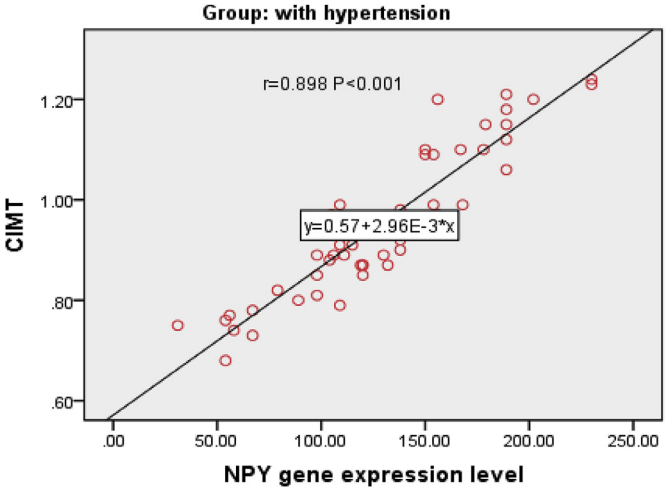


Fig. 4. a: Significant positive Correlation between NPY gene expression level and CMIT among hypertension group. b: Significant positive Correlation between NPY gene expression level and CMIT among without hypertension group.

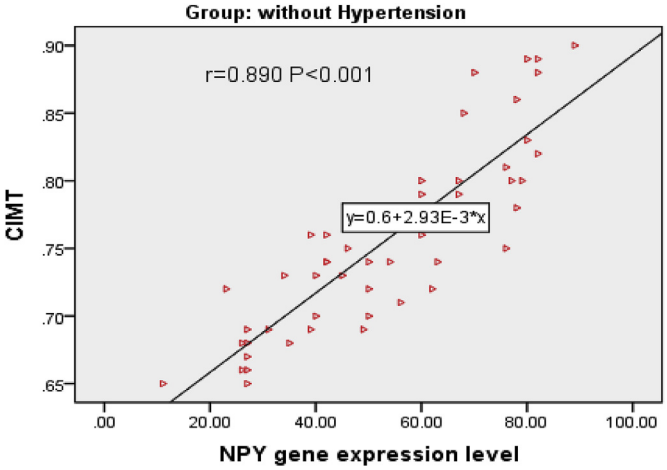


Fig. 4. (*continued*)

DNA. Cycling conditions was performed in 96-well plates as follows: 50 °C for 1 min (Pre-PCR read), then 95 °C for 10 min and 45 cycles of 95 °C for 15 s, 60 °C for 1 min (cycling), and 60 °C for 1 min (Post-PCR) (Fig. 1a) using the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA).

5. Quantitative assay of NPY gene expression by real-time PCR

Total RNA isolation was performed from whole blood by Direct-zol™ RNA MiniPrep kit, Zymo Research, followed by assuring RNA quality and purity. Extracted RNA was stored in −80 °C till time of use. First step, PCR was cDNA synthesis (reverse transcription step RT-PCR) using (*QuantiTect Reverse Transcription Kit,Qiagen, Applied Biosystems, USA, 2012*), using Applied Biosystems 2720 thermal cycler (Bioline, Singapore, USA). Second step, PCR was cDNA amplification (real-time PCR step): The cDNA was used in SYBR green based quantitative real-time PCR for quantification of IL-1 β geneexpression by (SensiFAST TM SYBR Lo-ROX Kit, Bioline), using the following designed primers (Midland,TX). 1- NPY primer sequence: Forward primer 5 ′- GCTGC GACAC TACAT CAACC -3 ′, Reverse primer 5 ′- AGTCT CATTT CCCAT CACCAC -3 ′ and 2- Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) primer sequence: Forwardprimer 5 ′ TGATGACATCAAGAAG GTGGTGAAG-3 ′, Reverse Primer 5 ′ TCCTTGGAGGCCATGTGGGCCAT-3 ′.Data analysis with Applied Biosystems 7500 software version 2.0.1. The relative quantification (RQ) of gene expressioncompleted using comparative ΔΔCt method where the amountof the target IL-1 β gene, is normalized to an endogenousreference gene (GAPDH) and relative to a control (Fig. 1b). Each run was completed using melting curve analysis to confirmspecificity of the amplification and absence of primer dimers.

6. Statistical analysis

Results were collected, tabulated, statistically analyzed by IBM personal computer and statistical package SPSS version 22.0. Two types of statistics were done. Chi-square test (x^2) is a test of significance used to study association between two qualitative variables. Odd ratio, describe the probability that people who are exposed to a certain factor will have a disease compared between the two groups. Mann-Whitney test for abnormally distributed quantitative variables comparing between two groups.

Kruskal-Wallis test for abnormally distributed quantitative variables, to compare between more than two studied groups, P-value < 0.05 was considered statistically significant.

7. Results

There was a significant increase in TG, TC, LDLc and CMIT values in chronic kidney patient with hypertension and without hypertension compared to control, while there was a significant decrease in HDLc compared to control (Table 1 &Fig. 2a and b).

There was a significant increase in NPY gene expression in patients groups compared to control, also there was a significant frequency increase in TC, CC and C allele genotyping of NPY gene in patients groups

Table 5
Distribution of lab investigations among the studied groups.

Kidney function tests	NPY SNP in Hypertension group			Kruskal-Wallis test	Post hoc value
	TT (I) (N = 7)	CT (II) (N = 24)	CC (III) (N = 19)		
	Mean ± SD	Mean ± SD	Mean ± SD		
Urea	66.14 ± 46.68	71.12 ± 34.26	60.05 ± 32.44	**2.88** **P = 0.237**	I vs. II = 0.155 I vs. III = 0.816 II vs. III = 0.174
Creatinine	7.82 ± 1.04	8.65 ± 1.82	7.71 ± 1.49	**F = 1.95** **P = 0.153**	I vs. II = 0.470 I vs. III = 0.986 II vs. II = 0.156
GFR	28.71 ± 11.91	31.12 ± 11.44	30.15 ± 11.94	**0.25** **P = 0.882**	I vs. II = 0.619 I vs. III0.750 II vs. III = 0.825
AST	22.28 ± 11.33	18.37 ± 12.84	25.21 ± 18.90	**2.60** **P = 0.272**	I vs. II = 0.264 I vs. III = 0.953 II vs. III = 0.147
ALT	21.42 ± 15.66	12.07 ± 7.64	18.04 ± 13.56	**8.01** **P = 0.018***	I vs. II = 0.014* I vs. III = 0.465 II vs. III = 0.031*
TG	199.71 ± 58.04	207.33 ± 50.75	205.94 ± 49.19	**F = 0.06** **P = 0.942**	I vs. II = 0.936 I vs. III = 0.959 II vs. II = 0.996
TC	178.0 ± 12.68	237.04 ± 33.18	226.10 ± 31.62	**F = 10.05** **P < 0.001***	I vs. II < 0.001* I vs. III = 0.003* II vs. III = 0.483
HDLc	38.14 ± 5.66	37.91 ± 4.84	40.15 ± 5.11	**F = 1.10** **P = 0.338**	I vs. II = 0.994 I vs. III = 0.643 II vs. III0.328
LDLc	99.91 ± 20.19	157.65 ± 41.96	144.75 ± 37.33	**6.24** **P = 0.004***	I vs. II = 0.001* I vs. III = 0.011* II vs. III = 0.275
CIMT	0.74 ± 0.03	0.88 ± 0.05	1.11 ± 0.08	**F = 100.21** **P < 0.001***	I vs. II < 0.001* I vs. III < 0.001* II vs. III < 0.001*

*significant.

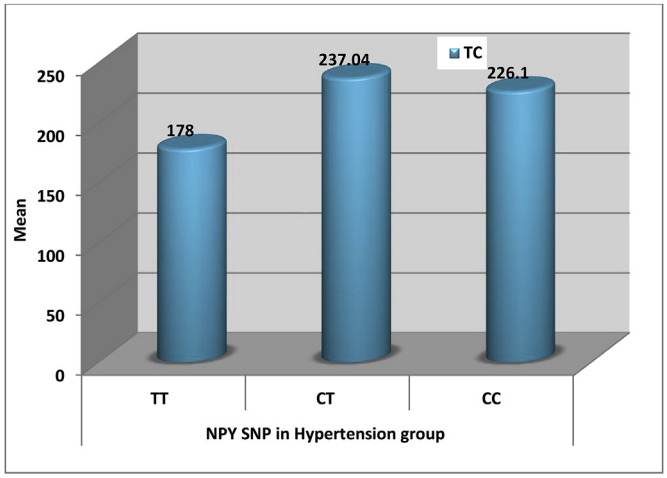


Fig. 5. a: distribution of Total cholesterol among the studied NPY SNP in Hypertension group, b: distribution of CMIT among the studied NPY SNP in Hypertension group.

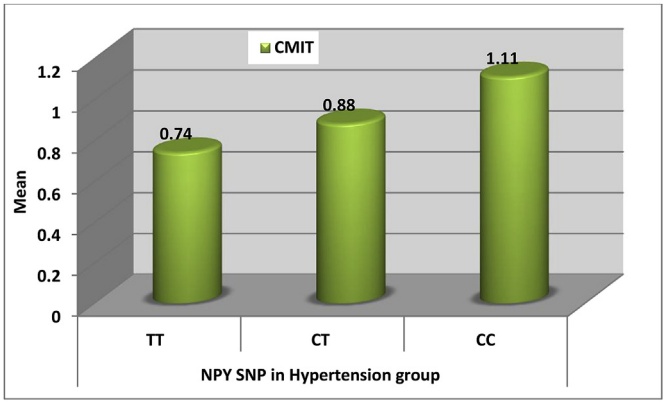


Fig. 5. (*continued*)

compared to control (Table 2 & Fig. 3).

There was a significant increase in CC genotype and C allele in patients groups with hypertension when compared with patients group without hypertension with odd ratio 3.26 and 1.77 respectively (Table 3).

There was a significant positive correlation between NPY gene expression level and CMIT among patients with and without hypertension groups compared to control, while there was a significant negative correlation between NPY gene expression level and LDLc among control group (Table 4 & Fig. 4a and b).

There was a significant difference among different NPY genotypes as regard ALT, TC, LDLc and CMIT values in the studied patients with hypertension group (Table 5 & Fig. 5a and b).

There was a significant increase of NPY gene expression in CC genotypes of NPY gene when compared to other two genotypes in two patients groups with and without hypertension (Table 6 and Fig. 6).

8. Discussion

Chronic kidney disease (CKD) is a worldwide health problem with a high economic rate to health systems and is an independent risk factor for cardiovascular disease (CVD). Entirely stages of CKD are related with increased risks of cardiovascular morbidity, premature mortality,

Table 6
Mean distribution of gene expression among the studied NPY SNP groups.

Groups	NPY SNP	NPY gene expression	Test	Post hoc value
		Mean ± SD		
With hypertension	TT	55.28 ± 12.07		< 0.001*(I vs. II- I vs. III- II vs. III)
	TC	113.16 ± 17.04	F = 100.31	
	CC	176.10 ± 26.49	P < 0.001*	
Without hypertension	TT	28.15 ± 7.35		< 0.001*(I vs. II- I vs. III- II vs. III)
	TC	55.67 ± 12.69	F = 66.88	
	CC	78.80 ± 6.10	P < 0.001*	
Controls	TT	4.20 ± 3.18		I vs. II = 0.121
	TC	2.70 ± 2.37	F = 4.61	I vs. III = 0.098
	CC	2.15 ± 2.10	P = 0.099	I vs. III = 0.290

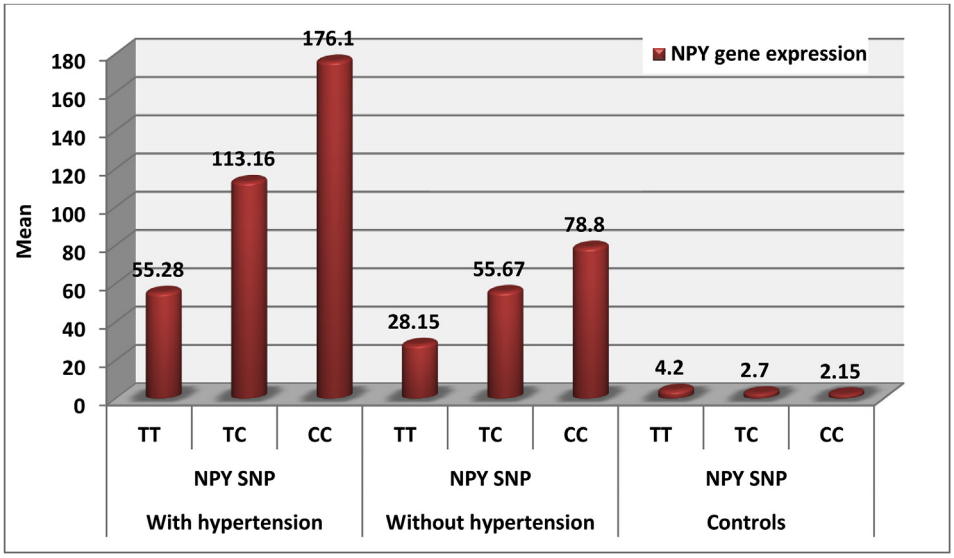


Fig. 6. distribution of NPY gene expression among the studied NPY SNP in the studied groups.

and/or decreased quality of life. [15].

Kidney failure is conventionally considered the most serious outcome of CKD and symptoms are usually caused by complications of reduced kidney function [16].

The *NPY* gene, comprising four exons, is sited on chromosome 7p15.1 and codes for a 36-amino acid peptide which is secreted via neurons [17].

The endogenous renal NPY is expressed not just in the end of sympathetic nerves but also and principally in the renal tubular cells itself and may have paracrine properties in the kidney, the endogenous NPY-system is also amenable to pharmalogical and genetic management in the kidney [18].

In present study, there is a significant increase in NPY gene expression in patients groups compared to control, In accordance with these results [19] found that plasma NPY protein level linked with proteinuria and quicker CKD expansion besides with a higher hazard of kidney failure. Which may describe by the sympathetic system and/or properties intrinsic to the NPY molecule, including interference with innate immunity.

This study shows a significant difference increase in distribution of CC genotype and C allele for NPY gene in patients of chronic kidney disease with hypertension when compared to patients of chronic kidney disease without hypertension with odd ratio (3.26 and 1.77 respectively) with concomitant increase in NPY gene expression.

Matched with the current study a Pilot studies in CKD patients with resistant hypertension show that sympathetic denervation associates with hypertension control and GFR stabilization [20].

Also, the study of [21] found that measured sympathetic activity is closely related with the GFR and with proteinuria in CKD patients.

The study of [22] found that genetic variations of a biomarker of sympathetic activity like the chromogranin gene associate with a substantially non-proteinuric disease like nephrosclerosis.

In the present study, there is a significant positive correlation between NPY gene expression level and CIMT among chronic kidney disease patients with hypertension and without hypertension, with highest level of TC, LDLc and CMIT among CC genotype of NPY gene.

In accordance with this results, the study of [23] found that NPY genotype T-399C, considered as risk factors of hyperlipidaemia and carotid atherosclerosis and the study of [24] stated that human NPY-mediated gender-difference in the regulation of blood pressure. It was reported that long-term administration of NPY in the subcutaneous infusion could induce cardiac dysfunction and cardiac hypertrophy of which may be mediated by the Ca2+/CaM-dependent CaN pathway and p38 mitogen-activated protein kinase (MAPK) signal pathway in rats.

Also, the study of [25] found that activation of the Y2 receptor can stimulate lipid accumulation by the protein kinase A, mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways.

In present study, There was a significant difference among different NPY genotypes as regard total cholesterol (TC), LDLc and CIMT values in the studied patients with hypertension group.

In several studies, genetic variation in the NPY gene has been associated with higher low-density lipoprotein cholesterol and serum cholesterol levels [26], with obesity [27] and with increased risk for diabetes mellitus type 2 [28].

Correlational studies have examined potential SNPs in the promoter, introns, signalsequence, translated polypeptide chain, and 5' untranslated region in the NPY gene [29]. Inside the promoter region of NPY, the rs17149106 SNP was associated with high incidence of obesity in American health care professionals [27].

Another NPY promoter SNP, rs16147, has a mixed association. A positive association with obesity was found in Malaysian [30] and Spanish [31] along with a higher BMI from newborns to adulthood in a German population [32].

Also, the T1128C polymorphism in the NPY gene lead to a variation in the synthesis, trafficking, and/or secretion of the peptide [33] was originally associated with elevated levels of serum cholesterol but has also been linked to atherosclerosis and diabetes [34, 35]).

9. Conclusion

A significant association was found between CC genotype and C allele of NPY at rs16147 and risk of developing CKD with increase in NPY gene expression and a significant positive correlation between NPY gene expression level and CMIT. A significant prevalence of CC genotype and C allele of NPY at rs16147 in patients with hypertension may suppose that increase in NPY gene expression in patients carry CC genotypes at rs16147 might have an impact on CMIT and lipid profile lead to hypertension.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100666.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Ethical approval

Research Involving Human Participants. The study was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent, and the Ethics Committee of Faculty of Medicine, Menoufia University approved the study protocol.

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