Research article

Association between genetic variants in *ANGPTL4* (T266M) and *MC4R* genes predisposing to obesity

Zainab Adel Abbas

Department of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq

(Received: August 2022 Revised: October 2022 Accepted: December 2022)

Corresponding author: **Zainab Adel Abbas.** Email: za5474068@gmail.com

ABSTRACT

Introduction and Aim: Recently, obesity has begun to be seen not only as a physical state of an individual but also as an illness in and of itself. It is possible that there is not much research done on the exact variants or genes that put people at risk for obesity in Iraq. The aim was to sight the genetic variants predisposing to obesity in Baghdad population by screening the associated ANGPTL-4 and MC4R genes.

Materials and Methods: A total of 124 individuals aged between 15-67 years participated in this study that was prospective, descriptive, and cross-sectional in nature. Blood collected from each individual was analyzed for biochemical parameters such as fasting blood glucose, random blood glucose, HbA1c, thyroid function tests (TSH) and complete blood count. Genomic DNA was subjected to amplification of the *ANGPTL4* gene. The amplified product was subjected to restriction digestion using the enzyme *Nla*III and further genotyped.

Results: This study found no correlation between ANGPTL-4 (T266M) and MC4R rs13447324 among obese population studied in Baghdad, Iraq.

Conclusion: The study found no conclusive evidence linking the ANGPTL-4 T266M gene to obesity in the Baghdad population. The study also discovered that people of Baghdad did not have an association between MC4R rs13447324 and obesity. However, larger sample size investigations are required.

Keywords: Genetic variants; *ANGPTL4*; *MC4R*; obesity.

INTRODUCTION

Angiopoietins are glycosylated proteins that bind to the tyrosine kinase with Ig and epidermal growth factor (TIE domains) receptors playing an important role in embryonic and postnatal angiogenesis (1). There are several proteins that are closely related to angiopoietins referred to as angiopoietin-like proteins ANGPTLs (2). Structurally, the ANPTLs (except Angptl8) have domain architecture like angioproteins and have a characteristic protein structure that contain an N-terminal coiled coil domain, followed by a coiled-coil oligomerization domain and a C-terminal fibrinogen-like domain (3). ANPTLs in addition to regulating angiogenesis are also involved in several metabolic functions such as lipid metabolism, inflammation, hematopoietic stem cell activity, and cancer cell invasion (2,3). Among the ANPTLs, ANGPTL4 also referred as fasting-induced adipose factor (Fiaf), PPAR gamma-induced angiopoietin-related protein (PPAR gamma-AR), and PPARG angiopoietin-related protein has recently emerged as a factor that significantly modulates plasma triglyceride levels (3) overexpression of which leads to enhanced lipolysis, fatty acid oxidation and reduction in adipose tissue mass (4). ANGPTL4 concentrations in blood serum have effects on glucose homeostasis, lipid metabolism, insulin levels and preventing tumor cell adhesion and migration (3).

Overweight individuals generally have a malfunctioning leptin-melanocortin pathway. The melanocortin system coordinates responses to inputs from the nervous system, the endocrine system, and the metabolic system. The brain melanocortin system is a key regulator of energy balance, and any dysfunction in this system can lead to obesity (5). The melanocortin receptors (MC1R-MC5R) have a distinct distribution pattern and distinct physiological role. Brain and spinal cord are rich in expression of MC3R and MC4R, both of which are neural MCRs. The melanocortin 4 receptor encoded by the MC4R protein, has been shown to play a crucial role in nutritional health by suppressing hunger and participating in the regulation of energy homeostasis Studies with MC4R-knockout mice have been shown to be associated to metabolic disorders such obesity, hyperinsulinemia, hyperphagia, and hyperglycemia (6) Similarly, deletion of MC4R has been associated to increased body fat, leading to liver steatosis without atherogenic diet (7).

Several *MC4R* single nucleotide polymorphisms as well as allelic variants have been detected in metabolically healthy and unhealthy obese humans from different populations (8). More than 200 variants for this gene have been identified with the

frequency of variants ranging between 0.5 to 8.5%, In humans, several MC4R alleles have been associated with increased risk of developing obesity as compared to non-carriers (9). In this study we aimed to screen ANGPTL-4 (T266M) and MC4R genes in obese individuals and investigate the variants associated with these genes in obese population of Baghdad.

MATERIALS AND METHODS

The study included 124 (64 females and 60 males) participants aged between 15-67 years, who sought outpatient consultations at the Al-Kindy teaching hospital, the Center of Endocrines and Diabetes/Rusafa, and many private clinics and laboratories in Baghdad, Iraqi between December 2019- September 2020. Participant data such as name, age, sex, chronic diseases, BMI, antibiotic treatment, and other information was collected.

Inclusion criteria involved individuals with elevated BMIs and irregular waist measurements that were thought as being overweight or obese were required to stop using antibiotics and smoking for at least a month before sampling. Smokers, chemotherapy patients, COVID-19 virus positive patients, and any patient with elevated total white blood cells and/or thrombocytopenia/thrombocytosis were excluded.

Blood (5ml) was drawn from everyone, transferred to a tube containing EDTA. Blood was analyzed for biochemical parameters such as fasting blood glucose, random blood glucose, HbA1c, thyroid function tests (TSH) and complete blood count. A portion of the blood was stored at -20ºC for extraction of genomic DNA.

PCR-AFLP for *ANGPTL4* gene

The T266M polymorphism was genotyped by using the PCR-amplified fragment length polymorphism (PCR-AFLP) method. This method required the use of specific primer sets in conjunction with the *NlaIII* restriction enzyme (10). The primers used are: *ANGPTL4* rs1044250 Forward primer: 5′-TGCCTCA TGGAGTGGCCTCT- 3′and *ANGPTL4* rs1044250 Reverse primer: 5′-TGTCCTCGCCACCCAGGT-3′. The PCR master mix (20 µl) consisted of PCR buffer 5X, dNTPs mixture 200 µM each, forward primer 1pmol reverse primer 1pmol and Taq DNA polymerase 5U/ µl. Genomic DNA template (2µl of 100 ng) was added to the reaction mixture, while to control tube nuclease free H2O (2 μl) was added instead of template DNA. PCR was performed in a thermal cycler (Thermo Fisher Scientific, USA). programmed as follows: 94 ºC 4 minutes, 94 ºC 1 minute, 56 ºC 1 minute, 72 ºC 1 minute 35 cycles and 72 ºC 7 minutes. PCR amplified products were electrophoresed on 2 % agarose gel at 5V/cm for 3.5 hours, stained and the bands visualized. The bands with molecular size 165 bp representing the presence of *ANGPTL4* were excised *and* restricted digested with *Nla*III restriction enzyme. The restriction enzyme *Nla*III was prepared and concentrated as per the manufacturer's (Biolabs, UK) instructions. Restriction products were analyzed on 3% agarose gel. The expected digested products consist of: 156+ 9 bp for C allele and 94+ 62 + 9 bp for T allele.

PCR-AFLP for *MC4R* rs13447324

Genotyping of *MC4R* rs13447324 variant was done using the PCR-AFLP (amplified fragment length polymorphism) method by using specific primer sets; *MC4R* rs13447324 forward primer: ATCAATTCAGGGGGACACTG and Reverse primer AACGCTCACCAGCATATCAG and *Nhe*I restriction enzyme (Promega/ USA) (11). Master mix was prepared in a total volume of 25 µl per reaction. Buffer (5X), dNTPs mixture 200 µM, forward primer 5 pmol reverse primer 5 pmol and Taq polymerase 5U/ µl, Genomic DNA template (2µl of 100 ng) was added to the reaction Eppendorf tube, no template control (NTC) tube was added containing all PCR master mix components, and Nuclease free H2O (2 μl) were added instead of template DNA. Eppendorf tubes containing PCR master mix reaction were transferred to the thermal cycler that is programmed as follows: 94 ºC for 2 minutes/ 1 cycle, 94 ºC for 1 minute, 60 ºC for 30 seconds, 72 ºC for 1 minute (30 cycles) and final extension 72 ºC for 7 minutes. Amplified PCR products were electrophoresed on 1.5 % agarose gel. The appearance of bands with molecular size 307 bp amplicon represented the presence of *MC4R* variant. The PCR products were digested through *MC4R* c.124 G > T mutation with restriction enzyme *Nhe*I and incubated at 37°C for 3 hours. Restriction reaction was prepared as manufacturer's instructions with final volume of 20 µl and a concentration of PCR product equal to 1µg/ µl. Restriction reaction was incubated at 37°C for 3 hours, then inactivated by heat inactivation at 65°C for 15 minutes. The digested products loaded on 2.5% agarose gel at (5V/cm) for 2.5 hours. Appearance of 163bp + 44 bp fragments referred to the presence of MC4R c.124 G > T mutation. Numerous hereditary illnesses, including adenocarcinoma (12, 13), chronic myeloid leukemia (14), and beta-thalassemia (15), have been successfully diagnosed using the molecular approach and in the treatment of certain malignancies (16). On the other hand, this method was utilized to diagnose several harmful microorganisms in addition to hereditary illnesses (17), therefore, polymerase chain reaction (PCR) is recommended for detecting genetic variations in the ANGPTL4 (T266M) and MC4R genes that are associated with the diagnosis of obesity.

Statistical analysis

SPSS ver.20 software (SPSS, Inc., Chicago, IL, USA) was used to analyze continuous variables. The mean and standard error calculated is expressed as the mean

± standard error. The statistical analysis was performed using the student's one-way analysis of variance (ANOVA) test. $P \leq 0.05$ was measured to indicate a statistically significant difference.

RESULTS

Genotyping of T266M polymorphism by PCR-AFLP

T266M polymorphism was carried out through the application of the PCR-AFLP method. Only four out of ninety-one people (4.3%) had this variation. Fig. 1 displays the outcome of performing PCR-AFLP on this variation, which may be found in Fig.2.

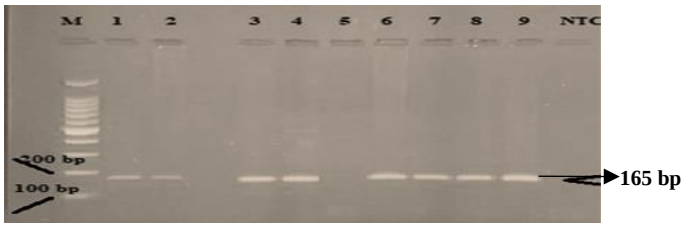

Fig. 1: PCR amplification of 165bp of *ANGPTL4 gene* **M**: DNA ladder (100 bp); Lanes 1-9: Samples positive for *ANGPTL4* (165 bp); NTC: Negative control

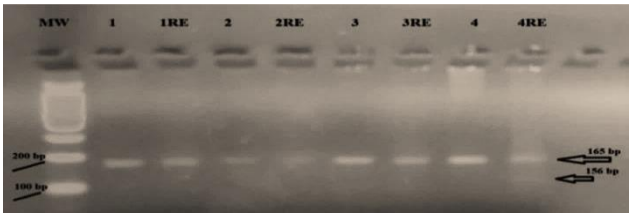

Fig. 2: Agarose gel electrophoresis of PCR amplified restriction enzyme *Nla*III digested products for *ANGPTL4* MW: 100 bp DNA ladder; Lanes 1-4: PCR amplified products of ***ANGPTL4*** (165 bp). Lane 1RE-4RE: PCR amplified products of ***ANGPTL4*** treated with *Nla*III restriction enzyme. Lane 4RE: 156 bp is the digested product of ***ANGPTL4*** which refers to the presence of T266M variant.

Association between obesity classes and *ANGPTL4* (T266M) polymorphism

In this study, the association between obesity classes of included subjects and the presence of T266M polymorphism was mentioned in Table 1. A total of 124 people participated in the experiment, however 33 of them saw no improvement in their condition, thus they were not included in the statistical analysis.

Genotyping of *MC4R* rs13447324 c.124 G > T by PCR-AFLP

By using PCR-AFLP (Amplified-Fragment Length Polymorphism) method, *MC4R* was digested with *Nhe*I restriction enzyme. Only 3/ 92 (3.2%) were having this variant. The result of PCR-AFLP for this variant was shown in Fig. 3

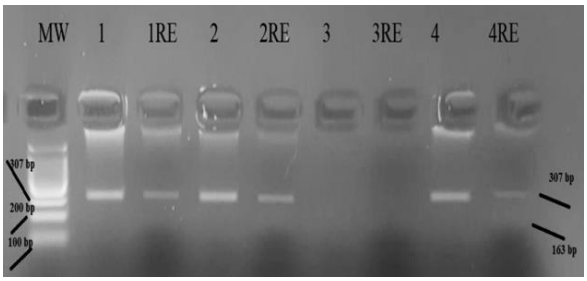

Fig. 3: Agarose gel electrophoresis of PCR digested products with restriction enzyme NheI of *MC4R c.124 G > T* mutation. Lanes 1-4: Amplified PCR products of *MC4R* variant (307 bp). Lane 1RE-4RE: PCR amplified products of *MC4R* digested with *Nhe*I restriction enzyme. Lane 4RE: 163 bp arrowed is the digested product of *MC4R c.124 G > T* mutation. MW: 100 bp DNA ladder.

Association between obesity and *MC4R* rs13447324

Table 2 shows that no statistically significant association exists between the obese groups and MC4R rs13447324. A total of 124 participants were tested, however 32 produced no results and were therefore omitted from the analysis.

DISCUSSION

Results in this study showed no correlation between ANGPTL4 T266M polymorphism and obesity. The findings of this study agree with a study by Talmud *et al*., (18) who examined the impact of this mutation on triglyceride levels using 2772 samples and found that the mutation had a minimal impact on adipose tissue. In contrast, Smart-Halajko *et al*., (19) reported a positive correlation to exist between

Table 1: The association between *T266M* mutation and obesity classes

T266M	Lean *n* = 16	Normal *n* = 14	Overweight *n* = 5	Class I *n* = 12	Class 2 *n* = 22	Class 3 *n* = 22	Total *n* = 91	*p*
Positive	1	1	0	2	0	0	4	0.226 C NS
Negative	15	13	5	10	22	22	87	

n: number of cases; C: chi-square test; NS: not significant. Note: > 20 % of cells had expected count of < 5

Table 2: The association between *MC4R* rs13447324 mutation and obesity classes

MC4R RE	Lean *n* =15	Normal *n* =13	Overweight *n* = 4	Class I *n* = 16	Class 2 *n* = 20	Class 3 *n* = 24	Total *n* = 92	P value
Positive	0	0	0	0	2	1	3	0.472 C NS
Negative	15	13	4	16	18	23	89	

n: number of cases; C: chi-square test; NS: not significant. Note: > 20 % of cells had expected count of < 5

ANGPTL4 levels with fat mass and body but found no correlation between the T266M variation and body fat tissue weight in middle-aged men.

A study by Matsunaga *et al*., 2020, showed that LPL inhibitors and deficiency or overexpression of any one of them results in hypotriglyceridemia or hypertriglyceridemia, which in turn affects adiposity (20) .In a Chinese study, Tong *et al*., discovered that the SNP rs1044250 in the ANGPTL4 gene was linked to an elevated risk of obesity (21) Previous studies conducted were based on the hypothesis that the variant rs1044250 (T266M), located in the C-terminal region of ANGPTL4 is highly conserved across human, rat, and mouse species and probably has a functional role. Results showed that mutations within rs1044250 can alter cardiovascular risk by reducing the amount of ANGPTL4 activity in endothelial cells resulting in an increase in both angiogenesis and arterial permeability (10). The SNP rs1044250 promotes abdominal fat formation and WC by decreasing lipolysis, increasing triglyceride levels, and disrupting the integrity of WAT-hepatic lymphoid tissue, all of which contribute to an increased appetite (21). The results of the current investigation found no connection between MC4R rs13447324 and obesity. Reports suggest mutations in MC4R cause obesity that is inherited in a co-dominant fashion (9). In contrast, the results of this investigation did not find any link between this SNP and obesity, which is in line with the findings of an earlier study conducted in Denmark. (22). Our results are contrary to earlier reports that have shown mutations in MC4R gene to be associated with obesity (23). Another study stressed the significance of the MC4R gene in causing childhood obesity (24). Many Danish overweight and obese children and adolescents failed to reduce their weight even after using many treatments due to mutations unresolved in MC4R gene (25). Results for MC4R SNP undertaken in this study showed the SNP to be not associated with obesity, the observation which was previously reported by Moazzam-Jazi *et al*., (26). Europeans are more likely than Middle Easterners to be overweight because of this SNP. The melanocortinergic circuit associated with energy homeostasis is integrated by MC4R. In the hypothalamus, the paraventricular nucleus is where the satiety- and energy expenditure-inducing peptides -MSH, cocaine and amphetamine-regulated transcript (CART), and other related peptides bind to MC4R during meal intake. On the other hand, the MC4R antagonist neuropeptide AgRP (agouti-related peptide) promotes both an increase in hunger and a decrease in energy expenditure in a low energy state (27). This is the first study undertaken to examine the role of *MC4R* rs13447324 in causing obesity in Iraq. Even though the sample size was smaller than anticipated due to lack of funds and constrained study time, the research revealed that this SNP is not common in Iraqi population.

CONCLUSION

This is a first study in Iraq where obesity-related genotyping of ANGPTL-4 T266M and MC4R rs13447324 has been investigated. Even though there was no conclusive evidence linking these gene changes to obesity, more research in Baghdad, Iraq, with a bigger population is necessary.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Ward, N.L., Dumont, D.J. The angiopoietins and Tie2/Tek: adding to the complexity of cardiovascular development. Semin Cell Dev Biol.2002; 13(1):19-27.
2. Oike, Y., Yasunaga, K., Suda, T. Angiopoietin-related/angiopoietin-like proteins regulate angiogenesis. Int J Hematol., 2004; 80: 21-28.
3. Biterova, E., Esmaeeli, M., Alanen, H.I., Saaranen, M. Ruddock, L.W. Structures of Angptl3 and Angptl4, modulators of triglyceride levels and coronary artery disease. Sci Report 2018; 8(1): 1-12
4. Cai, Y. C., Yang, H., Wang, K. F., Chen, T. H., Jiang, W. Q., Shi, Y.X. ANGPTL4 overexpression inhibits tumor cell adhesion and migration and predicts favorable prognosis of triple-negative breast cancer. BMC cancer. 2020;20: 878.
5. Adan RA, van Dijk G. Melanocortin receptors as drug targets for disorders of energy balance. CNS Neurol Disord Drug Targets. 2006;5(3):251-261.
6. Hammad, M. M., Abu-Farha, M., Hebbar, P., Cherian, P., Al Khairi, I., Melhem, M., *et al*., *MC4R* Variant rs17782313 Associates with Increased Levels of DNAJC27, Ghrelin, and Visfatin and Correlates with Obesity and Hypertension in a Kuwaiti Cohort. Front Endocrinol (Lausanne), 2020;11: 437.
7. Han J, Li Q. MC4R deficiency in pigs' results in hyperphagia and ultimately hepatic steatosis without high-fat diet. Biochem Biophys Res Commun. 2019; 520(3):651-656.
8. Gao L, Wang L, Yang H, Pan H, Gong F, Zhu H. *MC4R* single nucleotide polymorphisms were associated with metabolically healthy and unhealthy obesity in Chinese northern Han populations. Int J Endocrinol. 2019; 2019:1-9.
9. Farooqi IS, Yeo GSH, Keogh JM, Aminian S, Jebb SA, Butler G, Cheetham T, O'Rahilly S. Dominant, and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. J Clin Invest. 2000;106(2):271-279.
10. Abid, K., Trimeche, T., Mili, D., Msolli, M. A., Trabelsi, I., Nouira, S., *et al*., ANGPTL4 variants E40K and T266M are associated with lower fasting triglyceride levels and predicts cardiovascular disease risk in Type 2 diabetic Tunisian population. Lipids Health Dis, 2016; 15: 63.
11. Drabkin, M., Birk, O. S., Birk, R. Heterozygous versus homozygous phenotype caused by the same MC4R mutation: novel mutation affecting large consanguineous kindred. BMC Med Genet., 2018; 19: 135.
12. Ali, S.M., Lafta, B.A., Al-Shammary, A.M., Salih, H.S. In vivo oncolytic activity of non-virulent Newcastle disease virus Iraqi strain against mouse mammary adenocarcinoma. AIP Conference Proceedings, 2021, 2372, 030010.
13. Ali, S.M., Laftah, B.A., Al-Shammary, A.M.,Salih, H.S. Study the role of bacterial neuraminidase against adenocarcinoma cells in vivo. AIP Conference Proceedings,2021,2372,030009.
14. Ahmed, A.A., Khaleel, K.J., Fadhel, A.A., Al-Rubaii, B.A.L. Chronic Myeloid Leukemia: A retrospective study of clinical and pathological features. Bionatura, 2022, 7(3), 41.
15. Al-Musawi, A. H. O., Aziz, H. M., Khudair, S., Saleh, T. H. Molecular characterization of HBB gene mutations in beta-thalassemia patients of Southern Iraq. Biomedicine. 2022; 42(5):1040-1043.

16. Rasoul, L.M., Marhoon, A.A., Albaayit, S.F.A., Ali, R.W., Saleh, T.H., Al-Rubaii, B.A.L. Cytotoxic effect of cloned EGFP gene on NCI-H727 cell line via genetically engineered gene transfer system. Biomedicine, 2022;42(5):938-942.
17. Abdulkaliq Awadh, H., Hammed, Z.N., Hamzah, S.S., Saleh, T.H., AL-Rubaii, B.A.L. Molecular identification of intracellular survival related *Brucella melitensis* virulence factors. Biomedicine.2022; 42(4):761-765.
18. Talmud, P. J., Smart, M., Presswood, E., Cooper, J. A., Nicaud, V., Drenos, F., *et al.*, ANGPTL4 E40K and T266M: effects on plasma triglyceride and HDL levels, postprandial responses, and CHD risk. Arterioscler Thromb Vasc Biol, 2008; *28*: 2319-2325.
19. Smart-Halajko, M. C., Robciuc, M. R., Cooper, J. A., Jauhiainen, M., Kumari, M., Kivimaki, M., *et al.*, The relationship between plasma angiopoietin-like protein 4 levels, angiopoietin-like protein 4 genotype, and coronary heart disease risk. Arterioscler Thromb Vasc Biol, 2010; *30*: 2277-2282.
20. Matsunaga, A., Nagashima, M., Yamagishi, H., Saku, K. Variants of Lipid-Related Genes in Adult Japanese Patients with Severe Hypertriglyceridemia. J Atheroscler Thromb, 2020; *27*: 1264-1277.
21. Tong, Z., Peng, J., Lan, H., Sai, W., Li, Y., Xie, J., *et al*., Crosstalk between ANGPTL4 gene SNP Rs1044250 and weight management is a risk factor of metabolic syndrome. J Transl Med., 2021; 19: 72.
22. Kring, S. I., Larsen, L. H., Holst, C., Toubro, S., Hansen, T., Astrup, A., *et al*., Genotype-phenotype associations in obesity dependent on definition of the obesity phenotype. Obes Facts, 2008; 1: 138-145.
23. Stutzmann, F., Tan, K., Vatin, V., Dina, C., Jouret, B., Tichet, J., *et al*., Prevalence of melanocortin-4 receptor deficiency in Europeans and their age-dependent penetrance in multigenerational pedigrees. Diabetes. 2008; *57*: 2511-2518.
24. Doulla, M., McIntyre, A. D., Hegele, R. A., Gallego, P. H. A novel MC4R mutation associated with childhood-onset obesity: A case report. Paediatr Child Health. 2014; 19: 515-518.
25. Trier, C., Hollensted, M., Schnurr, T.M, Lund, M.A.V., Nielsen, T.R.H., Rui, G., *et al*., Obesity treatment effect in Danish children and adolescents carrying Melanocortin-4 Receptor mutations. Int J Obes (Lond). 2021; 45: 66-76.
26. Moazzam-Jazi, M., Sadat Zahedi, A., Akbarzadeh, M., Azizi, F., Daneshpour, M. S. Diverse effect of MC4R risk alleles on obesity-related traits over a lifetime: Evidence from a well-designed cohort study. Gene. 2022; *807*: 145950.
27. Salum, K. C. R., de Souza, G. O., Abreu, G. M., Campos Junior, M., Kohlrausch, F. B., Carneiro, *et al*., Identification of a Rare and potential pathogenic *MC4R* variant in a Brazilian patient with adulthood-onset severe obesity. Front. Genet. 2020; 11: 608840.