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Leptin rs7799039 polymorphism is associated with multiple sclerosis risk in Kuwait

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ABSTRACT

Background: Leptin association with Multiple sclerosis (MS) pathogenesis and MS related clinical characteristics is inconsistent. Here, we investigated whether two common variants in leptin (*LEP*) and leptin receptor (*LEPR*) genes influence MS risk and leptin levels in MS patients.

Methods: In a case-control study including 169 MS patients and 100 controls we examined the association of leptin in MS. Blood samples were used for DNA extraction and plasma retrieval. Taqman genotyping assays were used for *LEP* rs7799039 and *LEPR* rs1137101 genotyping, and enzyme-linked immunosorbent assay for plasma leptin level.

Results: Leptin levels were significantly lower in MS patients compared to controls (β = 0.157, 95%CI: 0.033–0.26, p = 0.012). *LEP* rs7799039AA associated with MS risk (OR: 2.52; 95%CI: 1.35–4.67, p = 0.003). None of the assessed markers associated with MS disability, severity or response to treatment.

Conclusion: *LEP* rs7799039AA is a risk factor for MS in our Kuwaiti population, and leptin levels are lower in MS patients compared to healthy controls. Our findings suggest future studies must consider all factors influencing leptin levels to resolve its controversial involvement in MS pathogenesis or progression.

1. Introduction

Leptin is a hormone produced by adipose tissues that has a well-established role in metabolic homeostasis (Chan et al., 2003). The majority of leptin related clinical studies focused on its association with obesity, the metabolic syndrome and diabetes (Farr et al., 2015; Kaur, 2014; Tsai, 2017). In recent years, neuroendocrine functions of leptin have been expanded to include effects on the immune and central nervous systems (CNS) (Francisco et al., 2018; Harvey, 2007). Leptin functions in immunity and the CNS are supported by evidence from investigating its function and levels in immune-related and neurodegenerative pathological conditions (Abella et al., 2017; de Candia and Matarese, 2018; McGuire and Ishii, 2016). Multiple sclerosis (MS) a chronic demyelinating autoimmune disorder involves T-cell mediated auto-immune response to myelin in the CNS. This response initiates a cascade of pro-inflammatory factors and recruits inflammatory cells to site of attack. This autoimmune process is thought to be repeated in the form of MS attacks or episodes that gradually diminishes the healing and regenerative repertoire of the CNS resulting in permanent CNS lesions. Leptin has been shown to modulate the immune response by activating immune cells such as T-lymphocytes, and mediating the release of pro-inflammatory factors (Fernandez-Riejos et al., 2010). Moreover, increased body mass index (BMI) which directly correlates to increased leptin level, has been shown to associate with increased risk of MS (Kavak et al., 2015; Munger et al., 2009). Therefore, it has been postulated that leptin might be involved in the pathogenesis of MS due to its suggested role in immune-metabolism regulation and its reported role in various autoimmune disorders (Francisco et al., 2018). Several reports have shown leptin levels to be elevated in MS patients with active disease, and that leptin levels are directly related to MS severity, progression and response to treatment (Batocchi et al., 2003; Rotondi et al., 2013). However, reports on the association of leptin with MS are inconsistent with several reports refuting the association in favor of other adipocytokines (Hietaharju et al., 2010; Kvistad et al.,

Abbreviations: ANOVA, analysis of variance; BMI, body mass index; CIS, clinically isolated syndrome; CNS, central nervous system; EDSS, expanded disability status scale; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; IQR, inter-quartile range; LEP, leptin; LEPR, leptin receptor; MS, multiple sclerosis; MSSS, multiple sclerosis severity score; NS, not significant; SNP, single nucleotide polymorphism

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2018). It is known that leptin levels are affected by gender and BMI which may contribute to the discrepancy in its association with MS (Evangelopoulos et al., 2014). Moreover, it has been shown that variants in the leptin (*LEP*) and leptin receptor (*LEPR*) genes may influence levels of circulating leptin level and predispose to obesity although these reports are inconsistent (Ghalandari et al., 2015). Specifically, a *LEP* polymorphism (rs7799039) at the 5' end of the leptin gene and a missense variant in *LEPR* (rs1137101, Q223R) have been widely investigated for their association with different diseases in addition to body fat mass (Hoang et al., 2014; Hu et al., 2016; Jiang et al., 2004; Mendez-Hernandez et al., 2017; Yang et al., 2016). Therefore, we hypothesized that leptin levels are altered in MS under the influence of genetic changes in leptin related genes or MS pathogenesis. In this study, we investigated leptin levels and variants in *LEP* and *LEPR* genes in a sampled MS cohort to determine the association of leptin and leptin related genetic variants with MS risk and clinical variables.

2. Materials and methods

2.1. Sample collection

MS patients' non-fasting blood samples were collected at Dasman Diabetes Institute's MS clinic, whereas healthy controls' non-fasting blood samples were collected at Mubarak hospital's phlebotomy unit. All blood samples were collected during morning clinic sessions between 7 and 11 am following an average of 2 h after last meal in EDTA coated vacutainers (Becton Dickinson, NJ, USA). This study's protocols were approved by Dasman diabetes institute ethical review committee which adheres to the declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. A random population sample of 169 MS patients and 100 healthy control individuals provided written consent, demographic information and a blood sample. All study protocols and objectives were fully explained to all participants before securing their informed written consent. MS patients' inclusion criteria were as follows; a detailed clinical history (MS age of onset, disease duration, expanded disability status scale (EDSS) score, and treatment history), being a Kuwaiti citizen, and an MS diagnosis duration of ≥ 2 years. Healthy controls' exclusion criteria were; having a family history of MS, and a diagnosis of any disorder including autoimmune or neurodegenerative disorder, and being non-Kuwaiti. All study participants underwent a brief demographics questionnaire inclusive of age, sex, and smoking status. Height (m) and weight (kg) measurements were recorded to compute participants' body mass index (BMI) measurement. Patients who underwent bariatric surgery were excluded from this study. Collected blood samples were centrifuged at $2500 \times g$ at room temperature for 10 min, and plasma fractions were collected and stored at $-80\,^{\circ}C$ until use.

2.2. LEP and LEPR variant genotyping

DNA extraction was performed on isolated buffy coat layers using Qiagen DNA mini kit according to manufacturer's protocol (Qiagen, Germany). Extracted DNA was assessed for quantity and quality using a spectrophotometer and 1% DNA gel electrophoresis, respectively. Taqman *LEP* (rs7799039) and *LEPR* (rs1137101) single nucleotide polymorphism (SNP) genotyping assays were used to assess genotype distribution among study cohorts (Applied Biosystems, CA, USA). In summary, 50 ng of DNA from every sample were used for genotyping using ABI7500 Fast Real-time PCR system (Applied Biosystems, CA, USA). Genotype allelic discrimination was determined by SDS v1.4.1 software (Applied Biosystems, CA, USA). Ten samples representing the three genotypes for each SNP were sequenced to validate genotyping assay specificity and accuracy using ABI 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

2.3. Leptin level assay

Abcam human leptin enzyme-linked immunosorbent assay (ELISA) was used (Abcam, Cambridge, UK). In brief, a 96-well plate precoated with anti-leptin capture antibody antibodies was equilibrated along with kit reagent to room temperature. Kit provided leptin standard and plasma samples were diluted using sample diluent and added to assigned wells. A capture and detector antibody cocktail was prepared using kit provided antibody diluent and added to standard/sample preloaded wells. Plates were incubated at room temperature for 1 h on a plate shaker set at 400 rpm. Incubation was terminated by aspirating unbound reaction mixtures and washing three times with kit supplied wash buffer PT. After washing all residual liquid was removed by blotting and TMB substrate was added to each well and incubated in the dark for 10 min on a Dynatech Vari-shaker set at 400 rpm (Dynatech Labs, NV, USA). Stop solution was added and plates were incubated for 1 min on a plate shaker to ensure uniform mixing. Reaction endpoint optical density measurements were recorded at 450 nm. Intra-assay coefficients of variation was $< 10\%$, and inter-assay coefficient of variation was $< 11\%$.

2.4. Data analysis

Leptin levels were non-normally distributed as assessed by D'Agostino and Pearson normality tests ($p < 0.0001$ for both cohorts) and were log transformed and checked for normality using the same test ($p > 0.05$ for both cohorts). Student *t*-test and Analysis of variant (ANOVA) test were used to compare inter-cohort means and to assess differences in leptin levels among groups respectively. Fisher exact tests and linear regression analysis was used to determine dependent variable association while adjusting for confounding factors known to influence leptin levels including sex, age, BMI and smoking status. Fisher exact and chi-square tests were used to analyze investigated SNPs allelic and genotype association with MS risk. Hardy–Weinberg equilibrium was performed on both SNPs in the two cohorts. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 25 (IBM, IL, USA). Differences were considered statistically significant at a level of *p*-value ≤ 0.05.

3. Results

MS patients and healthy control cohorts demographics, clinical characteristics and leptin levels are shown in Table 1. Age, sex and BMI are established factors affecting leptin levels and we found these factors influencing leptin levels in both cohorts when analyzed separately except for age in the MS cohort ($p = 0.23$). High leptin levels were found; in older individuals ($p = 0.029$ in controls), in females higher than males ($p < 0.001$ in both cohorts), and in individuals with high BMI indices ($p < 0.001$ in both cohorts). Leptin levels between the two cohorts did not differ significantly ($p = 0.098$). However, after adjusting for multiple factors (sex, BMI, age, smoking status) separately suggested their influence was contributing significantly to leptin levels in the two cohorts. After adjusting for these factors collectively when comparing leptin levels between the two cohorts we found leptin levels to be higher in healthy controls than MS patients ($\beta = 0.157$, 95%CI: 0.033–0.26, $p = 0.012$). Approximately 70% of MS male patients were current smokers, whereas only 14.3% female MS patients were current smokers. Smoking associated with lower leptin levels in the MS cohort ($\beta = -0.26$, 95%CI: -0.4 to -0.12, $p < 0.0001$) without any adjustment for confounding factors.

The assessed variants in *LEP* (rs7799039) and *LEPR* (rs1137101) were tested for their agreement with Hardy–Weinberg equilibrium in both cohorts. In healthy controls both rs7799039 and rs1137101 were in Hardy–Weinberg equilibrium ($p = 0.6$ and 0.9 respectively). Similarly, in the MS cohort rs7799039 and rs1137101 were in Hardy–Weinberg equilibrium ($p = 0.55$ and 0.62 respectively). *LEP*

Table 1
Control and MS patients' demographics and leptin levels.

Criteria	MS (n = 169)	Controls (n = 100)	p-value
Sex (%)			
Male	57 (33.7)	34 (34)	NS[a]
Female	112 (66.3)	66 (66)	
Age (average [range])	32.2 (21–62)	30.3 (21–60)	NS
BMI[b] (average [range])	27.1 (16.1–53.2)	28.25	NS
Current smokers (%)	56 (33.1)	(19.8–54.4)	NS
MS type (RRMS/SPMS/	154 / 12 / 3	22 (22)	
PPMS)	2 [1–3]	-	
EDSS (median [IQR[c]])	3.69 [2.1–5.24]	-	
MSSS (median [IQR])		-	
Treatment (%)	71 (42)	-	
Fingolimod	34 (20)		
Natalizumab	53 (31)		
Other (Interferons, dimethyl	11 (7)		
fumarate)			
None			
Leptin level, ng/mL (median	4.08	5.3 [3.3–8.35]	0.012
[IQR])	[1.43–8.04]		

[a] NS: not significant.
[b] BMI: body mass index.
[c] IQR: inter-quartile range.

Table 2
LEP rs7799039 and *LEPR* rs1137101 allelic and genotype distribution in MS patients and healthy controls.

SNP criteria	MS (n = 169)	Healthy controls (n = 100)	p-value
SNP allelic frequency (%)			
LEP rs7799039			
A	178 (52.7)	86 (43)	0.032
G	160 (47.3)	114 (57)	*NS
LEPR rs1137101			
A	228 (67.5)	129 (64.5)	
G	110 (32.5)	71 (35.5)	
Genotype frequency (%)			
LEP rs7799039			
AA	52 (30.8)	15 (15)	0.014
AG	74 (43.8)	56 (56)	NS
GG	43 (25.4)	29 (29)	
LEPR rs1137101			
AA	81 (47.9)	43 (43)	
AG	66 (39.1)	43 (43)	
GG	22 (13)	14 (14)	

* NS = not significant.

(rs7799039) allelic distribution was significantly different between the two cohorts (p = 0.032), whereas *LEPR* (rs1137101) was not (p = 0.51). *LEP* rs7799039 genotype distribution was significantly different in the MS cohort compared to healthy controls (p = 0.014) with genotype AA associating with MS risk in an autosomal recessive disease model (OR: 2.52; 95%CI: 1.35–4.67, p = 0.003) (Table 2). Dividing cohorts by sex did not result in any sex-specific SNP association with MS risk.

Neither SNP affected our reported leptin level association between the cohorts. However, *LEPR* rs1137101AA showed borderline effects on lowering leptin levels between the two cohorts (p = 0.088) (Fig. 1). *LEPR* rs1137101AA influence was persistent in MS females when compared to healthy females (p = 0.026), but we could not assess its effect among males as the sample size of males in both cohorts was too small to produce convincing results. Intra-MS cohort analysis of SNPs and leptin level with MS clinical variables produced very few associations. *LEP* rs7799039 did not influence leptin levels in MS patients alone (p = 0.6) or when adjusted for sex, age, BMI, and with (p = 0.57) or without smoking status (p = 0.56). Whereas, *LEPR* rs1137101 had a borderline association with leptin levels (p = 0.077) when analyzed alone. When adjusted for age, BMI and smoking status genotype GG

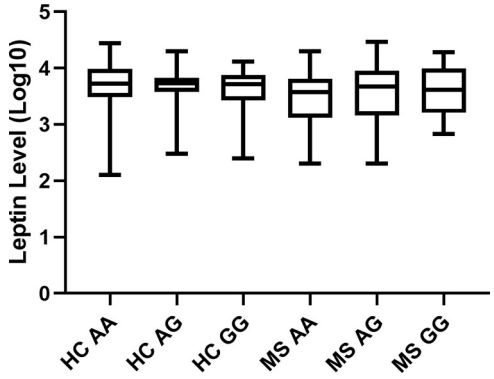

Fig. 1. Leptin levels in the two cohorts presented according to *LEPR* rs1137101 genotypes.

mildly associated with increased leptin levels (β = 0.096, 95%CI: 0.003–0.2, p = 0.044) with additional adjustment for sex the significance was lost (p = 0.055). Both SNPs did not associate with EDSS or MSSS scores neither did leptin levels when adjusted for sex, age, and BMI.

Discussion

Kuwait has a high prevalence of obesity along with an increasing MS prevalence (Al-Haifi et al., 2013; Alroughani et al., 2014). Leptin levels control body weight by inhibiting food intake and facilitating energy expenditure, and are higher in subjects with a high body fat content. Leptin has immune modulatory functions and is known to be produced by immune cells (Conde et al., 2010). Therefore, leptin association with MS is theoretically plausible. Here, we report novel findings for leptin and its associated *LEP* rs7799039 variant in a Kuwaiti MS cohort. Our finding of lower leptin levels in MS patients compared to controls is novel and unfortunately adds to the controversy surrounding leptin in MS. The majority of published literature supports an increase or no difference in MS patients leptin levels in comparison to control subjects (Batocchi et al., 2003; Chatzantoni et al., 2004; Emamgholipour et al., 2013; Kraszula et al., 2012; Messina et al., 2013; Rotondi et al., 2013). However, it is worthy to mention that each of these studies had various limitations. Most of the reported studies so far have a small MS patient sample size (n < 100) except Emamgholipour et al. (n = 191). Batocchi et al. focused on therapy influenced leptin levels specifically, so MS patient selection was biased toward treatment with interferon-beta. While Emamgholipour et al. and Messina et al. did not correct for the influence of smoking, BMI or sex which are all proven to affect leptin levels. Kraszula et al. had a very low sample size and focused only on RRMS patients using serum samples which exhibit volume displacement effect possibly making leptin more concentrated (Yu et al., 2011). Whereas Chatzantoni et al. and Rotondi et al. found no differences in leptin levels in their case-control studies. However, Chatzantoni et al. did report lower leptin levels in treatment naïve or methylprednisolone treated MS patients. A meta-analysis conducted using these and other case-control studies of leptin levels in MS also suggested leptin levels are higher in MS than healthy controls (Xie et al., 2018). The meta-analysis however did not acknowledge the limitations of the included studies and lack of uniformity across their studies' parameters as shown by tests of heterogeneity for multiple factors. Moreover, one of their included studies had sample bias saturation for clinically isolated syndrome (CIS) and 49.4% MS smokers and thus their leptin levels should have been adjusted for smoking and CIS excluded from the meta-analysis (Evangelopoulos et al., 2014). Our present study is not without limitation for we have used non-fasting blood samples since we were ethically bound to not influence patient's treatment regimens and most patients preferred having eaten before their intravenous treatments. However, we did attempt to standardize the time of blood sampling

after the last meal (~2 h) across the two cohorts to minimize its contribution to leptin level variations. Another limitation is that our MS cohort had over representation of RRMS patients compared to SPMS and PPMS. However, this is a reflection of the statistical prevalence of MS sub-types within MS populations (Zuvich et al., 2009). Our study strength is that we have a relatively moderate sample size and have assessed and corrected for most factors that may affect leptin levels.

The suggested contribution of leptin in MS is as an adipose tissue pro-inflammatory byproduct recruiting immune cells and release of cytokines (Huitema and Schenk, 2018). It is plausible that low leptin level is a marker for positive response to MS treatments administered, however a recent report refutes this possibility (Kvistad et al., 2018). Alternatively, according to the World Health Organization Kuwait 2016 statistics and other reports approximate that 42.4% of Kuwaiti adolescents are obese (Rey-Lopez et al., 2019). It is possible that MS patients under the burden of an MS diagnosis and treatment have lower BMI and less adipose tissue hence the low leptin levels compared to seemingly healthy controls from a population where obesity is prevalent. In fact, the association of obesity with MS risk was abolished when we compared MS patients to seemingly healthy obese population. Moreover, we did not find any obesity effect on disability as MS obese category patients had lower disability (EDSS average 1.96) than normal and underweight (EDSS average 2.42) or overweight MS patients (EDSS average 2.35). It is possible that physical activity contributed to the lack of obesity association with disability as physical activity has been shown to reduce MS related disability (Charron et al., 2018). We have initially attempted to assess physical activity in our cohort but the response rate was too low (60% in MS and 40% in controls) to be meaningful (data not shown). However, we did notice that 48.5% of overweight and 40% obese MS patients who answered the questionnaire stated current physical activity averaging 5 h per week. In addition, 42% of obese MS patients were current smokers which is known to lower leptin levels.

The investigation of *LEP* and *LEPR* variants for their association with MS risk has been previously reported albeit with contradicting results (Farrokhi et al., 2016; O'Doherty et al., 2009; Rey et al., 2011; Yousefian et al., 2018). Our finding of *LEP* rs7799039A associating with MS risk in our MS cohort is novel. However, we could not find any influence of rs7799039AA genotype on plasma leptin levels. Rs7799039 is a promoter variant in the *LEP* gene and allele G has been shown to associate with higher leptin levels and obesity (Dasgupta et al., 2015; Mammes et al., 1998). Moreover, our investigation into the effects of this variant in healthy controls detected a borderline association of allele A with lower leptin levels ($p = 0.06$). Conversely, the association of *LEPR* rs1137101GG with higher leptin levels we found is well established through studies confirming its association with obesity (Eldosouky et al., 2018; Kasim et al., 2016; Yiannakouris et al., 2001).

In conclusion, our findings suggest *LEP* rs7799039A is a risk factor for MS in our population, and that it may have affected the low leptin levels detected in our MS cohort. In addition, we replicated the established association of *LEPR* rs1137101GG with higher leptin levels in an MS cohort. However, it is worthy to note that leptin level investigation in an obesity prevalent population may limit the value of using it as a risk marker or predictor for MS.

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Declaration of Competing Interest

None.

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