Leptin and Leptin Receptor Polymorphism in Egyptian Diabetic Children with Latent Toxoplasmosis

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Declaration of interest

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Abstract

Background: Toxoplama gondii has been linked serologically to Diabetes mellitus. There is a vicious cycle between toxoplasmosis and diabetes mellitus as Toxoplasma gondii infection increases susceptibility to acquiring diabetes and diabetes mellitus patients are more susceptible to opportunistic infections such as toxoplasma gondii. Toxoplasmosis can induce an increase in leptin secretion via its cell surface receptor. The aim of this study is to investigate the effect of Leptin receptor polymorphism in diabetic children with latent toxoplasmosis in a cohort of Egyptian children.

Methods: Fifty patients with diabetes mellitus type I and 50 controls were included in this study. Toxo IgM and IgG, Serum level of leptin, C peptide were determined by Enzyme Immuno Assay. Glycosylated hemoglobin in blood was determined by colorimetric assay. Genotyping was carried out by the PCR and RFLP.

Results: Toxoplasma was more frequent in diabetic children group then in control group. Diabetic children were more associated with R allele compared to with Q allele. Serum leptin, C peptide and blood glucose level were significantly higher in positive *Toxoplasma.* RR distribution and R allele were significantly higher in positive *Toxoplasma*.

Conclusions: These results suggest that leptin receptor polymorphism influences leptin level, which modulate inflammation and immune response to *T. gondii* infection and may have a role in pancreatic pathology in diabetic children. Knowledge of these variants in T1DM might contribute to a better understanding of the role of inflammation in the etiology and progression of this disease.

Keywords: Children, Diabete, ,ELISA, Leptin, PCR, Polymorphism, Toxoplasma.

Running title: Leptin Polymorphism in Diabetic Children with Toxoplasmosis

1. Introduction

Toxoplasma gondii is a coccidian parasite with its fecal-oral cycle occurs exclusively in cats [1]. In human, toxoplasmosis can be transmitted by ingestion of oocysts by oral route, ingestion of tissue cysts in undercooked meat, transplacental, blood transfusion or organ transplantation [2]. Due to its easy and multiple mode of transmission, more than one third of humanity catches toxoplasmosis [3]. During acute toxoplasmosis, necrotizing lesions found in many human organs as lung, liver, spleen, heart and pancreas[1]. However, with intact immunity, acute phase is mostly resolved with T helper 1 immunity with residual latent tissue cysts and infection is usually asymptomatic [4]. Toxoplama gondii has been linked serologically to Diabetes mellitus (DM) [5]. There is a vicious cycle between toxoplasmosis and diabetes mellitus as Toxoplasma gondii infection increases susceptibility to acquiring diabetes and diabetes mellitus patients are more susceptible to opportunistic infections such as toxoplasma gondii [6], [7], [8].
Adipocytes derived Leptin, is a protein previously thought to control food intake only. Recently pleotropic of leptin role in many metabolic processes was established. One of the most important functions of leptin is the control of inflammatory process as activation of macrophage activation and release of cytokines [9].
Toxoplasmosis can induce an increase in leptin secretion [10] via its cell surface receptor [11]. So, single-nucleotide polymorphisms in this receptor as substitution of Adenine by Guanine at nucleotide 668 result in substitution of glutamine by arginine at codon 223 in exon 6 (Q223R) of the LEPR gene which encodes the extracellular portion of leptin receptor. This change can lead to change in signaling and function of leptin receptor and result in high serum leptin level [12].

The aim of this study is to investigate the effect of Leptin receptor polymorphism in diabetic children with latent toxoplasmosis in a cohort of Egyptian children.

2. Material & methods

Fifty patients from Mansoura University Children Hospital, with diabetes mellitus type I were diagnosed according to the criteria of the American Diabetes Association (ADA) [13]. The

patients, with normal weight, are 28 males and 22 females, with ages ranging from 3 to 15 years (mean 12.42 ± 4.64). Patients presented other congenital metabolic diseases, under nutrition, HIV infection, other parasitic disease, obese patients, any other parasitic disease except toxoplasmosis are excluded.

In addition, 50 healthy volunteers (with matched ages and gender) without symptoms of acute or chronic infection and with no family history of Diabetes were included in the study after prior consent.

Stool examination was done, to exclude parasitic diseases, by using direct smear, Formol-Ether concentration method. Acid fast stain were used for Coccidea, Gomori's trichrome stain,Weber's trichrome stain for Microsporidia and agar plate culture for excluding Strongyloides stercoralis [14]. Toxo IgM and IgG assessment were done using the Diapro Diagnostic Bioprobes Srl (Milano, Italy) ‘‘Capture” Enzyme Immuno Assay. Serum level of leptin was measured using DRG leptin Sandwich - enzyme linked immunosorbent assay kit (DRG Diagnostics, GmbH, Germany). Glycosylatedhemoglobin in blood was determined by quantitative colorimetric kit from STANBIO laboratory company procedure NO: 0350 (North main street.Taxas).Serum levels of C peptide were determined by immunosorbent assay kit from Calbiotech company catalog No: CP179s.

For leptin receptor polymorphism, high molecular weight DNA was extracted from EDTA-blood samples (3 ml) taken from every participant in the study, using GFX blood DNA purification kit (Amersham Biosciences UK Limited). Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) based genotyping was carried out using gene-specific primers. The primer to amplify the Q223R region was designed using the integrated DNA technology (IDT) tool, 5’ GGCCTGAAGTGTTAGAAGAT 3’ (forward) and 5’ CTGCTCTCTGAGGTGGGAAC 3’ (reverse). The amplified products were restricted with three specific restriction endonucleases. For Q223R, MspI site was created for its variant allele and the fragments of 173 and 469 bp, were produced and visualized using 2% agarose gel.

2.1 Ethical consideration

Approval of Institutional Review Board (IRB) was obtained at faculty of Medicine, Mansoura University. An informed verbal and written consent from children parents to participate in the

study with a full right to withdraw was obtained with assurance of confidentiality and anonymity of the data.

2.2 Statistical analysis

Data were analyzed with SPSS version 21. The normality of data was first tested with one-sample Kolmogorov-Smirnov test. Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test. Continuous variables were presented as mean ± SD (standard deviation) for normally distributed data and Median range for non-parametric data. The two groups were compared with Student *t* test (parametric data) and Mann–Whitney test (non parametric data). ANOVA test was used for comparison of means of more than two groups in parametric data and Kruskal Wallis test was used for comparison of medians of more than two groups in non parametric data. Significant variables entered into Logistic regression model using the Forward Wald statistical technique to predict the most significant determinants and to control for possible interactions and confounding effects. For all above mentioned statistical tests done, the threshold of significance is fixed at 5% level (p-value).

3. Results

Table 1 shows significant increase of HbA1c and blood glucose level, also a significant decrease of C peptide level in diabetic group compared to control group (p value < 0.001). On the other hand no significant difference was found between cases and control group regarding serum leptin level **(**p value >0.05).

In the diabetic children, 36% were homozygous for the wild-type (QQ), 54% were heterozygous (QR) and 10% were homozygous for the mutant (RR) genotype while in control (54 %) were homozygous for the wild-type (QQ), (46 %) were heterozygous (QR) and none were homozygous for the mutant (RR) genotype. The distribution of QR and RR were higher in diabetic children group compared to control group (p=0.028). Also, diabetic children was more associated with R allele (61.7%) compared to (45%) with Q allele.

Concerning the presence of toxoplasma, it was more frequent (p= 0.009) in diabetic children group (42%) then in control group (18%).

Table (1): Comparison between diabetic children and control groups regarding the studied parameters

Items	Diabetic children group (n=50)		Control group (n=50)		Test of sig.	p-value
Serum leptin (ng/ml)						
Median (Min-Max)	1.30 (0.2-43)		2.45 (1.10-5.60)		Z=0.16	0.874
C peptide (ng/ml)						
Median (Min-Max)	0.13 (0.09-10.26)		1.75 (0.1-3.0)		Z=6.67	<0.001**
HbA1c						
Mean ± SD Min-Max	8.04±2.50 4.5-12.8		4.71±0.57 4.1-6.59		t=9.13	<0.001**
Blood glucose (mg/dl)						
Median (Min-Max)	178 (131-466)		99.5 (90-200)		Z=8.18	<0.001**
Leptin receptor polymorphism						
QQ QR RR	n=18 n=27 n= 5	36.0% 54.0% 10.0%	n=27 n-23 0	54.0% 46.0% 0.0%	χ^2=7.12	0.028*
Toxoplasma						
Positive Negative	n=21 n=29	42.0% 58.0%	n=9 n=41	18.0% 82.0%	χ^2=6.85	0.009*

Table 2 shows that high C peptide level was associated with Q allele (p=0.036) while R allele was associated with high serum leptin (p=0.015) and blood glucose level (p=0.004) and with positive toxoplasma (p=0.001). Also, cases was more associated with R allele (61.7%) compared to (45%) with Q allele (p=0.031).

Table (2): Relation between leptin receptor polymorphism alleles and other parameters

Items	Q allele (n=140)		R allele (n=60)		Test of sig.	P /Pc
Age/years						
Mean ± SD Min-Max	12.57±4.18 3-18		11.18±4.92 3-18		t=2.036	0.043*/0.08
Sex						
Male Female	77 63	55.0 45.0	35 25	58.3 41.7	χ^2=0.189	0.663
s. leptin (ng/ml)						
Median (Min-Max)	2.25 (0.2-43)		3.8 (0.8-43)		Z=2.43	0.015*/0.03*
C peptide (ng/ml)						
Median (Min-Max)	1.4 (0.09-10.26)		0.24(0.09-2.3)		Z=2.093	0.036*/0.07
HbA1c						
Mean ± SD Min-Max	6.18±2.39 4.1-12.8		6.82±2.55 4.1-12.8		t=1.703	0.090
Blood glucose (mg/dl)						
Median (Min-Max)	110 (90-466)		150 (94-466)		Z=2.873	0.004*/0.016*
Toxoplasma						
Positive Negative	n=32 n=108	22.9% 77.1%	n=28 n=32	46.7% 53.3%	χ^2=11.33	0.001*/0.002*
Groups						
Diabetic children Control	n=63 n=77	45.0% 55.0%	n=37 n=23	61.7% 38.3%	χ^2=4.67	0.031*/0.06

Pc= Bonforroni corrected P value (Number of comparison x P value).

Table 3 shows that no significant difference between positive and negative toxoplasma regarding age, sex, and HbA1c. On the other hand, serum leptin, C peptide and blood glucose level were significantly higher in positive toxoplasma ($p < 0.05$).

RR distribution and R allele were significantly higher in positive toxoplasma (16.7%, 46.7%) compared to (0%, 22.9%) negative toxoplasma (p=0.002, 0.001 respectively).

Table (3): Comparison between positive and negative toxoplasma regarding the studied parameters

Items	Positive toxoplasma (n=30)		Negative toxoplasma (n=70)		Test of sig.	p-value
Age/years						
Mean ± SD Min-Max	11.88±5.08 3-18		12.32±4.05 3-16.5		t=0.482	0.631
Sex						
Male Female	14 16	64.7% 53.3%	42 28	60.0% 40.0%%	χ^2=1.51	0.218
s. leptin (ng/ml)						
Median (Min-Max)	3.8 (0.8-43)		2.40 (0.2-43)		Z=2.65	0.008*
C peptide (ng/ml)						
Median (Min-Max)	1.4 (0.1-10.26)		0.14 (0.09-3.0)		Z=2.197	0.028*
HbA1c						
Mean ± SD Min-Max	6.03±2.24 4.1-11.7		6.60±2.58 4.1-12.8		t=1.130	0.261
Blood glucose (mg/dl)						
Median (Min-Max)	150 (101-466)		110.00 (90-389)		Z=2.35	0.019*
Leptin polymorphism						
QQ QR RR	10 15 5	33.3% 50.0% 16.7%	35 35 0	50.0 50.0 0.0	χ^2=12.96	0.002*
Allele Q R	32 28	53.3% 46.7%	108 32	77.1% 22.9%	χ^2=11.33	0.001*

Regression analysis **(Table 4)** showed that high serum leptin level, high C peptide level and R allele were independently associated with positive toxoplasma (OR=1.07, 1.52 and 2.95 respectively). After multivariate regression analysis and adjusting the confounding factors, the most significant predictors for positive toxoplasma were high serum leptin (OR=1.06) and R allele (OR=3.46).

Table (4): Logistic regression analysis of independent predictors of positive toxoplasma

Independent predictors	Univariate regression			Multivariate regression	
	β	P-value	OR (95%CI)	P-value	OR (95%CI)
s. leptin (ng/ml)	0.065	<.001**	1.07 (1.03-1.11)	0.021*	1.06 (1.01-1.12)
C peptide (ng/ml)	0.424	0.049*	1.52 (0.96-2.41)	-	-
Blood glucose (mg/dl)	0.001	0.658	1.001 (0.997-1.005)	-	-
Allele Q (r) R	1.083	0.001*	2.95 (1.55-5.61)	0.001*	3.46 (1.66-7.42
Constant Model χ^2 % correctly predicted	-1.436 56.5, P =<0.001 77 %				

4. Discussion

This study shows no significant difference between diabetic and control children concerning serum leptin level, which is agreed with [15], [16], [17]. On the other hand some authors reported that leptin is a risk marker of diabetes [18], [19] while others reported an inverse relation between leptin level and diabetes [20], [21]. There is an inverse relation between leptin level and insulin secretion as proved by animal studies [22] due to an underlying leptin resistance mediated by obesity in type II DM [23].

As regard toxoplasma, it was more frequent in diabetic children group compared to in control group. Infectious aetiology as Helicobacter pylori [24], Coxsakie B4 virus [25], T. gondii [26] had been linked to diabetes mellitus (DM). Many theories to explain to this linkage as: T. gondii tachyzoites can cause direct necrosis of pancreatic inslet cells [26], residual bradyzoites in tissue cysts in pancreatic β-cells leads to autoimmunity with a resulting diabetes [6], [8], immunosuppression found in diabetes can reactivate bradyzoites in tissue cysts to active tachyzoites [27].

On the other hand, high serum leptin and Blood glucose level were associated with R allele while high C peptide was associated with Q allele. Exonic polymorphisms in the LEPR gene, namely Q223R polymorphism cause a change in charge (Glutamine [Q] to Arginine [R]) at codon 223 [28]. This polymorphism occurs in a region which encodes the extracellular domain of leptin receptor, affects the function of the receptor, and impairs the ability of leptin to bind to its receptor, lead to higher leptin level [29]. High leptin level is associated with lower insulin secretion [22] thereafter higher blood glucose. Q allele is associated with lower leptin level so it is associated with relatively higher insulin level and C peptide.

This study reveals that children with positive toxoplasma show a significant elevation in serum leptin, C peptide and blood glucose level. Also, a logistic regression analysis lead to that the independent predictors of positive toxoplasma were high serum leptin level, R allele and high C peptide level.

Leptin plays an important role in Th1 cell activation and in the products of Th1 like elevated levels of IL-2, IFN-y and TNF-α [30]. Also, increased production of leptin has been reported as a component of the acute-phase response to inflammatory stimuli; its secretion is regulated by the proinflammatory mediator TNF-α [9], [31]. In rat, Toxoplasma gondii infection can cause an increase in leptin secretion without changing body weight in a period of 4 weeks [10]. Cellular immunity is the main control mechanism in T. gondii infections and the released cytokines, in turn, increase leptin expression in adipose tissue in a vicious cycle [32], [33].

Zhu et al. reported that insulin in combination with D-glucose has an additive effect on intracellular T. gondii growth. This fact can explained the higher percentage of C peptide (denoting insulin) and blood glucose which flourish toxoplasmosis in diabetic children aggravating pathology related to this pathogen[6].

After multivariate regression analysis and adjusting the confounding factors, the most significant predictors for positive toxoplasma were high serum leptin and R allele. This can be explained by the fact that R allele is associated with higher leptin level, which mediate inflammation and secretion of type 1 cytokines and may have a role in pancreatic pathology in diabetic children as immune response to T. gondii infection. This complex varies from individual to individual due to high level of heterogenity in genetic background [34]. An exacerbated and persistent

inflammatory immune reaction mediated by IFN-γ stimuli (type 1 cytokines) would lead to a noxious cellular effect on host tissue [35].

In conclusion, Leptin receptor polymorphism influences leptin level, which modulates inflammation and immune response to T. gondii infection and may have a role in pancreatic pathology in diabetic children. Knowledge of these variants in T1DM might contribute to a better understanding of the role of inflammation in the etiology and progression of this disease.

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