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Studying the Association of Genetic Polymorphism in *FTO* Gene with Maternal Obesity and Metabolic Phenotypes in a Sample of Iraqi Pregnant Women

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Abstract

The prevalence of overweight and obesity is rising worldwide, particularly among women of reproductive age. The fat mass and obesity-associated protein (*FTO*) gene is known to be linked with obesity. The goal of this study was to investigate whether the *FTO* rs1421085 genetic polymorphism is a predictor for maternal obesity and to evaluate its association with obesity–related metabolic phenotypes in a sample of Iraqi pregnant women. Group of 62 overweight/obese and 32 healthy non-obese pregnant women were included in this study. Genotyping of *FTO* rs1421085 gene variant was determined by tetra-primer amplification refractory mutation system-polymerase chain reaction (Tetra-primer ARMS–PCR). Metabolic phenotypes included fasting glucose (FG), glycated hemoglobin (HbA1c), lipid profile, fasting insulin, leptin (LEP), leptin receptor (LEPR), LEP/LEPR ratio, and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). Statistical analysis revealed that participants with CC and CT genotypes increase the odds of being overweight/obese (odds ratio, OR: 5.232, confidence interval, CI 95% 1.467-18.659, *P*= 0.011) and (OR: 3.006, 95% CI: 1.034-8.742, *P*= 0.043) respectively. Furthermore, each copy of the risk allele (C) increased the odds of being overweight/obese (OR: 3.3051, 95% CI: 1.697-6.437, *P*= 0.0004). Analysis also revealed that *FTO* rs1421085 polymorphism was associated with higher levels of TC, LDL-C, LEP and LEP/LEPR ratio and decreased FG levels across the study population.

Keywords: Maternal obesity, *FTO*, Phenotype, Gene polymorphism, Tetra-primer ARMS–PCR, Iraq

1. Introduction

A major public health challenge in the 21st century is the epidemic of obesity across all spectrums of age groups (Desai *et al.*, 2013), particularly among women of reproductive age. Obesity is a major risk factor for the development of cardiovascular disease (CVD), a leading cause of death (Ugwuja *et al.*, 2013). As for maternal overweight and obesity, they are well-known risk factors for pregnancy complications (Leddy *et al.*, 2008), high birth weight and of an infant being large for gestational age (Tanvig *et al.*, 2013). In Iraq, the number of obese and overweight people has markedly increased. In 2015, Iraqi Ministry of Health estimated that 30.6% of Iraqi adult females were overweight, while 42.6% of adult females were obese (Iraqi Ministry of Health, Annual Statistical Report, 2016).

The susceptibility to obesity is thought to result from a combination of genes, behavior, and environment (Tanvig, 2014). Hundreds of genes were found to be as contributing to obesity (Butler, 2016). The fat mass and obesity associated (*FTO*) gene is one of these genes that is mainly expressed in the hypothalamus (Gerken *et al.*, 2007; Hsiao and Lin, 2016). It codes alpha-ketoglutarate dependent dioxygenas (*AlkB*), which is a nuclear protein of the *AlkB* related non-haem iron and 2-oxoglutarate-dependent oxygenase superfamily (Sanchez-Pulido and Andrade-Navarro, 2007). The variant rs1421085 of the *FTO* gene were first identified by Dina *et al.* (2007) as potentially functional single nucleotide polymorphism (SNP) of the *FTO* gene that was consistently strongly associated with early-onset and severe obesity for the C allele. Variants in the fat mass and obesity-associated (*FTO*) gene have been associated with obesity and obesity-related phenotypes in different populations.

Although data from different populations including Europeans found the strongest association between signal in the first intron of the *FTO* gene for SNP rs1421085 with early-onset and morbid adult obesity (Meyre *et al.* 2009), the presence of this variant in Iraqi population has not been examined.

The aim of this study was to perform a case–control association study and evaluate the association between the *FTO* rs1421085 polymorphism and the susceptibility to obesity for a sample of Iraqi pregnant women. We also undertook an analysis to provide a quantitative assessment of *FTO* rs1421085 polymorphism association with metabolic biomarkers that are known from epidemiological studies to be associated with higher body mass index (BMI) and an increased risk of type 2 diabetes and cardiovascular disease, and insulin resistance (Freathy

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et al., 2008). Alteration of hormones involved in appetite and energy homeostasis is associated with disturbances of eating behavior and obesity (Chearskul *et al.*, 2012). Insulin and leptin (LEP) are two of the most commonly identified hormones in the regulatory control of food intake, body weight and metabolism (Benyshek, 2007).

These obesity related metabolic biomarkers include fasting glucose (FG), glycated hemoglobin (HbA1c), lipid profile, two obesity related hormones (insulin and LEP), leptin receptor (LEPR), and homeostasis model assessment-insulin resistance (HOMA-IR).

2. Materials and Methods

2.1. Study population

The Scientific Committee of Chemistry Department, College of Science at Mustansiriyah University and the Ethics Committee of Al-Eluia Teaching Hospital for Birth in Baghdad, Iraq approved the current study (approval number 3355/21/MG). The objectives and methodologies were explained to all participants and verbal consent had been taken. The study included 94 pregnant women enrolled at Al-Eluia Teaching Hospital in Baghdad, Iraq for delivery between February and May 2017. Participants (from the same ethnicity) with normal pregnancy were selected, under supervision of obstetrician, based on their weight. All participants are residents of Baghdad, Iraq. Medical records were reviewed to obtain medical history and to determine eligibility pertaining to maternal age, obstetric complications during pregnancy. Gestational age (number of completed weeks of pregnancy) was estimated based on ultrasound scans. Women were excluded if the pregnancy was complicated by medical conditions such as hypertension, diabetes, infection diseases or smoking or if they were taking any medications that could affect their weight.

Two main groups included, 32 pregnant women ageing 18-35 years, with a normal BMI (below 25 kg/m^2) served as controls and 62 pregnant women ageing 18-49 years with abnormal BMI (greater than or equal to 25 kg/m^2) served as patients (Overweight/Obese) group. Both groups were matched in gestational age (37-39 weeks); however, they differed in age.

2.2. Anthropometric measurements

Weight and height, were taken by a nurse for further BMI calculation. Women were weighed after delivery with a well-calibrated digital scale. All participants were barefooted with minimal clothes. Weight was measured in kilograms with an accepted error of 0.1 kg. Height was measured in centimeters with tape measures in standing position. BMI was defined as weight in kilograms divided by the square of height in meters (World Health Organization, 2018).

2.3. Samples collection

Fasting blood samples (10 mL) were obtained from each mother just before delivery by venipuncture using disposable syringe. Whole blood (5 mL) was placed in ethylene diamine tetra-acetic acid (EDTA)-coated tubes for genomic DNA extraction and for measurement of HbA1c. Another 5 mL of blood was placed into gel tubes and left for at least 15 min at room temperature. Then, it was centrifuged at 3000 ×g for 10 min to collect sera. The serum was divided into aliquots in Eppendorf tubes and stored at -20 C° until use for metabolic measurements.

2.4. Metabolic measurements

Quantitative measurement of HbA1c was performed by fluorescence immunoassay (FIA) system with iCHROMA™ Reader. FG was determined by colorimetric enzymatic method (ECM) using kit supplied by Biosystems, Spain. Lipid profile (total cholesterol TC, triglycerides TG, high-density lipoprotein-cholesterol (HDL-C) was determined by ECM using kits supplied by Linear Chemicals S.L, Spain. Low-density lipoprotein-cholesterol LDL-C and very low-density lipoprotein-cholesterol (VLDL-C) were estimated using the Friedewald equation (Friedewald *et al.*, 1972). Quantitative measurement of human serum levels of LEP, insulin, and LEPR was performed using an ELISA kits supplied by KOMA BIOTECH INC. Korea, Monobind Inc., USA and My Biosource, USA respectively. HOMA-IR is a method used to quantify insulin resistance calculated from FG and insulin using Matthew's formula (Matthews *et al.*, 1985).

2.5. Genotyping

Genomic DNA was isolated from EDTA whole blood samples using the gSYNC™ DNA Extraction Kit provided by Geneaid Biotech Ltd, Taiwan according to the manufacturer's protocol and stored at –20 °C. DNA concentration was quantified with the NanoDrop (BioDrop, United Kingdom). Genotyping of *FTO* rs1421085 SNP was performed by using the tetra-primer amplification refractory mutation system–PCR (ARMS–PCR). This method uses four primers in a single PCR to determine the genotype; two non-allele-specific primers which amplify the region that comprises the SNP named outer primers, and two allele-specific primers (inner primers) which will produce the allele-specific fragments (Ahlawat *et al.*, 2014). The primers designed in this study for genotyping of *FTO* gene rs1421085 are shown in Table 1.

PCR reaction was performed in a total volume of 10 µL containing 1 µL genomic DNA, GoTaq® G2 Green Master Mix, 2X (Promega, USA). GoTaq®G2 DNA Polymerase is supplied in 2X Green GoTaq®G2 Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$. 10 pmol of each inner and outer primers (BIONEER, Korea) and PCR graded water (Promega, USA).

The thermocycling protocol: Initial denaturation at 95ºC for 2 min followed by 35 cycles of 30 sec at 95ºC, 30 sec at 51.6ºC, and 1 min at 72ºC followed by a final extension for 2 min at 72ºC. Products were run on a 2% agarose gel stained with ethidium bromide against 5µL of 25-2000 bp DNA ladder (BIONEER, Korea).

Table 1. The primers designed for genotyping of *FTO* gene rs1421085

Gene, SNP	Primers	Primer Sequences 5'-3'	Tm (°C)	Length (bp)	Primer Conc. (Pmol)	Ta (°C)	Expected Amplicons (bp)
FTO gene rs1421085	outer forward, OF	GGTTGTAATGAAGTTTTAGGCCTCA	58.5	25	10	51.6	450, internal standard
	outer reverse, OR	AGCCATCCATCAGGTTAAATAAATG	56.7	25	10		
	inner forward IF,T-allele	TAGCAGTTCAGGTCCTAAGGCATTAT	60.6	26	10		213, T allele
	inner reverse IR, C-allele	CAAATTCTCATCAGACACTTAATCACTG	58.1	28	10		291, C allele

Tm Melting Temperature; **Ta** Annealing Temperature**; bp** base pair

2.6. Statistical analysis

Statistical Package for Social Sciences (SPSS Inc. Chicago, IL, USA, version 19.0) was used for data analysis. The quantitative variables (measured parameters) were expressed as means ± standard deviation (SD), whereas qualitative variables (genetic polymorphisms) were expressed as absolute numbers and frequencies. Genotype and allele frequency distributions were compared among patients and control groups using the Chi-square ($\chi 2$) test or fisher exact test assuming codominant, dominant, and recessive models of inheritance. Data was also analyzed to calculate the odds ratio (OR) and 95% confidence interval (CI) to determine the risk of obesity associated with the risk allele. General linear model (GLM) was conducted to analyze the impact (partial eta-squared η_p^2) of the *FTO* gene SNP on anthropometric and metabolic traits. This test was performed while controlling for age factor. A value of ($\eta_p^2 = 0.01$) defines small, ($\eta^2 = 0.06$), defines medium, and ($\eta^2 = 0.14$) defines large effects (Lakens, 2013). As a significant age differences were detected between groups, all analyses were adjusted for age. A value of ≤0.05 was considered statistically significant. Web-ASSOTEST - a web-based version of ASSOTEST available on www.ekstroem.com (Wrzosek *et al.*, 2016) - was used to determine whether observed genotype frequencies are consistent with Hardy Weinberg equilibrium (HWE) test.

3. Results

The Tetra-ARMS PCR product of *FTO* (rs1421085) gene polymorphism was analyzed by agarose gel electrophoresis. T allele generated 213 bp product-size and C allele generated 291 bp product-size, while two outer primers generated a product size of 450 bp which represents the internal standard. Results are demonstrated in Figure 1.

For the control samples, the genotype distribution for the *FTO* rs1421085 SNP was in HWE (*P*>0.05) (Salanti *et al.*, 2005). However, for patients, the genotype distribution for *FTO* rs1421085 SNP showed significant deviations (P < 0.05) from HWE.

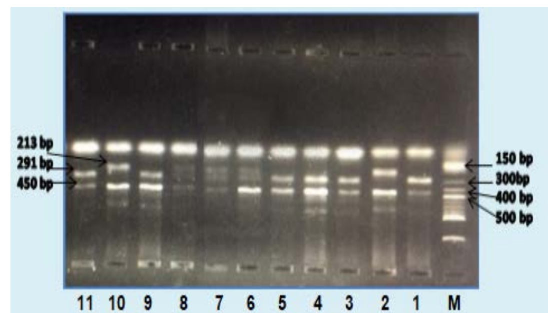

Figure 1. Genotyping of *FTO* gene polymorphism (rs1421085) by Tetra-ARMS PCR on 2% agarose gel electrophoresis. Lanes M: DNA Marker. Lanes 1,3, 4,5, 9 and 11: CC genotype. Lanes: 2 and 10: TT genotype. Lanes, 6,7and 8: CT genotype.

The OR (adjusting for age) and their 95% confidence interval (CI), and corresponding *p*-value with genotypes and alleles frequencies for *FTO* rs1421085 gene polymorphism as well in control and patients females are presented in Table 2.

Table 2: Alleles and genotypes frequencies of the *FTO* rs1421085 gene variant with odd ratio (OR) values among control and over-weight/ obese patients.

Model	Control n (%)	Patient n (%)	OR (CI 95%)	*P*- value
Genotype				
TT	20 (62.5%)	19 (30.6%)	Reference	-
CT	8 (25.0%)	21 (33.9%)	3.006 (1.034-8.742)	0.043*
CC	4 (12.5%)	22 (35.5%)	5.232 (1.467-18.659)	0.011*
Dominant				
TT	20 (62.5%)	19 (30.6%)	Reference	-
CC/CT	12 (37.5%)	43 (69.4%)	3.772 (1.485-9.584)	0.005**
Recessive				
TT/CT	28 (87.5%)	40 (64.5%)	Reference	-
CC	4 (12.5%)	22 (35.5%)	3.344 (1.010-11.076)	0.048*
C/T Allele				
T Allele	48	59	Reference	-
C Allele	16 (25%)	65(52.42%)	3.3051 (1.697-6.437)	0.0004**

*****$P < 0.05$, ******$P < 0.01$, OR: is the odds ratio after adjustment for age between the control and patients females. CI: 95% confidence interval.

The frequencies distribution of rs1421085 genotypes in patients group was 19 (30.6%) for TT, 21(33.9%) for CT, and 22 (35.5%) for CC. In control group, the distribution of genotypes was 20 (62.5%) for TT, 8 (25.0%) for CT, 4 (12.5%) for CC. Statistical analysis revealed that participants with the homozygous mutant (CC) genotype significantly increased the odds of being overweight/obese (OR: 5.232, CI 95% 1.467-18.659, *P*= 0.011), while heterozygous (CT) genotype was associated with increased risk for obesity (OR: 3.006, 95% CI: 1.034-8.742, *P*= 0.043). It was observed that the presence of risk allele (C) had significantly higher odds of being overweight/obese (OR: 3.3051, 95% CI: 1.697-6.437, *P*=0.0004) than females with the T allele only. The statistical analysis also revealed that both dominant and recessive models of rs1421085 significantly increased the odds of overweight/obese (OR: 3.772, 95% CI: 1.485-9.584, *P*= 0.005) (OR: 3.344, 95% CI: 1.010-11.076, *P*= 0.048) respectively.

The association between the *FTO* rs1421085 SNP and metabolic traits across study population was determined by a comparison study using codominant (genotype test) and dominant (increased risk in CC or CT *vs.* TT) models of inheritance of the *FTO* rs1421085 polymorphism and presented in Table 3.

Table 3. Impact of the *FTO* rs1421085 genetic polymorphism on maternal characteristics assuming different models of inheritance across the study population.

Parameter	TT	TC	CC	P^a value	Dominant Model (CT+CC vs. TT)	P^b value
BMI kg/m^2	26.77±4.81	28.94±4.94	30.49±4.58	0.018*	29.67±4.79	0.006*
HbA1C %	5.25±0.36	5.19±0.36	5.29±0.35	0.715	5.24±0.36	0.788
FG mg/dl	95.01±23.08	80.91±21.70	92.19±30.85	0.054	86.22±26.74	0.027*
TC mg/dl	146.84±34.76	163.6±44.41	172.93±44.93	0.065	167.99±44.45	0.025*
TG mg/dl	136.82±56.34	134.56±41.92	155.38±65.34	0.422	144.36±54.66	0.603
HDL-C mg/dl	52.08±14.55	51.29±13.84	49.95±13.13	0.422	50.66±13.39	0.603
VLDL-C mg/dl	27.36±11.27	26.91±8.38	31.07±13.07	0.840	28.87±10.93	0.638
LDL-C mg/dl	67.399±32.69	85.39±44.98	91.90±38.29	0.051	88.45±41.68	0.017*
LEP ng/mL	22.27±10.28	27.44±7.75	28.10±7.24	0.029*	27.74±7.45	0.008*
LEPR ng/mL	36.36±12.99	34.95±12.63	39.58±14.25	0.563	37.08±13.46	0.914
LEP/LEPR	0.62±0.318	0.87±0.38	0.828±0.431	0.022*	0.85±0.40	0.006*
Insulin µIU/mL	8.18±3.83	8.65±6.49	11.497±7.27	0.103	9.96±6.94	0.189
HOMA-IR	2.02±1.40	1.85±1.62	2.55±1.86	0.316	2.17±1.76	0.705

a. *P* value obtained by age adjusted multiple comparisons (TT/AA/AT). b: *P* value obtained by age adjusted comparing the women of the TT Vs. AT+AA genotypes. * *P* significant at the 0.05 level, ** *P* significant at the 0.01 level

Women carrying the C risk allele CT+CC of rs1421085 have a significantly ($P<0.05$) higher BMI, TC, LDL-C, LEP and LEP/LEPR ratio compared to TT genotypes carriers. FG significantly decreased in CT+CC carriers vs. TT. On the other hand, there were no associations between *FTO* rs1421085 polymorphism and HbA1C, TG, HDL-C, VLDL-C, LEPR, and HOMA-IR among the entire study group.

Further analysis was conducted to assess whether the *FTO* rs1421085 SNP is correlated with obesity-related traits across patients (overweight/obese) group. Statistical analysis revealed (data not shown) that rs1421085 SNP only significantly associated with increased TC, LDL levels, and decreased levels of FG and HOMA-IR, while no significant effect of this SNP was observed on BMI, LEP/LEPR ratio, HbA1C, LEP, insulin, LEPR, TG, VLDL-C, and HDL-C among patients group.

Assuming co-dominant model of inheritance revealed that the SNP has a significant medium impact on BMI (ηp^2=**0**.085, $p<0.05$), FG (ηp^2=0.070, $p<0.05$), LEP (ηp^2=0.085, $p<0.05$), and LEP/LEPR (ηp^2=0.091, $p<0.05$). When assuming the dominant model (CT+CC), however, it was found that the SNP has a significant medium impact on BMI (ηp^2=0.079, $p<0.05$), LDL-C (ηp^2=0.067, $p<0.05$), LEP (ηp^2=0.085, $p<0.05$), and LEP/LEPR (ηp^2=0.089, $p<0.05$), and a significant, yet small, impact on both TC and FG (ηp^2=0.059, $p<0.05$) each.

4. Discussion

In general, the HWE test assumes that the genotypes are sampled from the general population, and therefore the HWE tests are performed based on the controls (Feng *et al.*, 2014). Wittke-Thompson *et al.* (2005) suggest that if a deviation from HWE in cases or in both cases and controls is detected, it does not necessarily imply genotyping errors. Therefore, to detect any genotyping errors, we randomly selected 24% of the samples to re-genotype. The obtained results were 100% identical. Thus, we believe that the deviation from HWE in cases cannot be attributed to the genotyping errors. There are many possible factors for disequilibrium, which may cause significant deviations from HWE, either by influencing the distribution of genes in the population or by altering the gene frequencies. These factors include gene flow, small population size, and non-random mating (Turnpenny and Ellard, 2016). Disequilibrium can also arise from population substructure or inbreeding (Graffelman and Weir, 2016). All that could provide an explanation for the deviations from HWE.

In recent decades, obesity has become a global health epidemic. It is increasingly recognized to have the potential to influence the next generation through effects on women of reproductive age (Black *et al.*, 2013*;* Ng *et al.*, 2013). The *FTO* gene leads to an increased risk of obesity by influencing a central regulation of food intake (Hsiao and Lin, 2016). Some reports suggest that it affects food intake, as carriers of the risk allele tend to choose high energy and palatable food (Almen *et al.*, 2013). FTO was previously studied across Iraqi diabetic patients and reported an association of variants in exon 2 with diabetes. (Ramadhanzaidan *et al.,* 2011). In our study, we found that *FTO* rs1421085 polymorphism was associated with increased risk for maternal obesity, higher levels of TC, LDL-C, LEP and LEP/LEPR ratio and decreased FG levels across the study population. Among overweight/ obese women, however, the SNP only associated with increased TC, LDL levels, and decreased levels of FG and HOMA-IR. Interestingly, this leads to an idea that presence of this variation may have a protective role against FG elevation and eventually against developing type 2 diabetes (Janghorbani and Amini, 2012). These results were inconsistent to Attaoua *et al.*, (2009) who reported that genotypes of *FTO* were correlated with insulin resistance, and homozygous C/C was positively

correlated with an increase in insulin resistance over the value predicted by the increase in BMI.

Although, these findings partially deviate from the previous comparative studies of (Solak *et al.*, 2014; Abdel Rahman *et al.*, 2018), they reported that no significant association was found when comparing the rs1421085 genotypes in terms of BMI measurements among obese subjects.

Recent evidence suggested that *FTO* variants directly affect adipocyte function through targeting IRX3 and IRX5 (Sobalska-Kwapis *et al.*, 2017). Compared with risk-allele carriers (C), non-risk-allele carriers (T) exhibited reduction in IRX3 and IRX5 expression. These results showed that *FTO* gene mutations promote increased expression of IRX3 and IRX5 (Yang *et al.*, 2017). In a study examining the mechanistic basis of the association between the *FTO* region and obesity, Claussnitzer *et al.* (2015) found that the rs1421085 T-C alteration disrupts a conserved motif for the regulatory gene ARID5B repressor. This can cause depression of a potent preadipocyte enhancer that ultimately alters the function of adipocytes, which shift from energy-dissipating beige adipocytes to energy-storing white adipocytes with a 5-fold reduction in mitochondrial thermogenesis. This process results in imbalance of body energy, lipid accumulation and subsequent obesity without a change in physical activity or appetite (Yang *et al.*, 2017). Claussnitzer *et al.* (2015) concluded that the *FTO* SNP rs1421085 represents the causal variant that disrupts a pathway for adipocyte thermogenesis involving ARID5B, IRX3, and IRX5, providing a mechanistic basis for the genetic association between *FTO* and obesity.

Other potentially modifiable factors that cause overweight and obesity are lifestyle factors such as physical inactivity and eating habits (Mogre *et al.,* 2014). These factors could not be controlled and might cause bias.

It is noteworthy that the current study is the first study that examines the associations between rs1421085 polymorphism and obesity represented by BMI and related metabolic phenotypes represented by elevation in TC, LDL, and LEP/LEPR in a sample of Iraqi female population. Finally, it is important to point out to the limitations of the study like generalizability as the samples were collected from one hospital only; therefore, further investigations in other regions of Iraq are necessary to generalize these results among Iraqi female population.

5. Conclusion

No data on the relation between *FTO* rs1421085 polymorphism and obesity in Iraqi population is available. Thus, our findings provide the first evidence about the association of the rs1421085 SNP, found in first intron of *FTO*, with maternal obesity elevated metabolic phenotypes including TC, LDL-C, LEP and LEP/LEPR ratio and decreased levels of FG in a sample of Iraqi female population. The findings from our study have an important health implication. It may be speculated that Iraqi females of reproductive age may be at risk of CVD, considering that obesity and overweight are accompanied by unfavorable blood lipids patterns, Thus, weight lessening is necessary and desirable to reduce comorbidities that might later need a very intensive medical treatment, leading to rising health care costs. As *FTO* rs1421085 SNP is associated with decreased levels of FG among study population and decreased levels of FG and HOMA-IR among overweight/obese group, presence of this variation may, therefore, play a protective role against diabetes.

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