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Original Article

Screening of Exons 4-9 Polymorphisms of *FTO* Gene in Endometrial and Ovarian Cancers

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Abstract

Endometrial and ovarian tumours are almost mechanistically affected by reproductive hormones. Ovarian cancer may be explained as metastatic or synchronous primary ovarian cancer, and the specific diagnosis is a challenge. This study aimed to investigate the mutations in fat mass and obesity-associated (*FTO)* genes and investigated the association of these mutations with the risk of endometrial and ovarian cancers as well as with cancer grade and stage. Blood samples were collected from 48 endometrial and ovarian cancer cases and 48 healthy women. Genomic DNA was extracted, and PCR was done to amplify *FTO* exons 4-9. Sanger sequencing identified 6 different novel mutations submitted to DDBJ: p.W278G and p.G284G in exon 4, p.S318I and p.A324G in exon 5 and two mutations in intron 4. Other mutations were also detected in *FTO* gene sequencing results, rs112997407 in intron 3, rs62033438, rs62033439, rs8048254 and rs8046502 in intron 4. The novel p.W278G, p.S318I and p.A324G mutations were predicted to be damaging. We did not find a significant association for all variables with cancer risk or clinical stage and grade except for rs62033438 variants, which showed a significant association with cancer grade, especially AA genotype (OR= 15, 95% CI:1.32 -169.88, P= 0.03). In conclusion, the statistical analysis did not clarify whether *FTO* mutations are implicated in cancer. Further studies with more samples are recommended to provide a more accurate picture of the correlation between *FTO* mutations and endometrial and ovarian cancer susceptibility.

Keywords: Screening, FTO, Endometrial cancer, Ovarian cancer

1. Introduction

Endometrial and ovarian tumours are almost mechanistically affected by reproductive hormones. Ovarian cancer may be explained as metastatic or synchronous primary ovarian cancer, and the specific diagnosis is a challenge (1). The study of genetic predisposition has become one of the priorities of scientific research interested in knowing the causes of cancer spread in the world in general and in Iraq in particular (2).

Fat mass and obesity-associated (*FTO*) gene is the first gene that has been proven to be associated with obesity (3). FTO protein is an AlkB-like 2-oxoglutarate—dependent nucleic acid demethylase, 3-methyl thymidine and 3-methyl uracil in nuclear acids, which are its specified substrates (4). It favours single-stranded nuclear acids, hence methylated RNA instead of DNA. N6-methyladenosine in mRNA is the molecular target of *FTO* (5).

FTO was described as the first N 6 -methyladenosine (m6A) demethylase of eukaryotic messenger RNA (mRNA), and the roles of *FTO* in adipogenesis and tumorigenesis have been associated with its m6A demethylase activity (6). The functions of m6A

modification as a gene expression post-transcriptional modulator are controlling mRNA stability or enhancing mRNA translation efficacy through its discrimination of different m6A reader proteins (7). The primary role of *FTO* in cancer evolvement and drug resistance targeting *FTO* prevents therapeutic potential in treating *FTO* overexpressed cancers. *FTO* inhibitors have been experienced in vitro and in vivo and show powerful antitumor effects in treating glioblastoma and breast cancer (8) (Figure 1).

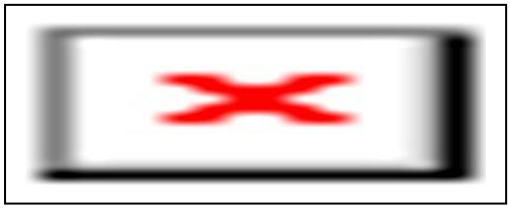

Figure 1. *FTO* as an m 6 A demethylase and its role in RNA m 6 A modification, *FTO* participates in overweight/obesity and cancers due to its post-transcriptionally expression regulation of its critical target genes. Different inhibitors suppress the therapeutic potential to treat *FTO*-overexpressing cancers. MA, meclofenamic acid; 2HG, 2-hydroxyglutarate; C12, Compound 12 (9).

The location of *the FTO* gene is on chromosome 16q12.2, with nine exons (Figure 2). The expression of *FTO* is abundant in the hypothalamus, which regulates food intake, signifying its potential role in satiety (10). Studies have also indicated that *FTO* is associated with cancer independent of obesity (11).

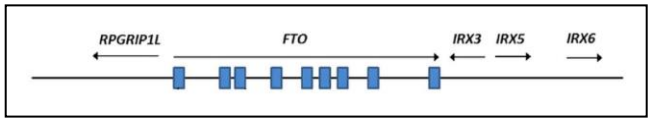

Figure 2. *FTO* gene and its adjacent genes, the blue rectangles represent exons. This image has been modified from Reitz, Tosto (15)

Earlier studies have confirmed that *FTO* can stimulate several intracellular signalling pathways essential in carcinogenesis comprising signal transducer and activator of transcription 3 (STAT3), phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), cyclin D1 and matrix metalloproteinases (MMPs) (12).

Both *FTO* mRNA and protein expression can be stimulated through PI3K/Akt and MAPK pathways by Estrogen in endometrial cancer; in estrogen-dependent endometrial cancer, *FTO* was a target protein of Estrogen; this is what was shown in Zhang, Zhou (13).

A study shows that in ovarian tumours and cancer stem cells, *FTO* expression is inhibited, which in its role inhibits the self-renewal of ovarian cancer stem cells and suppressed tumorigenesis in vivo, in which the *FTO* demethylase activity is essential (14).

This study aimed to investigate the mutations in fat mass and obesity-associated (*FTO)* genes and investigated the association of these mutations with the risk of endometrial and ovarian cancers as well as with cancer grade and stage.

2. Materials and Methods

Forty-eight endometrial and ovarian cancer cases were recruited from the Oncology and Hematological Disease Center in Al- Sader Teaching Hospital – Al-Basrah Province South of Iraq. At the same time, 48 healthy women matched to cases by age (37 to 77 years) were randomly selected. Genomic DNA Mini Kit (Geneaid, Taiwan) has been used for genomic DNA extraction from peripheral blood leukocytes.

2.1. PCR and Sequencing

Screening of *FTO* gene exons 4-9 was performed using typical PCR cycle conditions using 6 sets of primers to amplify all exons and exon-intron boundary regions of *FTO* (16) (Table 1). PCR mixture was achieved by adding 1 µl from each DNA template (≈ 40 ng), forward and reverse primers (10 pmol) to 12.5 µl from GoTaq Green master mix (Promega, USA), then the reaction was adjusted to 25 µl with deionized distilled water. The following program was used for amplification: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds; 58°C for 30 seconds; and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. The amplification efficiency was checked by agarose gel 2% with ethidium bromide and visualized under ultraviolet transillumination.

Table 1. Primers were used for amplification and sequencing, and expected PCR fragments

Target	Forward primer (5`-3`)	Reverse primer (5`-3`)	Fragment size (bp)
Exon 4	ACACCTGGCCTATTTAAGG	CAACAAGAGTGAAGCTCC	560
Exon 5	AGCATCCCCTTTCACTCTTC	ACTTGTCAACGGCATTTTCC	751
Exon 6	TGAATTCACAGCCAGGGAC	CACAATTCTTGAAAGTCTTGCC	571
Exon 7	TGCCAGCTTACACTGGGAAC	TCCTGGCTATACCCATCACC	426
Exon 8	GGCCATCATTTACTGCATTG	ATTAATGTAGGTGCCGTGGG	441
Exon 9	CCTCCCGTGGATTAATTTCC	CAACGTTGGAGGAGAAAAGC	250

Sanger sequencing was performed for all PCR products with the same forward PCR primers (Macrogen Co., Korea)

2.2. Software

Sanger sequencing results were manipulated with CodonCode Aligner (V 7.1.2) and then aligned with other published sequences in NCBI using the Basic Local Alignment Tool (BLAST). Novel alleles were submitted to the DNA Database of Japan (DDBJ). Fisher exact software was used to determine the *P*-value to evaluate the differences in variation frequencies between both groups; the non-significant *P*-value was > 0.05. Prediction of the

possibility impact of amino acid substitutions on the function and stability of human proteins was performed by PolyPhen-2 software (17).

3. Results

In our study, *FTO*gene sequencing allowed the identification of 6 different novel mutations submitted to DDBJ, which showed in table 2: p.W278G and p.G284G in exon 4, p.S318I and p.A324G in exon 5 and two mutations in intron 4.

Table 2. Novel submitted variables to DDBJ

Codon change	Position	Accession number
-	Intron 4	LC616379
AGT>ATT	Exon 5	LC616380
GCA>GGA	Exon 5	LC616381
TGG>GGG	Exon 4	LC616382
GGT>GGG	Exon 4	LC616383
-	Intron 4	LC616384

All coding mutations are located at the catalytic domain of *the FTO* gene (Figure 3). No polymorphisms were identified in exons 6-9. Thirty-five and 41 sequence results were succeeded from 48 samples for both patients and control, respectively. We observed noticeably that the mutations in coding regions except p.S318I which was identified in five healthy subjects and one patient were the least and rarest; on the other hand, the majority of mutations, as well as the most frequent, were located in introns, especially intron 4.

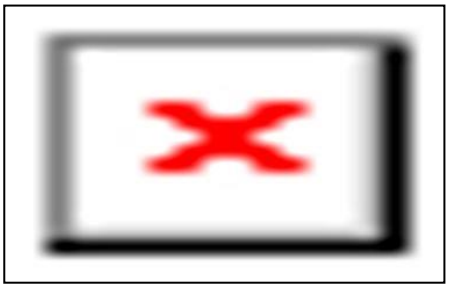

Figure 3. schematic diagram elucidates missense mutation positions at the catalytic domain. Non-coding mutations are not represented; this image has been modified from a previous study (16)

PolyPhen-2 software analysis was used for interaction expectations with substitution information and integration of protein structure; a high score of 1.0 resulted in the novel p.W278G substitution predicting damaging effect in exon 4. While the predicted protein damaging effect, score was 0.99 for p.S318I and p.A324G mutations. The resulting variations sequence chromatograms are shown in figure 4, which demonstrate its positions and genotypes.

The genotype and allele frequencies of *FTO* gene polymorphism in cancer patients and control are summarized in table 3. The statistical analysis of detected genotypic frequencies did not display a significant association for all variables.

We observed that rs112997407 SNP in intron 3 frequency was higher in the control group (*P*=0.17, 95% CI: 0.02-2.12), the similar thing for p.W278G mutation which was recorded in 2 control individuals (*P*=0.19, 95% CI: 0.01- 4.01). The other novel exon 4 mutation (p.G284G) was detected in cancer women with 4 clinical stages and pathological grade (Table 4). The frequencies of the CA genotype were 10.87% and 12.77% for control and cancer patients, so there was no significant difference. Regarding rs62033438 and rs62033439 variants, we also noticed that the genotypic and allelic frequencies did not differ statistically between the study groups. The odd ratio of GG genotype for rs62033438 was 2.31 with *P* 0.13 (95% CI: 0.72- 7.38); also, 53.19% of patients were carrying AG genotype against 43.48% among the control group (OR=2.22, 95% CI: 0.81- 6.08, *P*=0.09). No significant relationship between rs62033439 polymorphisms and cancer risk (OR=2.31, 95% CI: 0.72- 7.38, *P*=0.16) for the GG genotype, while the odd ratio for the GC genotype was 1.83 (*P*=0.17, 95 CI: 0.69-4.89), G allele was present in 53.19% from cancer patients comparing with 42.39 % from the control group (OR=1.32, 95% CI: 0.73- 2.40, *P*=0.09). We used other primer pairs for amplifying and sequencing exon 5 and its boundaries, so only 35 and 41 samples from cancer patients and the control group resulted. Of cancer patients, 8.57% carried rs8048254 in intron 4 (OR=3.75, 95% CI: 0.37-37.80, *P*=0.25). We did not observe a significant association between rs8046502 and LC616379 with cancer risk (*P*=0.30, 95% CI: 0.64- 4.10) and (*P*=0.15, 95% CI:0.09-1.59), respectively. We recorded a missense mutation in exon 5 (p.S318I), but there was no association with cancer risk depending on statistical analysis (*P*=0.14, 95 CI: 0.02- 1.91). In two patients, other missense mutations were also detected in exon 5 (p.A324G), but the difference in frequency was not significant (OR=6.19, *P*=0.20, 95% CI: 0.29- 133.48).

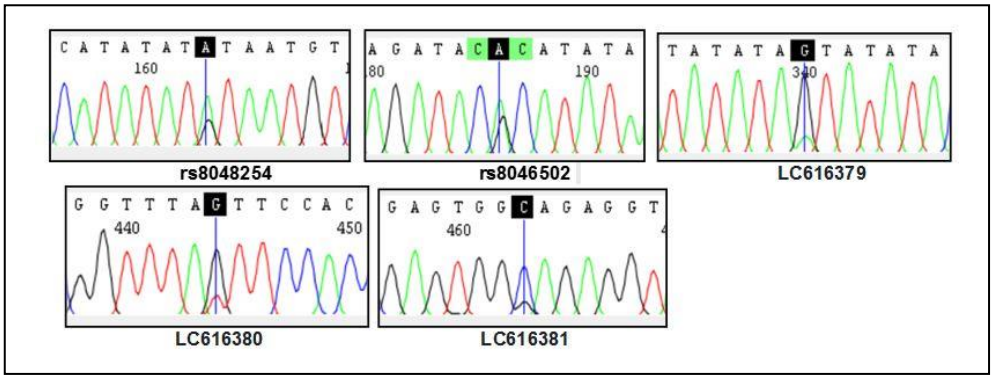

Figure 4. sequencing chromatogram showing mutations locations, detected genotypes and accession number

Table 3. *FTO* gene variants distribution and association with endometrial and ovarian cancer patients

Variation position	Location	SNP ID / Accession No.	Control (48)	Patients (48)	[a]OR	(95% CI)	[b]*P*-value
c.781-99	Intron 3	rs112997407 AA AG	42 (87%) 4 (13%)	46 (97.83) 1 (2.17%)	1.00 0.23	Ref. 0.02- 2.12	0.17
c.861 p.W278G	Exon 4	LC616382 TT TG	44 (95.65%) 2 (4.35%)	47 (100%) 0	1.00 0.19	Ref. 0.01- 4.01	0.24
c.881 p.G284G	Exon 4	LC616383 TT TG	46 (100) 0	46 (97.83) 1 (2.17%)	1.00 3	Ref. 0.12- 75.56	0.5

Variation position	Location	SNP ID / Accession No.	Control (48)	Patients (48)	[a]OR	(95% CI)	[b]*P*-value
		LC616384					
c.144+17	Intron 4	CC	41 (89.13%)	41 (87.23%)	1.00	Ref.	
		CA	5 (10.87%)	6 (12.77%)	1.20	0.34- 4.24	0.62
		rs62033438					
		AA	16 (34,78%)	9 (19.15%)	1.00	Ref.	
c.144+37	Intron 4	GG	10 (21.74%)	13 (27.66%)	2.31	0.72- 7.38	0.13
		AG	20 (43.48%)	25 (53.19%)	2.22	0.81- 6.08	0.09
		A allele	52 (56.52%)	43 (45.74%)	1.00	Ref.	
		G allele	40 (43.48%)	51 (54.26%)	1.54	0.86- 2.75	0.09`
		rs62033439					
		CC	16 (34.78%)	10 (21.28%)	1.00	(Ref.)	
c.144+117	Intron 4	GG	9(17.39%)	13 (27.66%)	2.31	(0.72- 7.38)	0.16
		GC	21 (45.65%)	24 (51.06%)	1.83	(0.69- 4.89)	0.17
		C allele	53 (57.61%)	44 (46.81%)	1.00	(Ref.)	
		G allele	39 (42.39%)	50 (53.19%)	1.32	(0.73- 2.40)	0.09
			n= 41	n= 35			
		rs8048254					
c.925-225	Intron 4	AA	40 (97.56%)	32 (91.42%)	1.00	Ref.	0.25
		AG	1 (2.44%)	3 (8.57%)	3.75	0.37- 37.80	
		rs8046502					
c.925-200	Intron 4	GG	27 (65.85%)	19 (54.29%)	1.00	Ref.	0.30
		AG	14 (34.15%)	16 (45.71%)	1.62	0.64- 4.10	
		LC616379					
c.925-54	Intron 4	AA	33 (80.49%)	32 (91.43%)	1.00	Ref.	0.15
		AG	8 (19.51%)	3 (8.57%)	0.39	0.09- 1.59	
c.953 p.S318I	Exon 5	LC616380					
		GG	36 (87.8%)	34 (97.1%)	1.00	Ref.	
		GT	5 (12.2%)	1 (2.9%)	0.21	0.02- 1.91	0.14
c.971 p.A324G	Exon 5	LC616381					
		CC	41 (100%)	33 (94.29%)	1.00	Ref.	0.20
		CG	٠	2 (5.71%)	6.19	0.29- 133.48	

a: Odd ratio; *b*: *P*-value calculated depending on fisher exact test; CI: confidant interval.

Table 4. Pathological grade and clinical stages of less frequent mutations

Variation	Patient	Stage	Grade
rs112997407	#1	1	1
LC616383	#1	4	4
rs8048254	#1	3	2
	#2	3	2
	#3	1	1
LC616379	#1	1	1
	#2	2	3
	#3	1	1
LC616380	#1	3	2
LC616381	#1	1	1
	#2	2	4

3.1. Association of *FTO* Gene Polymorphisms with Pathological Grade and Clinical Stage

This section analyzed the relationship between the most frequent *FTO* variants and pathological grades of endometrial and ovarian cancer. Patients have been divided into grades I/II and III/IV (Table 5). The rs62033438 variants were significantly associated with cancer grade, especially the AA genotype (OR=15, 95% CI: 1.32 -169.88, *P*=0.03), 64.29% of cancer patients were carrying GA genotype (OR=6.80, 95%CI: 0.75 - 60.76, *P*=0.09). Furthermore, no significant association was noticed between rs62033439 genotypes and grade (CG vs CC (OR=2,95% OR: 0.40-9.52, *P*=0.40), GG vs CC (OR=0.42, 95% CI: 0.06 - 3.21, *P*=0.40). The rs8046502 variant was found in 63.64% of cancer patients compared with 37.50% from the control group, but this difference was insignificant (OR=2.92, 95% CI: 0.66-12.82, *P*=0.16) (Table 5).

We examined the association of *FTO* variables with the endometrial and ovarian cancer stages (Table 6). All *FTO* polymorphisms do not exhibit a significant association with the clinical stage of endometrial and ovarian cancer.

Table 5. Association of *FTO* variants with pathological grades

Variants	Grade I/II	Grade III/IV	OR	95% CI	*P*-value
LC616384					
CC	26 (81.25%)	15 (100%)	1.0	Ref.	
CA	6 (18.75%)	0	0.13	(0.007 - 2.5)	0.18
rs62033438					
GG	12 (37.50%)	1 (6.67%)	1.0	Ref.	
GA	16 (50%)	9 (64.29%)	6.80	(0.75 - 60.76)	0.09
AA	4 (12.5%)	5 (35.71%)	15	(1.32 -169.88)	0.03
rs62033439					
CC	7 (22.58%)	3 (18.75%)	1.0	Ref.	
CG	13 (41.94%)	11 (68.75)	2	(0.40- 9.52)	0.40
GG	11 (35.48%)	2 (12.50%)	0.42	(0.06 - 3.21)	0.40
rs8046502					
GG	15 (62.50%)	4 (36.36%)	1.0	Ref.	
GA	9 (37.50%)	7 (63.64%)	2.92	(0.66 - 12.82)	0.16

Table 6. Association of *FTO* gene variants with clinical stages

Variants	Stage I/II	Stage III/IV	OR	95% CI	*P*-value
LC616384					
CC	23 (85.19%)	18 (0.90%)	1.0	Ref.	
CA	4 (14.81%)	2 (10%)	0.63	(0.11- 3.89)	0.63
rs62033438					
GG	7 (25.93%)	6 (30%)	1.0	Ref.	
GA	17 (62.96%)	8 (40%)	0.55	(0.14- 2.18)	0.85
AA	3 (11.11%)	6 (30%)	**2.33**	(0.40- 13.60)	0.35
rs62033439					
CC	4 (15.38%)	6 (28.57%)	1.0	Ref.	
CG	15 (57.69%)	9 (42.86%)	0.40	(0.08 1.81)	0.23
GG	7 (26.92%)	6 (28.57%)	0.57	(0.11- 3.04)	0.51
rs8046502					
GG	8 (44.44%)	11 (64.71%)	1.0	Ref.	
GA	10 (55.56%)	6 (35.29%)	0.44	(0.11-1.70)	0.23

4. Discussion

Numerous worldwide publications have examined the association of *FTO* SNPs with the risk of various types of cancer (18-21); thus, investigating whether *FTO* SNPs are associated with cancer risk became essential and exciting. It has been found that *FTO* modifies different RNAs, which are also related to different biological processes such as cell cycle, survival, proliferation, stem cell maintenance, tumour growth, migration, invasion and self-renewal (13, 22). FTO functioned as a positive modulator of cervical cancer cell migration and proliferation by its effect on the translation efficacy of Myc and E2F1 (23). The association between FTO and Estrogen in endometrial cancer has been described (13); it was also evidenced that FTO nuclear localization promoted by Estrogen and the last advanced signalling pathway of mammalian target of rapamycin (mTOR) in endometrial carcinoma, hence stimulating cancer cells proliferative activity (24). By downregulation of the *FTO* gene, it was observed that ovarian tumour cell regeneration was inhibited as well as suppression of tumorigenesis in vivo through cAMP signalling blocking (22).

This study investigated the correlation between *FTO* polymorphisms and potential gynaecological cancer susceptibility and cancer stage and grade, so we screened exons 4-9 of *the FTO* gene and exon-intron boundaries. No polymorphisms were detected in *FTO* exons 6-9. Most of the detected mutations were in intron 4; two were novel (LC616384 and LC616379) beside previously recorded variations (rs62033438, rs62033439, rs8048254 and rs8046502). We did not find a significant association between *FTO* polymorphisms and endometrial and ovarian cancer risk. Coding mutations were also detected in our study; three changed the amino acid (p.W278G in exon 4, p.S318I and p.A324G in exon 5). These newly detected missense mutations were not having a statistically significant association with cancer risk. The positions of coding variants were at the catalytic domain of *FTO* protein; a predicting damaging effect was yielded when we used PolyPhen-2 software analysis for interaction expectations with substitution information and integration of protein structure. It has been concluded that dysregulation of *FTO* makes it considered a tumour-suppressive or has an oncogenic role in human cancers (25). We also examined the association of *FTO* polymorphisms with cancer stage and grade; however, only the AA genotype of rs62033438 variation in intron 4 showed a significant correlation with pathological grade of gynaecological cancer (P=0.03). Among less frequent mutations, LC616383 in exon 4 was found in women with stage and grade 4 endometrial cancer (Table 4). Studies also concluded that *FTO* correlates with cancer therapeutic response; they found enhancement of cervical squamous cell carcinoma chemo-radiotherapy resistance by *FTO* (26). FTO SNPs regulate adjacent genes (RPGRIP1L, RBL2, IRX3, IRX5), which involve cancer incidence differently (27). In the future, we need to study more samples and focus on the most variable regions of the *FTO* gene so there will be no statistical bias in the results. It is also important to examine the effect of these mutations on gene expression, its association with cancer stage and grade, and the patient's response to treatment.

We investigated in this study the association between *FTO* exons 4-9 mutations and the risk of endometrial and ovarian cancer. No significant association was detected except rs62033438 variation in intron 4, which showed a significant correlation with the pathological grade of gynaecological cancer (P=0.03). No variations were detected in exons 6-9. More studies are needed to elucidate the association of other *FTO* exons with cancer risk.

Authors' Contribution

A. N. A. collects the samples from the oncology and Hematological Disease Center, M. K. A. and R. A. A. out the majority of experimental work, especially DNA extraction, Electrophoresis, PCR amplification and

other techniques. A. I. A. and M. K. A. did most sequence data analysis and resulting writing.

All authors have read and agreed to the published version of the manuscript.

Ethics

Due to the samples being collected from human patients, the committee of Ethical Standards of the University of Basrah and the Ministry of Health depended on all samples. All participants signed a written informed approval form using a designed questionnaire.

Conflict of Interest

The authors declare that they have no conflict of interest.

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