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Evaluation mucin 1 polymorphism and expression with infertility in Iraqi females

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Abstract

Mucin-1 is a protein coding gene which located on chromosome 1 q22 part. The current study focuses on MUC 1 polymorphism and expression with infertility in Iraqi females. This study included 72 females divided into: forty infertile women and thirty-two fertile women. The present study found three SNPs in Mucin-1 gene and has been determined in Iraqi females. The rs145224844 showed three genotypes CC, CA, AA. No statically significant differences among the three genotypes between fertile and infertile were found. The odds ratio referred that C allele acting as protective allele while A allele could have the ability to associate with the disease. While rs139620330 showed three genotypes TT, TC, CC with no significant differences between fertile and infertile. The odds ratio referred that the C allele could have the susceptibility to association with the disease, but T allele decreases this susceptibility acting as protective agent. Finally, rs144273480 showed three genotypes CC, CA, AA with a high significant difference between fertile and infertile. The odds ratio referred that the A allele could have the susceptibility to association with the disease, but C allele decreases this susceptibility acting as protecting agent. Gene expression for mucin-1 increased within infertile women, while it decreased in fertile women.

Keywords: Iraqi females; infertility; polymorphism; mucin 1

Introduction

Mucins is a large glycoprotein, and composed of various amino acids skeleton: alanine, proline, glycine and threonine, serine (PTS), which allow a N and O glycosylation (Schnaar, 2016; Authimoolam and Dziubla, 2016; Chaudhury *et al*, 2015). MUC1 is a Protein coding gene, generates a 122102 DA protein made up of 1255 amino acids with liner of DNA, this gene located on chromosome 1 q22 part (Liu and Zeng, 2020). MUC1 covers the surface of all epithelial cells (Nath and Mukherjee, 2014; Bose and Mukherjee, 2020; Gao *et al*, 2020) .It protects the cells from severe environmental conditions by forming a protective barrier across the mucosal surface. It possesses intracellular signaling roles in cancer cells and is important in cancer development (Joshi *et al*, 2015; van Putten and Strijbis, 2017; Ballester *et al*, 2021: Dhar and McAuley, 2019). MUC1 is a membrane bound mucin and This large transmembrane O-glycoprotein (approximately 200 kDa) with a rigid structure that extends up to 200–500 nm

from the cell surface of primarily epithelial and hematopoietic cells (Hattrup and Gendler, 2008). Because of its extracellular domain, MUC1 acts as a barrier to protect cells in healthy tissues (Kashyap and Kullaa, 2020). MUC1 can help regulate cell shedding and adhesion during metastasis; protect the apical cell membrane of epithelial cells from rupture, harmful environments, and immune attack; provide resistance to stimuli; inhibit immune responses through receptor shielding; and act as a decoy receptor for invading pathogens (van Putten and Strijbis, 2017). It also helps with lubrication, hydration of the cell surface, and protection against degradative enzymes (Fig2-7A), (Kasprzak and Adamek, 2019**;** Marczynski *et al*, 2021; Kosmerl *et al*, 2021). MUC1 expression and its influence on disease processes, MUC1-CT has played an anti-inflammatory function in numerous airway infections. Infertility is a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse (Akalewold *et al*, 2022), and prevalence rate increased by 0.37% per year for females it is considered global reproductive health problem for infertility had increased from 1990 to 2017 (Sun *et al*, 2019). There is significant genetic diversity in features related to prevalent diseases and reproductive age, both of which influence female fertility (Coignet *et al*, 2017; Laisk-Podar *et al*, 2015; Perry *et al*, 2015). Mucin 1 (MUC1) is expressed on the apical surface of uterine epithelia and acts as a barrier against microbial infection and enzymatic attack. In most species, MUC1 loss at implantation sites appears to be essential for embryo attachment and implantation. The expression of MUC1 is regulated by progesterone (P) and proinflammatory cytokines, including interferon and TNF (Dharmaraj[2] *et al*, 2010).

Keywords: Mucin 1, Infertility, Gene expression, polymorphism.

Materials and Methods

Current study involved 72 females, Fourty females were infertile with age range from (18 – 45 years) and thirty-two were fertile with age range from (15- 48 years) recruited from Arab Iraqi females. The samples collected from Al-Yarmouk Hospital /Infertility Department/in Baghdad/Iraq, Ministry of health, Baghdad Health Department /Al – Kerkh. The period of collecting samples was from November /2021 to march/2022). Blood samples (5 ml) were collected from each female for both groups. The blood sample were collected into an EDTA tube to RNA and DNA extraction.

DNA extraction and genotyping

The DNA was extracted from whole blood of study groups subjects by using protocol in *EasyPure*® Blood Genomic DNA Kit (Catalog No.EE121). The genotyping of the MUC1 was carried out using polymerase chain reaction (PCR) and sequencing. The PCR cycles for 875bp segment, the reaction began at 94°C for 5 minutes, then 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 3 minutes and a final extension at 72°C for 5 minutes. The primers was used in DNA sequencing and gene expression showed in (Table 1) for 875bp segment. The primers was investigated by IDT (Integrated DNA Technologies Company, USA). Agarose gel 1.5% was used for observing PCR products.

Table 1: *Specific primer sequences for DNA sequencing and real time PCR*

Primer	Sequence (5′→3′ direction)	Templet length	reference
MUC1 Human qPCR expression Primer			
Forward	CCAGCACCGACTACTACCAA	875 bp	Shen *et al.*, 2015
Reverse	CCAGCTGCCCGTAGTTCTTT		
GAPDH Human qPCR expression Primer			
Forward	GAAATCCCATCACCATCTT	165bp	Saeed *et al*, 2021
Reverse	GAGCCCCAGCCTTCTCCATG		

RNA extraction and cDNA synthesis

RNA was extracted from blood samples with Triazole, using the Trans Zol Up Plus RNA Kit. (TransGen, biotech .ER501-01). Purity is measured by nanodrop (Thermo Fisher Scientific, USA). Total RNA was reversely transcribed to complementary DNA (cDNA) using EasyScript® One-Step gDNA Removal and cDNA Synthesis Super Mix Kit. The procedure were carried out in a reaction volume of (20 µl) according to the manufacturer's instructions. The total RNA volume was (4 µl) to be reversely transcribed, oligo Dt18 as primer and RNA extraction was performed to cDNA synthesis by thermal cycler and each random primer, incubated for 10 minutes for 25°C and for Anchored oligo (dT)18 primer and GSP, incubated at 42°C for 15 minutes (for qPCR) Incubate at 85°C for 5 seconds to inactivate enzymes.

DNA sequencing

The purified PCR products 875 bp of the analyzed MUCIN 1 gene was sent to Macrogen Company in Korea for DNA sequencing. Furthermore, the nucleotide sequences was compared to the information in gene bank of the National Center for Biotechnology Information (NCBI) web site databases using the BLAST search tool.

Quantitative Real Time PCR (qRT–PCR)

The expression levels of *MUC 1* gene were estimated by the reverse transcription-quantitative polymerase chain reaction (qRT-PCR) method, which is a sensitive technique for the quantification of steady-state mRNA levels. The way to confirm the expression of target gene, quantitative real time qRT-PCR SYBR Green test was used.

The MUC 1 and GAPDH genes expression levels and folds change were quantified by measuring the cycle threshold (Ct) employing the 2xqPCR Master Mix Kits components. Every reaction was done in a duplicate. The required volume of each component was calculated according to Table 2.

Table 2: *Components of quantitative real-time PCR used in MUC1 and GAPDH genes expression experiment.*

Components	20 µl rxn
qPCR green master mix	10.0
Nuclease free water	5
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
cDNA	3

Statistical Analysis

WINPEPI computer program (version 11.63) was used to estimate the statistical significance of the P values calculated with Fisher's exact test as well as the Odds Ratio that was assessed by a special $\chi 2$ formula Abramson, 2011 (Rodriguez *et al*, 2009). Hardy-Weinberg equilibrium was tested by chi squared test that was done using OEGE – Online Encyclopedia for Genetic Epidemiology studies (Barrett *et al*, 2005).

Results and Discussion

Genotyping Result

Figure 1 show the bands indicated the genomic DNA by the gel electrophoresis, while figure 2 show gel electrophoresis of amplified DNA products 875bp primer of MUC 1 gene.

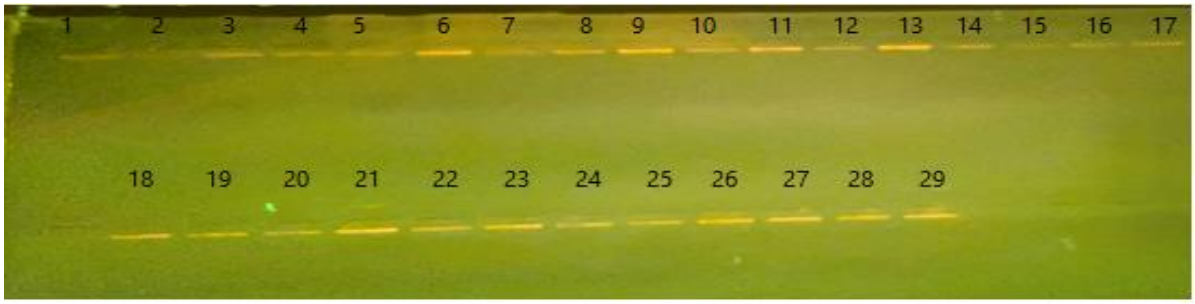

Figure 1: Gel electrophoresis of genomic DNA extraction from blood, 1 % agarose gel at 70 voltages for 45 mints

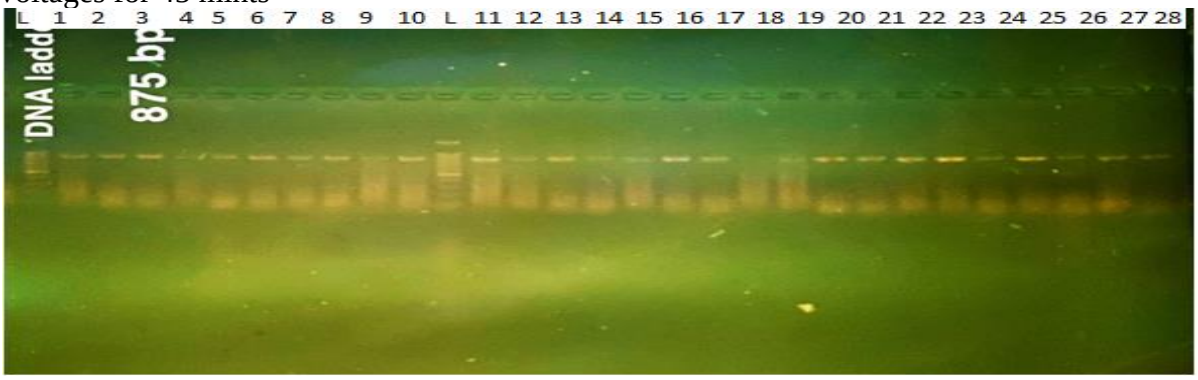

Figure 2: Amplification product of MUC 1 after electrophoresis on 1.5 % agarose at 70 volts for 1:30 hours

Lane L= DNA ladder marker, Lane (1-10) =infertile group, Lane (11-28) = fertile group

Table 3 showed there were no significant differences found in the genotype distribution and allele frequency between the fertile and the infertile groups of the SNP rs145224844. But the odds ratio for the C allele was 1.00 while the odds ratio for the A allele was 6.3 Which indicating that the A allele could have the ability to associate with the disease, but C allele decreases this susceptibility, So allele C acting as protective allele. This means females with allele A from SNPs rs145224844 may tend to be infertile.

Table 3: *Comparison of the Genotype and Allele Frequencies detected by Hardy-Weinberg equilibrium law of MUC gene polymorphism rs145224844 between fertile group and infertile groups*

***MUC1* Polymorphism rs145224844**	**Frequencies (%)**		**P value**	**Odd ratio (95% CI)**
	fertile group (n=)	**infertile Graves Group (n=)**		
Codominant				
CC	) 4 % (n=28.93	70.0 % (n=21)	---	1.00 (Reference)
CA	3.3 % (n=1)	10.0 % (n=3)	0.2	4.0 (0.3-41.2)
AA	).3 % (n=1 3	20.0 % (n=6)	0.06	8.0 (0.8-71.5)
Allele				
C	95.0 % (n=57)	75.0 % (n=45)	---	1.00 (Reference)
A	5.0 % (n=3)	25.0 % (n=15)	0.005	6.3 (1.7-23.2)

Significant differences P ≤ 0.05*and non-significant P> 0.05.

The observed genotype frequencies in both fertile and infertile was higher than those predicted by the Hardy-Weinberg Equilibrium theory Table 4. Both infertile and fertile groups exposed significance levels of $\chi 2$ value; they were 12.6408 and 16.1333 respectively. These values did not agree with H.W.E with significant defenses. The departure from H.W.E. show that this locus may undergo to evolutionary selection in Iraqi population.

The population of study (fertile and infertile groups) revealed that CC is the common genotype, while the CA and AA genotype declined in frequency.

Table 4: *Expected Frequencies of Mucin 1 rs145224844 Genotypes Using Hardy-Weinberg Equilibrium for the expected frequencies of genotypes.*

Groups		CC	CA	AA	χ2
fertile genotype	observed	28	1	1	12.6408*
	Expected	27.075	2.85	0.075	
infertile genotype	observed	21	3	6	16.1333*
	Expected	16.875	11.25	1.875	
Total observed		49	4	7	

Significant differences (χ2 >3.84) between observed and expected frequencies for both fertile and infertile group

Distribution of the muc1 rs139620330 genotype and allele frequency are shown in Table 5. There were no significant differences in the genotype distribution and allele frequency between the fertile and the infertile groups. However, the odds ratio for the T allele were 1.00 while the odds ratio for the C allele were 5.3 indicating that the C allele could have the susceptibility to association with the disease, but T allele decreases this susceptibility acting as protective allele. This means females with allele A from SNP rs139620330 may tend to be infertile.

The observed genotype frequencies in both fertile and infertile was higher than those predicted by the Hardy-Weinberg Equilibrium theory Table 6. Both fertile and infertile exposed significance levels of χ2 value; was 0.0086 in control and 18.3372 in patient respectively. The fertile agree with H.W.E. did not departure from H.W.E. while the infertile departure from H.W.E. So, this locus may undergo to effect of genetic factors in Iraqi population.

The population of study (fertile and infertile groups) revealed that TT is the common genotype in study Iraqi population, while the TC and CC genotypes declined in frequency.

Table 5: *Comparison of the Genotype and Allele Frequencies detected by Hardy-Weinberg equilibrium law of MUC gene polymorphism rs139620330 between fertile group and infertile group*

***MUC1* polymorphism rs139620330**	**Frequencies (%)**		**P value**	**Odd ratio (95% CI)**
	fertile group (n=)	**infertile Graves Group (n=)**		
Codominant				
TT	96.7 % (n=29)	90.0 % (n=27)	---	1.00 (Reference)
TC	).3 % (n=1 3	3.3 % (n=1)	0.9	1.0 (0.06-18.0)
CC	).0 % (n=0 0	6.7 % (n=2)	0.2	5.3 (0.02-11.6)
	Alleles distribution			
T	98.3 % (n=59)	91.7 % (n=55)	---	1.00 (Reference)
C	1.7 % (n=1)	8.3 % (n=5)	0.1	5.3 (0.06-4.7)

Significant differences P ≤ 0.05*and non-significant P> 0.05.

Table 6: *Expected Frequencies of Mucin 1 rs139620330 Genotypes Using Hardy-Weinberg Equilibrium for the expected frequencies of genotypes.*

Groups		TT	TC	CC	χ2
fertile genotype	observed	29	1	0	0.0086 NS
	Expected	29.0083	0.9833	0.0083	
infertile genotype	observed	27	1	2	18.3372*
	Expected	25.2083	4.5833	0.2083	
Total observed		56	2	2	

Significant differences (χ2 >3.84) between observed and expected frequencies for both

Fertile and infertile group

The genotype rs144273480 distribution and allele frequency between the infertile and the fertile group are shown in table 7. There were a highly significant differences in the genotype distribution and allele frequency between the infertile and the fertile groups. Furthermore, the odds ratio for the C allele were 1.00 while the odds ratio for the A allele were 8.1 indicating that the A allele could have the susceptibility to association with the disease, but C allele decreases this susceptibility. This means females with allele A from SNP rs144273480 may tend to be infertile**.**

The observed genotype frequencies in both fertile and infertile was higher than those predicted by the Hardy-Weinberg Equilibrium theory Table 8. Fertile healthy group showed significant departure from H.W.E. while infertile group showed non significant from H.W.E. Levels of $\chi 2$ value; was 6.4668 in fertile and 0.068 in infertile respectively. The departure from H.W.E. may show that this locus may undergo to evolutionary selection in Iraqi population.

The population of study (fertile and infertile groups) revealed that CC is the common genotype comparing to low frequency of the CA and AA genotypes.

Table 7: *Comparison of the Genotype and Allele Frequencies detected by Hardy-Weinberg equilibrium law of MUC gene polymorphism rs144273480 between fertile group and infertile group*

***MUC1* Polymorphism rs144273480**	**Frequencies (%)**		**P value**	**Odd ratio (95% CI)**
	fertile group (n=)	**infertile Graves Group (n=)**		
Codominant				
CC	90.0 % (n=27)	50 .0 % (n=15)	---	1.00 (Reference)
CA	3.3 % (n=2)	40.0 % (n=12)	0.004	10.8 (0.2-5.4)
AA	).3 % (n=1 3	10.0 % (n=3)	0.1	5.4 (0.05-5.6)
Allele distribution				
C	95.0 % (n=57)	70.0 % (n=42)	---	1.00 (Reference)
A	5.0 % (n=3)	30.0 % (n=18)	0.001	8.1 (0.2-2.9)

Significant differences P ≤ 0.05*and non-significant P> 0.05.

Table 8: *Expected Frequencies of Mucin 1 rs144273480 Genotypes Using Hardy-Weinberg Equilibrium for the expected frequencies of genotypes.*

Groups		**CC**	**CA**	**AA**	**$\chi 2$**
fertile genotype	observed	27	2	1	6.4668*
	Expected	26.1333	3.7333	0.1333	
infertile genotype	observed	15	12	3	0.068
	Expected	14.7	12.6	12	
Total observed		56	2	2	

Significant differences ($\chi 2$ >3.84) between observed and expected frequencies for both fertile and infertile group

Mucin gene showed significant differences in different SNPs or locus so this gene may relate with infertile. This study goes with previous study submitted by Saeed who emphasized that polymorphism in different locus in gene mucin 1 could be significantly related with infertile (Saeed *et al*, 2021)**.** Moreover, polymorphism in present study agree with result of previous study about Korean women which recorded polymorphism in these locus (Kim *et al*, 2020).

Furthermore, another report about mucin-1 SNP polymorphism 568 recorded that the MUC1 A/G polymorphism at its 568 sight disrupts the physiological functions of MUC1 which is important to the physiological protection of gastric mucosa. Thus present study provided

evidence that may identify the MUC1 A/G polymorphism at 568 sight, as a potential genetic factor which leads to an increase in susceptibility for infertility through alteration of MUC1 expression in the population that carry the A allele (Xu *et al*, 2009)**.**

Moreover, polymorphism rs144273480 and rs145224844 in current study agree with result of previous study about MUC 1 there were a significant increase of MUC1 rs4072037 CC genotype and C allele frequencies was observed in Anti Synthetase Syndrome-Interstitial Lung Disease (ASSD) patients compared to healthy control. Likewise, MUC1 rs4072037 TC and CC genotypes and C allele frequencies were significantly different between ASSD-ILD+ and IPF patients. Additionally, serum KL-6 levels were significantly higher in ASSD patients comparing with healthy controls. Nevertheless, there were no significant variations in serum KL-6 levels were found between ASSD-ILD+ and IPF patients. Our findings suggest that the presence of MUC1 rs4072037 C allele increases the risk of ASSD and it could be a useful genetic biomarker for the differential diagnosis between ASSD-ILD+ and IPF patients (López-Mejías *et al*, 2021).

In addition, the current study disagree with local Iraqi study published in 2020, which found a significant difference in the heterozygous (AG) and homozygous mutant type (AA) genotype frequencies in (rs6165) SNPs of the Follicle Stimulating Hormone Receptor gene (FSHR) SNP in infertile Iraqi women, with primary amenorrhea occurring more frequently in fertile women than in infertile women, and the ESR1 gene variation are associated with infertility (Nijeeb *et al,* 2020).

Moreover, polymorphism in present study disagree with result of previous study about Northern Iranian of gastric cancer which recorded that The G allele at rs4072037 of MUC1 gene was linked with a significant decreased gastric cancer risk. A significant decreased risk of gastric cancer was observed in people with AG genotypes of *MUC1* polymorphism. Genetic models show there was no significant link between the PSCA 5057C > T polymorphism and the risk of gastric cancer (Alikhani *et al*, 2020) .

The current study disagrees with a previous Spanish study which found no link between infertility and polymorphism variation in 60-bp VNTR of the MUC1 gene (Goulart *et al*, 2004).

The present study agree with a previous Iraqi study submitted by Hassab with her team who recorded higher significant of LIF gene polymorphism with failure of implantation for Iraqi infertility women during IVF (Hassab *et al*, 2021)

Gene expression for MUC 1

As shown in Table 9 the mean Ct of MUC1 gene for the infertile, fertile respectively (21.072, 23.384*), while the mean of Ct for GAPDH gene expression for the fertile was 23.702, and for the infertile was 23.772, as show in table 9.

Ct of Some research samples as show in Figure 3, while Figure 4 showed the melting curve. Present study GAPDH gene has been used as a standard gene for comparison in MUC 1(figure5). The present study found that MUC1 expression significantly high in infertile females comparing to fertile females, as shown in table 9. Current study the fold change of MUC1 is 5.220* which is more than 1, Therefore it is positive associated with infertility.

A fold change is a measure that describes how much a quantity varies from one measurement to the next. It is defined as the ratio between the two quantities; if the fold change is equal to or less than 1, it is negative; if the fold change is greater than 1, it is positive (Warden *et al*, 2013).

MUC1 expression in the endometrium was revealed to be negatively associated with implantation in studies conducted in Brazil and Spain (Dentillo *et al*, 2007; Altmäe *et al*, 2009).

The present study agree with previous study in Baghdad city submitted by Saeed with her team who recorded that the MUC1 fold change was positive changes in infertile women, and This Comparison may refer to a concluded that increase folding of muc1 may related with infertility (Saeed *et al*, 2021).

Furthermore, Present result about muc1 goes with a previous paper, which submitted by Dharmaraj with his team who reported a comprehensive expression profile of MUC1, MUC4 in normal endometrium during the menstrual cycle and in eutopic and ectopic endometrium of women with and without endometric, besides these mucins do not vary significantly during the menstrual cycle. There was little difference in MUC1 expression between eutopic endometrial and ectopic endometriotic tissues. (Dharmaraj[1] *et al*, 2014).

MUC1 expression in the endometrium is linked to embryo implantation failure. This result is consistent with recent study results published in the United Kingdom in 2021, which show that MUC1 helps to endometrial cell surface binding (Francis *et al*, 2021), as well as the same conclusion published in Iranian population study(Mojarrad *et al* , 2013).

This result disagree with previous study about infertility submitted by Gipson with his colleagues who recorded that gene folding of MUC 1 was significant comparing between fertile and infertile (Gipson *et al*, 2008).

The current study agree with previous study submitted by Hassab with his colleagues that recorded fold change refer to a highly increasing in LIF gene expression level among failure implantation subgroup of Iraqi Arab infertility females during IVF (Hassab *et al*, 2021).

Table 9: *Fold of MUC1 gene expression Depending on 2-ΔΔCt Method*

groups	Means Ct of MUC1 875	Means Ct of GAPDH	ΔCt (Means Ct of MUC1 875 - Means Ct of GAPDH)	Mean ΔCt Calibrator (MUC1 875 - ct GAPDH	ΔΔCt	2-ΔΔCt	experimental group/ Control group	Fold of gene expression
infertile group	21.072	23.772	-2.7	-1.24	-1.46	2.751083636	2.751/0.527	5.220*
fertile group	23.384*	23.702	-0.318	-1.24	0.922	0.527776859	0.527/0.527	1.000

Significant different p≤0.05, no significant p≥0.05, NS= no significant

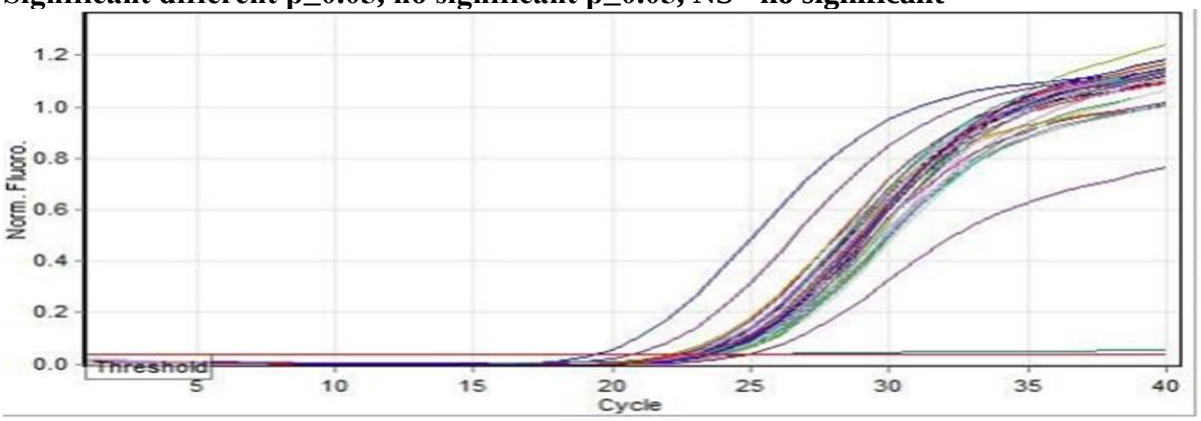

Figure 3: MUC1 dissociation curves by qPCR Samples included study group. The photograph was taken directly from Qiagen Rotor gene qPCR machine

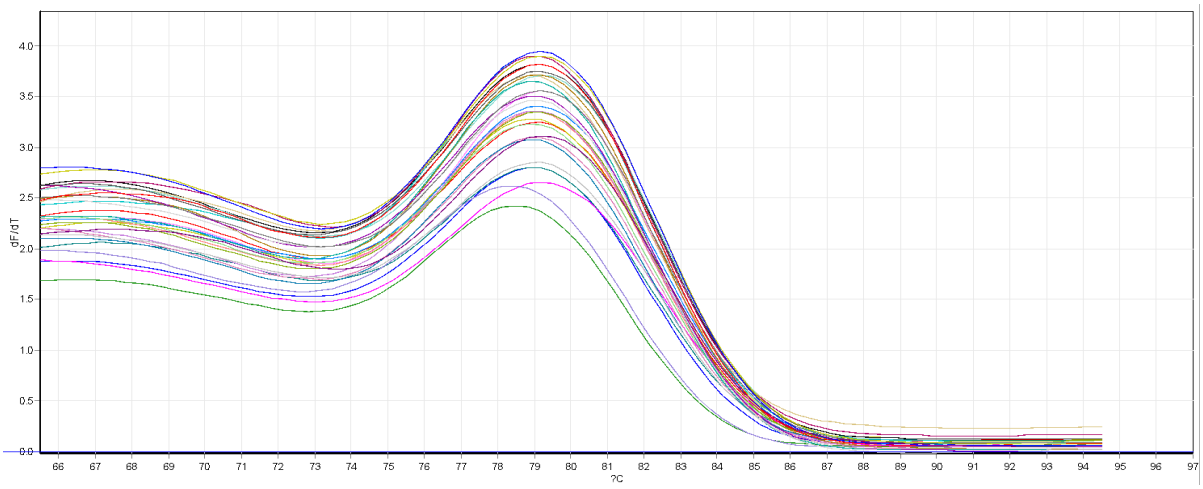

Figure 4: -MUC1 amplification plots by qPCR. Samples included study groups. The photograph was taken directly from Qiagen Rotor gene qPCR machine

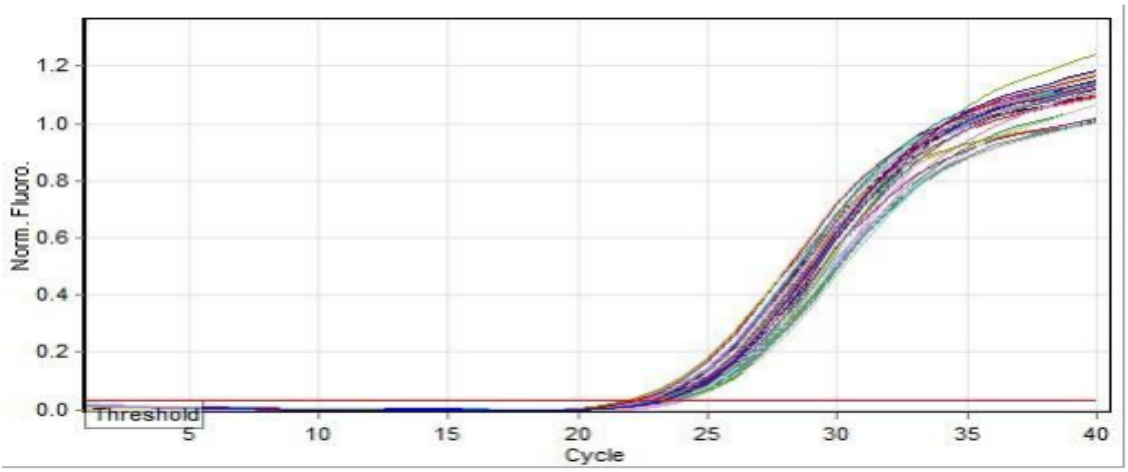

Figure 5: GAPDH amplification plots by qPCR Samples included all study groups The photograph was taken directly from Qiagen Rotor gene qPCR machine.

Conclusion

The CC genotype of rs 145224844 is the common genotype in population of study, The allele A of rs145224844 is etiological factor associated with the disease while allele C is the protective allele. the TT genotype of rs 139620330 is the common genotype in population of study and the allele C is etiological factor associated with the disease while allele T is the protective allele. The CC is the common genotype in population of study at rs 144273480 polymorphism and the allele A is etiological factor associated with the disease while allele C is the protective allele.

Mucin-1 gene expression increased within infertile women, while it decreased in fertile women.

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