A Study of Gene Expression of Inhibin and Activin Subunits (INHA,INHBA) in the Placenta of Women with Missed Miscarriage

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Abstract

Missed Miscarriage (MM) was a special type of spontaneous Miscarriage that the embryo or fetus has already died but remained in the uterus for days or weeks and with a closed cervical ostium . Patients might present with or without subtle clinical symptoms such as vaginal bleeding or abdominal pain. Missed Miscarriage, occuring in approximately 8–20% of clinically confirmed intrauterine pregnancies, was often confirmed using ultrasonography.

In this study, We used bioinformatics analysis to identify hub genes. Verified the expression of the genes inhibin subunit alpha(INHA) and inhibin subunit beta A(INHBA) in women placenta with MM .Placental tissues were obtained from therapeutic abortion (8-16 weeks, first trimester) and it was kepped in shield solution at -20 c. Genomic RNA from tissues samples were extracted by using Direct-zol™ RNA MiniPrep Kit(USA),then INHA and INHBA genes was amplified through via Reverse Transcription-Pol merase Chain Reaction(RT-PCR).We used The Livak method, also known as the $2^{-\Delta\Delta Ct}$ method, is a widely used approach for analyzing data from quantitative real-time PCR (qPCR) experiments to calculate changes in gene expression levels. This method, simplifies the process of quantifying gene expression by normalizing the number of target gene copies to an endogenous reference (usually a housekeeping gene(ACTB)) and then relative to a control .

The results showed INHBA folding showed a significant decrease in MM with mean 1.07 ± 0.87, p = 0.000025 From the women undergoes with normal pregnancy 3.55 ± 2.03 and for the INHA the mean of women experienced miscarriage 1.22 ± 0.85, p = 0.012835 compared to the mean of normal status 1.8 ± 1.58, which is slightly low.

The significant p-values for both INHA and INHBA folding suggest that the structural properties of these domains are altered in the context of miscarriage. This alteration could reflect changes in protein function or regulation that are critical during early pregnancy.

Keywords: INHA ,INHBA genes, ACTB , Missed Miscarriage, RT-qPCR

Introduction

Missed abortion (MA) is a special type of spontaneous abortion manifested as that intrauterine embryo stops growing and dies, but embryo and trophoblastic tissue are not spontaneously evacuated from uterine

cavity in early pregnancy [1,2,3].Patients might present with or without subtle clinical symptoms such as vaginal bleeding or abdominal pain. Missed abortion, occurring in approximately 8–20% of clinically confirmed intrauterine pregnancies [4], was often confirmed using ultrasonography. In the process of MM, placental tissue closely adheres to the uterine wall, causing difficulty in curettage [5]. It makes women confronting with the surgery and facing the risk of severe bleeding and complications associated with anesthesia. Previous studies have shown that there are multiple etiologic factors involved in the occurrence of MM, including genetic and uterine abnormalities, endocrine and immunological dysfunctions, infections, nutritional and environmental factors, psychogenetic factors, and endometriosis [6, 7, 8]. However, the pathogenesis of the disease is currently unclear.

MM was undoubtedly a huge physical and psychological setback for women with fertility requirements. Therefore, early identification of women at high risk of MM was pivotal, which might aid in providing possible theoretical basis for implementing clinical measures to prevent MM[9,10].

The placenta is the essential organ of mammalian pregnancy and errors in its development and function are associated with a wide range of human pathologies of pregnancy[11].

Inhibin and activin are glycoprotein molecules belonging to the transforming growth factor family and both appear to participate in the establishment of pregnancy[12]. Inhibin is a dimeric disulfide-linked glycoprotein molecule consisting of an α and a βsubunit [13]. All inhibins share a common α subunit (18–20 kD) and are classified as inhibin A or B depending on the type of β subunit (βA, 13 kD; βB, 15 kD). Activin is

also a disulfide-linked molecule but consists of two β subunits. Depending on the type of β subunit, activin is classified as activin A (βA βA dimer), activin AB (βA βB dimer), or activin B (βB β B dimer) [14,15].

Inhibin A is encoded by two different genes: the inhibin alpha (INHA) gene and the inhibin beta A (INHBA) gene. The INHA gene is located on Chr 2q35 and consists of two exons with a total length of 1,083 bp linear mRNA , while the INHBA gene is located on chr7p14.1 with a total length of 1,275 bp linear mRNA and consists of five exons [16] . Its codes for the beta A subunit. When these two subunits combine, they form inhibin A, which is a hormone involved in various reproductive processes[15]. The INHA and INHBA genes are part of the transforming growth factor-beta (TGF-beta) superfamily and play a crucial role in the regulation of follicle-stimulating hormone (FSH) secretion and other aspects of reproductive physiology[17,18,19].

Activin A is encoded by the inhibin beta A (INHBA) gene. This gene codes for the beta A subunit of activin, which, when dimerized, forms activin A. Activin A is part of the transforming growth factor-beta (TGF-beta) superfamily and is involved in various biological processes, including cell growth, differentiation, and reproductive physiology[,1217].

MATERIALS AND METHODS

Design of Study: MM and Normal pregnancy women with an age range from 18 to 40 years were involved in this study after strict application of the exclusion criteria. Data were collected through direct interviews with the patient . Written consent was obtained from each patient participating in this study to fulfill the international research ethical criteria.

This study was conducted at Bint AL-Huda Hospital in Thi-Qar, especially, in the Women's Emergency , Wahj Al DNA Laboratory in Karada / Baghdad and Biochemistry Laboratory in the College of Science (University of Thi-Qar). at the period between 6/11/2023 to 16/8/2024.

The controls and patients were divided into two groups:

1. Control group: included fifty (50) supposed healthy subjects aged (18-40 years)were obtained dataset from the GEO database(NCBI).
2. MM Patients: included fifty (50) with MM aged(18- 40 years).

Tissue samples from MM group were taken . Pregnant women with chronic disease such as (hypertension , DM , HD , AD) , anomaly of reproductive system , virus infection (TORCH) test and pregnant women under any drugs.were excluded , Moreover, the patients who had genetic defects or hereditary diseases associated with spontaneous abortion in their family history were excluded .The tissues were frozen immediately in shield solution and stored at −40°C .

RNA extraction [20]

Total RNA is effectively isolated from a placenta tissue sample by useing TRI Reagent(also sold as TRIzol) solution and tissues can be homogenized in 300 µl of TRI Reagent® and mix thoroughly for 5 minutes. To remove particulate debris, centrifuge should be performed at 10,000-16,000 x g for 30 seconds and transfer the supernatant into an RNase-free tubeو then the RNA were purification by the Direct-zol™ RNA MiniPrep (the Direct-zol™ RNA MiniPrep provides a streamlined method for the purification of up to 50 µg (per prep) of high-quality RNA directly from samples in TRI Reagent) .

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a laboratory technique combining reverse transcription of RNA into DNA (in this context called complementary DNA or cDNA) and amplification of specific DNA targets using polymerase chain reaction (PCR) .A pair of 18-24 bp oligonucleotide primers that are complementary to the unique sequences on each DNA strand flanking the target region are required for PCR.[21]

Conversion of the RNA to cDNA [22]

For the conversion of the RNA to cDNA, specify the target MiRNA and lengthen the MiRNA, the PrimeScriptTM RT Kit (#RR037A) was used .PrimeScriptTM RT reagent Kit is designed to perform the reverse transcription optimized for real-time RT-PCR. It uses PrimeScriptTM RTase, which features excellent extendibility and makes fast, efficient cDNA template synthesis for Real Time PCR possible. The step experimental procedure is simple and suitable for high throughput analysis. This kit can be used in combination with Real Time PCR reagent, SYBR® Premix.

The Specific Primers of IHNA Gene

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- ACTACTGCCACGGGAACTGT- 3'	58.2	55	65
Reverse	5'- GGAGTAGCCACCATCAGAGG - 3’	59.3	50	

The Specific Primers of IHNBA Gene

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'-GGTATGTGGAGATAGAGGATGAC-3'	58.2	55	105

Reverse	5'- TCCTGGCTGTCCTGAC- 3’	59.3	50	

The Specific Primers of ACTB Gene

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- AGTTGCGTTACACCCTTTCTTGAC - 3'	60	55	171
Reverse	5'- GCTCGCTCCAACCGACTGC - 3’	60	50	

Any existing qPCR assay performed efficiently using standard cycling conditions may be converted to a fast qPCR assay with KAPA SYBR FAST qPCR Kits. Typically, minimal re-optimization of reaction parameters is required. After completion of the reaction mixture preparation, the tubes placed in the thermal cycler instrument and programmed as in the table below;

Table (1) The Steps of RT-PCR

Step	Temp. (°C)	Time	Cycle	Scaning
Enzyme activation	95 °C	05:00 min	Hold	
Denaturation	95.0 °C	00:20 sec	40	
Annealing	60.0 °C	00:20 sec		
Extension	72.0 °C	00:20 sec		

STATISTICAL ANALYSIS OF REAL-TIME PCR DATA

All statistical analysis was done using The Livak method, also known as the $2^{-\Delta\Delta Ct}$ method, is a widely used approach for analyzing data from

quantitative real-time PCR (qPCR) experiments to calculate changes in gene expression levels to INHA and INHBA genes .

Also the statistical analysis were used with the statistical package for the social sciences SPSS for windows (version 17.0, SPSS Inc, Chicago, III) . The results were expressed as mean ± standard deviation (mean ± SD) with LSD (least significant difference). Analysis of variance (ANOVA) was used to compare parameters in different studied groups. P-values ($P \leq 0.05$) were considered statistically significant.

RESULTS AND DISCUSSION

The causes of miscarriage remain a serious concern in reproductive health. It's important to understand the biological mechanisms that cause miscarriage, which is also important for improving clinical outcomes and providing evidence for affected women. This study aimed to investigate the placental gene expression in women who have experienced miscarriage, providing intuitions into the molecular pathways that may leads to such a manner, and may represents a cause of the disease .In this study, the disease group represents women who have experienced MM, while the control group indicate of women with a normal pregnancy during the first four months(7 to 16 week).

The major aim of this structural bioinformatics study is to examine expression and fold ability changes between disease and control groups in these regions. These domains are involved in the function and regulation of proteins, particularly for viral strategies and immune evasion. [23,24]

The following figures represents comparative analysis of the INHA and INHBA genes folding data made from disease group and control group.

As both INHA and INHBA genes had regular statistically significant changes between control and disease groups, its looks like to be regulated in response to the disease.

Gene expression was successfully determined using Real time PCR (qRT-PCR) technology with the use of a specific primer, and the accuracy of the magnification of the gene product was noted by the Cycler Threshold (**CT**) values, and the appearance of gene expression was noted in combination with the Cybergreen dye and the rise of the curve above the threshold line with the increase in the device's glow .

The gene expression of INHA and INHBA genes was calculated using the ($\mathbf{2^{-\Delta\Delta Ct}}$) method based on the normalization correction process using the conservative gene ACTB[25], and the Cycler Threshold (**CT**) values of the target genes were analyzed using the **CT** values of the conservative or standard gene ACTB housekeeping in all experimental treatments and the control group to complete the correction process in gene expression.[26]

The gene expression levels of the gene showed clear differences in the levels of gene expression in the MM and the control group, as the gene expression results for the two genes INHA and INHBA showed a clear decrease (down regulation) in missed miscarriage samples compared to the result of gene expression For the control group, according to the Livak method, and the accuracy of amplification of the gene product was observed at the cycle threshold value (**Ct**) for the two targeted genes. The results showed that there is an inverse relationship between the **Ct** values and gene expression .

The higher its value, the lower the gene expression. (It is inversely related to the amount of starting template, which means that a high **Ct** value indicates lower levels of gene expression or amplification gene)[27,28,29].as in the figure(1),(2),(3),(4)

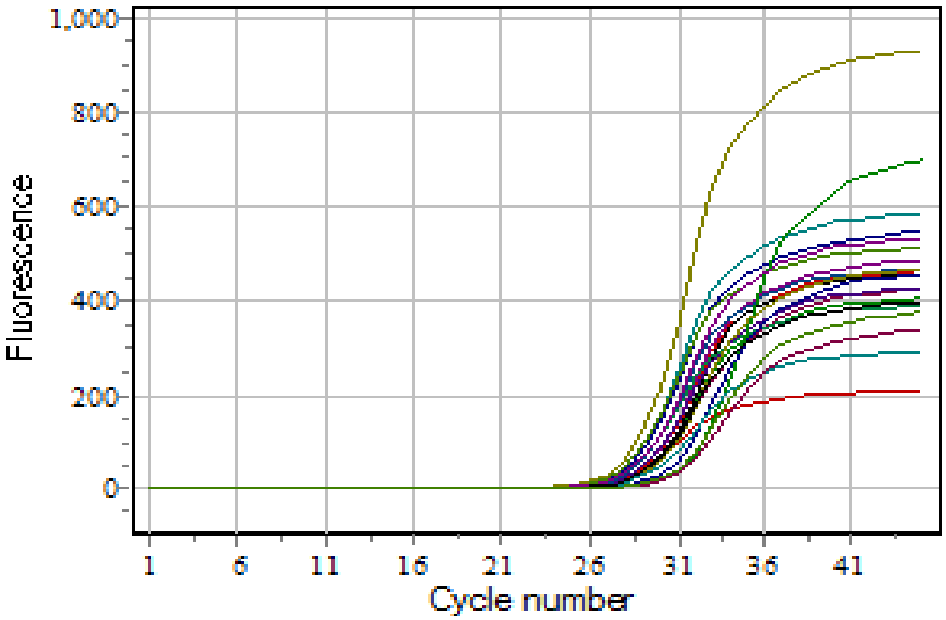

Figure (1) INHA gene curve in aborted women

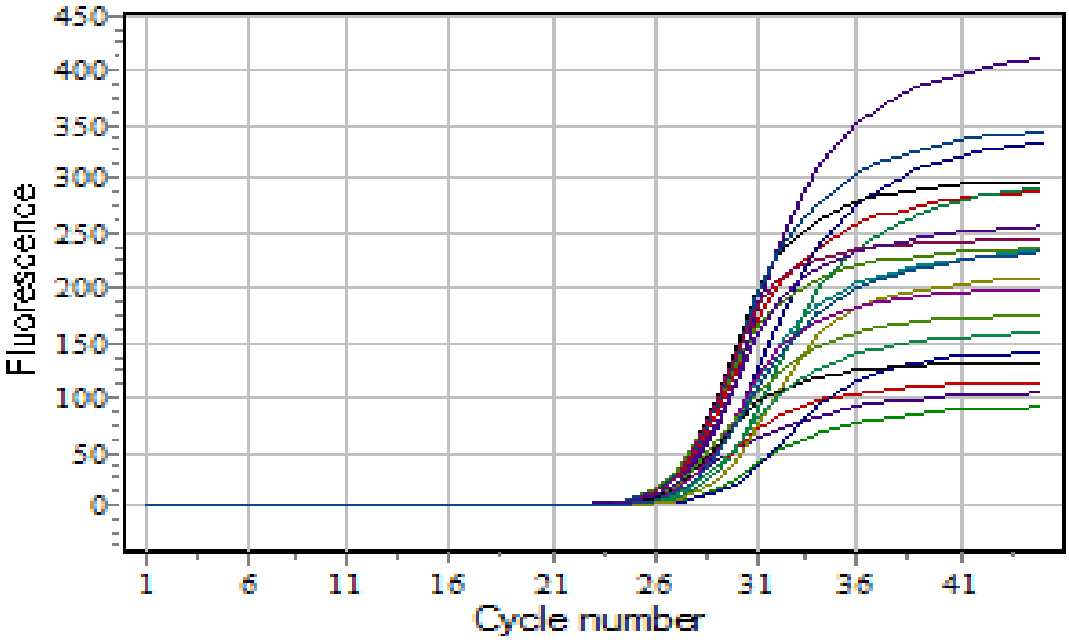

Figure (2) INHA gene curve in the control group

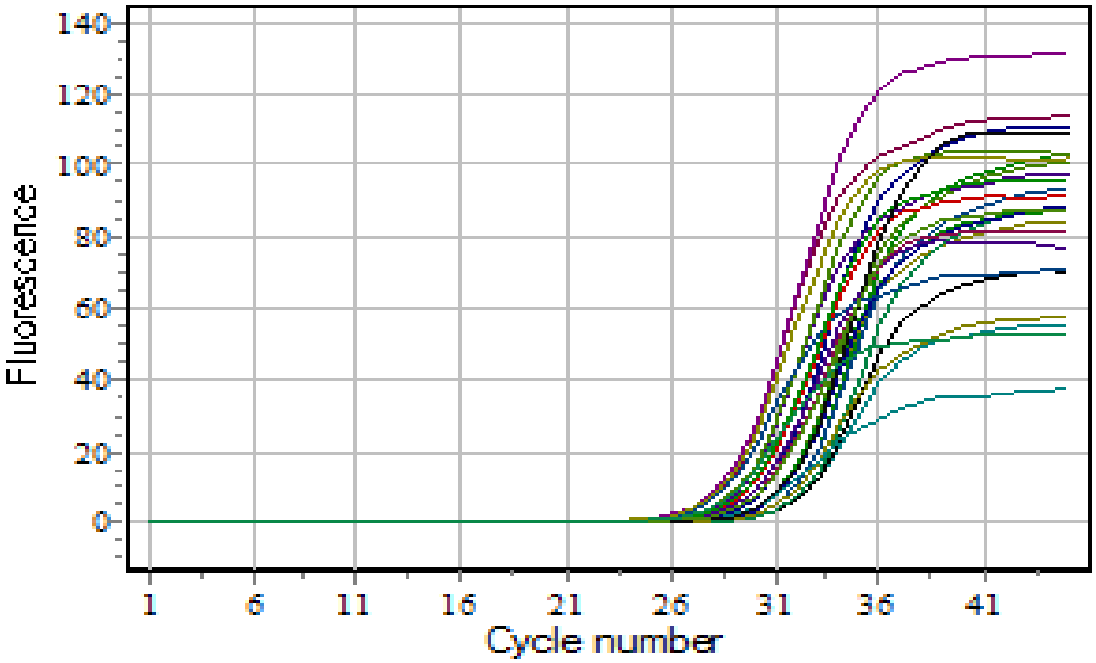

Figure (3) INHBA gene curve in aborted women

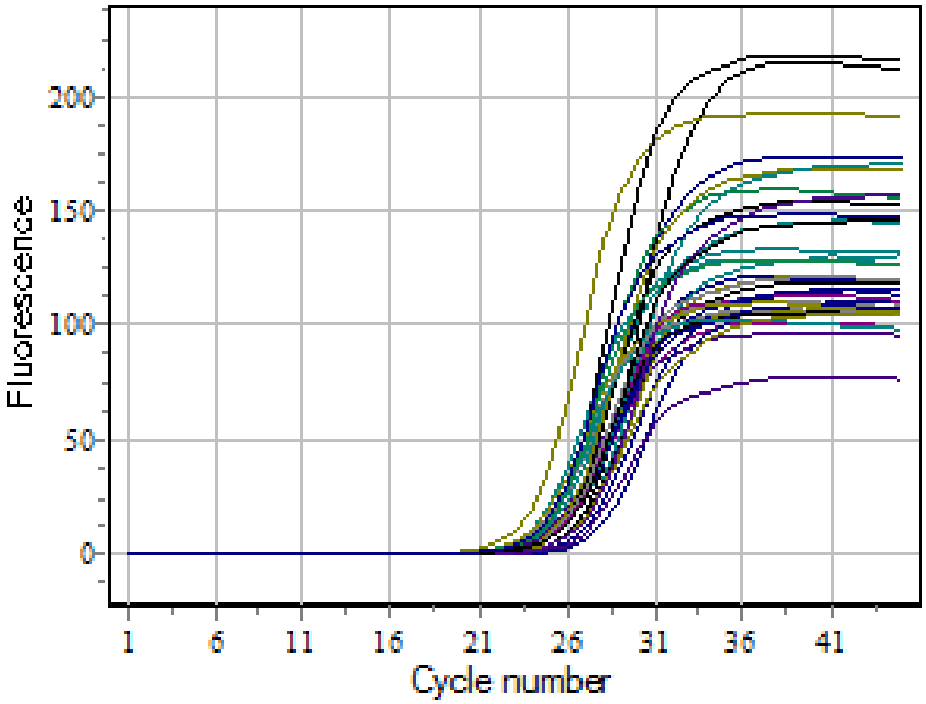

Figure (4) INHBA gene curve in the control group

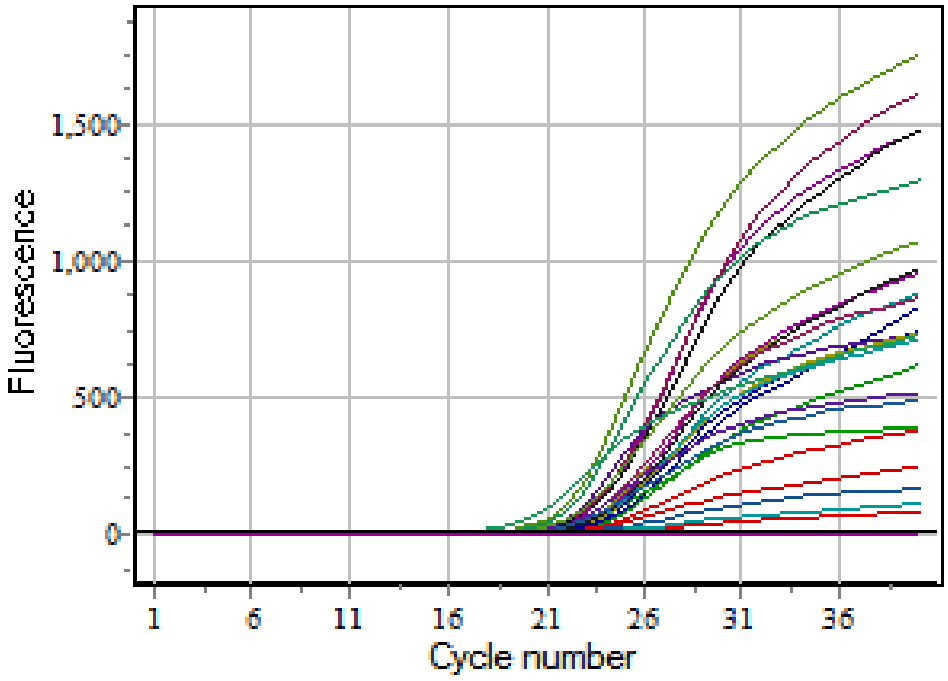

Figure (5) ACTB gene (housekeeping gene) curve

INHBA folding shows a significant difference with higher mean 3.55 ± 2.03 From the women undergoes with normal pregnancy 1.07 ± 0.87, p = 0.000025 and for the INHA the mean of women experienced miscarriage 1.8 ± 1.58, compared to the mean of normal staus 1.22 ± 0.85, p = 0.012835, which is slightly elevated .

Table(2)Compared means of placental gene expression of the studied groups

Parameters	Groups	N	Min	Max	Mean ± Std.	p - value
INHBA Folding	Disease	50	0.09	3.4	1.07 ± 0.87	0.000025
	Control	50	0.27	7.14	3.55 ± 2.03	
INHA Folding	Disease	50	0.19	3.03	1.22± 0.85	0.012835
	Control	50	0.21	6.76	1.8 ± 1.58	

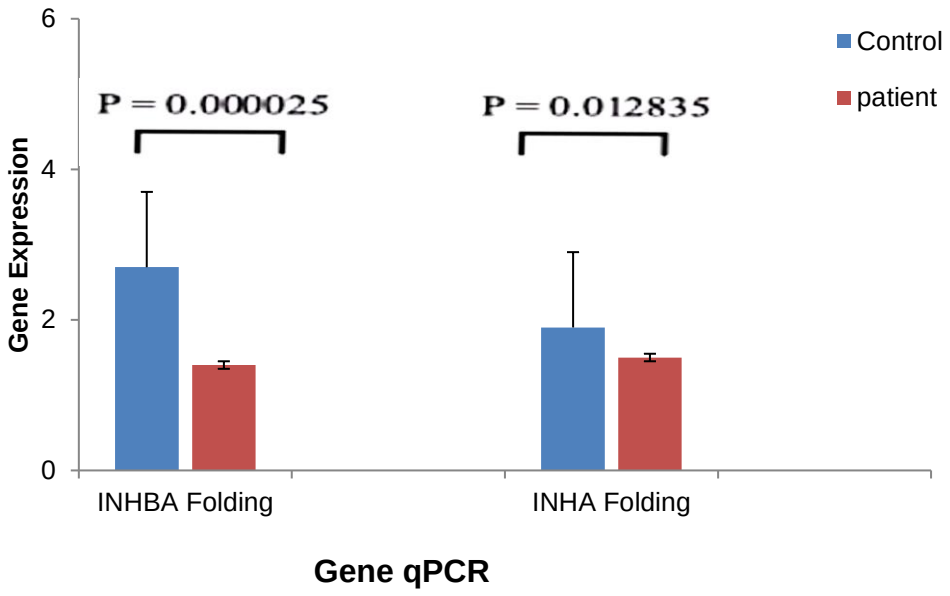

Fig. (6) Gene expression of INHA and INHBA genes between patients and control

The significant p-values for both INHA and INHBA folding suggest that the gene expression of both inhibin-A and activin-A hormones are decreased in the women with missed miscarriage. The decrease in the concentration of these two peptides leads to a decrease in the secretion of the FSH hormone. Juan Xie et al ;2021 showed that the decrease in the FSH hormone may be a cause of miscarriage.[30]

Also, any dysregulation or imbalance of these proteins may related to miscarriage and impair the ability of the human body to support pregnancy endometrial receptivity specifically in the first semister. [31]

Knöfler et al., also, discussed the importance of placenta as nessesity for successful pregnancy, and abnormal gene expression alteration in placental cells can lead to early pregnancy failure (miscarriage). [32]

Inhibins and activins are members of the transforming growth factor (TGFβ) superfamily, that includes the TGFβs, inhibins and activins, bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs)[33]. The family members are expressed throughout the human body, and are involved in the regulation of a range of important functions. The precise regulation of the TGFβ pathways is critical . The inhibins and activins regulate aspects of the male and female reproductive system,[34] therefore, it is not surprising that most of the diseases associated with abnormalities of the inhibin and activin genes are focused on reproductive disorders and reproductive cancers.[35-39] It is likely that inhibins and activins will be shown to play more important roles in a range of human genetic diseases in the future.

The clear decrease in the INHA and INHBA genes were showed a (down regulation) in MM samples the role of inhibin-A and activin-A in the regulation of placenta and kepping on pregnancy. Although the reduction

of inhibin-A and activin-A bioactivity are clearly not the only cause, evidence suggests that this change may serve as a susceptibility factor, increasing the likelihood of MM.

CONCLUSION

The results appears that there was a marked correlated among gene expression of INHA and INHBA genes ,(its coded both of inhibin-A and activin-A piptides) and MM. Decreased gene expression of INHA and INHBA genes was an independent relative risk for MM in inhabitance. Further studies are needed to explore the potential mechanisms by which role of this genes to MM.

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