

Mutations and Variants Analysis of Inhibin Gene Subunits in Women with Premature Ovarian Failure

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Abstract : This study aimed to illustrate the role of inhibin gene as a candidate gene in women with premature ovarian failure (POF). In this study, sixty women who suffered from POF; and have menopause at least 8 months after the last period, whose ages range between (20-39) years, venous blood samples were collected from these patients who are admitting to the Children and Maternity Hospital and Al-Hilla teaching hospital in Hilla city/Iraq. Also, forty women with no history of menopause with age range approximately matched to that of patients were included as control group. The genotyping was carried out by using PCR, RFLP and SSCP techniques to identify variants in some of candidates genes associated with POF. So regarding to *INHα1* gene, the result showed that there is a significant differences between patients and control groups that related to the presence this subunit. The result of RFLP to investigate the variant in this gene showed that most of patients and all control were homozygous for the wild type G allele, and G allele frequency was (92.8%) in patients group and (100%) in control group whereas A allele frequency was (7.2%) POF patients group and (0%) in the control group. The GG genotypes have frequency (85.7%) in POF and (100%) in control group, whereas the AG was found to be (14.3%) and (0%) in patients and control respectively. The results of *INHβA1*, *INHβB1* and *INHβB2* mutational screening, it was found that there is a significant differences between presence and absence of this gene in patients and control group at (P value 0.01) for *INHβA1*, while no one of patients and control groups show missing of *INHβB1* and *INHβB2* subunits. Using SSCP technique to investigate the presence of variants in *INHβA1*, *INHβB1* and *INHβB2* genes. The results illustrate that there is a significant differences in conformational changes of these gene subunits in patients and control subjected to this study. And this gave an indicator to relationship of the variants in these subunits with arising of POF in women in our population.

Keywords : POF, Mutation, Variants, inhibin α and β .

I. Introduction

Premature ovarian failure (POF) is an early ovarian malfunction different from normal menopause, which disturbs production of follicles resulting in amenorrhea under the age of 40 in 1-3% of reproductive age women (Pouresmaeiliet al., 2014). Affected women show menstrual problems followed by an elevated level of gonadotropins, such as follicle stimulating hormone (FSH) ≥ 40 IU/L and hypoestrogenism for an average four months, measuring serum FSH is a routine diagnosis procedure for the disease (Cox and Liu, 2014;

Al-Sabbaghet al., 2016). The studies confirmed that the multifactorial and heterogeneous biological events including infection, autoimmune disorders and metabolic factors are likely responsible for the disorder development; in 90% of observed cases, the etiology is unknown and the disease is defined as idiopathic POF (Bidet et al., 2011; Shelling, 2010).

The candidate gene approach to conducting genetic association studies focuses on associations between genetic variation within pre-specified genes of interest and phenotypes or disease states; these genes are most often selected for study based on a priori knowledge of the gene's biological functional impact on the trait or disease in question, and certain mutations will directly impact the function of the gene in question, and lead to the phenotype or disease state being investigated (Patnala et al., 2013).

Also (Caburet et al., 2012). told that, although POF may be due to metabolic, autoimmune, infectious or iatrogenic causes, compelling evidence suggests that, certain forms of the disease have a genetic etiology, and specific genetic alterations or mutations have been associated with syndromic or non-syndromic forms of the disease.

The first serious point in conducting candidate gene studies is the choice of an appropriate candidate gene, that may plausibly play a significant role in the process or disease under investigation; as soon as investigators have designated a candidate gene, they must decide, which polymorphism could be most useful for testing in a relation study; to this end, they must recognize existing gene variants and detect which of those variants give proteins with altered functions, that may guidance the trait of interest (Patelet et al., 2016). The inhibin is a candidate gene for mutational investigation in humans, specified its important role in regulating ovarian function either as, a negative modulator of pituitary FSH synthesis or as a paracrine factor (Persani et al., 2010). As the *INHα* G769A mutation is usually a heterozygous mutation, it is probable that, any consequence this change might have a role on inhibin biological function while would not be a whole loss of function; it is proposed that in mutation carriers, a decline in inhibin function by 50%, would have effects at two levels (i) fetal gonadal development, and (ii) in the regulation of normal folliculogenesis and ovulation;

a fall in inhibin biological potency could possibly obstruct the normal development of the fetal ovary, continuing to affect ovarian function and development after birth (Makanji *et al.*, 2008).

Also (Yoon *et al.*, 2012) postulated that , variation in the INH gene can lead to a diminished bioactivity or decreased level of inhibin , which may also affect the action of FSH and the reproductive efficacy of women , namely premature depletion of ovarian follicles , and may aid as a susceptibility factor for POF . A raised serum FSH level and low inhibin β level in the early follicular phase has also been stated to associate with reproductive ageing and contracted ovarian reserve (Mutlu and Erdem, 2012). As well as (Chapman *et al.*, 2015) strongly recommend that , the suggestion of the missense mutation of the INH α gene as a genetic marker for ovarian failure ; thus that urges germline studies of other significant molecules networked about inhibins .

II. Materials and Methods :

1. Clinical samples : This case control study involved women diagnosed with premature ovarian failure , they were referred to the-Children and Maternity Hospital and AL-Hilla teaching hospital in Iraq , all subjects underwent a standard diagnostic work-up to rule out any verifiable cause of POF prior to inclusion into the study .

A. Patients : Sixty patients participating in this case control study were related to women who suffered from POF ; these women have menopause at least 8 months after the last period , whose ages range between (20-39) years, venous blood samples were collected from these patients under supervision of specialist gynecologist .

B. Control: Venous blood samples from 40 women as a control that had normal menstrual cycle , with no history of menopause with age range approximately matched to that of patients .

2. Genomic DNA Extraction From Fresh Blood : The procedure was achieved according to the method recommended by the manufacturing company (Favorgen) with some modification (by special communication)

The primers used for the amplification of fragment genes were listed in Table (1).

Table (1): Primers sequences and PCR conditions :

Gene	Primer sequence (5'-3')	Size (bp)	PCR conditions	Reference
Inhibin alpha	F: GGCCACACTCGGACCAGAC R: AGCCACAAACCATGACAGTAG	244	94°C 1min 1x	(14)
INH α 1			94°C 2min 58°C 1min 30x 72°C 1min	
Inhibin beta A	F: GCAGGAGCAGATGAGGAAAAGGGAG R: CGCATGCGGTAGTGGTTGAT	302	72°C 8min 1x	
INH β A1				
Inhibin beta B	F: CGTGGTGCCGGTGTTCGTGGAC R: GCCGGTGGGTCTATGAT	202		(14)
INH β B1				
INH β B2	F: GCACCCACCGGCTACTACG R: TCCCGCTTGACGATGTTGT	218		

3. Statistical Analysis : This study used statistical analysis that included the calculation of mean values and percentage. The statistical package for the Social sciences version 18 (SPSS Inc., Chicago, USA) was used for statistical analysis. The allele frequency of INH α 1 gene polymorphism was tested and analyzed by Fisher's exact test [(Allele $\times$ 2) + mixed / (Total $\times$ 2)] . The association between genotype and risk of POF was estimated by calculation of Chi square , P value and odd ratio (OR) with 95% confidence interval (95%CI). A p-value $\leq$ 0.05 was considered as significant .

Results and discussion :

POF Diagnosed Women : Only 60 patients with POF were included in this study who admitted to the-Children and Maternity Hospital and AL-Hilla teaching hospital for surgery . These women have menopause at least 8 months after the last period , whose ages ranging from (20-39) years . Besides 40 healthy women , that had normal menstrual cycle , with no history of menopause were also included as control group . According to the clinical findings of the gynecologist and according to the hormones levels (FSH , LH and E2) the results of POF patients were scored .

The results showed that , most of women with POF have a high levels of FSH and LH and low level of E2 . And the levels of FSH and LH are highly a significant at (p value < 0.05) . Moreover , the levels of E2 decreased significantly in women with POF .

The products of PCR (amplicons) for INH gene subunits (INH α 1, INH β A1, INH β B1 and INH β B2) were obtained by using specific primers for each subunits . And the variants analysis were detected either by Restriction Fragments Length Polymorphisms (RFLP) technique or by Single Strand Conformational Polymorphisms (SSCP) .

1. RFLP technique:

The products of INH α 1 were sized at 244bp as shown in figure (1) . There were only four patients give negative results for this gene , which may be attributed to the absence or mutation (deletion) for this gene subunit , table (2) .

The study show the presence of significant differences between patients and control groups at (P value=0.01) . This

may give idea to attributable of lacking this gene with arising of POF .

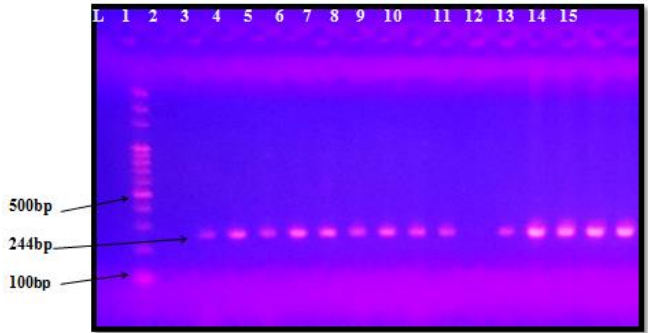


Figure (1): Gel electrophoresis of 2% agarose for INHα1PCR products visualized under U. V light after staining with ethidium bromide.; lane 1-10: from blood of POF patients , lane 11-15: from blood of control. The size of product is 244bp. Lane 1 show negative results for the gene presence , while all other lanes show positive results . L: 100 bp marker
Table (2) : The presence and absence of INHα1 gene subunit in patients and control groups .

Groups	Gene INHα1 Subunit		Chi-square	P value	OR
	Presence	Absence			
Patients(n=60)	56(93.3%)	4(6.7%)	2.778*	0.01*	1.71
Control(n=40)	40(100%)	0			

*significant at P-value≤ 0.05
 To show the variants in the INHα1 among the women patients with POF at the genetic locus 769 . The PCR products were subjected to RFLP technique , by using Bbv1 enzyme . And the analysis of this variant showed that the wild gene was digested into 3 fragments of 85, 25 and 134bp . In the presence of homozygote G769A variant the enzyme recognition site is abolished, and hence yields only two fragments of 85 and 159bp. Heterozygote carriers will have all four fragments (159, 134, 85 and 25bp). The 25bp fragment was undetectable on the gel as it is too small , figure (2) .

This result was showing that most of patients and all control were homozygous for the wild type G allele. Although, there were significant differences in the genotype between patients and controls (p value=0.01). Also it was found that the G allele was predominant than A allele in both patient and control. Table (3) . The result showed that G allele frequency was (92.8%) in patients group and (100%) in control group whereas A allele frequency was (7.2%) POF patients group and (0%) in the control group as show in table (4).

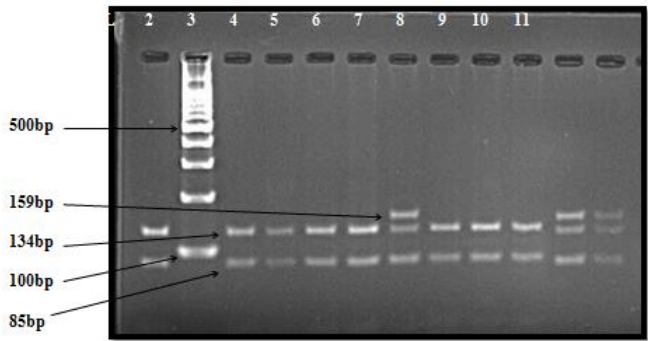


Figure (2): Gel electrophoresis of 2% agarose showing the restriction digestion patterns of INHα1 polymorphisms of gene using Bbv1 enzyme. Lanes 1,2,3,4,5,7,8 and 9 refer to homozygous wild type (GG) with 134bp , 85bp and 25bp . Lane 6, 10 and 11 show heterozygous genotype (GA)with 159bp, 134bp , 85bp and 25bp. L: DNA ladder =100bp.
Table (3): Genotype distribution of 769 G/A polymorphism in INHα1 gene subunit in POF patients compared with the control groups .

Genotype	Patients	Control	Chi-square	P value	OR
GG	48 (85.7%)	40(100%)	6.234*	0.01*	0.545
AG	8 (14.3%)	0			
AA	0%	0%			
Total	56	40			

*significant at P-value≤ 0.05

Table (4): Allele frequencies of 769 G/A polymorphism in

Allele frequency	Patients	Control
G	92.8%	100%
A	7.2%	0%
* Fisher exact test		

In case of INHα1 gene, since GG is predominant which may represent the wild type and the significant differences between patient and control means that , presence this variants is related to POF and have a role in stimulation this disease .

Alteration of wild type INH gene , incorporates all forms of alterations containing insertion ,deletions , missense and point mutations in the coding and noncoding regions ; deletions may involve of the entire gene or only a portion of the gene , and point mutations may results in frameshift mutations , stop codons or amino acid substitutions (Shelling, 2004).

The variant was the result of a G→A missense substitution at nucleotide 769 that changes codon 257 from GCT to ACT, resulting in an alanine to threonine amino acid substitution in the INH α 1 gene subunit (Shelling et al., 2000). The substitution has been postulated to impair the binding of inhibin to its putative receptor [Makanjiet al., 2014]. And this leading to a subsequent inability to activate the consequent signal transduction pathway and deregulate the FSH level by negative feedback ; alternative hypotheses for a functional effect of this transition mutation include preventing cleavage of the mature peptide, dimer formation or altering glycosylation (Fallahianet al.,2009; Mutluand Erdem, 2012).

The females lacking INH display an interesting phenotype , where histological investigation of their ovaries , show the presence of healthy follicles at all stages of folliculogenesis , till the late pre-antral stage, after which there is formation of granulosa cell tumors ; this demonstrates a crucial role of inhibin as a effective modulator of ovarian granulosa cell proliferation and , observed that in the absence of INH, there are uncontrolled granulosa cell proliferation , can resulting in ovarian tumors (Makanji et al., 2008).

The INH α 1 G769A variant was first informed in (7%) of patients with POF compared with (0.7%) in controls ; this mutation has also been found in patients from India and Italy; besides this , transition in the INH α 1 gene was identified in (16.7 percent) of Iranian patients with POF (Fallahian et al., 2009).

The results of this study as compared to other studies were accepted with (Chapmanet al.. 2015),(Mutlu and Erdem, 2012) which reported 10.5% of the sporadic POF cases as compared with 0.005% controls carried the INH α 1 G769A mutation . Another such study in the Indian population has revealed the presence of INH α 1 G769A and an additional three novel new missense mutations , the presence of the INH α 1 G769A mutation was significantly greater in women with POF although two of the controls were also carriers (Prakashet al., 2010). The mutational analysis of the INH α 1 can be used as a definite genetic marker for early diagnosis of POF, and patients can be offered genetic counseling to plan their conception at an early age of reproduction(Chand et al., 2007).

The independent studies produced different results and hypothesis generating findings were not replicated across several studies .The study of this variant was extended to include a larger cohort of Italian and German subjects and no difference in mutation frequency was observed between POF patients and control subjects , so they demonstrated that no association between the INH α 1 G769A mutation and the risk of POF (Corre et al., 2009). It was found neither in POF patients from Korea nor in patients from Auckland ,this mutation can correlated to POF(Makanjiet al., 2014). While, in the New Zealand and Indian populations there is a positive risk difference due to the INH α 1 G769A mutation between POF and control subjects(Zintzara, 2009). Furthermore , the population diversity influenced the effect sizes of G769A polymorphism and there was inconsistency of genetic

effects across ethnicities (Europeans and Asian Indians) (Zintzaras andLau, 2008a).

The overall lack of association between the polymorphisms and POF might be due to other unidentified functional mutations that exist in the INH α 1 gene that affect the susceptibility to POF ; in addition, other genes (such as FSHR and FOXL2) involved in the FSH pathway may affect the risk of POF (Beysen et al., 2009; Laissue et al., 2008; Simpson , 2008). Reduced production of INH , or production of mutant forms of INH is linked to several ovarian diseases , containing premature ovarian failure and polycystic syndrome (Kim et al., 2012). As in many other genetic disorders, the relationship between INHA1 G769A and POF is not simple, and the genetic variant may be acting more like a susceptibility allele other than a disease associated mutation with 100% penetrance , and studies have shown the incidence of asymptomatic carriers suggesting incomplete penetrance and /or a multigenetic causes of POF (Chand et al., 2010).

The genetics of POF basically relies upon the designing and undertaking of association rigorous candidate gene studies ; in future, studies should be planned with the idea of being incorporated with other similar studies , and the analysis also gives the opportunity to place each study in the context of all others and to investigate why studies reach different conclusions (Zintzaras and Lau, 2008b).particularly , the 769A variant is quite rare in all populations and all the studies comprised in the analysis involved a small number of subjects and consequently, the analyzed sample may not be very representative of the different populations considered ; though, the frequency of the variant will have to be determined in a larger number of samples (Zintzara, 2009).

2. SSCP Technique :

The mutational screening analysis using SSCP technique were performed for the remaining inhibin gene subunits (INH β A1 , INH β B1 and INH β B2) . For INH β A1 the size of amplicon was at 302 bp figure (3) . There were also only four of patients show absence of the gene subunit , table (5) as compared to control group , who showing the presence of this subunits for each individuals . The results found there is a significant differences between presence and absence of this gene in patients and control group at (P value 0.01) , so the lacking of this gene subunit in women may correlated to POF disease

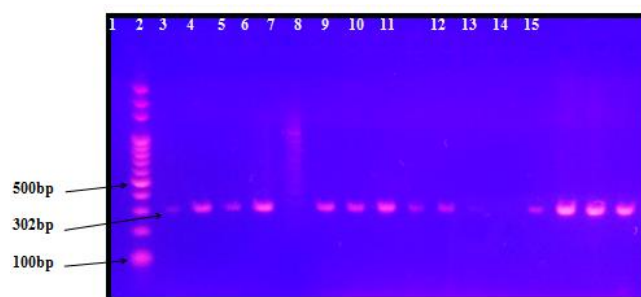


Figure (3): Gel electrophoresis of 2% agarose for INH β A1 PCR products visualized under U.V. light after staining with ethidium bromide.; lane 1-11: from blood of POF patients, lane 12-15: from blood of control. The size of product is 302bp . Lane 5 and 11 show negative results for this gene subunit . L: 100 bp marker

Table (5) : The presence and absence of *INHβA1* gene subunit in patients and control groups .

Groups	Gene <i>INHβA1</i> subunit		Chi-square	P value	OR
	Presence	Absence			
Patients(n=60)	56(93.3%)	4(6.7%)	2.778*	0.01*	1.71
Control(n=40)	40(100%)	0			

*significant at P-value≤ 0.05

While for *INHβB1* the product size of this gene subunit is about 202bp . No one of patients and control groups show missing of gene subunit figure (4)

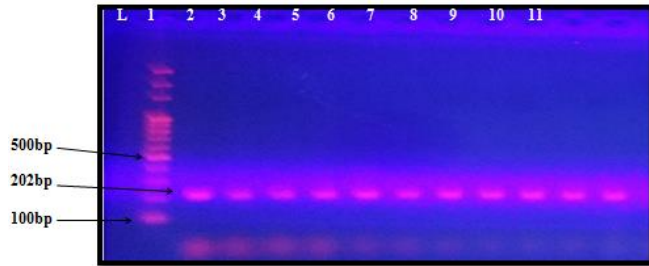


Figure (4): Gel electrophoresis of 2% agarose for *INHβB1* PCR products visualized under U.V. light after staining with ethidium bromide.; lane 1-8: from blood of POF patients, lane 9-11: from blood of control. The size of product is 202bp . L: 100 bp marker

And the last gene subunit of inhibin , *INHβB2* the product of the amplicon of this gene subunit is 218bp , and also no one of patients and control groups show the absence of this subunit figure (5)

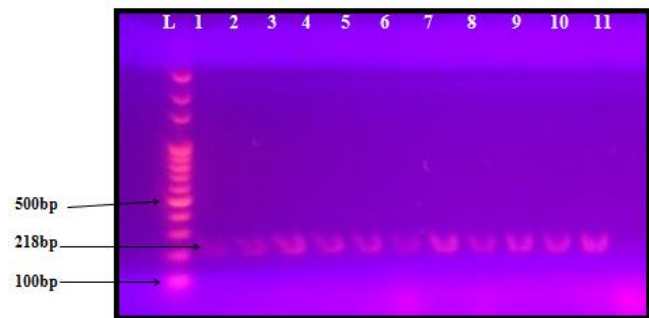


Figure (5): Gel electrophoresis of 2% agarose for *INHβB2* PCR products visualized under U.V. light after staining with ethidium bromide.; lane 1-8: from blood of POF patients, lane 9-11: from blood of control. The size of product is 218bp . L: 100 bp marker

The different banding patterns observed on the SSCP gels are indicative of the different conformations taken on by the ssDNA when separated in a acrylamide gel with electrophoresis . For SSCP it is essential that samples were denatured prior to loading

For *INHβA1* after SSCP only 4 patients and 10 patients show conformational changes in DNA pattern on PAGE as four and one bands respectively figure (6) . As well , there are only two show changes in DNA patterns as four band , and four have one band in control group , the details shown in table (6)

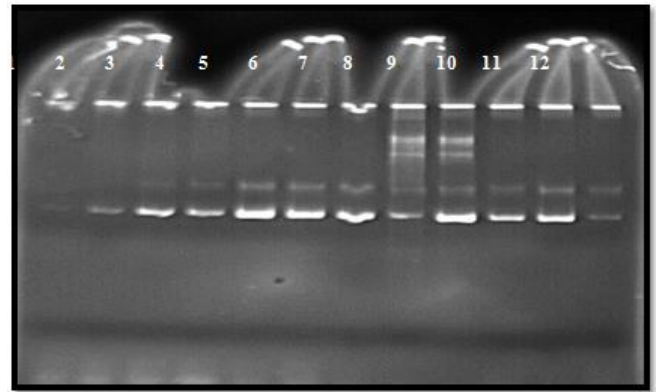


Figure (6): Polyacrylamide gel electrophoresis for variants detection of *INHβA1* using SSCP technique , visualized under U.V. light after staining with ethidium bromide ; lane 1-9: from blood of POF patients, lane 10-12: from blood of control. Lane 1 and 2 show conformational changes as 1 band , while lane 8 and 9 show conformational changes as 4 bands . Line 3,4,5,6,7,10,11 and 12 show migrational patterns as 2 bands

Table (6) : The conformational changes in migration patterns for *INHβA1* gene subunit .

Groups	Conformational changes as four bands	Conformational changes as one band	Chi-square	P value	OR
Patients(n=56)	4(7%)	12(21.4%)	2.558*	0.02*	2.267
Control(n=40)	2(5%)	4(10%)			

*significant at P-value≤ 0.05

While for *INHβB1* , following the SSCP technique we found 10 patients show migrational changes as one bands and there are only 4 patients show conformational changes with extra bands , figure (7) . And the study found that, only two of control group with one bands in PAGE. The results shown in table (7)

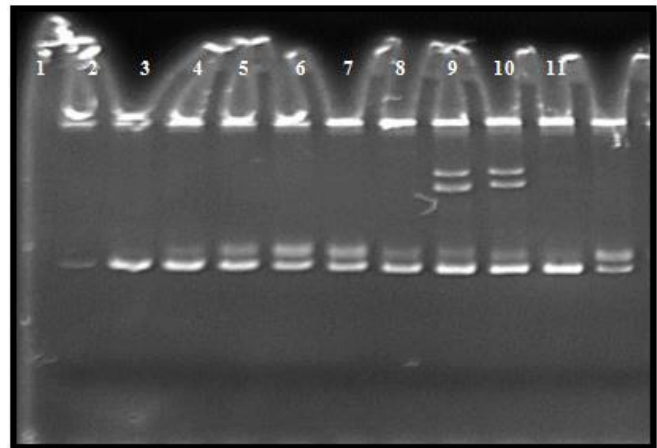


Figure (7): Polyacrylamide gel electrophoresis for variants detection of *INHβB1* using SSCP technique , visualized under U.V. light after staining with ethidium bromide ; lane 1-9: from blood of POF patients, lane 10-11: from blood of control. Lane 1,2 and 10 show conformational changes as 1 band , while lane 8 and 9 show conformational changes as 4 bands . Line 3,4,5,6,7 and 11 show migrational patterns as 2 bands .

Table (7) : The conformational changes in migration patterns for *INHβB1*

Groups	Conformational changes four bands	Conformational changes as one band	Chi-square	P value	OR
Patients(n=60)	4(6.7%)	10(16.7%)	6.349*	0.04*	5.783
Control(n=40)	0	2(5%)			

*significant at P-value ≤ 0.05

After the SSCP technique , figure (8) , it found that , only six patients with migrational changes as compare with control which have no changes in their patterns on PAGE , table (8) .

There is a significant differences in conformational changes of these gene subunits in patients and control subjected to this study. And this gave an indicator to relationship of the variants in these subunits with arising of POF in women in our population .

Table (8) : The conformational changes in migration patterns for *INHβB2* gene subunit .

Groups	Conformational changes with extra bands	Chi-square	P value	OR
Patients(n=60)	6(10.3%)	4.255*	0.03*	0.574
Control(n=40)	0			

*significant at P-value ≤ 0.05

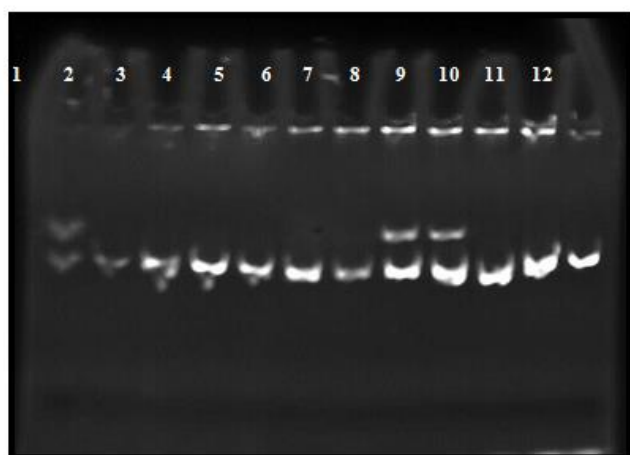


Figure (8): Polyacrylamide gel electrophoresis for variants detection of *INHβB2* using SSCP technique , visualized under U.V. light after staining with ethidium bromide ; lane 1-9: from blood of POF patients, lane 10-12: from blood of control. Lane 1,8 and 9 show conformational changes as 2 bands , while lane 2,3,4,5,6,7,10,11 and 12 show migrational patterns as 1 bands

Although , there is a change in the patterns of 6 patients ; but these changes do not give rise to the new variation , because of silent mutation occurred in this locus mentioned by(Shelling,2004) who found that ,the migrational shift detected in the *INHβA1* fragment may be caused by a silent substitution ; this variant did not change the amino acid sequence of the *INHβA* subunit . In *INHβA* and *INHβB* , although mutations with supposed functional significance have been found , population screens have not shown these to be common causes of POF (Bretherick, 2008). As well as , the fragments *INHβA1*, *INHβB1* and *INHβB2* did not expose any migration variants in any of the patient samples when compared against individuals with the wild type (Shelling *et al.*, 2000). The overexpression of *INHβB*

subunit could rescue the *INHβA* null embryonic lethal phenotype , and mice were generated , in which the mature region of the *INHβA* subunit gene was replaced with the corresponding mature region of the *INHβB* subunit gene (Chand *et al.*, 2007).

Also (Maet *al.*, 2015) stated down , that any mutation has no effect of *INH* , because the mutation is silent ; generally, this mutation does not affect the original amino acid sequence or the carrier's ovarian function , however, the *INHβB* mutation may be included in the alteration of ovarian reserve and ovarian function ,and *INHβ* subunit genes , *INHβA* and *INHβB* mutations with supposed functional significance have been found . Furthermore significant mutations were not found in the *INHβA* or *βB* subunit genes , although a silent transition variant was detected in the *INHβA* gene (Chandet *al.*, 2010).

This result was not come with other results achieved by (Mutlu and Erdem, 2012) who showed that , mutations in *INHβA* and *INHβB* did not reveal any association with ovarian failure , and it is difficult to determine what is the role of the silent mutation in ovarian failure ; where they observed that , the propeptide region , had not lead to alteration in the structure of the mature peptide chain.

Moreover (Di Pasquale *et al.*, 2004) mentioned that , the *INHβA* and *INHβB* genes did not show any association except the rare variants , in which *INHβA* gene revealed a missense variant in individuals with POF and , while the *INHβB* gene revealed a silent variant in women with POF . Detection of mutation prior to the development of ovarian failure allows the carriers to make informed decisions concerning reproductive options ; early detection would provide a good opportunity for early intervention such as replacement of the inhibin hormone in women to delay the onset of ovarian failure (Hassan *et al.*, 2012).

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