Original Article

Genetics of Female Infertility: Molecular Study of Newborn Ovary Homeobox Gene in Poor Ovarian Responders

Osamah Batiha, Nour Alhoda Alahmad, Amer Sindiani[1], Khaldon Bodoor, Sherin Shaaban, Mohammad Al-Smadi[2]

Department of Applied Biological Sciences, Jordan University of Science and Technology, Irbid, [1]Department of Obstetrics and Gynecology, Jordan University of Science and Technology, Irbid, [2]Department of Reproductive Endocrinology and IVF Unit, King Hussein Medical Center, Amman, Jordan

ABSTRACT

Background: Newborn ovary homeobox (*NOBOX*) gene plays a critical role in the transcriptional regulation of oocyte-specific genes. Previous studies have demonstrated a pathogenic effect of *NOBOX* variants on premature ovarian insufficiency (POI) patients. Poor ovarian response (POR) is a risk factor for POI. Therefore, genetic variants in the *NOBOX* gene may also be studied as risk factors for POR development. **Aims:** The aim of the study is to investigate the association between seven known *NOBOX* single-nucleotide polymorphisms (SNPs) and POR in Jordanian females. **Settings and Design:** This was a case–control study of 60 females with POR for controlled ovarian hyperstimulation and 59 healthy females with no history of reproductive problems. Blood samples were collected from the participants and seven SNPs of *NOBOX* gene were screened. **Subjects and Methods:** DNA was extracted from blood samples. Polymerase chain reaction with primers specific for seven known SNPs in *NOBOX* gene was used to amplify the specified region within the gene followed by Sanger sequencing. **Results:** The seven SNPs investigated in this study, namely, rs77587352 (c.271G>T, p. Gly91Trp), rs7800847 (c.349C>T, p. Arg117Trp), rs193303102 (c.907C>T, p. Arg303X), rs193303103 (c.1025G>C, p. Ser342Thr), rs193303104 (c.1048G>T, p. Val350Leu), rs201947677 (c.1064G>A, p. Arg355His), and rs146227301 (c.1856C>T, p. Pro619Leu), only represent the wild-type allele in both females with POR and healthy participants. **Conclusions:** The results show that only monomorphic genotype of the *NOBOX* variants was found in Jordanian females studied.

KEYWORDS: *Female infertility, newborn ovary homeobox gene, poor ovarian response*

INTRODUCTION

Ovarian reserve describes the female reproductive potential in terms of both quality and quantity of oocytes.[1,2] Decreased or diminished ovarian reserve represents the premature loss of oocytes and poor oocyte development, a common condition occurs naturally after the age of 40 leading to menopause.[3,4] Younger females with decreased ovarian response are usually referred to reproductive clinics and undergo a controlled ovarian hyperstimulation (COH) protocol to obtain mature oocytes. In some cases, they achieve a poor response to COH manifested by low number of retrieved oocytes or complete absence of oocytes. Females with such condition

are diagnosed with poor ovarian responders (POR). More than one-third of females undergoing assisted reproductive technology (ART) is diagnosed with POR.[5]

The prevalence of POR in different countries ranges between 9% and 24%.[6] In 2007, the American Society for Reproductive Medicine and the Society for ART reported that at least 50% of canceled *in vitro* fertilization (IVF) cycles were due to POR.[7]

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The etiology of POR is unknown; however, age, advanced endometriosis, ovarian surgery, pelvic adhesion, smoking, and high body mass index are considered to be risk factors associated with low ovarian reserve.[8]

Furthermore, POR is linked to mutations in genes involved in oocyte production. For example, several studies identified an association between genetic polymorphisms in the gonadotropin hormone genes and their receptors and POR.[9]

Newborn ovary homeobox gene (*NOBOX*) is an oocyte-specific homeobox gene which is expressed in mice primordial and growing oocytes[10] and is essential for mice folliculogenesis. Several studies point to an essential role for *NOBOX* in oocyte development such as the lack of the gene is linked to the loss of postnatal oocyte, blocks the transition from primordial to growing follicles, and downregulates oocyte preferential genes such as *Oct4* and *Gdf-9*.[11]

NOBOX gene is located on chromosome 7q35 and is composed of 10 exons.[12]

NOBOX transcript in humans is present in the ovary, testis, and pancreas. *NOBOX* expression in the oocyte was detected from the primordial stage of the ovarian follicle until the MII stage of mature oocyte.[13] Microarray analysis of newborn mouse ovaries lacking *NOBOX* gene showed that 28 oocyte-specific genes were downregulated more than five-fold, whereas only five oocyte-specific genes were upregulated.[14] Some of these affected genes have specific roles in signaling pathways related to oocyte developments such as actin binding (*Ttid* gene), aldehyde dehydrogenase (*E330034G19Rik* gene), aldehyde reductase (*Aldrl6* gene), arginine deiminase (*Padi6* gene), exocytosis (*Rims1* gene), histone/oocyte specific (*H1foo* gene), microtubule movement (*Dnahc8* gene), notch signaling pathway (*Jag1* gene), olfactory receptor (*Olfr976* gene), oligoadenylate synthetase (*Oas1e, Oas1d, Oas1c*, and *Oas1h* genes), protein kinase (*Mos* and *2610028F08Rik* genes), Ring finger protein (*Rfpl4* gene), secreted factor (*Fetub, Astl, Gdf9,* and *Oosp1* genes), Solute carrier (*Slc6a20* gene), and transcription (*Pou5f1, 1700008J08Rik,* and *Sall4*). Others they do not seem to have a clear function (*C86187, D5Ertd577e, BC052883, Nlrp4f, Nlrp14, Oog1, BG071013, D9Ertd414e, BM229829, D11Ertd636e, AK005675, E130009J12Rik,* and *Nlrp4c* genes).[14] The results of these studies confirm the critical role of the *NOBOX* gene in oocyte gene regulation and oogenesis. In addition, it implies that mutations in *NOBOX* may lead to POR and infertility.

The association between *NOBOX* and premature ovarian insufficiency (POI) disorder has been studied in several populations [Table 1].[15-20] POI is a condition of amenorrhea, estrogen deficiency, and menopausal follicle-stimulating hormone (FSH) levels in young women (<40).[21]

Poor ovarian response (POR) to gonadotropin is an indicator of ovarian failure, whereas poor responders have a higher chance of being diagnosed with POI.[22-25]

In this study, we investigated the frequency of seven known *NOBOX* single-nucleotide polymorphisms (SNPs) [Table 2] in females with POR. The selected SNPs have been previously associated with POI disease in different populations.[15-17,19]

Subjects and Methods

Selection and description of participants

This case-controlled prospective study included 152 POR patients. Blood samples were collected from different IVF centers in Jordan (King Hussein medical city, Istishari hospital, Prince Rashid Hospital, Islamic hospital, and Al-Amal Maternity Hospital) in the period of 2014–2017.

Sixty participants suffering from POR were included with the age range of 20–46 years according to the European Society of Human Reproduction and Embryology criteria.[4] Patients with at least two of the following standards were included [Supplementary Table]: (1) anti-Müllerian hormone (AMH) level <1.1 ng/ml, (2) FSH level more than 10 mIU/ml and E2 level 25–75 pg/ml on day 3 of a normal menstrual cycle, (3) the antral follicle count (AFC) is <9, and (4) the number of retrieved meiosis II oocytes is <5. Patients >40 years were included in this study only when they were diagnosed with POR before the age of 40 and were still trying ART at the time of sample selection. Ninety-one samples were excluded due to incomplete data or due to known infertility causes such as pelvic surgery, ovarian cysts, radiation therapy, and chemotherapy. Despite the long duration of sample collection, sample size was limited because of the scarcity of the disease in Jordan and the stringency of the selection criteria.

Control group included 59 whole blood samples collected from normal fertile females with age range of 24–39 years whom were able to carry out a normal pregnancy without the need to undergo any ART, and they have no medical history of pelvic surgeries, ovarian cysts, radiation therapy, and chemotherapy.

Control samples were collected from different private clinics in Amman, (King Hussein medical city and King Abdullah university hospital). The Institutional Review

Table 1: Newborn ovary homeobox gene variations in premature ovarian insufficiency in different populations

Country (ethnicity)	Sample size		Region of sequence	Sequence variation	Allele frequency for mutation (%)				Global MAF*	References
	Patients	Controls			Patients		Controls			
					Hetero-zygote	Homo-zygote	Hetero-zygote	Homo-zygote		
Japan	30	20	Exons 2-6	-	-	-	-	-	-	Zhao et al.,[20] 2005
USA (white women)	96	278	Exons 1-10	c. 1064G>A	1.01	0	0	0	-	Qin et al.,[28] 2007
China	200	200	Exons 4-6	-	-	-	-	-	-	Qin et al.,[19] 2009
France (Caucasian, Senegalese, and Bantu)	178	362	Exons 1-8	c. 271G>T	1.2	0	0	0	0.01 (T)	Bouilly et al.,[17] 2011
				c. 349C>T	1.6	0	0	0	0.02 (T)	
				c. 907C>T	0.6	0	0	0	-	
				c. 1025G>C	2.2	0	0	0	-	
				c. 1048G>T	0.6	0	0	0	-	
France (Caucasian or African)	213	362	Exons 1-10	c. 131G>T	2.4	0	0	0	0.01 (T)	Bouilly et al.,[15] 2015
				c. 271G>T	3.0	0	0	0	0.01 (T)	
				c. 331G>A	0.6	0	0	0	<0.01 (A)	
				c. 349C>T	1.2	0	0	0	0.02 (T)	
				c. 1112A>C	0.6	0	0	0	<0.01 (C)	
				c. 1856C>T	1.2	0	0	0	0.01 (T)	
Tunisia (98% Arab)	125	200	Exons 1-10	c. 271G>T	0.8	0	0	0	0.01 (T)	Bouali et al.,[16] 2016
				c. 349C>T	2.4	0.8	0	0	0.02 (T)	
				c. 1856C>T	2.4	0	-	-	0.01 (T)	
China	96	211	Whole exome	c. 567delG					-	Li et al.,[18] 2017

*Data obtained from 1000 genomes project phase 3. MAF=Minor allele frequency

Board at Jordan University of Science and Technology and King Abdullah University Hospital (KAUH) approved the study, and informed written consent was obtained from all patients and controls.

DNA extraction and sequencing

Genomic DNA was extracted from whole blood samples using Puregene Blood Core Kit B (Qiagen) according to the manufacturer's instructions.

Seven SNPs have been studied in *NOBOX* gene covered by three different pairs of primers. All primers were designed through SnapGene software using the *NOBOX* gene sequence obtained from the Ensembl genome browser according to the transcript ID number (ENST00000467773.1). The primers were synthesized at Princess Haya Biotechnology Centre, Irbid, Jordan. Primers sequences, products size, included polymorphisms, and their cycling conditions are listed in Table 3.

The polymerase chain reaction (PCR) products were loaded in 2% agarose gel in 1X TBE buffer at 120V for 45 min to determine the product size. 50 bp DNA ladder was used to determine the band sizes, and the gel was stained with ethidium bromide then displayed by GelDoc-It 310 imaging system (UVP, USA). PCR products were purified using ExoSAP-IT PCR Product

Table 2: A summary of the seven studied single-nucleotide polymorphisms in this study

dbSNP ID	Sequence variation	Position	Amino acid change	Gene consequence
rs77587352	c. 271G>T	chr7:144401890 (GRCh38.p12)	p. Gly91Trp	NOBOX: Missense variant
rs7800847	c. 349C>T	chr7:144401541 (GRCh38.p12)	p. Arg117Trp	NOBOX: Missense variant
rs193303102	c. 907C>T	chr7:144400250 (GRCh38.p12)	p. Arg303X	NOBOX: Stop gained
rs193303103	c. 1025G>C	chr7:144400132 (GRCh38.p12)	p. Ser342Thr	NOBOX: Missense variant
rs193303104	c. 1048G>T	chr7:144399863 (GRCh38.p12)	p. Val350Leu	NOBOX: Missense variant
rs201947677	c. 1064G>A	chr7:144399847 (GRCh38.p12)	p. Arg355His	NOBOX: Missense variant
rs146227301	c. 1856C>T	chr7:144397460 (GRCh38.p12)	p. Pro619Leu	NOBOX: Missense variant

SNP=Single-nucleotide polymorphisms, NOBOX=Newborn ovary homeobox

Table 3: Primer sequences used for polymerase chain reaction in the study and their cycling conditions

Primer number	Primer sequence (5'-3')	Product size	Polymorphisms included		Program
			DNA variation	Sequence variation ID	
NB.E3-4	F: TCTCTTTGTCTTCCTGGTCCA	519	rs77587352	c. 271G>T	94°C 60s
	R: GCGGCTTCTTCTCTCCTGA		rs7800847	c. 349C>T	59.5°C 60s
					72°C 60s
					35 cycles
NB.E5-6	F: AAGTTTCTTCTTCTTTCAGATCAGCT	552	rs193303102	c. 907C>T	94°C 60s
	R: AGGGGCTGCAGGATTGT		rs193303103	c. 1025G>C	59.5°C 60s
			rs193303104	c. 1048G>T	72°C 60s
			rs201947677	c. 1064G>A	35 cycles
NB.E10	F: TCCTGGAGTGACCCCTGTTTGC	204	rs146227301	c. 1856C>T	95°C 30s
	R: CTTGCTGAGTAAGGGCCCAGT				60.9°C 30s
					72°C 30s
					35 cycles

Table 4: Selection categories and numbers of implicated samples

	FSH/AMH	FSH/AMH	FSH/AMH	FSH/AMH
AFC/MII	0	4	1	0
AFC/MII	9	19	1	0
AFC/MII	3	3	1	13
AFC/MII	0	0	6	0

Parameter is included. AFC=Antral follicle count, FSH=Follicle-stimulating hormone, AMH=Anti-Müllerian hormone, MII=Metaphase II oocytes

Cleanup kit (Affymetrix) and then loaded in the ABI 310 Genetic Analyzer (ABI Prism310, Applied Biosystems) at Princess Haya Biotechnology Centre. ChromasPro software (Technelysium Pty Ltd) was used to analyze the sequencing data.

RESULTS

Seven genetic variations in the *NOBOX* gene in 60 POR females with an average age of 33.6 + 6.4 years (20–46) were compared to 59 healthy fertile females under the age of 40 (24–39). Table 4 summarizes the selection categories and the numbers of participants investigated.

The average AMH level of the cases involved was 0.344 + 0.257 ng/ml (0.03–0.98), FSH level was 19.55 + 13.7 mIU/ml (10.6–62.6), whereas the average AFC number was 3.52 + 1.64 (0–6) and 2.25 + 1.27 (0–4) for the average number of meiosis II oocytes retrieved after stimulation . According to the sequencing analysis, there were no differences between cases and controls in all seven SNPs, all of them had the wild-type alleles. Table 5 summarizes the results of the studied SNPs in both cases and controls. In general, environmental endocrine disruptors, tobacco, genetic mutations, endometriomas, ovarian surgery, chemotherapy, and short menstrual cycles are factors that affect the stimulation process in assisted reproduction cycles.[26] In this study, we excluded samples with a history of endometriomas, ovarian surgeries, and chemotherapy.

DISCUSSION

The process of oogenesis is tightly regulated by a set of genes, which guarantee the proper production of competent oocytes ready for fertilization. Among these genes are the *NOBOX*, which has been found to play important roles in oogenesis and folliculogenesis.[12,27] In this work, we studied the prevalence of seven SNPs

Table 5: Summary of sequencing results of the common newborn ovary homeobox single-nucleotide polymorphisms among all samples

dbSNP ID	Sequence variation	Amino acid variation	Location	Allele frequency (%)					
				Patients with POR			Control group		
				Wild type	Hetero-zygote	Homo-zygote	Wild type	Hetero-zygote	Homo-zygote
rs77587352	c. 271G>T	p. Gly91Trp	Exon 3	100	0.0	0.0	100	0.0	0.0
rs7800847	c. 349C>T	p. Arg117Trp	Exon 4	100	0.0	0.0	100	0.0	0.0
rs193303102	c. 907C>T	p. Arg303X	Exon 5	100	0.0	0.0	100	0.0	0.0
rs193303103	c. 1025G>C	p. Ser342Thr	Exon 5	100	0.0	0.0	100	0.0	0.0
rs193303104	c. 1048G>T	p. Val350Leu	Exon 6	100	0.0	0.0	100	0.0	0.0
rs201947677	c. 1064G>A	p. Arg355His	Exon 6	100	0.0	0.0	100	0.0	0.0
rs146227301	c. 1856C>T	p. Pro619Leu	Exon 10	100	0.0	0.0	100	0.0	0.0

POR=Poor ovarian response

in the *NOBOX* gene and found that both cases (POR patients) and control groups represented the wild-type allele in all investigated SNPs; therefore, we exclude the role of *NOBOX* in POR in the studied Jordanian cohort.

Several previous studies have reported the association between *NOBOX* polymorphisms and ovarian failure syndromes such as primary ovarian insufficiency.[14,16,17,28] This association is not surprising as *NOBOX* is known as an important early regulator of both folliculogenesis and oogenesis processes. However, the relationship between *NOBOX* and POR has not been investigated till now. Nevertheless, several studies have shown that poor response can be an early sign of ovarian failure;[22-25] therefore, we determined to study the role of *NOBOX* in females with poor response to COH.

Several polymorphisms in the *NOBOX* gene have been associated with POI in populations of the USA, France, Tunisia, and Han China.[15-18,28] In a Tunisian study, mutations were found in 6.4% in patients and none of them were found in control group. In France, 5.6% of the patients with POI displayed heterozygous NOBOX mutations. Another study in France found a NOBOX loss-of-function mutation in 6.2% of POI cases.

However, in other studies in Japan and China, they found no association between *NOBOX* SNPs and POI.[19,20] The discrepancy between these studies could be due to differences in ethnicity, selection criteria, differences in sample size, and the analysis of different regions of the *NOBOX* gene.

Despite the importance of the evaluation of the ovarian reserve in determining the outcome of the assisted reproductive procedures, still few indicators/biomarkers are being used. Physiological markers such as high FSH and low AMH are commonly used as indicators of the ovarian reserve.[4,8] Hormones such as FSH, LH, and their receptors are important for folliculogenesis;

therefore, the association between SNPs in these genes and poor response has been studied.[9,29] One of these polymorphisms in the promoter of FSHB gene (rs10835638; c.-211G>T) has been associated with lower FSH level and late or longer menopause.[30] FSH receptor is important for the FSH action, and some genetic polymorphisms have been associated with ovarian response.[31,32] Finally, genetic variants in the luteinizing hormone biological function subunit β have been associated with poor response to hormonal stimulation during ART procedures which qualifies this gene as a good indicator of ovarian response.[33,34]

Lifestyle such as smoking and taking oral contraceptive pill also has directly effects on ovarian reserve and hormone levels regardless of genetic associations.[35]

There are some limitations of this study. First, it included a small sample size which may prevent polymorphisms detection. Second, the samples were collected from different IVF centers which may cause variations in the results of hormone levels. In future studies, we recommend to collect the samples from one IVF center and to investigate the roles of other genes, in addition to incorporate females responded normally to COH as controls.

Poor response is considered a big challenge for gynecologists as well as patients to achieve successful pregnancy and to overcome infertility problems; therefore, more studies should be followed to understand the pathophysiological etiologies' behind this condition. Molecular investigation for variants in genes important in folliculogenesis and ovulation will help in optimizing better treatment of POR cases and help to improve pregnancy outcomes.

To our knowledge, this is the first study in Jordan studying the genetic causes of POR. We can exclude the probability of *NOBOX* variations pathogenicity for POR until proven otherwise.

Conclusions

This is the first study exploring *NOBOX* gene variations in poor ovarian responders. We did not find any link between POR and seven previously studied SNPs in *NOBOX* gene (rs77587352, rs7800847, rs193303102, rs193303103, rs193303104, rs201947677, and rs146227301) in a subset of Jordanian females. We recommend investigating the role of other genes in the future.

Conflicts of interest

There are no conflicts of interest.

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Supplementary Table: Clinical data of patients included in this project

Patient#	Age	FSH	E2	AMH	AFC	M I	M II
1	30	14.2			4	0	1
2	38	13.6				0	0
3	21	13.3				0	3
4	39	12.2				0	1
5	25	13.4			7 (empty)	0	0
6	25	6.8			5	0	0
7	32	10.6				0	4
8	33	14.2				0	2
9	26	52		1.2		2	1
10	40	10.6			6	0	2
11	33	7.08			4	0	3
12	37				5	1	4
13	28	5.54			4	0	4
14	34	11.1		0.4			
15	38	20.1		0.06			
16	27	11.4		0.05	2	0	2
17	39		579.86	0.03	0	0	1
18	31	27		1.1		1	2
19	33	5	811.07	0.5		0	2
20	29	11		0.4			
21	36			0.23			
22	37	6.91	3453	0.1	2		
23	32	10.8				0	1
24	33	18.4	53.1			0	3
25	41			0.3		0	1
26	38			0.98		0	3
27	40			0.3	4	2	2
28	39	10.6		0.3	2		

Contd...

Supplementary Table: Contd...

Patient#	Age	FSH	E2	AMH	AFC	M I	M II
29	42			0.9		3	3
30	40	5.7		0.4		0	3
31	31			0.4		0	4
32	42	26.7		0.1			
33	41			0.4		0	2
34	30	62.6		0.23			
35	31	31.8		0.3			
36	39	6.3		0.2		0	0
37	31			0.9	low AFC		
38	42			0.1		2	0
39	39	4.1		0.4	low AFC	5	4
40	38			0.2		0	2
41	35			0.4		1	3
42	40	2.6		0.1		0	3
43	30			0.15	1		
44	41			0.1		0	0
45	46			0.4		0	2
46	31	7.4		0.2		0	1
47	43			0.1		0	3
48	32	2.6		0.8		0	2
49	36			0.3		0	3
50	29	4.6			4	0	4
51	23	5.6			4	1	3
52	28	1.6			5	0	3
53	25	7.93			4	0	4
54	45	7.1		0.46		0	2
55	30	9.8			4	0	2
56	22	7.57			4		
57	26	3.9			4		
58	30	8.39			3		
59	35	5.9			1		
60	22	15.07		0.86	2		