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Original article

Association of recurrent spontaneous abortion with polycystic ovarian syndrome under the influence of killer immunoglobulin like receptors

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ABSTRACT

Aims: The primary aim of the current study is to decide if genetic polymorphisms of the killer immunoglobulin-like receptor (KIR) and human leukocyte antigen (HLA-C) genes are related to recurrent spontaneous abortion (RSA) in the Saudi women who are affected with polycystic ovarian syndrome (PCO) and in PCO patients without RSA.
Materials and methods: The study was conducted in Riyadh and included 199 cases divided as follows: 69 PCO Saudi women with an RSA history, i.e. three or more spontaneous abortion cases, 65 patients were exclusively with PCO and 65 healthy controls, typed for 17 KIR genes and the HLA-C1 and HLA-C2 allotypes using polymerase chain reaction-sequence-specific primer methodology.
Results: The study reported that the recurrences of KIR3DL1, 2DS4ins, 2DL2, and KIR2DS2 significantly reduced among RSA-PCO women in comparison with healthy controls (OR = 337.45, 39.08, 2.4, 4.59 with $p < 0.01$) and a connection with maternal HLA-C genotypes was also examined. Furthermore, the analysis of KIR-HLA-C combinations demonstrated a preventive impact of KIR2DS2, 2DL2, 2DL2/2DL3, 2DL3 with its counterpart HLA-C1 ligand in both homozygote or heterozygote combinations. Such findings were also reported in PCO women. With regard to recurrences, KIR2DL1 marked the highest significant frequenting in both PCO women and RSA-PCO women in comparison with control in the presence of HLA-C2 and homozygote form (HLA-C2C2). However, this frequency decreases in the absence of HLA-C2 ligand. In addition, KIR2DS2, KIR2DL3 significantly increased in the absence of HLA-C1 ligand in both PCO group and RSA-PCO group when compared with healthy cases.
Conclusion: It can be concluded from the results of the study that the KIR genes (2DS2, 3DL1, 2DL2, 2DL3) may play a vital role in the protection opposing PCO and RSA in the presence of HLA-C1 ligand which ensures successful pregnancy, and KIR2DL1 is a risk factor for PCO and RSA with HLA-C2 ligand.

1. Introduction

Human pregnancy can be interrupted at different stages causing abortion, and this occurs frequently to some cases. These repeated pregnancy interruptions, as stated in Kalousek (1993), can be described in a variety of ways, including recurrent spontaneous abortion (RSA), recurrent pregnancy loss (RPL), unexplained recurrent spontaneous abortion (URSA). In their operational definitions, most of the above-mentioned terminologies describe the terminus of pregnancy which happens more than two times for a particular patient prior to her six-months pregnancy (Kalousek, 1993; Su et al., 2018; Mansour et al., 2020). Fu (2015) reported that the frequency of RSA among fertile women is approximately 5%. Some studies revealed that Spontaneous Abortion or the failure of pregnancy without any external interference prior to five months influences up until 20% of the recognized pregnancy cases. (Griebel et al., 2005; Abd- Ellatef et al., 2018).

According to Dandan and Jian (2016), the underlying causes behind RSA which affected women are still unidentified. Furthermore, roughly 60% of RSA causes are unexplored

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(Jaslow et al., 2010) and the greatest number of them are possibly linked with immunological abnormalities (Jaslow et al., 2010; Su et al., 2018).

More than 50% of the RSA reported cases are associated with innate immunity, and this chiefly attributed to NK cell quantity or activity issues (Zhang, 2010). In order for any pregnancy condition to continue effectively, NK cells should support the harmful attack of the fetal trophoblast and the growth of spiral arteries. (Hiby et al., 2010). Moreover, the interaction of NK cells with HLA produced by extravillous trophoblast cells facilities both the harmful attack and placenta's formation (Colucci, 2017). KIRs are glycoproteins categorized according to their forms and purposes. Every KIR molecule has two or three external immunoglobulin domains (called 2D molecules and 3D molecules), a transmembrane segment, and an internal tail which is either short or long (Varla-Leftherioti, 2004). The KIR gene family is located within that leukocyte receptor cluster on chromosome 19q13.4. Accordingly, the sequences of KIR and content genes haplotypes are classified as either A or B. Firstly, the A haplotype contains six inhibitory receptor centers (namely 2DL1, 2DL3, 2DL4, 3DL1, 3DL2, and 3DL3), with merely one stimulating KIR (2DS4). Secondly, the B haplotype is divided into several subgroups, which differ principally in their stimulatory receptor combinations (Vilches and Parham, 2002; Middleton et al., 2003; Middleton et al., 2005; Mansour et al., 2020). A bulk of studies found that KIR genes are of 16 identified types, seven of them are inhibitory receptors KIR2DL1-3, KIR2DL5, and KIR3DL1-3; and six genes are activating receptors KIR2DS1-5 and KIR3DS1. Furthermore, KIR2DL4 is the only activating/inhibitory receptor gene, whereas KIR2DP1 and KIR3DP1 are considered pseudogenes because they do not carry out any functional KIR receptor (Borrego et al., 2002). According to Trowsdale and Moffett (2008), KIR–HLA combinations are essential for early placentation and sufficient maternal blood flow to the fetus resulting in effective reproduction.

Despite the fact that approximately 3000 HLA-C alleles are identified, KIRs are classified into two groups: HLA-C1 and HLA-C2, which are distinguished by an amino acid dimorphism at position 80. Many genetic studies have associated KIRs and their ligands to the result of a number of infectious, autoimmune, and malignant disorders (Khakoo,2010).

Studies conducted on KIRs, such as (Xiong et al., 2013; Al Omar et al., 2015; Osman et al., 2016; Alomar et al., 2017), have been recently connected to cancer and autoimmune diseases in the Saudi population. However, some research studies revealed that there is a relationship between PCOS and the rise of spontaneous abortion rate (Terhi and Piltonen, 2016). Besides several research reports concluded that PCOS is a female-specific endocrine disease characterized by inordinate amounts of androgen production (Nina et al., 2013; Chen et al., 2017).

The causes of PCOS are still undiscovered. In their review of the pathophysiology of PCOS, (Nina et al., 2013) revealed that a woman's risk of developing PCOS is affected by both her natural surroundings and heredity. ZafariZangeneh et al. (2017) concluded that patients with PCOS exhibit symptoms of low-grade inflammation. Moreover, Sala Elpidio et al., (2018) in their recently published paper found that PCOS has an impact on the endocrine system and is associated with low-grade inflammation. These cells have the same phenotype as the regular NK cells (Gong et al., 2017). The NK cells amount reduced during the normal mensuration period proliferative stage but increased after ovulation in the phase of secretion, and all these cells stop living a couple of days prior to menstrual cycle (King, 2000; Trundley and Moffett, 2004; Yang et al., 2011). Through the release of inflammatory elements, NK cells can assist the endometrium to revive, individualize and mend (Norman and Brännström, 1996; Trundley and Moffett, 2004; Berbic et al., 2014). PCOS Endometrial signs are believed to be uterine NK cells. (Piltonen, 2016). Compared to normal women, patients who suffer from PCOS have fewer NK cells in the phase of secretion of the cell cycle, which may cause disruptions in the role NK cell effector (Matteo et al., 2010). Consequently, a homeostasis instability occurs in the woman's reproductive system and PCOS symptoms, e.g. menstruation dysregulation and sterility (Matteo et al., 2010; Yang et al., 2011; Berbic et al., 2014).

2. Materials and methods

2.1. Subjects

As stated previously, the blood samples of the current study were taken from 199 Saudi women with PCOS and PCO women with recurrent spontaneous RSA-PCO, and control individuals. The blood samples were gathered from the cases in King Khalid University Hospital. The collection of samples lasted roughly for a year, i.e. from January 2019 until January 2020. The 199 patients diagnosed differently: 65 with PCOS, 69 with PCOS and RSA, and 65 subjects representing the healthy control groups. The samples were taken from women whose ages range from 18 to 45 years old. The research commenced with 3 ml samples of venous blood draws (i.e. standard venipuncture) from all the 199 participants included in the study, i.e. patients and controls. As far as ethical considerations are concerned, the researcher received and official permission from both King Khalid University Hospital's medical ethics committee and the ethics committee of King Saud University. Furthermore, all controls and patients formally consented to participate in the current study.

2.2. KIR and HLA-C typing and genomic DNA extraction

In accordance with the manufacturer's guidelines, a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) were employed to isolate genomic DNA from peripheral blood. KIR genotyping was conducted based on the manufacturer's instructions utilizing a KIR polymerase chain reaction (PCR)-sequence-specific primer commercial typing kit (MiltenyiBiotec, Inc., Cologne, Germany), containing a kit includes a panel of locus-specific oligonucleotide primers that can be used to individually amplify 17 KIR genes separately (15 genes and two pseudogenes) besides common 2DS4 variants. These primers were also employed for HLA-C1 and HLA-C2 typing as revealed by Tajik et al. (2010). Omar et al. (2016), found that PCR was applied for every reaction. The whole PCRs were executed via a thermal cycler machine T100™ (Bio-Rad, United States). The PCR commodities were electrophoresed in two percent agarose gels spotted with ethidium bromide and monitored on a UV transilluminator using gel documentation to examine the presence or absence of gene-specific amplicons system (BioDocAnalyze system from Biometra).

2.3. Statistical analysis

In order to identify the occurrence of every KIR, HLA-C ligand, and KIR-KIR-ligand collection in the patient and control groups of the study, direct counting method was employed. The statistical program Sigma Plot version 11 was utilized to analyze the data obtained. The X^2 and the two-tailed Fisher exact test with Bonferroni correction was employed to decide if there were any differences between each KIR gene group distribution, HLA-C genotype, and KIR/HLA-C collection between the groups. Statistical significance was regarded as a $p \leq 0.05$. It estimated the magnitude of the impact by computing odds ratios (ORs) and their 95 percent confidence intervals.

3. Results

In the current case-control research, the distribution of KIR genotypes and the HLA-C allotype ligands among females with PCO was scrutinized. This includes at the minimum three inexplicable RSAs, PCO patients, and females without PCO and RSA history.

Table 1 demonstrates the comparative distribution of the percentages of the 17 KIR genes for both the PCO group and control. In this research, every single KIR gene was observed in both groups, and the framework genes existed in each healthy control woman. In comparison with control group, the PCO group significantly acquired lower frequencies for the 3DL1,2DL2, 2DS1, 2DS2, and 2DS3 genes. The control group recorded (OR = 109.33, 5.10, 2.51, 2.96 and 3.67, respectively, with $p < 0.05$).

Nevertheless, the 2DL1,2DL5 and 2DS4ins genes constituted a significantly higher frequency in the PCO group contrasted with control group (OR = 0, 0, 0.10 with $p < 0.05$).

As displayed in Table 2, when compared to the control group, HLA-C1 was examined at a significantly lower frequency in the PCO group (OR = 7.37 with $p < 0.05$). Furthermore, there were significantly lower frequencies for the heterozygote (HLA-C1C2) formation in the PCO female group compared with control group (OR = 0.19 with $p = 0.0001$). Yet, among the PCO group, significantly higher frequencies for the HLA-C2 and the homozygote form (HLA-C2C2) were detected in the PCO group in comparison with the control group (OR = 5.91, 3.09 with $p < 0.05$).

Table 3 summarizes the findings of the investigation of the combined relationship of the KIR genes and the HLA-C ligand with PCO. The analysis also revealed that the 2DL1,2DL2 and 2DL3 genes had a connection with some combinations with the HLA-C ligand. Accordingly, a preventive impact was detected for 2DL2,2DL2/2DL3,2DS2 with HLA-C1 ligand. Also, there were significantly lower frequencies for them in PCO when compared to control group (OR = 25.65, 17.7, 10.5 with $p < 0.05$ respectively). Moreover, 2DL2,2DL2/2DL3 in the existence of its C1 ligand in the homozygote (HLA-C1C1) formations were noticed. In addition, there were significantly lower frequencies in PCO as opposed to the control group (OR = 9.45, 10.285 with $p = 0.001$, 0.0005 respectively). Besides, a preventive impact was detected for 2DL1, 2DL2,2DL3, 2DL2/2DL3, and 2DS2 with their ligands HLA-C1 in heterozygote (HLA-C1C2) forms (OR = 3.66, 30.5, 4.81, 22.6, 18,4 with $P < 0.05$ respectively). Conversely, the 2DL1 with its HLA-C2 ligand in the homozygote (HLA-C2C2) reported significantly higher frequencies in PCO compared to the control group (OR = 0.27, $p = 0.001$). 2DL1 was detected higher frequencies in PCO in comparison with control in the disappearance of HLA-C2 (OR = 0.381, $p = 0.03$). Nevertheless, 2DS2, 2DL3 showed significantly higher frequencies in PCO when compared to the control in the disappearance of HLA-C1 (OR = 0.24, 0.05 with $p < 0.05$).

Additionally, the comparative distribution of the percentages of the 17 KIR genes for the RSA-PCO and control groups is illustrated in Table 4. Based on the analysis of the current research, all KIR genes were identified in both groups, and the framework genes existed in every single healthy woman in the control group. Among the RSA-PCO group, KIR3DL1, 2DL2 genes were considered significantly lower frequent in comparison with the control group (OR = 337.45, 4.59 with $p < 0.05$). Yet, the 2DL1 gene acquired a significantly higher frequency in the RSA-PCO group. In addition to this, the frequency of 2DS4dl reached a higher level in the RSA-PCO group (OR = 0.0722 with $p = 0.001$). Conversely, 2DS4ins and 2DS2 genes were listed at significantly lower frequencies than the control group (OR = 39.08, 2, 49 respectively with $p < 0.05$). The occurrence of 2DS5, 3DS1 genes showed significantly higher frequency in the RSA-PCO group than the control one (OR = 0.4428, 0.2102 with $p = 0.032$, 0.0001 respectively).

Table 5 demonstrates the distribution of HLA-C groups and genotypes. It reveals that HLA-C1, HLA-C1C2 in the RSA-PCO group include significantly lower frequencies if compared with the control group (OR = 4.13, 2.58 with $p = 0.0001$, 0.01 respectively).

Apart from that, Table 6 reports the results obtained from the analysis of the combined association of the KIR genes and HLA-C ligands with RSA-PCO. The 2DL2 and 2DL3 genes were detected to be linked with some combinations with the HLA-C ligand. Accordingly, a preventive impact displayed 2DL2, 2DL3, and 2DL2/2DL3 in the existence of its C1 ligand HLA-C1 and the homozygote (HLA-C1C1) formations. Compared to the control group (OR = 5.34, 12.2, 4.51, 4.87, 5.30 $p < 0.05$, respectively), the frequencies in the RSA-PCO group were significantly lower. Furthermore, the preventive impact was detected for 2DS2 with its ligand HLA-C1 (OR = 5.4 with $P < 0.05$). Conversely, the 2DL3 and 2DS2 in the existence of HLA-C1 ligand had significantly higher frequencies in the RSA-PCO group when compared to the control one (OR = 0.13, 0.34 with $p = 0.00001$, 0.01 respectively). Yet, 2DL1 indicated higher frequencies in the RSA-PCO group than their counterparts when HLA-C2 exists and the homozygote (HLA-C2C2) formations (OR = 0.45, 0.24 with $p = 0.05$, 0.0001 respectively). In spite of this, 2DS2 ,2DL2 ,2DL3, 2DL2/2DL3 genes recorded significantly lower frequencies in RSA-PCO group in comparison with the control one in the appearance of its C1in the homozygote formation (HLA-C1C2) (OR = 3.08, 3.63, 2.4, 3.13 $p = 0.006$, 0.006, 0.02, 0.02).

Table 1
Comparison of killer immunoglobulin-like receptor gene frequencies between polycystic ovarian syndrome women and controls.

Genes	PCO No.	CONTROL No.	OR	CI 95%	P
3DL2	65	65	NA	NA	1
3DL3	65	65	NA	NA	1
2DL4	65	65	NA	NA	1
3DP1	65	65	NA	NA	1
2DL1	65	53	0	0-NaN	0.0003
2DL3	56	53	0.710	0.276–1.821	0.634
3DL1	24	64	109.333	14.238–839.561	2.10E−15
2DS4 dl	56	54	0.789	0.303–2.054	0.809
2DS4 ins	65	35	0	0-NaN	2.30E−11
2DL2	22	47	5.104	2.416–10.780	1.98694E−05
2DL5	62	44	0.101	0.029–0.361	6.4235E−05
2DS1	11	22	2.512	1.098–5.744	0.043
2DS2	33	49	2.970	1.410–6.255	0.006
2DS3	18	38	3.675	1.764–7.655	0.0007
2DS5	17	18	1.081	0.498–2.348	1
3DS1	17	10	0.513	0.215–1.228	0.194
2DP1	63	65	Infinity	NaN-Infinity	0.496

Table 2
Frequencies of the killer immunoglobulin-like receptor ligand C1/C2 in polycystic ovarian syndrome women and healthy control groups.

	PCO n = 65			CONTROL n = 65					
Gene/genotype	positive	freq	negative	positive	freq	negative	OR	CI 95%	P
C1	18	27.70%	47	48	74%	17	7.373	3.395–16.008	2.28E−07
C2	44	68%	21	48	74%	17	5.916	2.769–12.639	3.61815E−06
C1C2	10	15.38%	55	31	48%	34	0.199	0.087–0.458	0.0001
C1C1	8	12.30%	57	17	26%	48	0.396	0.157–0.998	0.074
C2C2	34	52.30%	31	17	26%	48	3.097	1.482–6.470	0.004

Table 3
Distribution of the frequencies of killer immunoglobulin-like receptor genes in presence and absence of their C1C2 ligand between polycystic ovarian syndrome patients and controls.

	PCO n = 65			Control n = 65					
Kir Ligand	positive	Negative	freq	positive	Negative	freq	OR	CI 95%	P
2DL2 + C1+	3	62	4.6	36	29	55.3	25.655	7.294–90.233	9.70E−11
2DL2/3 + C1+	3	62	4.6	30	35	46	17.714	5.039–62.270	3.58E−08
2DS2 + C1+	7	49	10.7	39	26	60	10.5	4.124–26.735	6.93E−08
2DL2 + C1C1+	2	63	3	15	50	23	9.45	2.064–43.269	0.001
2DL2/3 + C1C1+	2	63	3	16	49	24.6	10.286	2.257–46.872	0.0005
2DS2 + C1C1+	5	60	7.7	11	54	16.9	2.444	0.798–7.486	0.180
2DL2 + C1C2+	1	64	1.5	21	44	32.3	30.546	3.962–235.505	1.8735E−06
2DL2/3 + C1C2+	1	64	1.5	17	48	26	22.667	2.914–176.293	4.9416E−05
2DS2 + C1C2+	2	63	3	24	41	36.9	18.439	4.134–82.24	1.1174E−06
2DL2 + C1−	19	46	29.2	10	55	15	0.440	0.186–1.040	0.090
2DL2/3 + C1−	15	50	23	7	58	10.7	0.402	0.152–1.065	0.100
2DS2 + C1−	26	39	40	9	56	13.8	0.241	0.102–0.570	0.001
2DL3 + C1+	15	50	23	40	25	61.5	5.333	2.486–11.442	1.5943E−05
2DL1 + C2+	43	22	66	43	22	66	1	0.484–2.068	1
2DL3 + C1C1+	6	59	9.2	14	51	21.5	2.699	0.966–7.540	0.087
2DL3 + C1C2+	9	65	13.8	26	39	40	4.815	2.046–11.329	0.0001
2DL3 + C1−	42	23	64.6	6	59	9.2	0.056	0.021–0.149	3.61E−11
2DL1 + C2C2+	34	31	52.3	15	50	23	0.274	0.129–0.582	0.001
2DL1 + C1C2+	10	55	15	26	39	40	3.667	1.588–8.466	0.003
2DL1 + C2−	21	44	32.3	10	55	15	0.381	0.163–0.892	0.039
2DS1 + C2+	7	58	10.7	15	50	23	2.486	0.939–6.581	0.100

Table 4
Comparison of killer immunoglobulin-like receptor gene frequencies between recurrent spontaneous abortion women who have poly-cystic ovarian syndrome and control groups.

Genes	RSA-PCO No.	CONTROL No.	OR	CI 95%	P
3DL2	69	65	NA	NA	1
3DL3	69	65	NA	NA	1
2DL4	69	65	NA	NA	1
3DP1	69	65	NA	NA	1
2DL1	69	53	0	0	9.55983E−05
2DL3	55	53	1.124	0.477–2.653	0.830
3DL1	11	64	337.455	42.253–2695.107	2.26E−25
2DS4 dl	68	54	0.072	0.009–0.577	0.002
2DS4 ins	2	35	39.083	8.820–173.175	4.84E−12
2DL2	25	47	4.596	2.210–9.557	3.16756E−05
2DL5	56	44	0.486	0.219–1.079	0.079
2DS1	21	22	1.169	0.566–2.417	0.714
2DS2	34	38	2.498	1.195–1.195	0.018
2DS3	34	38	1.449	0.732–2.868	0.304
2DS5	32	18	0.443	0.215–0.910	0.032
3DS1	32	10	0.210	0.092–0.479	0.0001
2DP1	68	65	Infinity	NA-Infinity	1

4. Discussion

To the researcher's best knowledge, the current study is regarded the first investigation on the relationship between KIR gene polymorphism and polycystic ovarian syndrome with frequent spontaneous abortion as well as the relationship between KIR gene polymorphism and polycystic ovarian syndrome with no RSA in the Arabian ethnic group. The study asserts that there is a connection between individual KIRs and some of their combinations with the HLA-C maternal ligand and the success versus failure of pregnancy and PCO in a Saudi population. The results also indicated recurrent existence of the KIR genes, 3DL1, 2DL2, 2DL3, 2DS1,2DS2 among the healthy group in comparison with the PCO group. The highest and significant relationship with prevention opposing PCO was recorded with the 3DL1,2DL2,2DL3,2DS1,2DS2 genes. The same finding was reported by Faridi et al., (2009), that study revealed that KIR2DL3 was protective factor. Interestingly, the 2DL2,2DL3, 2DS1,2DS2 molecules

Table 5
Frequencies of the killer immunoglobulin-like receptor ligand C1/C2 in Recurrent spontaneous abortion women who have poly-cystic ovarian syndrome and control groups.

	RSA-PCO n = 69			CONTROL n = 65					
Gene/genotype	positive	freq	negative	positive	freq	negative	OR	CI 95%	P
C1	28	40.60%	41	48	74%	17	4.135	1.987–8.603	0.0001
C2	56	81%	13	48	74%	17	0.656	0.289–1.486	0.407
C1C2	18	26.00%	51	31	48%	34	2.583	1.251–5.334	0.012
C1C1	10	14.40%	59	17	26%	48	2.090	0.876–4.983	0.131
C2C2	38	55.00%	31	17	26%	48	0.656	0.289–1.486	0.407

Table 6
Distribution of the frequencies of killer immunoglobulin-like receptor genes in presence and absence of their C1C2 ligand in recurrent spontaneous abortion women who have poly-cystic ovarian syndrome and control groups.

	RSA-PCO No. (%) – n = 69			Control-n = 65					
Kir Ligand	positive	Negative	freq%	positive	Negative	freq	OR	CI 95%	P
2DL2 + C1+	13	56	18	36	29	55.3	5.348	2.459–11.627	1.37053E−05
2DL2/3 + C1+	11	58	15.9	30	35	46	4.520	2.014–10.142	0.0001
2DS2 + C1+	15	54	21.7	39	26	60	5.4	2.532–11.516	8.40913E−06
2DL2 + C1C1+	4	65	5.7	15	50	23	4.875	1.524–15.597	0.006
2DL2/3 + C1C1+	4	65	5.7	16	49	24.6	5.306	1.669–16.871	0.003
2DS2 + C1C1+	5	64	7.2	11	54	16.9	2.607	0.853–7.971	0.111
2DL2 + C1C2+	8	61	11.5	21	44	32.3	3.639	1.477–8.968	0.006
2DL2/3 + C1C2+	7	62	10	17	48	26	3.137	1.204–8.172	0.023
2DS2 + C1C2+	11	58	15.9	24	41	36.9	3.087	1.362–6.994	0.006
2DL2 + C1−	12	57	17.3	10	55	15.3	0.864	0.345–2.161	0.818
2DL2/3 + C1−	11	58	15.9	7	58	10.7	0.636	0.231–1.756	0.452
2DS2 + C1−	22	47	31.8	9	56	13.8	0.343	0.144–0.817	0.015
2DL3 + C1+	8	61	11.5	40	25	61.5	12.2	5.008–29.722	1.04E−09
2DL1 + C2+	56	13	81	43	22	66	0.454	0.205–1.002	0.052
2DL3 + C1C1+	8	61	11.5	14	51	21.5	2.093	0.814–5.385	0.162
2DL3 + C1C2+	15	54	21.7	26	39	40	2.4	1.125–5.118	0.025
2DL3 + C1−	30	39	43.4	6	59	9.2	0.132	0.050–0.347	1.07037E−05
2DL1 + C2C2+	38	31	55	15	50	23	0.245	0.116–0.517	0.0002
2DL1 + C1C2+	18	51	26	26	39	40	1.889	0.909–3.925	0.0997
2DL1 + C2−	13	56	18.8	10	55	15.3	0.7832	0.317–1.935	0.652
2DS1 + C2+	15	54	21.7	15	50	23	1.08	0.479–2.434	1

engage with the C1 ligand. The existence of both genes (2DL2/2DL3) has a correlation with the reduced PCO. Moreover, the frequency of PCO was reported with KIR2DL1, KIR2DL5 genes. It is worth mentioning that the biological significance of KIRs in vivo relies on if these receptors simultaneously exist in the individuals with their ligands. A combined examination asserted that the KIR2DS2,2DL2, 2DL3,2DL2/2DL3 genes contain a strong preventive impact with its specific HLA-C1 allele in homozygote and heterozygote forms. In addition to this, this prevention was not efficient anymore in the disappearance of its ligand that was detected in KIR2DS2 and KIR2DL3 with the absence of HLA-C1. During the KIR-HLA-C interaction impact, the activation of KIR2DL1 displayed a defective impact whenever its HLA-C2 ligand disappeared. On the other hand, the highest and significant connection with prevention against unsuccessful pregnancy in PCO women was detected with the 3DL1,2DS4ins,2DL2, 2DL2/2DL3, 2DL3 and 2DS2 genes, and they have a powerful preventive impact with its specific HLA-C1 allele both in homozygote and heterozygote formations. Besides, this prevention disappeared when the absence of its ligand noticed in KIR2DS2 within the missing of HLA-C1. The activating KIR2DL1 displayed a defective impact whenever its HLA-C2 ligand did not exist. As far as the frequency of repeated abortion correlation to PCO is concerned, the higher risk with 2DL1, 2DS4dl,2DS5, and 3DS1 was detected. There was modern study agree with our finding this study revealed that the KIR genes and their HLA ligands were considered as the markers of the immunopathogenesis of the disease, KIR3DS1 and KIR2DS4-*del* in homozygosis were associated with susceptibility to PCOS (Sala Elpidio et al., 2018). Additionally, much research has revealed that PCOS patients have many endometrial abnormalities. This could explain some of the adverse endometrium-related results in these patients. Terhi and Piltonen (2016) pointed out that PCOS and an increase of miscarriage rate may coincide. It is worth noting that no single up to now has investigated the relationship between polycystic ovarian syndrome with frequent abortion that shows a task for the KIR gene and HLA-C ligands. However, there was a single study on PCO with KIRs revealed that PCOS has an impact on the endocrine system and is associated with low-grade inflammation (Sala Elpidio et al., 2018). Low-grade inflammation is scrutinized in PCOS women (ZafariZangeneh et al., 2017). NK cells in the uterus are regarded to be endometrial signs for PCOS (Piltonen, 2016). Unlike normal women, PCOS patients have declined figures of NK cells within the secretory stage of the cell cycle resulting in interruptions that possibly happen within the effector of NK cells functions (Matteo et al., 2010), leading to a homeostatic imbalance of the female reproductive system, culminating in PCOS manifestations such as menstruation dysregulation and sterility (Yang et al., 2011; Berbic et al., 2014). NK cells are parts of the protection of the female reproductive system, folliculogenesis, ovulation, and the menstrual cycle. On the surface of NK cells, the KIR control the stimulation and function of those cells after engaging with HLA class I ligands (Sala Elpidio et al., 2018). That research, which included 197 Brazilian women participants: 93 patients 104 controls, identified higher frequencies of KIR3DS1-Bw4 and homozygotic KIR2DS4-del in patients in comparison with controls, proposing that this locus confer susceptibility to PCOS. Also, a lower frequency of KIR2DS4-full was recorded in patients, which demonstrates that the KIR and its HLA ligands

were associated with PCO development (Sala Elpidio et al., 2018). This finding is in accordance with our results in the current study. Concerning RSA, there is a recent Saudi study in the women population conducted by Mansour et al. (2020) discovered that the KIR2DS2 and KIR2DL5A were significantly lower among RSA women in comparison to healthy controls. Besides, the study explained that the analysis of KIR-HLA-C combinations demonstrated a preventive impact of KIR2DS2 with its equivalent HLA-C1 ligand in both homozygote and heterozygote combinations, which is consistent with our results. Contrary to our results, a recent Turkish study in a women population with Unexplained Recurrent Pregnancy Loss (URPL) found a significant association between the KIR genotypes and URPL. It showed that the KIR 2DL1, 2DL2, 2DL3, 2DL4, 2DS1, 2DS2, 2DS4, and 2DS5 polymorphisms are related to URPL. The 2DS3 genotype was not identified in neither the case nor control group (Ay et al., 2019). Another study found that the role of KIR3DL1 in RSA was statistically significant ($p = 0.044^*$; OR = 0.833; fixed), (Akbari et al., 2020). Faridi et al. (2009) reported a significantly more preventive impact of this gene. In the current study, the researchers noticed that the KIR2DL1 constituted the highest significant risk for PCO with the RSA group and in PCO without the RSA group in the existence of its ligand HLA-C2. This finding is consistent with Hiby et al. in 2004, whose study revealed that the interaction between extra villous trophoblast and uterine NK cells, and the combination of fetal HLA-C2 and maternal AA KIR genotype, which is anticipated to provide the strongest inhibitory signals to NK cells. C2 is related more closely to its equivalent KIR than C1, and maternal NK cells of the AA genotype can express the inhibitory C2 receptor (KIR2DL1) but not the stimulating KIR that possibly carry compensatory stimulating signals when compared illness condition and maternal KIR genotype for every single pregnancy case including a C2 fetus (Hiby et al., 2004).There was another study agree with our finding which explained by Akbari et al., (2020),this study revealed that the presences of HLA-C2 with KIRs considered a risk factor for RSA. Interestingly. The findings of the current study is consistent with the Northern Irish study conducted by Flores et al. (2007) which included 227 participants: 139 control and 88 patients. Their study revealed that RSA patients presented a low number of KIR inhibitory receptors, particularly KIR2DL2. The former is described to contain a stronger inhibitory capacity than 2DL3 through its higher affinity to C1 (Flores et al., 2007). However, there is still no clear explanation concerning the increased frequency of the AA genotype with a predominance of inhibitory receptors in a subset of RSA patients. The NK activity occurred due to a balance between impeding and stimulating receptors. In this context, 2DL1, representing the highest inhibitory receptor, exists in all AA patients, and the interaction 2DL1–HLA-C2 is the one that better supports to an unfavorable balance in NK indication. Yet, AA patients will be homozygous for 2DL3, that is opposing 2DL2, will indicate a lesser amount of interaction with C1 ligands and, consequently, a weaker inhibition. Thus, the declined frequency of 2DL2 existing in RSA possibly support the total balance between activation and inhibition that impede NK activity become a stimulating condition that may contribute to the loss of pregnancy (Flores et al., 2007). In their study which involved 259 Caucasian participants: 160 RSA and 99 control, among KIR2DL2 patients with RPL, Yang et al., (2020) found that the less co-expression of HLA-C1C1 and higher frequencies of HLA-C2C2 in the partners are linked to RPL. This study is also consistent with the current research. Besides, in Han Chinese population in 110 RSA and 105 control indicated the high rate of HLA-C2C2 in KIR AB and KIR BB women possibly decline the expression of the KIR2DL1 receptor and cause powerful stimulation impact, which breaks the self-regulation of NKs and stimulates NK-mediated immunological dismissal of the fetus(Su et al., 2018). Additionally, the study found that women who had frequent natural abortion are three times greater than or equal to the frequency of KIR3DL1 which was significantly lower, and the BB haplotype frequency was significantly higher in comparison with the control group (Su et al., 2018). Apart from that, Varla-Leftherioti et al. (2005) found a lower frequency of inhibitory KIRs (inhibitory 2DL1, 2, and 3) in the peripheral blood and decidual tissue of women suffering multiple miscarriages, compared with productive women. In the current study, the researchers found that the HLA-C2 increased in patient group, is consistent with the study carried out by Hiby et al. (2010), which revealed that trophoblast expresses both paternally and maternally inherited HLA-C surface proteins and that maternal KIR AA frequencies are high in affected pregnancies only when the fetus has more group HLA-C2 genes (C2). Wilczyńska et al. (2019) observed that the susceptibility to RSA increased strongly at the presence of HLA-C2 and KIR Bx genotypes, which we explored in our result also. To conclude, PCOS is still ambiguous; a great deal of information concerning different features of etiology and controlling are inadequately comprehended. Accordingly, further research is required to advance the existing body of knowledge on PCOS. As the earlier research evidenced PCOS and an increase of the natural abortion rate had been suggested to coincide (Terhi and Piltonen, 2016), we revealed that in our research, but further investigation is needed in this field and more justifications are required.

Conflicts of interest

All authors declare that they have no conflict of interests in this manuscript.

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