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INSR gene Exome Sequencing Results in patients with PCOS

PKOS'lu Hastalarda *INSR* geni Ekzom Dizileme Sonuçları

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Öz

Giriş ve Amaç: Polikistik over sendromu (PKOS), içerisinde insülin direnci, infertilite, hiperandrojenizm gibi bulgularının yer aldığı bir endokrinopatidir. Genetik alt yapısı çeşitli genlerde bulunan varyasyon ve mutasyonlarla araştırılmaya devam etmektedir.
Gereç ve Yöntemler: Bu çalışmada AE-PKOS kriterlerine göre prospektif 16 PKOS hastasının klinik ve laboratuvar bulguları değerlendirip ve *INSR* geni ekzom dizileme yöntemi ile analiz ettik.
Sonuçlar: Çalışmamızda *INSR* genine ait toplamda 4 novel varyasyonu ortaya çıkardık. Bunlar; NM_000208.4:c.974+82_974+83insT, NM_000208.4:c.974+107_974+108insC, NM_000208.4:c.653-79A>G ve rs1449625253 intronik varyasyonlarıdır. Toplamda 41 çeşit varyasyonu içeren 196 varyasyon ortaya çıkardık. *INSR* geninde bulduğumuz 27 bilgi verici SNP kullanılarak ilk kez bu çalışmada, yüksek linkaj eşitsizliği skoru (r^2=1) gösteren 2 farklı üçerli tag SNP bloğunu saptadık. Bunlar; rs2963-rs2245649-rs2245655 ile rs6413502-rs41509747-rs73498780 bloklarıdır. Ayrıca, en sık görülen rs7508516 ve hastalarımızda da gözlemlediğimiz ve PKOS'dan sorumlu olabilecek rs2059807 gibi varyasyonların MAF değerlerini ortaya çıkardık.
Tartışma: Çalışma Türkiye'de PKOS hastalarının *INSR* genlerinin ekzom dizileme yöntemi ile araştırıldığı ilk çalışmadır. *INSR* genine ait tag SNP'lerin ilk kez ortaya çıkarıldığı ve yeni aday varyasyonları bulduğumuz çalışma literatüre önemli veriler sunmaktadır.

Anahtar kelimeler: PKOS, tag SNP, *INSR* geni, linkaj eşitsizliği, ekzom dizileme

Abstract

Objective: Polycystic ovary syndrome (PCOS) is an endocrinopathy that includes findings such as insulin resistance, infertility and hyperandrogenism. Its genetic background continues to be investigated with variations and mutations in various genes.
Material and Methods: In current study, we evaluated the clinical and laboratory findings of 16 prospective PCOS patients according to AE-PCOS criteria and analyzed the *INSR* gene by exome sequencing method.
Results: In our study, we revealed a total of 4 novel variations of the *INSR* gene. These are NM_000208.4:c.974+82_974+83insT, NM_000208.4:c.974+107_974+108insC, NM_000208.4:c.653-79A>G and rs1449625253 and are all intronic variations. In this study, using the 27 informative SNPs that we found in the *INSR* gene, we detected two different triple tag SNP blocks showing high linkage disequilibrium score (r^2 = 1) for the first time. These blocks are rs2963-rs2245649-rs2245655 and rs6413502-rs41509747-rs73498780. We also revealed the MAF values of variations such as rs7508516, which is the most common, and rs2059807 (0.25) which we observed in our patients and may be responsible for PCOS.
Conclusion: The study is the first study in Turkiye in which the *INSR* genes of PCOS patients were investigated by exome sequencing method. The study, in which the tag SNPs of the *INSR* gene were revealed for the first time and we found new candidate variations, provides important data to the literature.

Keywords: PCOS, tag SNP, *INSR* gene, linkage disequilibrium, exome sequencing

1.Introduction

Polycystic ovary syndrome was first associated with the polycystic structure of the enlarged ovary with symptoms of obesity, hirsutism, infertility and amenorrhea in 1936 [1]. It is reported that the worldwide prevalence of PCOS reaches up to 13%. The presence of polycystic ovaries on ultrasonography, infertility, acne, amenorrhea or oligoamenorrhea, hirsutism, hyperandrogenism, obesity and insulin resistance are among the clinical and laboratory findings of PCOS [2,3]. Metabolic diseases such as hepatic steatosis, glucose intolerance, dyslipidemia, type 2 diabetes and hypertension are involved in the etiology of PCOS. [4].

Clinical diagnosis of PCOS can be made based on the decisions taken at the Androgen Excess and Polycystic Ovary Society (AE-PCOS) conferences in 2008, as well as the National Institute of Health (NIH-1990), Rotterdam-2003 and AE-PCOS criteria [5–7].

Various variations of many genes have been studied in patients with PCOS. These include genes responsible for ovarian and adrenal steroidogenesis [8–10] and genes involved in insulin signaling [11–18]. Genetic studies that can relatively explain insulin resistance in PCOS began in 1990 with the discovery of various variations in the *INSR* gene by Conway and colleagues [19]. In our study, we aimed to investigate *INSR* gene (MIM *147670) mutations and variations in 16 PCOS patients, whom we determined according to AE-PCOS-2008 criteria, by exome sequencing method. We evaluated the frequencies of the variations we found in our patient population.

2.Material and Methods

We isolated the gDNA from the peripheral blood of 16 adult PCOS patients, which we determined according to the AE-PCOS criteria, with the help of a commercial kit (Pure Link™). We sequenced appropriate dilutions (20 ng/µl) of the genetic material, including the *INSR* gene (NM_000208.4) and UTR regions, by preparing the ION AmpliSeq Custom panelFor this, we amplified the total number of nucleotides into 29 amplicons with a length of 4589 nucleotides. After evaluating the variants by taking account into Genome Reference Consortium Human Build 37 (GRCh37, a.k.a hg19), we analyzed them in the Integrative Genomics Viewer (IGV, version 2.16.1) panel. By employing University of Santa Cruz California (UCSC) and the Genome Aggregation Database (gnomAD) databases, we organized them with the help of Microsoft® 365 Excel®. We compared the *INSR* gene (NCBI gene ID:3643) variations per patient, the types of variation profiles, their frequencies, and their rates in databases. We calculated and presented the average age, homeostasis model assessment-estimated insulin resistance (HOMA-IR) values, and bodymass index (BMI) values of our patients as mean ± standard deviation. We performed haplotype analyzes using the internet-based GVS Server 150 [20]. We compared the linkage disequilibrium (LD) scores of SNPs in normal populations by using one of internet based web site [21] with our own LD scores.

The results were interpreted as mean±sd or as percentages, when appropriate. We analyzed data using descriptive statistical methods (mean, standard deviation, median, frequency) by using Microsoft Excel (2013).

3.Results

The average age of our patients was 22.3±4.3 HOMA-IR values were 2.6±1.9, and BMI was 26.8±5.3 kg/m^2. While the ratio of those with a BMI >25 kg/m2 was 62.5%, of those with a HOMA-IR value >2.5 was 31.5%.

The total number of variations in the *INSR* gene was 196 and the average number of variations was 12.3±5.1 The number of variations found was 41. 70.7% of the variations were intronic, 26.8% were synonymous variants and 2.4% were missense mutations (Table 1).The most common SNPs are rs7508518 (100%, n=16), rs2252673 (87.5%, n=14), rs2059807 (81.25%, n=13), rs2059806 (56.25%, n=9) and rs2860178 (56.25%, n=9) variations were seen (Figure 1). We detected 8 different variations that were found only one time in patients. These are the variations rs1972808679, rs140361295, rs73498780, rs41509747, rs764580348, rs199750451 and rs56012021. The patient with the highest variation is the PCOS3 (22 variations), and the patient with the least variation is PCOS12 (5 variations) (Figure 2). We found different variations involving position of chr19:7,184,672. These variations; one of which is novel (chr19:7184672G>A, Figure 3), the other of which is unknown frequency (rs1974375611, chr19:7184672-7184674GGG>AAT, Figure 4). Novel variation (G>A) was seen isolated in 3 of our patients (PCOS2, 3 and 6) and together with rs1974375611 in 4 of our patients (PCOS1, 4, 5 and 8). Novel variation (rs1449625253) has not been hit before in any population that was investigated in TOPMED data set but has rs number without unknown reason while reference allel (guanine) has been found in all individuals. The frequency of the rs1974375611 variation in our patient population was found to be 25% (n = 4).

Table 1. Forty one *INSR* gene variations in 16 patients with PCOS

Variant	Position	Rs Number	Positive case/Population Size	Ratio (%)	Variant Type
1	chr19:7117487	11883325	8377/31342	28.3	intronic
2	chr19:7117506	11883202	8881/31326	28.3	intronic
3	chr19:7119636	367614576	7639/272522	<1	intronic
4	chr19:7119651	184752862	8881/31326	28.3	intronic
5	chr19:7119653	187956009	2339/31020	7.5	intronic
6	chr19:7119658	13306448	2988/31318	9.5	intronic
7	chr19:7125297	1799817	63149/280798	22.5	Benign synonymous
8	chr19:7125297	1799815	14073/282414	<1	Benign synonymous
9	chr19:7132397	1972808679	1/140254	<1	intronic
10	chr19:7141505	73498780	1481/31384	<1	intronic
11	chr19:7141775	2229431	17926/282848	6.3	Benign synonymous
12	chr19:7142064	140361295	256/30780	<1	intronic
13	**chr19:7150418**	**2252673**	28384/31348	75	intronic
14	chr19:7150491	41509747	6441/282176	<1	intronic
15	chr19:7152717	6413502	4823/280838	<1	intronic
16	chr19:7163230	2245648	64019/282402	22.7	intronic
17	**chr19:7166109**	**2059807**	19271/31132	61.9	intronic
18	chr19:7166388	2229429	57214/282048	20.3	Benign synonymous
19	chr19:7167951	2860177	72548/282414	25.7	Benign synonymous
20	chr19:7163140	2245655	28509/282808	10	Benign synonymous
21	chr19:7163154	2963	28488/282812	10	Benign synonymous
22	chr19:7163214	2245649	28436/282566	10	intronic
23	chr19:7166138	3815902	67808/282608	23.9	intronic
24	chr19:7166376	2059806	71704/282234	25.4	Benign synonymous
25	chr19:7167817	1366234	6575/31342	20.9	intronic
26	chr19:7163065	2962	18781/282776	6.6	Benign synonymous
27	chr19:7170505	7252268	62920/281962	22.3	intronic
28	chr19:7170517	2860178	132152/282196	46.8	intronic
29	chr19:7172526	41412545	116/31366	3.5	intronic
30	chr19:7184238	6510959	6343/31232	20.3	intronic
31	chr19:7184243	41315074	4286/31258	13.7	intronic
32	chr19:7184721	13306455	4382/30928	14.2	intronic
33	chr19:7184275	2860179	4099/31312	13	intronic
34	chr19:7184518	891087	28054/282304	9.9	Benign synonymous
35	chr19:7184669	76480566	1/162106	<1	intronic
36	chr19:7184672	Novel*	0/11862	0	intronic
37	**chr19:7184673**	**1974375611****	**4/16 (current study)**	**25**	**intronic**
38	chr19:7184674	199750451	47/168800	<1	intronic
39	chr19:7184675	57380348	60/20519	<1	intronic
40	chr19:7267390	56012021	697/282854	<1	Benign synonymous
41	**chr19:7293898**	**7508518**	30501/30504	99.9	Missense variant

Table 2. Three novel variations in *INSR* gene in the current study

Patient number	Chromosome position	Frequecy	Suggested nomenclature
2,4,6-9,11,13-15	Chr19:7184241	62.5%	NM_000208.4:c.974+82_974+83insT
2,4-11,13-15	Chr19:7184266	75%	NM_000208.4:c.974+107_974+108insC
2-7	Chr19:7184715	37.5%	NM_000208.4:c.653-79A>G

In our haplotype analysis (Table 3), which we carried out using 27 suitable SNPs that were informative for all patients at the same time, we found 2 different sets of 3 SNPs that could be used as tag SNPs (figure 5). We found that the variations rs2963-rs2245649-rs2245655 and rs6413502-rs41509747-rs73498780 have the highest LD scores (r^2=1) among themselves (figure 6). We showed that among the twenty-seven SNPs, the SNPs with the highest heterozygosity values were the rs3815902 (G/A), rs2059806 (T/C) and rs2860178 (A/G) changes, respectively.

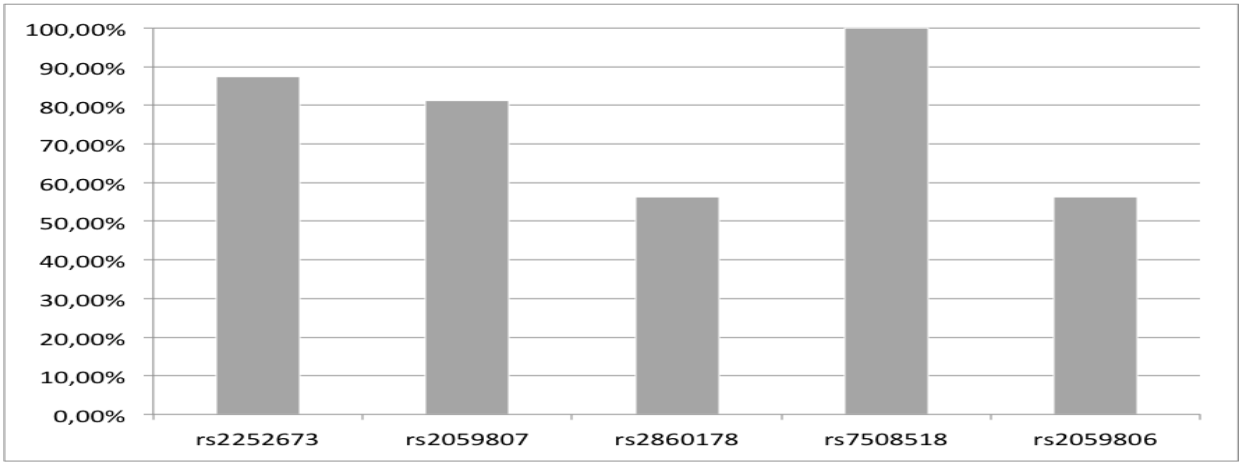

Figure 1. The most common variations of *INSR* gene in PCOS patients in current study

Table 3. Allelic calculation of 27 variations that were used in haplotype analysis

SNP*	Allele	Minor Allele	MAF (%)	Heterozygosity	Hardy-Weinberg Chi-Square
rs1847522862	A/ G	A	19	0.3	0.85
rs187956009	A/ G	A	13	0.22	0.33
rs13306448	T/ C	T	13	0.22	0.33
rs1799817	A/ G	A	16	0.26	0.55
rs1799815	A/ G	A	9	0.17	0.17
rs1972808679	G/ A	G	6	0.12	16
rs73498780	T/ G	T	3	0.06	0.02
rs2229431	A/ G	A	9	0.17	6.39
rs140361295	T/ C	T	3	0.06	0.02
rs2252673	C/ G	C	22	0.34	3.26
rs41509747	A/ G	A	3	0.06	0.02
rs6413502	T/ G	T	3	0.06	0.02
rs2962	A/ G	A	6	0.12	0.07
rs2245655	G/ T	G	13	0.22	2.94
rs2963	A/ G	A	13	0.22	2.94
rs2245649	C/ T	C	13	0.22	2.94
rs2245648	C/ T	C	16	0.26	1.34
rs2059807	A/ G	A	25	0.38	7.11
rs3815902	G/ A	G	47	0.5	2.22
rs2059806	T/ C	T	44	0.49	0.91
rs2229429	A/ G	A	25	0.38	0
rs7252268	A/ C	A	25	0.38	0
rs2860178	A/ G	A	38	0.47	0.64
rs41412545	A/ C	A	9	0.17	0.17
rs6510959	A/ G	A	28	0.4	0.82
rs41315074	G/ C	G	22	0.34	0.12
rs2860179	T/ C	T	13	0.22	2.94

*monomorphic sites (rs7508518) were excluded.

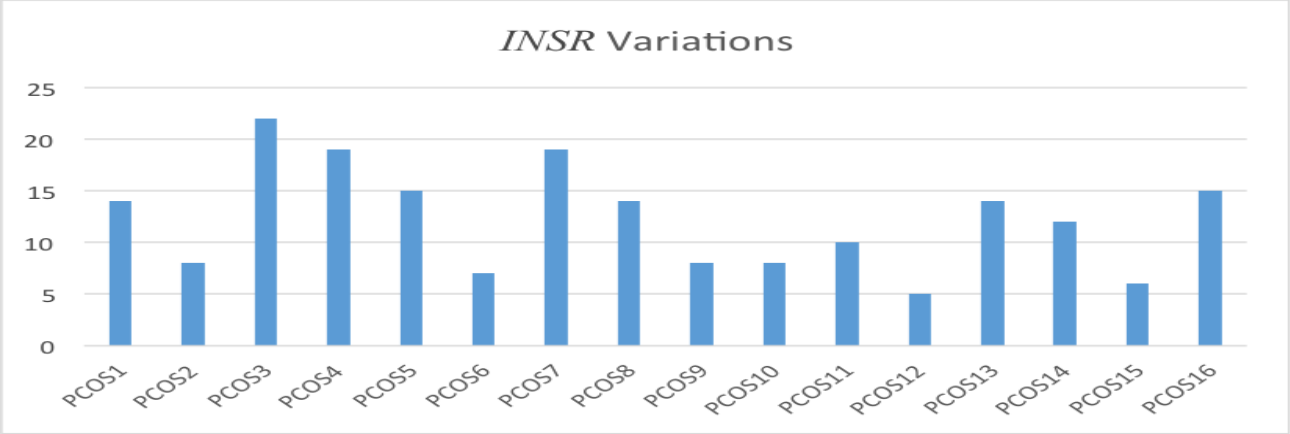

Figure 2. *INSR* gene variation counts vs PCOS patients

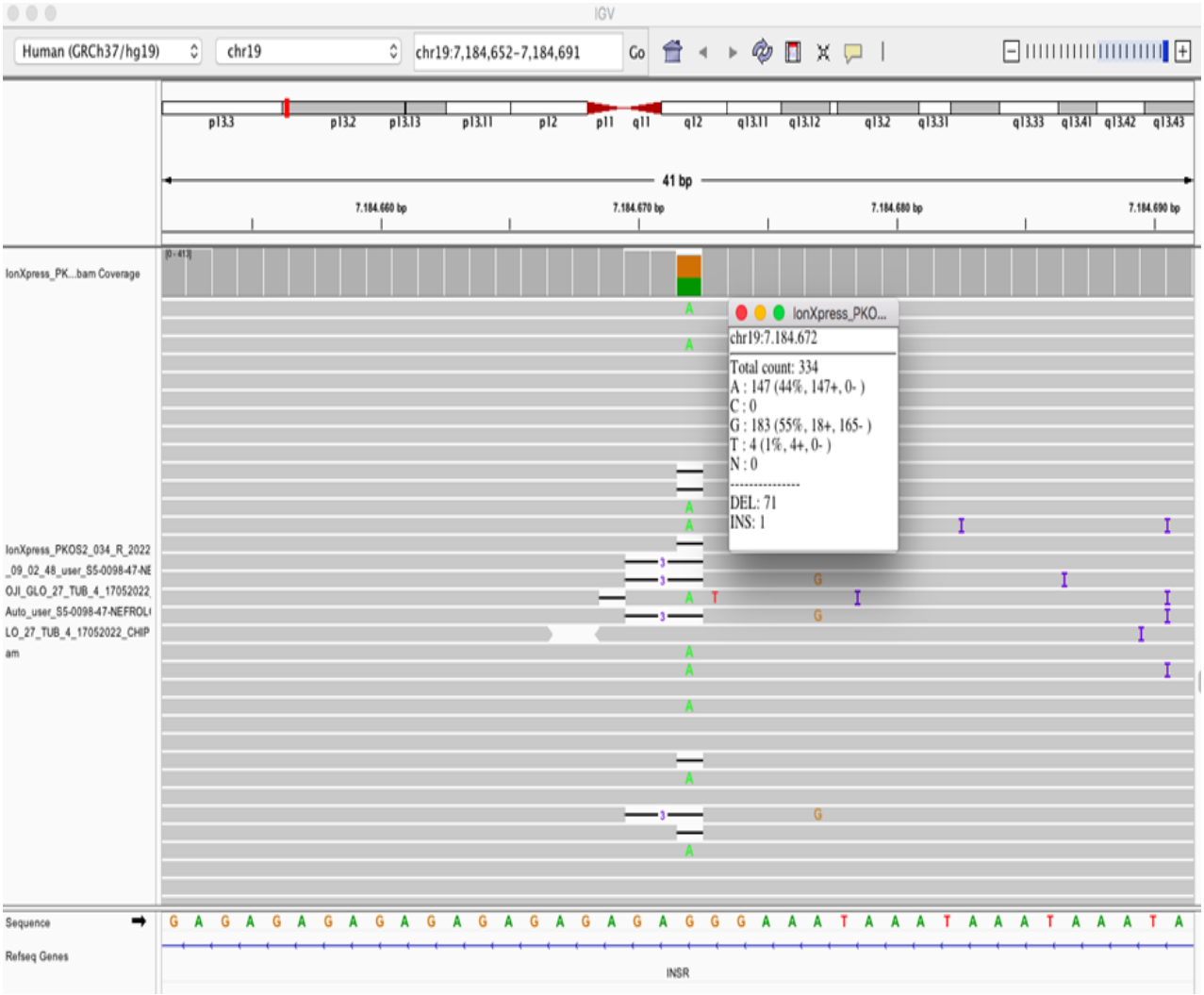

Figure 3. Representation of novel intronic variation at position chr19:7184672G>A by IGV

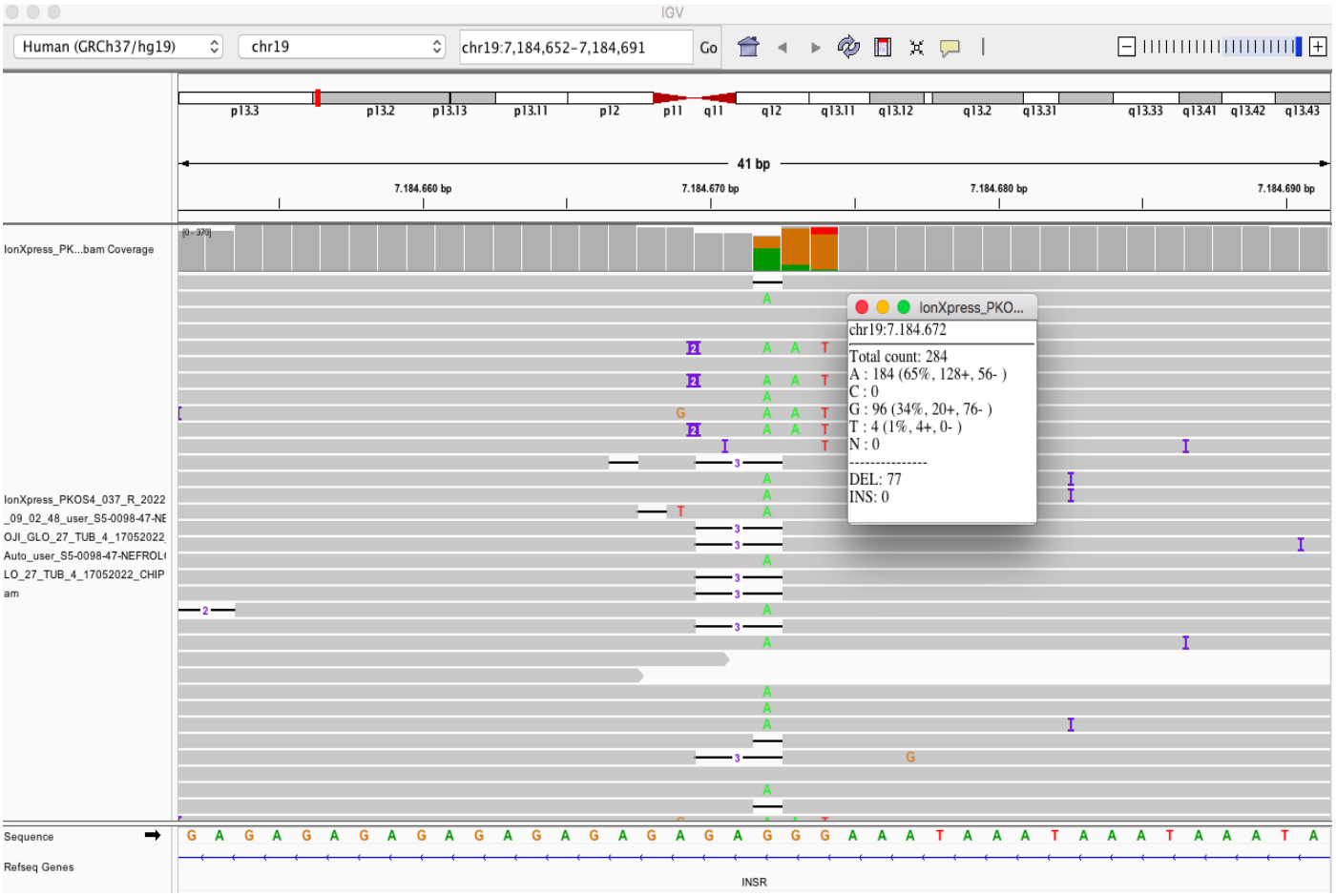

Figure 4. Representation of rs1974375611 by IGV

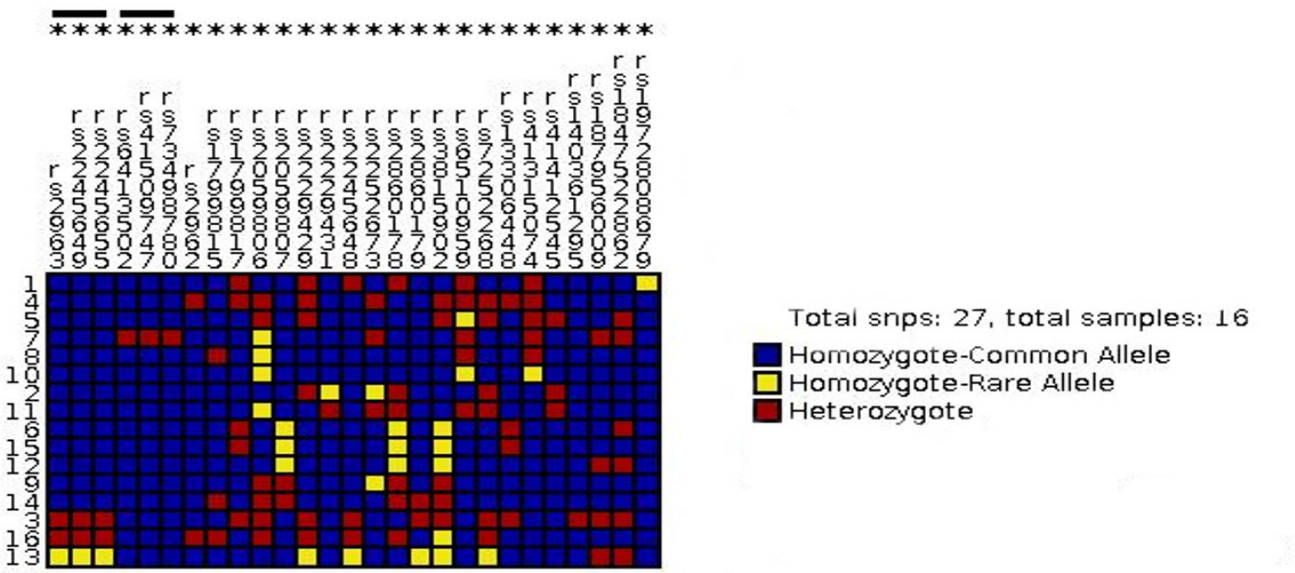

Figure 5.Graphical display of tag SNPs of *INSR* gene

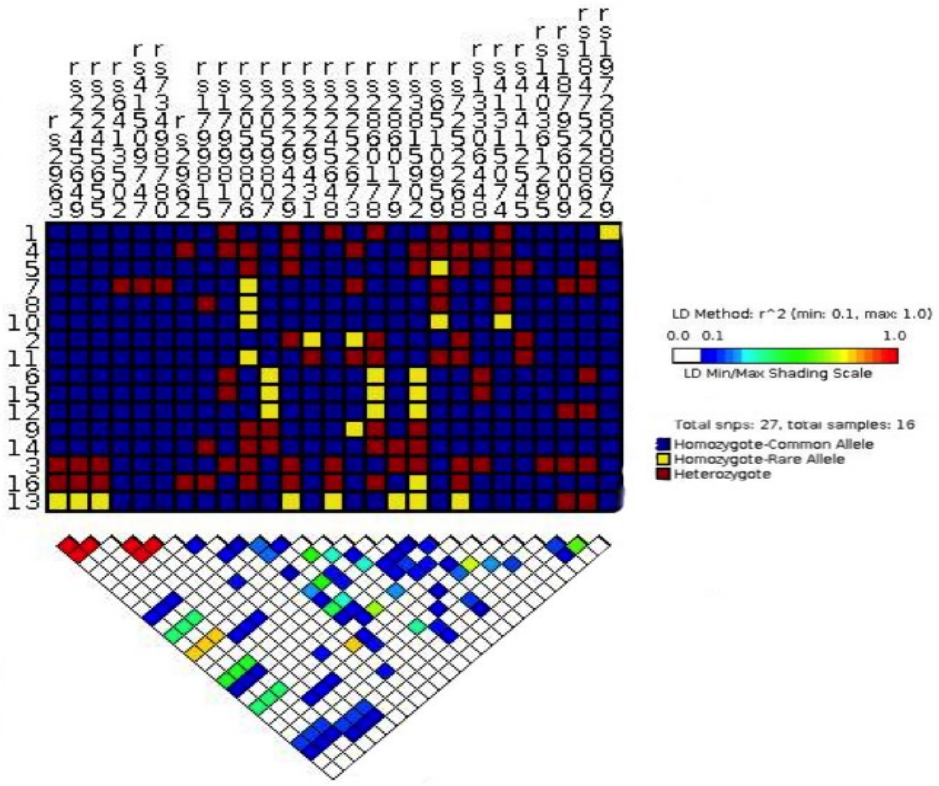

Figure 6.Graphical display of linkage disequilibrium between SNPs of *INSR* gene

Discussion

Insulin resistance is one of the investigated phenotypes that is at the center of PCOS [22,23]. Twin and family studies have shown that insulin resistance, which is included in PCOS, may be a genetic component of PCOS [24]. Similarly, the fact that daughters of women with PCOS also have insulin resistance findings supports the heredity of insulin resistance in PCOS [25]. In our study, we evaluated *INSR* gene variations on 16 patients with PCOS.

Rs7508518 (NC_000019.9:g.7293898G>C) is the most common (100%) homozygous variation in all our PCOS patients. Since the frequency of this variation in the normal population is close to 100%, it should be considered as a benign polymorphism. Because rs7508518 is in the category of non-pathogenic SNPs even in Leprechanuism syndrome (OMIM*2464200), which is congenitally accompanied by extreme insulin resistance and caused by mutations in the *INSR* gene. [26].

There are studies explaining the variations of rs2059807 and rs1799817 in insulin resistance in PCOS in European, Chinese and Indian women. [27,28]. Similarly, a meta-analysis study found that rs1799817 and rs2059806 may not be important in PCOS, but rs2059807 may be a candidate SNP for PCOS [29]. In a relatively small sampled case-control study of Iranian origin, it was shown that rs1799817 and rs2059806 were not responsible for PCOS [30]. In our PCOS patient population, we found the minor allele frequency (MAF) of the rs2059807 variation to be 25% and that of rs1799817 to be 16%. In the above studies, it was shown that the MAF value of rs2059807 was 39% and that of rs1799817 was 29% When we looked at our data, we revealed that the LD score of both SNPs (rs2059807 and rs1799817) was r^2=0.022. In related studies, both SNPs were found to show high linkage. (r^2=0.0035). We found that the status of our tag SNPs with high r^2 values was similarly high in different populations. It can be stated that among the 3 relevant SNPs, rs2059807 may be a candidate SNP for PCOS. In a case-control study originating from Turkiye, in which only a single SNP of the *INSR* gene was investigated, the allele frequency of rs1799817 in women with PCOS was found to be 25% (31). In that study, while there was a difference in BMI between the PCOS group (n = 44) and the control group (n = 50), it is noteworthy that there was no difference between the groups' fasting blood glucose and fasting insulin levels ($P > 0.05$).

The novel variant (chr19:7,184,672G>A) was seen for the first time in our cases and is included in the databases as rs1449625253. This region (chr19:7,184,672) is harboring several indel

variations. These variations in the highly heterogeneous region are those we did not include in the haplotype analysis. Since 9 of the other 10 variations were not informative for all patients and the last 1 (rs7508518) was homozygous and mutated in all our patients, haplotype analysis was able to performed by including 27 SNPs.

When we look for the databases to investigate the presence of other variations in the positions of the 3 intron variants we found; NM_000208.4:A>C change at position c.653-79 as (rs1974381527), NM_000208.4:c.974+ A>T change at position 107 (rs1432110005), NM_000208.4:C>T at position c.974+82 change (rs1974355109) has been reported at same 1/264690 (0.000004) frequencies [32].

In our study, *INSR* whole gene exome analysis was performed only in the patient group with PCOS, and all possible mutations and variations were investigated. In our study on 16 PCOS patients, we not only performed *INSR* haplotype analysis for the first time, but also found 4 new candidate SNPs. As a result of haplotype analyses, we revealed 2 different tag SNP clusters. It may be necessary to first perform segregation analyzes of the 4 SNPs we found and then reveal the MAF values in our own normal population.
The number of patients in our study and the lack of a control group from our own population can be seen as limiting factors. However, an important factor is that all of our PCOS patients were recruited using fairly homogeneous inclusion criteria according to the andojen excess (AE-2008) criteria. One study also studied 51 PCOS patients based on NIH criteria without involving control group and found that *CYP21A2* gene mutations which are causative for congenital adrenal hyperplasia (CAH) patients (33). Another research recruited 31 PCOS patients regarding Rotterdam criteria but not control group and found novel *FBN3* and *FN1* gene mutations . Lastly, related to *INSR* gene variations, an Indian inherited PCOS cohort with 43 patients revealed that rs2059807 was found to be pathogenic (33). We found same SNP (rs2059807) in *INSR* gene in 13 out of 16 samples in our relatively small PCOS patient cohort.

We report a total of 4 novel SNPs and 2 different tag SNPs in PCOS patients for the first time. In a future study where the *INSR* gene is associated with PCOS or another disease, it would be advantageous to investigate the presence of these tag SNPs. It will eliminate the need to study and sequence each SNP individually.

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