

RESEARCH ARTICLE

The Lys469glu/K469E Polymorphism of the Inflammatory Gene Intercellular Adhesion Molecule-1 Lacks any Apparent Role in the Polycystic Ovary Syndrome in Kashmiri Women: A Case Control Study

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Abstract

Background: Polycystic ovary syndrome (PCOS), associated with a state of low grade chronic inflammation, depends on multiple genetic and environmental factors. Elevated levels of inflammatory markers including intercellular adhesion molecule-1 (ICAM-1) have been demonstrated in affected women. Recent evidence indicates a significant linkage between chromosome 19p13 loci and multifactorial diseases that have an inflammatory component. The aim of this study was to assess the possible association of the lys469glu (K469E) polymorphism of the ICAM-1 gene located on chromosome 19p13 with risk of PCOS in Kashmiri women. **Material and Methods:** The K469E single nucleotide polymorphism (SNP) was analysed with DNA from peripheral blood leukocytes of 220 PCOS cases and 220 age matched non-PCOS healthy controls using PCR-RFLP. **Results:** Genotypic frequencies in cases were found to be 32 (14.5%) for EE, 98 (44.5%) for KE, and 90 (40.9%) for KK, with 130 (59.1%) for the KE+EE genotypes compared to healthy control values of 29 (13.2%) for EE, 113 (51.4%) for KE, 78 (35.5%) for KK and 142 (64.5%) for KE+EE combined. The odds ratios for the EE, KE and KE:EE genotypes were 0.95(95% CI= 0.53-1.71)[p= 0.88], 0.75(95% CI= 0.50-1.12)[p=0.168] and 0.79 (95% CI=0.53-1.16) [p= 0.23], no statistically significant differences being found between cases and controls ($\chi^2=2.07$; p=0.35). **Conclusion:** In conclusion, there was no apparent significant influence of the K469E polymorphism on risk of PCOS, or any clinical or laboratory parameters.

Keywords: Polycystic ovary syndrome- K469E Polymorphism- intercellular adhesion molecule-1

Asian Pac J Cancer Prev, **18 (11)**, 2925-2930

Introduction

The polycystic ovary syndrome (PCOS) is a common and complex endocrinopathy affecting 3-10% of women of reproductive age (Azziz et al., 2004; Nidhi et al., 2011). PCOS is associated with low-grade chronic inflammation (Dimitriadis et al., 2016; Nehir Aytan et al., 2016, Marciniak et al., 2016) and genes related to innate immunity and chronic inflammation may participate in its pathogenesis (Escobar-Morreale et al., 2005). Adhesion molecules are one of the main markers of low-grade inflammation and endothelial dysfunction (Blankenberg et al., 2003). There are soluble forms of adhesion molecules in circulation that allows assessment of the protein's concentration. During the last decade, several studies have analyzed the serum ICAM-1 levels in women with PCOS in an attempt to link PCOS with risk of cardiovascular diseases (CVD) and it has been reported that women with PCOS have significantly increased

ICAM-1 concentrations (Nasiek et al., 2004; Vrbikova J et al., 2005) suggesting ICAM-1 as a marker of low-grade inflammation, and a predictor of disease related to PCOS.

Genome-wide scans have predicted that PCOS susceptibility genes may reside over a broad region of chromosome 19p13.2 (Urbanek et al., 2005) ICAM-1 gene located in 19p13.3-p13.2 chromosomal region is a member of immunoglobulin superfamily of adhesion molecules. It is expressed on the surface of the endothelium cells, smooth muscle cells, macrophages and activated lymphocytes. ICAM-1 plays an important role in the adhesion of circulating leukocytes to the blood vessel wall and transendothelial migration to vascular intima (Hayflick et al., 1998). ICAM-1 binds to β_2 integrins of leukocytes, leukocyte function associated antigen-1 (LFA-1, Integrin $\alpha_L\beta_2$) and macrophage antigen-1 (MAC-1, Integrin $\alpha_M\beta_2$) (Springer TA, 1990). Fibrinogen could also be a ligand for ICAM-1 (Languino LR et al., 1993).

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A common genetic polymorphism (rs5498) at codon 469 in exon 6 of ICAM-1 gene resulting in substitution of an A with a G nucleotide and replaces lysine (K) with a glutamic acid (E) in ICAM-1 gene has been described (Vora et al., 1994). This polymorphism is suggested to affect mRNA splicing patterns that modify cell-cell interactions and influence inflammatory response (Iwao et al., 2004). To the best of our knowledge none of the study have examined the possible association between K469E polymorphism and development of PCOS in Indian women. However, several studies represented associations between K469E polymorphism and number of inflammatory diseases like inflammatory bowel disease (Papa et al. 2004), diabetes mellitus (Petrovic et al., 2008), peripheral occlusive arterial disease (Flex A et al., 2007), type 1 diabetes (Nejentsev et al., 2003) and coronary artery disease (Chou et al., 2015) in different populations. Therefore, the exon 6 of ICAM-1 gene represents a strong positional and biological candidate for the susceptibility to the development of inflammatory and metabolic diseases which may include PCOS. Hence the present study was undertaken to examine the association of K469E polymorphism on exon 6 of ICAM-1 gene with PCOS in Kashmiri (North Indian) women.

Materials and Methods

The study was recommended/approved by the Institutional ethics committee, Sher-i-Kashmir Institute of Medical Sciences, Soura, under IEC No: SIMS 131/IEC-SKIMS/2013-6479: dated 09-07-2013. 220 women fulfilling the criteria for PCOS were enrolled in the study. The assessment for standard criteria diagnosis of PCOS was based on the Rotterdam criteria which states 2 of the 3 features needs to be present to make the diagnosis of PCOS. These features includes (1) Oligo- or anovulation (< eight menstrual cycles in the presenting year) (2) Clinical and/or biochemical signs of hyperandrogenism and (3) Polycystic ovaries (either 12 or more follicles measuring 2–9 mm in diameter, or an ovarian volume of >10 cm³), with the exclusion of other etiologies (Non classic congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome). Non classic congenital adrenal hyperplasia (NCAH), cushing's syndrome, thyroid dysfunction, hyperprolactinemia, and androgen-producing tumors were ruled out by doing relevant investigation

All the PCOS patients belonged to Department of Endocrinology, Sher-i-kashmir Institute of Medical Sciences (SKIMS), Srinagar, Kashmir. The non-PCOS group represented 220 apparently normal women having regular menstrual cycles (21–35 days), displaying no evidence of clinical/biochemical hyperandrogenism, and having normal ovarian morphology on trans-abdominal ultrasonography. Controls were collected from various medical camps organized at colleges and at university of Kashmir. Women consuming any hormonal preparations or drug(s) known or suspected to affect reproductive or metabolic functions within 6 months of the study entry, or those having known diabetes mellitus, renal, hepatic, or cardiac dysfunction were also excluded from the study. The study was conducted over a period of two

years (January 2013 to January 2015). The study protocol was approved by the Institutional Ethics Committee and written informed consent was obtained from all the participants.

Clinical Assessment

All women underwent anthropometric assessment like measurement of height, weight, waist-hip circumference ratio and detailed systemic examination. Hirsutism assessment was done using modified Ferriman-Gallwey score by counting nine specified body areas. A score of > 8 out of a total of 36 was taken as significant.

Biochemical Analysis

Biochemical analysis includes oral glucose tolerance test (OGTT), insulin, triglycerides, low density lipoprotein (LDL), high density lipoprotein (HDL), cholesterol, liver function test and renal function tests. Oral glucose tolerance test (OGTT) was performed at 800–900 h after an overnight (10–12 h) fast. Blood samples were collected at 0, 60 and 120 min after an oral load of 75 -g anhydrous glucose dissolved in 200–300 ml of water. Blood samples (venous) were collected, separated in cold centrifuge at 4°C and aliquoted. The samples for glucose and lipid profile were analyzed on the same day.

Calculations

Insulin resistance was assessed by means of the fasting glucose/insulin ratio (GIR), homeostasis model assessment insulin resistance index (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI). The GIR values were calculated as fasting glucose (mg/dl)/fasting insulin (μIU/ml). The HOMA-IR index was calculated as [fasting insulin (μIU/ml) x fasting glucose (mg/dl)]/405. The QUICKI was calculated as $1/[\log \text{fasting insulin} (\mu\text{IU/ml}) + \log \text{fasting glucose} (\text{mg/dl})]$. High HOMA-IR, low QUICKI and low GIR scores denote insulin resistance (low insulin sensitivity). Body mass index (BMI) was calculated as body weight (kg) divided by body height squared (m²).

Hormonal analysis

Hormonal analysis included Leutinizing hormone (LH), Follicle stimulating hormone (FSH), Thyroxine (T4), Thyroid stimulating hormone (TSH), prolactin, testosterone, 17-hydroxy progesterone (17-OHP) and cortisol. 17-OHP to rule out non classical congenital adrenal hyperplasia and cortisol to rule out cushing's syndrome. T4 to rule out hypothyroidism, TSH to rule out hypothyroidism, Prolactin to rule out prolactinoma. Testosterone to diagnose hyperandrogenism and to rule out androgen secreting ovarian or adrenal tumours. The sampling was arranged in such a way so that the sample for LH, FSH, 17-OHP and testosterone was collected on 3rd to 7th day of the follicular phase of either spontaneous or progesterone induced menstrual cycle.

Laboratory analysis

Plasma glucose was measured by glucose-oxidase peroxidase method (GOD-POD, Nicholas Piramal Ltd.,

Mumbai, India) along with other biochemical parameters on autoanalyzer (Roche Hitachi 912, Minatoku, Tokyo, Japan). Hormonal assays were done by RIA/IRMA using commercial kits in duplicate and according to supplier protocol (Diagnostic Products Corporation, Los Angeles, CA and Immunotech and Medicorp Inc., Montreal Canada). Plasma insulin was measured by Electrochemiluminescence (Cobas e411, Roche Diagnostics Limited, Charles Avenue, West Sussex). Sensitivity, specificity, inter-assay, and intra-assay coefficients of variation were within the prescribed limits as per manufacturer's protocol.

DNA Extraction

DNA was extracted from peripheral blood leukocytes according to phenol-chloroform method. DNA extracted was qualitatively and quantitatively assessed by gel electrophoresis and by measuring optical density at 260nm and 280 nm by double beam spectrophotometer. The ratio of 260/280nm was calculated and only those DNA samples for which the ratio was 1.7-1.9 were considered for the experimental use.

ICAM-1 Codon 469 Genotyping

PCR amplification of a 226 bp fragment of ICAM-1 gene on exon 6 was performed with PCR-RFLP using two primers. Forward Primer K: 5-GGAACCCATTGCCCGAGC-3 and reverse primer E: 5-GGTGAGGATTGCATTAGGTC-3. 300ng genomic DNA was amplified in a total volume of 25µl of the reaction mixture containing 2.5µl of 10X PCR buffer, 2µl of MgCl₂, 0.5µl of 10 mM dNTPs, 0.5µl of 20nm primers, 0.3µl (1U) Taq DNA polymerase, 3µl genomic DNA, 15.7µl deionized water. PCR was carried out with an initial denaturation at 96°C for 1min and 30cycles of 20s of denaturation at 96°C, 50s of annealing at 64°C, 1min of extension at 72°C and final extension of 10min at 72°C. Subsequently, 10µl of PCR product of length 226bp was treated with 3U of Bsh1236I (FnuDII) in a total volume of 20µl (2µl of 10X buffer R+18µl of nuclease free water) and incubated for 16 hours at 37°C for optimum digestion. Upon restriction digestion KK(Lys/Lys) homozygous wild-type produces single band at 226bp, EE (Glu/Glu) homozygous mutant type produces two bands 139bp and 87bp, KE (Lys/Glu) heterozygous variant produces three bands at 226bp, 139bp and 87bp.

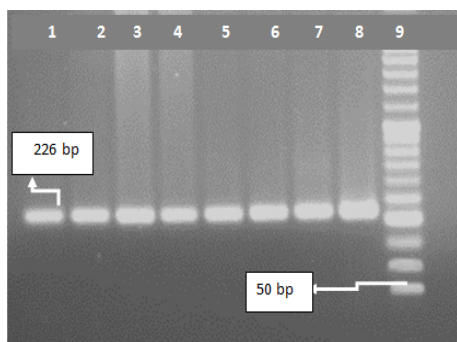


Figure 1. Representative Gel Picture Showing PCR Product Run on 2 % Agarose Gel. Lane 1-8; 226 bp PCR Product; Lane 9; 50 bp DNA Ladder.

The fragments were analyzed by 3% ethidium bromide stained agarose gel electrophoresis (Figure 1). Samples that failed to amplify were repeated and reanalyzed.

Statistical analysis

Data was statistically analysed for mean values and standard deviations in microsoft office excel. ANOVA and unpaired Student t-tests were used to compare the means of variables. Alleles and genotype frequencies in the case and control groups were compared using Chi-square and Fischer exact tests. Statistical significance was set at $p < 0.05$. The distribution of the genotypes in controls was compared with that expected from Hardy-Weinberg equilibrium (HWE) by the chi square (χ^2) test. $P > 0.05$ was considered to be consistent with HWE. Statistical analyses were performed using SPSS and vassarstats online software.

Results

Clinical characteristics of women with PCOS and control women are summarized in Table 1. The mean age of case and control women was comparable (23.04 ± 4.82 years in PCOS group versus 22.97 ± 3.58 years in control

Table 1. Clinical and Biochemical Characteristics of Cases and Controls

Variables	Cases N=220		Controls N=220		P value
	Mean	SD	Mean	SD	
Age (years)	23.04	4.82	22.97	3.58	0.86(NS)
FG score	13	3.23	7	2.33	<0.0001
BMI (kg/m ²)	23.87	3.03	23.43	3.31	0.14(NS)
Waist Hip Ratio	0.87	0.022	0.84	0.005	<0.0001
LH (IU/L)	8.06	1.97	6.13	1.2	<0.0001
FSH (IU/L)	6.66	1.23	6.94	1.99	0.07(NS)
Testosterone (ng/dl)	85.22	15.81	32.85	11.2	<0.0001
Blood glucose Fasting (mg/dl)	88.07	12.02	87.7	12.88	0.75(NS)
Blood glucose 1 Hr (mg/dl)	136	17.8	131.22	15.5	0.002
Blood glucose 2 Hr (mg/dl)	102.36	19.41	94.14	20.23	<0.0001
Insulin Fasting (µIU/ml)	20.8	5.22	10.81	7.72	<0.0001
FGIR	4.44	1.16	7.85	3.06	<0.0001
QUICKI	0.3	0.01	0.33	0.09	<0.0001
HOMA-IR	4.55	1.35	2.14	1.23	<0.0001
Cholesterol(mg/dl)	181.23	20.24	158.02	17.6	<0.0001
Triglycerides(mg/dl)	151.27	22.68	104.09	20.7	<0.0001
HDL(mg/dl)	43.01	6.21	48.05	7.81	<0.0001
LDL(mg/dl)	119.02	16.17	93.03	18.3	<0.0001

NS, Statistically Non significant ($p > 0.05$); SD, Standard Deviation; BMI, Body Mass Index; FGIR, fasting glucose insulin ratio; FG Score, Ferrimen Gallwey score. FSH, follicular stimulating hormone; HDL, High density lipoprotein; HOMA-IR, Homeostasis Model Assessment Insulin resistance index; LDL, Low density Lipoprotein; LH, luteinizing hormone; QUICKI, quantitative insulin sensitivity index

Table 2. Genotypic and Allelic Frequencies of ICAM-1 Gene Codon 469 among Cases and Controls and Their Association with Risk of PCOS

ICAM-1 Gene Codon 469	Variants	Cases (N=220)	Controls (N=220)	OR (95% CI) ; p* value	χ^2 ; p Value(Overall)
Genotypic Frequencies (N)	KK (Lys/Lys) -Wild	90(40.9%)	78 (35.45%)	1	2.07;0.35
	KE (Lys/Glu) -Heterozygous	98(44.54%)	113 (51.36%)	0.75 (0.50-1.12) ; 0.16	
	EE (Glu/Glu) -Variant	32(14.54%)	29 (13.18%)	0.95 (0.53-1.71); 0.88	
	KE+EE	130(59.09%)	142 (64.54%)	0.79 (0.539-1.16); 0.23	
Allelic Frequency (2N)	Lys (K allele)	278 (63.2%)	269 (61.1%)	1.0*	-
	Glu (E allele)	162 (36.8%)	171 (38.9%)	0.92 (0.70-1.20); 0.532	-

p*, Pearsons P value

Table 3. Anthropometric, Insulin Resistance and Lipid Profile Parameters of Cases in Accordance with the Genotypes of the ICAM1 K469E Gene Polymorphism

Variables	KK		KE		EE		p value
	Mean	SD	Mean	SD	Mean	SD	
BMI (Kg/m ²)	23.98	3.33	23.73	2.72	24.07	3.13	0.78 (NS)
Waist Hip Ratio	0.876	0.02	0.876	0.02	0.884	0.02	0.11 (NS)
Testosterone (ng/dl)	83.61	15.17	86.24	15.16	86.68	19.32	0.44 (NS)
Blood Glucose fasting (mg/dl)	86.97	11.52	88.88	12.73	88.76	11.27	0.52 (NS)
Glucose two hour (mg/dl)	102.16	21.43	102.82	16.76	103.8	21.53	0.91 (NS)
Fasting Insulin(μ IU/ml)	20.87	6.03	21.15	4.91	19.98	3.52	0.55 (NS)
FGIR	4.48	1.3	4.4	1.06	4.6	1.11	0.68 (NS)
HOMA-IR	4.49	1.46	4.67	1.36	4.39	1	0.51 (NS)
QUICKI	0.309	0.01	0.307	0.01	0.309	0.01	0.33 (NS)
Cholesterol (mg/dl)	179.01	20.97	180.73	19.97	188.02	17.54	0.09 (NS)
TG (mg/dl)	152.9	21.77	149.23	23.23	152.94	23.73	0.49 (NS)
HDL (mg/dl)	43.27	7.01	43.31	5.52	41.41	5.77	0.28 (NS)
LDL(mg/dl)	119.37	16.45	119.49	16.09	116.48	15.86	0.63 (NS)

NS, Statistically Non significant (P>0.05); SD, Standard Deviation

group; p=0.86) and was BMI (23.87± 3.03 in PCOS group 23.43 ±3.31 in control group; p=0.14). FG score, cholesterol, triglycerides, LDL, HDL, total testosterone, fasting insulin, FGIR, HOMA IR, and QUICKI showed significant differences between PCOS group compared

to control group (p=<0.0001). There was no significant difference in mean values of age, BMI, fasting glucose, and FSH among women with PCOS as compared to controls.

All PCOS cases and controls were genotyped for the ICAM-1 exon 6, codon 469 single nucleotide polymorphisms (SNP). The studied genotypes were almost equally distributed among cases and controls and the distribution was statistically insignificant. Genotypic frequency in cases was found to be 32(14.54%) for EE, 98(44.54%) for KE, 90 (40.9%) for KK and 130(59.09%) for KE+EE genotype compared to healthy controls

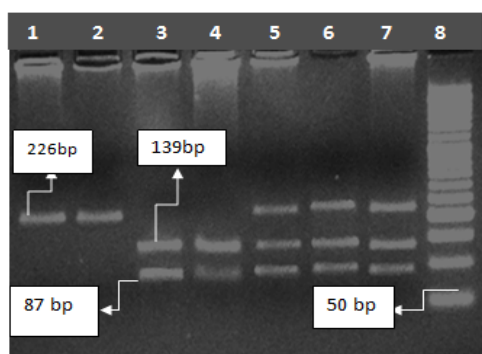


Figure 2. PCR-RFLP Results of Codon 469 K to E Substitution in ICAM-1 Gene. After treatment with Bsh1236I restriction enzyme, the original 226 bp PCR product was digested into 139 and 87 bp fragments when E allele existed. Starting from left, lane 1, 2 represents KK genotype (226bp), lane 3, 4 represents EE genotype (139bp and 87bp), lane 5, 6, 7 represents KE genotype (226bp, 139bp, 87bp). Lane 8 represents 50bp DNA size marker

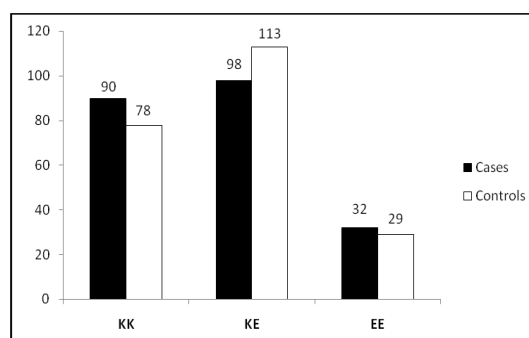


Figure 3. Histogram Showing Distribution of Genotypes among Cases and Controls

among which 29(13.18%) had EE, 113(51.36%) had KE, 78(35.45%) had KK and 142(64.54%) had KE+EE genotype. The odds ratio (OR) for EE genotype was 0.95 with 95% CI of 0.53-1.71 [$p=0.88$], for KE genotype OR was 0.75 with 95% CI of 0.50-1.12 with [$p=0.16$] and for KE+EE genotype OR was 0.79 with 95% CI of 0.53-1.16 [$p=0.23$]. The allelic frequency revealed statistically insignificant and almost equal distribution of E allele among cases and controls with OR=0.92; 95% CI= 0.70-1.20; [$p=0.53$]. The genotypic frequencies of ICAM-1 gene of codon 469 showed no difference in distribution between cases and the controls ($\chi^2=2.07$ and $p=0.35$) and was not statistically significant. The genotype frequencies among controls for ICAM-1 K469E SNP were found to be in agreement with HWE (i:e) $p>0.05$. The genotypic and allelic distributions of ICAM-1 K469E polymorphism for cases and controls are shown in Table 2.

Furthermore the association between polymorphism with that of the clinical and laboratory parameters was also carefully analysed. However, we did not find any significant association of genotypes with any of the PCOS characteristics (i:e) p value was >0.05 -shown in Table 3.

Discussion

In our study no difference was observed in the distribution of genotypic and allelic frequencies of K469E SNP of ICAM-1 gene between PCOS and controls, thereby, suggesting that the polymorphism in this codon may not be associated with the risk of PCOS in our population. Furthermore no significant association was observed between polymorphism with that of clinical and laboratory parameters. Our observations are in broad agreement with the previously published study (Vural et al., 2011) indicating that it is unlikely that this polymorphism plays a major role in determining susceptibility to PCOS. Contrary to our findings one of the recent study reported association of K469E polymorphisms with PCOS and metabolic comorbidities in obese women (Ojeda-Ojeda et al., 2016). Few studies that addressed the association of variants in the genes encoding adhesion endothelial molecules with PCOS rendered conflicting results (Lee et al., 2008; Kanmaz-Ozer et al., 2012).

K469E is a non-synonymous SNP and resides in the fifth immunoglobulin-like domain of ICAM-1. This domain may play a role in an immunodominant epitope of B lymphocytes and dendritic cells (Joling et al., 1994). In accordance with genetic association studies of this SNP in the previous reports (Papa et al., 2004; Petrovic et al., 2008; Flex et al., 2007; Nejentsev et al., 2003; Chou et al., 2015) it is suggested that this SNP can have a genetic and biological influence related to the pathogenesis of inflammatory diseases. Only two studies have been conducted on the association of K469E polymorphism with development of PCOS which restricts the comparison of our study to very few studies of this nature. However, several investigators have examined the genetic association between K469E ICAM-1 gene polymorphism and chronic inflammatory diseases which can provide indirect evidences for our study. A good example is the results of genetic association studies examining the relationship

between ICAM-1 polymorphism and type 1 diabetes. The ICAM-1 polymorphism was significantly associated with adult onset diabetes in a Japanese population (Nishimura et al., 2000) but not in Danish (Kristiansen et al., 2000) or Finnish families (Nejentsev S et al., 2000). Among Polish Caucasian patients with multiple sclerosis, the allelic frequency of ICAM-1 K469 was significantly increased (68% vs 49% in controls) (Mycko et al., 1998). In Italian patients with either polymyalgia rheumatica or giant cell arteritis, the K469E polymorphism was not significant (Salvarani et al., 2000). The allelic frequency of E469 was significantly increased among Palestinian and Jordanian patients with Behçet's disease (47.6% v 38.3% in controls) (Verity et al., 2000). These results are contradictory and so the significance of this gene may differ according to the type of inflammatory disease.

Mixed results from different populations (ethnic group) represent different gene pools, suggesting that gene-disease associations can be expected to vary between populations due to the differences in a complex genetic background.

Limitations

Our study was not free of limitations. ICAM-1 gene has 7 exons, from 2–6 exon, one non-synonymous SNP exists. Polymorphism on exon 4 and exon 6 of ICAM-1 gene have been widely explored for their implication in susceptibility in inflammatory disorders. Although we have published a study on polymorphism of exon 4 of ICAM-1 gene among PCOS women in Kashmir. However we could not assess other SNPs of ICAM-1 gene that can also have physiological role in this direction which further needs to be analysed.

In conclusion, to the best of our knowledge, this is the first study regarding the association of ICAM-1 K469E polymorphism with PCOS susceptibility in Kashmir valley. In our study neither the EE genotype nor the KK genotype was associated with PCOS. From our results we concluded that there is neither any association between K469E genotypes with susceptibility to PCOS nor with any of PCOS characteristics. Therefore, K469E polymorphism of the ICAM-1 gene may not be used as a genetic marker for PCOS. Also further replication study is required before the firm conclusion can be reached. However, the role of ICAM-1 gene alone as well as in combination with different clinical and laboratory parameters in our study possibly advocates the role of other genetic markers which could be responsible for the development of PCOS.

Sources of support

This work was supported by the research fund of the Department of science and technology (DST), women scientist scheme-A (WOS-A).

Project No

SR/WOS-A/LS-642/2012(G).

Acknowledgments

This work was supported by the research fund of the Asian Pacific Journal of Cancer Prevention, Vol 18 2929

department of science and technology (DST), women scientist scheme-A (WOS-A). Project No: SR/WOS-A/LS-642/2012(G). All authors have made a substantial contribution to the text submitted and approved the final manuscript. This paper has not been submitted or published elsewhere. The authors have declared no conflict of interest. We are thankful to all the patients and control subjects who voluntarily agreed to be the part of study.

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