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Molecular studies in Rheumatoid arthritis patients for determination of Cardiovascular disease (CVD) risk

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ABSTRACT

Among the serious complications people with rheumatoid arthritis (RA) experience, cardiovascular disease heads the list. Having RA doubles the risk of most heart problems, including heart attack, stroke and atherosclerosis - the build-up of fat, cholesterol and cellular debris (plaque) on blood vessel walls. Hence the present study targeted the I/D polymorphism in angiotensin 1 converting enzyme (ACE) gene using Insertion specific PCR and methylation status of angiotensin 1 converting enzyme (ACE) gene using methylation specific PCR in 30 RA patients confirmed by Rheumatoid factor (RF) and erythrocyte sedimentation rate (ESR) values. ID genotype was the predominant genotype in patients and controls. II genotype was higher in controls compared to patients. Methylation pattern analysis using MS-PCR suggested equal distribution of methylated and unmethylated regions of ACE gene in patients and controls thus suggesting lack of CVD risk. But in order to confirm, a higher sample size needs to be further probed.

Keywords: Angiotensin 1 converting enzyme (ACE), rheumatoid arthritis, Cardiovascular disease (CVD), Methylation.

INTRODUCTION

Rheumatoid arthritis is a chronic systemic inflammatory disease of undetermined aetiology, involving primarily the synovial membrane and articular structures of multiple joints. The disease is often progressive and results in pain, stiffness and swelling of joints. In later stages deformity and ankylosis develop. The process involves an inflammatory response of the capsule around the joints (synovium) secondary to swelling (turgescence) of synovial cells, excess synovial fluid, and the development of fibrous tissue (pannus) in the synovium. Rheumatoid factor (RF) is an antibody that can be found in the blood of 80% of people with rheumatoid arthritis. The insertion/deletion (I/D) polymorphism in ACE gene refers to an Alu repetitive sequence of 287 bp long, in intron 16, resulting in three genotypes, DD and II homozygotes and ID heterozygotes.

A significant relation between the number of methylated CpG sites and the ACE mRNA expression in various cell lines has been previously reported (Riviere *et al*., 2011). ACE gene is discussed as a common factor for the known relation between major depression and cardio- vascular disease, but the underlying mechanisms are poorly understood. Epigenetic alterations, probably via environmental influences as early stressful life events might be one explanation for these observations. The main objectives of the present study were to find an association between RA and CVD risk through analysing the ACE I/D genotype polymorphism and methylation pattern in ACE gene in Arthritis patients and to compare the obtained results with normal healthy controls using relevant statistical tools.

MATERIALS AND METHODS

Study participants and characterization

About 30 venous blood samples were collected from clinically confirmed RA patients in EDTA coated vacutainers from 'DEEPA MICROLAB' Erode, Tamil Nadu. The patients were categorized as RA based upon Rheumatoid factor (RF) values and ESR values. Thus obtained blood samples were brought to the laboratory and stored at 4^{o}C for DNA isolation.

Genomic DNA extraction

One mL of whole blood was lysed with 3mL of chilled RBC lysis buffer, vortexed for 1 minute and centrifuged at 4000rpm for 5minutes and the red supernatant was removed. This step was repeated twice to get white to pink pellet. To this 200µL of nuclei lysis buffer and 50µL of SDS were added. Followed by the addition of 3µL of proteinase K, the mixture was incubated at 65^{o}C for 2:30 hours. After this, 175µL of 5.3M Sodium chloride was added, centrifuged at 10,000rpm for 15minutes, supernatant carefully siphoned off and transferred to a new 2mL microcentrifuge tube. To this one mL of cold 100% ethanol was added and inverted ten times to precipitate the DNA. There after the tube was centrifuged at 1500 rpm for 10minutes, supernatant was removed and the pellet was resuspended in 75% alcohol. Centrifugation at 15000 rpm was performed to remove supernatant. The pellet was air dried and resuspended in 100-150µL of TE buffer and stored at -20^{0}C. The A_{260}/A_{280} values were checked to assess DNA purity through UV/Visible spectrophotometer.

Insertion specific – PCR

The isolated DNA samples were used for the amplification of ACE gene to find the distribution of DD, ID and II genotypes distribution in patients and controls using previously reported primer pairs (Tuncer *et al*., 2006). Forward primer 5'-CTGGAGACCACTCCCATCCTT 3'; reverse primer 5'-GATGTGGCCATCACATTCGTC3'. Thermal conditions for PCR consisted of initial denaturation at 95^{o}C for 10 min followed by 35 cycles of denaturation at 92^{o}C for 40 sec, annealing 58.1^{o}C for 30 sec, extension at 72^{o}C for 40 and a final extension at 72^{o}C for 10 minutes.

Bisulphite treatment of genomic DNA:

The DNA (up to 2µg) was digested with 20µL of restriction enzyme *Hha*I prior to bisulphite treatment. Then 2µL of 3M NaOH was added and denatured the DNA for 15 min at 37^{o}C. Further to the sample was added 208µL of urea/ bisulphite solution and 12µL of 10mM hydroquinone to make up final concentration of 5.36M urea, 3.44M bisulphite and 0.5% hydroquinone and the mixture was incubated overnight at 55^{o}C. After this, the mixture was purified using ready to use columns supplied by Helini Biomolecules, India.

Qualitative methylation-specific PCR analysis of ACE gene

To analyze the status of methylation of ACE gene, primers were designed using MethPrimer software programme from reference sequence of ACE gene obtained from NCBI. Criteria used as defined by the software: Island size > 100, GC Percent> 50.0, Obs/Exp> 0.60. There was no *Hha*I restriction site in ACE gene region studied as reported by NEB cutter. Primer pairs for methylated ACE, forward methylated primer 5'TATATTTTTATTTTCGGTAGGCGAC-3'; reverse methylated primer 5'-CTAAAAACCTAACGACCTCCGA-3'. Primer pairs for unmethylated ACE, forward unmethylated primer 5'- TATTTTTATTTTTGGTAGGTGATGT-3'; reverse unmethylated primer 5'-AACCTAAAAACCTAAC AACCTCCA-3'.

Thermal conditions for PCR consisted of an initial denaturation at 94^{o}C for 10 min, 38 cycles of denaturation at 95^{o}C for 40 sec, annealing at 49^{o}C for 30 sec, extension at 72^{o}C for 40 sec and a final extension at 72^{o}C for 7 minutes.

Statistical analysis:

Statistical analysis for ESR and genotypes were performed using Vassarstats statistical software

(http://vassarstats.net/). Allele frequency was calculated using the formula: Allele frequency = [(homozygous allele*2) + heterozygous alleles] / (total*2).

RESULTS

The demographics of patients and controls with ESR values were presented in Table 1. ESR values of patients and controls were compared using student's '*t*' test which revealed a significant difference ($p<0.0001$, Table 2).

Table 1: Demographics of patients with ESR values

Sample No	Age	Gender	Esr (mm/hr)	Patient / Control
S1	55	Male	20	Patient
S2	60	Female	20	Control
S3	48	Female	62	Patient
S4	60	Female	10	Control
S5	75	Male	22	Patient
S6	29	Female	8	Control
S7	31	Male	52	Patient
S8	55	Male	12	Control
S9	62	Female	44	Patient
S10	42	Male	7	Control
S11	63	Male	28	Patient
S12	39	Female	15	Control
S13	50	Female	34	Patient
S14	29	Male	7	Control
S15	42	Male	32	Patient
S16	12	Male	10	Control
S17	44	Female	36	Patient
S18	48	Female	16	Control
S19	61	Male	28	Patient
S20	53	Male	11	Control
S21	45	Female	86	Patient
S22	60	Male	14	Control
S23	52	Male	40	Patient
S24	62	Female	18	Control
S25	55	Female	38	Patient
S26	45	Female	16	Control
S27	51	Male	48	Patient
S28	52	Male	13	Control
S29	28	Female	32	Patient
S30	55	Female	7	Control
S31	43	Male	46	Patient
S32	50	Female	14	Control
S33	59	Female	110	Patient
S34	63	Male	12	Control
S35	55	Male	50	Patient
S36	55	Male	11	Control
S37	47	Female	92	Positive
S38	56	Female	15	Control
S39	16	Male	22	Patient

Table 1 : Continued…

Sample No	Age	Gender	Esr (mm/hr)	Patient / Control
S40	17	Male	8	Control
S41	42	Female	80	Patient
S42	76	Male	9	Control
S43	63	Female	28	Patient
S44	31	Male	12	Control
S45	39	Female	26	Patient
S46	33	Female	56	Patient
S47	60	Female	52	Patient
S48	56	Female	56	Patient
S49	53	Female	48	Patient
S50	57	Female	50	Patient
S51	60	Female	9	Control
S52	47	Female	10	Control
S53	75	Male	13	Control
S54	61	Male	17	Control
S55	44	Female	22	Control
S56	32	Male	9	Control
S57	33	Female	7	Control
S58	57	Female	21	Control
S59	53	Female	20	Control
S60	63	Female	24	Control

Table 2: Student's '*t*'- test comparison of ESR values

$Mean_a$- $Mean_b$	'*t*'	df	P	One- tailed	<.0001
-34.1667	-8.54	31.24		Two- tailed	<.0001

Table 3: ACE genotypes observed in patients and controls

RA patients	II Genotype	I/D Genotype	DD genotype
	3	20	7
Controls	12	12	6
	II/ID genotype OR 0.15 95% confidence interval from 0.0351 to 0.6418 P=.016	ID/DD OR 1.4286 95% confidence interval from 0.3876 to 5.2646 P=0.840	II/DD OR 0.2143 from 0.0403 to 1.1386 p=0.142

Table 4: Methylation pattern of ACE gene in patients and controls

	Methylated	Unmethylated	Methylated+ Unmethylated
RA patients	11	10	9
Control	12	10	8

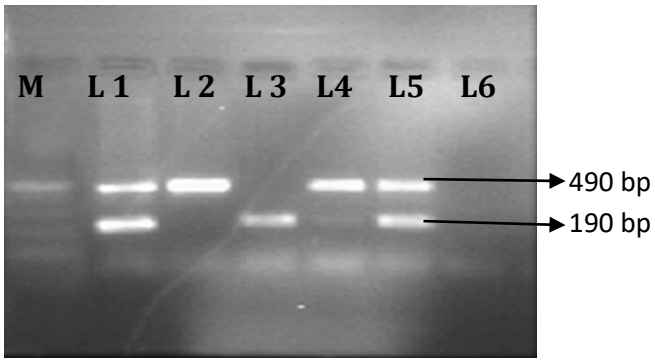

Figure 1: ACE I/D polymorphism in RA patients (positive)
M: 100 bp marker ; Lane: 1, 5- ID genotype ;
Lane: 2, 4- II genotype ; Lane: 3- DD genotype

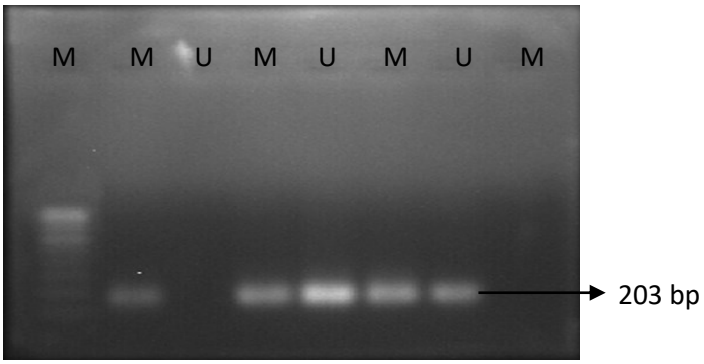

Fig 2: Methylation pattern in RA patients (positive)
Lane1 M: 100 bp Marker ; Lane 2, 4, 6: Methylated ; Lane 3, 5, 7: Unmethylated

Insertion – specific PCR

SNP analysis of ACE gene in RA patients revealed that there were 20 I/D genotypes, 3 II genotypes and 7 DD genotypes (Figure 1), whereas in controls there were 12 I/D genotypes, 12 II genotypes and 6 DD genotypes. Concerning the allele frequency both fitted in Hardy-Weinberg equilibrium with allele frequencies of 0.56 (patients) and 0.40 (controls) for D allele and 0.44 (patients) and 0.60 (controls) for I allele. The distribution of II genotype significantly differed between patients and controls ($p<0.05$, Table 3, Fig. 1).

Qualitative methylation specific PCR

ACE gene was found to be methylated in 36%, Unmethylated in 33.33% and both methylated and unmethylated in 30% of patients (Fig. 2). In controls, ACE gene was methylated in 40%, 33.33% were unmethylated and 26.66% had both methylated and Unmethylated (Table 4).

DISCUSSION

RA is a multiorgan chronic and complex disease with an autoimmune basis. Cardiovascular disease (CVD) is considered an extra-articular manifestation (EAM) (Sandoo *et al*., 2011) and a major predictor of poor prognosis of RA (Demaria 2002).

This study was undertaken to investigate cardio vascular disease risk in RA patients. Patients with RA are 30% to 60% more likely to suffer a CV event compared with the general population (Watson *et al*., 2003, Han *et al*., 2006), especially myocardial infarction (Turesson *et al*., 2004, Solomon *et al*., 2003, Wolfe *et al*., 2003). CVD accounts for 30–50% of all deaths in RA patients (Sandoo *et al*., 2011). Cardiovascular disease (CVD)-associated mortality risk is increased in both men and women with seropositive RA (Jonsson*et al*., 1999).

In the present study, 30 patient and 30 control samples were collected. Among the 30 patient samples 18 were females and 12 were males. RA is three times more frequent in women than men (Scott *et al.,* 2010). It usually strikes women between the ages of 25 to 50. Approximately 1.3 million American adults have RA, with women outnumbering men 2.5-to-1. From 1985 to 1994, the incidence of rheumatoid arthritis was 36.4 per 100,000 women, but from 1995 to 2004, that number increased by nearly half to 54 per 100,000 women. The incidence in men, however, stayed about the same, going from 28.6 to 29.5 per 100,000 over the same two decades.

ACE could be involved in the pathogenesis of cerebro-vascular disease by several biological mechanisms, including activation of angiotensin I and inactivation of bradykinin, resulting in decreased tissue perfusion, vascular smooth muscle cell growth, and stimulation of plasminogen activator inhibitor type 1. (Ehlers *et al*., 1989; Mizuno *et al*., 2003).

Distribution of both variant alleles (D and I) was similar among the patients and the controls. Similar distribution of the variant alleles led to lack of any significant association between the *ACE* ID polymorphism and CVD risk, either in homozygotes and heterozygotes or combined forms. In the present study D allele frequency was higher in patients and controls. Also ID/DD genotypes seemed to have a slight risk based on Odds ratio value. The distribution of the ACE genotypes differs between

races and it is used as a marker in population structure analyses (Barbalic*et al*. 2004).

Koreans and Japanese have reported a relatively low percentage of the DD genotype. The frequency of the D allele is 0.406 in the Koreans (Choung *et al*. 1999) and 0.33 in the Japanese population (Yoshida *et al*. 1995), contrast to Caucasians, where the D allele frequency is higher.

In methylation analysis of the current study, 11 were methylated, 10 were unmethylated and 9 were both methylated and unmethylated in RA positive samples whereas 12 were methylated, 10 were unmethylated and 8 were both methylated and unmethylated in controls. ACE promoter methylation has been found to affect serum ACE protein levels. Higher rates of ACE methylation have been found to lower ACE serum concentration and have a protective effect on the development of CVD. The differential DNA methylation patterns in the promoter region of the ACE gene seem to affect the expression of inflammatory CVD risk marker concentrations in patients with depression. Methylation pattern has been to have an influence on serum ACE protein expression in the sample and additionally on the amount of inflammatory risk markers for CVD, as ICAM-1, E-selectin and P-selectin in patients (Zill*et al*., 2012).

In conclusion, ESR proved to be a good marker for RA disease activity in patients compared to controls. ID genotype was the predominant genotype in patients and controls. II genotype was higher in controls compared to patients. Because of a more or less similar distribution of alleles, any association with cardiovascular disease risk could not be ascertained. Also methylation pattern analysis using MS-PCR suggested equal distribution of methylated and unmethylated regions in patients and controls, resulting in less significant findings. But in order to confirm the above findings, a higher sample size needs to be studied.

Conflicts of interest: The authors stated that no conflicts of interest.

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