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Full Length Research Paper

Elevated rheumatoid factor (RF) from peripheral blood of patients with rheumatoid arthritis (RA) has altered chromosomes in Coimbatore population, South India

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Rheumatoid arthritis (RA) is the most common systemic autoimmune disease affecting approximately 1% of the adult population worldwide and 0.75% in Indians. Rheumatoid factor (RF), a circulating antibody, is a key serum analyzer used in diagnosis of RA. Recent evidence indicates that chromosomal abnormalities (CA) has been used as a biological marker in the study of RA. The present study aims to identify the major and minor CA found among the RA patients in Coimbatore district, South India. The study was conducted in 110 samples (RA patients 55 and Controls 55) using G-banding protocol and the RA patients and controls were selected based on their RF value and they were categorized in to 2 groups based on their age. In this study it was found that there was a significant increase in RF value in the serum of group 2 RA subjects. The major alterations observed were deletions and translocations mainly in the chromosome 1, 2, 4, 6, 8, 9, 12, 16, 18 and minor aberrations like dicentrics, gaps, breaks and rings were observed in chromosomes 1, 6, 9, 18. Thus the results of this study predict that CA was one of the best biomarker for RA predictivity in women. Consequently, further identification of RA genetic risk factors should aid in elucidating the underlying mechanisms of autoimmunity, in general, and substantially impact drug discovery through the development of targeted diagnostics and therapeutics.

Key words: Rheumatoid arthritis, chromosomal alterations, rheumatoid factor.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease influenced by both genetic and environmental factors (Lynn et al., 1995 and MacGregor et al., 2000). The disease is characterized by inflamemation of the synovial tissue and local articular damage (Firestein, 2003). RA is a chronic systemic inflammatory illness with prevalence of approximately 0.75% in India (Malaviya et al., 1993). Disability in this inflammatory polyarthritis primarily stems from progressive bone erosion and co-morbidity with coronary artery disease, infection and lymphoma (Callahan and Pincus, 1995; Scott and Kingsley, 2006). As with many other autoimmune conditions, RA affects women more commonly than men. RA is the most common form of arthritis, affecting 0.3% to 1% of the adult population, mainly women after the age of 50 years (Symmons et al., 1994;

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Gabriel 2001). RA is associated with severe disability and substantial morbidity (Pincus, 1995; Sokka, 2003), and there is a growing recognition of premature RA-related mortality (Symmons et al., 1998; Mikuls et al., 2002; Gabriel et al., 2003; Sihvonen et al., 2004; Goodson et al., 2005; Hakoda et al., 2005). Rheumatoid Factor (RF), a circulating antibody to immunoglobulin G, is a key serum analyzer used in diagnosis of RA as well as an aid for the prognosis of RA-severity (Firestein, 2003). Although the etiology of RA is presently unknown, studies of RA heritability in two Northern European regions have demonstrated that an average of 60% of the disease variance can be attributed to genetic factors (MacGregor et al., 2000).

Recent evidence indicates that chromosomal abnormalities (CA) may also be of biological relevance in non-transformed cells, including synovial cells derived from patients with RA (Bonnici et al., 1992; Mertens et al., 1993; Ermis et al., 1993; 1995; Weiss et al., 1999 and Kehrer-Sawatzki et al., 1999).

The objective of this study was to identify the major and minor CA found among the RA patients in Coimbatore district, India. Furthermore, the RF was analyzed for all the RA patients and control subjects. Based on the RF value, the patients and controls were selected and their blood samples were collected and analyzed for CAs using Trypsin Giemsa banding in peripheral blood lymphocyte (PBL) culture. Since increase in age is one of the main factors for RA susceptibility, the RA subjects were categorized based on their age and compared to their controls.

MATERIALS AND METHODS

Selection of subjects

The study was carried out in Coimbatore population, South India as Coimbatore is a cosmopolitan city with huge populations and higher prevalence of RA. 55 RA patients and the control group consisted of 55 healthy normal subjects. All the subjects, were categorized into two groups (group 1 < 40 yrs and group 2 >41 yrs) who were recruited between 37 – 71 years of age. Informed written consent was obtained from all individuals, and an open questionnaire was directed towards the patients and controls to get the relevant details on age, life style factors, medical history, and number of delivery. An ethical clearance was obtained for this study from PSG Institute of Medical Research, Coimbatore and the blood samples were collected from the subjects and analyzed for RF value and CA.

Immunometric enzyme immunoassay for the quantitative measurement of IgG, IgM and IgA rheumatoid factors in serum

Sample collection and preparation

All serum samples are prediluted (in the ratio of 1: 100) with sample buffer. Therefore 10 µl of sample may be diluted with 1000 µl of sample buffer. Blood was collected by venipuncture into vacutainers and serum was separated by centrifugation after clot formation. All components of this test kit are supplied in a liquid format and ready to use, except the sample buffer and wash buffer.

Procedure

All components should be at room temperature before use. Dilute all patient samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 1000 µl of sample buffer in a polystyrene tube, mix well. Calibrators and controls are ready to use and need not to be diluted. Prepare a sufficient number of microplate modules to accommodate calibrators, controls and pre-diluted patient samples in duplicates. Pipette 100 µl of calibrators, controls and prediluted patient samples into the wells. Incubate for 30 min at room temperature (20 - 28°C). Discard the contents of the microwells and wash 3 times with 300 µl of wash solution. Dispense 100 µl of enzyme conjugate solution into each well. Incubate for 15 minutes at room temperature. Discard the contents of the microwells and wash 3 times with 300 µl of wash solution. Dispense 100 µl of TMB substrate solution into each well. Incubate for 15 minutes at room temperature protected from light. Add 100 µl of stop solution to each well of the modules and leave untouched for 5 min. Read the optical density at 450 nm. The developed color is stable for at least 30 min. Read optical densities during this time.

Chromosomal analysis

Cultures of leucocytes obtained from peripheral blood were set-up following the method of Moorhead et al. (1960). All chemical reagents were purchased from Sigma Chemicals, except colcemid that was obtained from Gibco Laboratory. Blood samples were set up to establish leukocyte cultures following standard procedures in our laboratory. 0.5 ml blood was added to 4.5 ml RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 1% streptomycin-penicillin, 0.2 ml reagent grade phyto-hemagglutinin, and was incubated at 37ºC. After 71 h, cultures were treated with 0.1 g/ml colcemid to block cells in mitosis. Lymphocytes were harvested after 72 h by centrifuging cells to remove culture medium (800 -1000 rpm), addition of hypotonic solution (KCl 0.075 M) at 37ºC for 20 min to swell the cells, and treated twice with Carnoy's fixative (3:1 ratio of methanol: acetic acid). Slides were carefully dried on a hot plate (56ºC, 2 min). Three days later, slides were stained using the Trypsin - Giemsa technique. For the CA analysis, 100 metaphases in first cell cycle were evaluated per subject under a microscope (100X) to identify numerical and structural CA. Observations were recorded on master tables and later transferred to a computer file.

Statistical analysis

All statistical analysis were performed by using the software SPSS for windows version 13. To asses the group statistics of the RA patients and controls for mean age, RF value and total CA and it was finally confirmed by using the ANOVA through, within and between groups of subjects and controls.

RESULTS AND DISCUSSION

In the present investigation, totally 110 subjects were selected which includes 55 RA subjects and 55 controls. Genetics can be applied to dissect different pathogenetic pathways in the etiopathogenesis of RA. In addition to traditional genetic factors, several environmental factors have been implicated as predictors of RA (Symmons and Harrison, 2000). Based on the age RA patients and controls were categorized in to two groups as Group 1 (<

40 years) and Group 2 (>41 years). The age of the RA patients and controls were selected as ±2 years and the subjects were all females and no males were recruited.

The 2- to 3-fold higher prevalence of the disease in women, primarily due to an increased female incidence before menopause, has been interpreted as indicating a role for hormonal or reproductive factors; about 70% of patients with RA are women.

RF is an antibody against the Fc portion of immune-globulin G. RF was first described by Waaler and Rose (1940), and Pike stated in 1949 that RF could be utilized as a diagnostic criteria in RA. In our study both subjects were monitored the RF value. Tables 1 and 2 shows the RF value and CAs found among the RA patients and controls. Table 3 shows the mean ± SD values for RA patients and their respective controls. The mean age of RA patients and controls in Group I and II were 38.7 ± 1.25 and 57.02 ± 7.48, 38.7 ± 1.16 and 57.64 ± 7.41 years respectively. The RF value of RA patients in Group II (48.38 ± 15.49 U/ml) was found to be higher, followed by Group 1 (28.7 ± 7.59 U/ml). The RF value in RA patients shows significant value when compared to their respective controls. The RF value in RA patients shows significant value when compared to their respective controls. Even in healthy people, RF levels increase with age, and positive reactions can be seen in 5% of young people and up to 25% of the elderly. RF is an important laboratory parameter because RF positive RA patients have more frequent joint deformity and extra-articular manifestation than RF negative patients. Also, the possibility of developing RA is high in healthy people with RF (Scott, 2000; Kaltenhauser et al., 2001). RF is a useful laboratory finding affecting the prognosis of RA (Fleming et al., 1976; Van der Heijde et al., 1988).

The conventional cytogenetic analysis of chromosomal aberration (CA) frequencies in PBL has been internationally standardized; furthermore, contrary to alternative measures, this biomarker has been validated in terms of its association with subsequent disease outcomes.

It is generally accepted that an increased frequency of CA in PBL indicates a clastogenic exposure and hence, at a population level, a statistical increase in cancer (Bonassi et al., 2000, 2004; Hagmar et al., 2004; Rossner et al., 2005). Table 4 displays the total number of major and minor CAs seen in both RA patients and controls. In RA patients the major CAs were found higher in chromosome 9 (Major CA n= 21 and minor CA n= 15), followed by chromosome 1 (Major CA n = 11 and minor CA n = 8). Only less number of aberrations was observed in chromosomes 2, 4, 6, 8, 12, 16 and 18 of the RA patient. Controls showed only less number of aberrations compared to the patient.

The major alterations observed were deletions and translocations mainly in the chromosome 9. Table 5 displays the p –value at 0.05 level significance between the RA patients and control groups analyzed for total CA and RF values.

Long arm 18 deletion syndrome (18q- syndrome) was first reported by de Grouchy et al. (1964). Chromosome 18 abnormalities have been reported in individuals with hypothyroidism, hypoparathyroidism, growth hormone deficiency, and insulin dependent diabetes mellitus (IDDM) (Gordon et al., 1995; Ghidoni et al., 1997; Dacou-Voutetakis et al., 1999).

Reports of chromosome 18 anomaly and autoimmune disease may represent chance associations or true genetic linkages. Given the number of patients reported with 18q deletion (approximately 100) the incidence of reported autoimmune disease appears to be increased over the general population. According to Jawaheer et al. (2002), Pascual et al. (2002); Brintnell et al. (2004), there is substantial evidence from linkage and association studies for a locus contributing to RA risk on chromosome 6p21. In a genome - wide screening of multicase families of RA patients, CA was observed in 1p13, 1q43, 6p21.3, 6q21 and 18q21 (Jawaheer et al., 2001; 2003; Mackay et al., 2002).

Chromosome 9q33.2 show strong and consistent association across three independent RA case-control studies (1732 cases/2502 controls), paralleling and extending the results of a whole-genome association study (Plenge et al., 2007) and a candidate gene study (Kurreeman et al., 2007). The major alterations observed in RA patients were deletions and translocations mainly in the chromosome 9. Interestingly in our study, Controls showed only less number of minor CA compared to the RA patients.

This was due to age, one of the main factors for increase in CA. Preliminary studies indicate that cytogenetic damage in humans accumulates with age (Tucker et al., 1994; Ramsey et al., 1995). The increase in the amount of chromosome aberrations related to age could be due to a gradual accumulation of aberrant cells in peripheral lymphocytes, a decreased efficiency in the recognition and repair of induced damage, or due to an increase in accumulated exposure to environmental clastogenic agents (Bender et al., 1988).

In our study, it was concluded that CA has been considered as one of the best biomarker for chronic inflammatory diseases like RA. Consequently, further identification of RA genetic risk factors should aid in elucidating the underlying mechanisms of autoimmunity, in general, and substantially impact drug discovery through the development of targeted diagnostics and therapeutics.

CA may be the consequence of the disease in RA patients. Chromosome 9 was found to have much consequence in the RA patients. In future the study may be extended by analyzing the synovial fibroblasts and at the molecular level. Identification and understanding of critical molecular pathways leading to RA development gives some fresh prospective in development of novel and more effective therapies for RA.

Table 1. Shows the Rheumatoid factor (RF) value and chromosomal alterations found among rheumatoid arthritis patients.

Par	A	G	RF value (U/ml)	Chromosome alterations (CA)													Total CA
				Chromosomes with major alterations									Chromosomes with minor alterations				
				1	2	4	6	8	9	12	16	18	1	6	9	18	
RA01	45	2	29						*						*		2
RA02	48	2	35	*									*				2
RA03	56	2	42	*													1
RA04	60	2	41			*											1
RA05	40	1	25							*					*		2
RA06	46	2	21														0
RA07	54	2	32				*								*		2
RA08	59	2	42							*			*				2
RA09	37	1	29						*							*	2
RA10	62	2	67						*					*			2
RA11	67	2	55	*											*		2
RA12	53	2	78				*		*						*		3
RA13	38	1	24									*					1
RA14	64	2	45	*											*		3
RA15	70	2	44						*							*	2
RA16	43	2	34			*							*				2
RA17	56	2	46						*		*		*				3
RA18	59	2	48			*								*			2
RA19	63	2	67	*											*		2
RA20	61	2	56						*								1
RA21	41	2	27							*							1
RA22	40	1	29						*								1
RA23	67	2	66			*											1
RA24	38	1	23									*					1
RA25	54	2	44						*								1
RA26	58	2	49	*											*		2
RA27	59	2	51		*						*						2
RA28	62	2	33						*								1
RA29	40	1	31						*				*				2
RA30	45	2	32					*									1
RA31	42	2	46	*													1
RA32	54	2	45			*									*		2
RA33	39	1	26						*								1
RA34	45	2	22	*					*				*		*		4
RA35	55	2	27			*											1
RA36	67	2	57									*			*		2
RA37	58	2	53			*											1
RA38	56	2	45				*										1
RA39	37	1	49				*									*	2
RA40	54	2	56	*					*				*				3
RA41	56	2	33		*												1
RA42	61	2	49				*										1
RA43	55	2	35									*			*		2
RA44	60	2	81						*								1
RA45	70	2	75						*								1
RA46	69	2	65						*					*			2
RA47	38	1	24	*											*		2
RA48	62	2	71			*											1

Table 1. Contd.

RA49	56	2	67		*						*						2
RA50	40	1	27	*				*				*				*	4
RA51	53	2	54						*								1
RA52	63	2	69						*								1
RA53	59	2	33						*				*				2
RA54	57	2	45						*						*		2
RA55	62	2	65				*								*		2

Table 2. Shows the Rheumatoid factor (RF) value and chromosomal alterations found among control subjects.

Par	A	G	RF value (U/ml)	Chromosome alterations (CA)													Total CA
				Chromosomes with major alterations									Chromosomes with minor alterations				
				1	2	4	6	8	9	12	16	18	1	6	9	18	
CN01	46	2	12												*		1
CN02	49	2	10														0
CN03	59	2	19														0
CN04	61	2	17														0
CN05	39	1	9														0
CN06	45	2	11														0
CN07	55	2	13														0
CN08	59	2	14							*							1
CN09	38	1	8														0
CN10	61	2	13														0
CN11	68	2	17														0
CN12	52	2	18														0
CN13	39	1	7	*									*				2
CN14	63	2	11														0
CN15	71	2	18														0
CN16	44	2	13														0
CN17	57	2	18														0
CN18	58	2	17														0
CN19	62	2	20														0
CN20	60	2	21														0
CN21	42	2	10														0
CN22	40	1	7										*		*		2
CN23	69	2	13														0
CN24	37	1	16														0
CN25	54	2	11														0
CN26	59	2	17														0
CN27	61	2	22		*									*		*	3
CN28	63	2	24														0
CN29	40	1	6														0
CN30	46	2	12														0
CN31	43	2	11											*			1
CN32	55	2	11														0
CN33	39	1	10														0
CN34	46	2	15											*			1
CN35	56	2	17														0

Table 2. Contd.

CN36	67	2	19									0
CN37	59	2	20								*	1
CN38	56	2	13									0
CN39	37	1	10		*							1
CN40	55	2	8									0
CN41	57	2	11							*		1
CN42	63	2	23									0
CN43	57	2	21									0
CN44	60	2	24									0
CN45	71	2	20			*						1
CN46	68	2	21						*			1
CN47	38	1	8									0
CN48	61	2	10									0
CN49	57	2	13									0
CN50	40	1	9									0
CN51	54	2	10					*				1
CN52	65	2	19									0
CN53	59	2	18									0
CN54	58	2	14									0
CN55	63	2	21								*	1

A – Age, G – Group, RA – Rheumatoid arthritis, RF – Rheumatoid factor, Group 1 - < 40 yrs age, Group 2 - >41 yrs age, * - Subjects with CA, Par – Particulars, CN – Controls.

Table 3. Shows the mean ± SD values for Rheumatoid Arthritis (RA) patients and their respective controls.

Particulars		Age (Yrs)	RF value (U/ml)	Total CA
RA patients	Group I	38.7±1.25	28.7±7.59*	1.8±0.92*
	Group II	57.02±7.48	48.38±15.49*	1.67±0.77*
Controls	Group I	38.7±1.16	9±2.79	0.5±0.85
	Group II	57.64±7.41	15.78±4.42	0.29±0.59

Values significant at $p<0.05$ level, RA patients – Rheumatoid arthritis patients, Yrs – Years, RF – Rheumatoid factor, U/ml – Units/milliliter, CA – Chromosomal aberrations.

Table 4. Shows the total number of major and minor CAs seen in both Rheumatoid Arthritis (RA) patients and controls.

Particulars		Chromosome number									Total CA
		1	2	4	6	8	9	12	16	18	
Major CA	RA patients	11	3	8	6	2	21	3	3	5	62
	Con	1	1	-	1	-	1	1	0	0	5
Minor CA	RA patients	8	-	-	3	-	15	-	-	4	30
	Con	3	-	-	4	-	3	-	-	3	13

CA – Chromosomal aberrations, RA patients - Rheumatoid arthritis patients, Con – Controls.

Table 5. Displays p – value at 0.05 level significance between the Rheumatoid Arthritis (RA) patients and control groups analyzed for total CA and RF values.

Particulars		p-value
Total CA	Group I	0.004121
	Group II	3.08e-15
RF- value	Group I	4.155 e-07
	Group II	<2.2 e-16

RF – Rheumatoid factor, P- value – Probability value.

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