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PCR BASED G3M(11) AND G3M(21) ALLOTYPING AMONGST RHEUMATOID ARTHRITIS PATIENTS

SHOBHA R. DHIMAN*, DIVYA KHANNA**, GURLOVELEEN KAUR**, PALKI SAHIB KAUR** AND PRAVEEN P. BALGIR**

*Department of Human Biology,
**Department of Biotechnology, Punjabi University, Patiala - 147 002, Punjab, India.

ABSTRACT

G3m(11) and G3m(21) allotypes have been analyzed in a group of Rheumatoid Arthritis patients and Normal Controls belonging to Punjabi population of North Western India. The PCR amplified DNA fragment, comprising a major portion of the second and third constant region domains CH^2 and CH^3 of the human IgG3 heavy chain gene, with the help of a pair of primers, was digested with restriction endonuclease Rsa1, and the pattern so generated was analyzed on PAGE (12%) and visualized with silver staining. Individuals homozygous for G3m(11) and G3m(21) and those heterozygous for G3m(11, 21) revealed differences in three codons for the amino acid residues. Individuals homozygous for G3m(11) have Proline, Phenylalanine and Serine at these positions, while those homozygous for G3m(21) showed Leucine, Tyrosine and Asparagine at the corresponding positions. However, the heterozygous G3m(11, 21) individuals displayed the mixed pattern for, both G3m(11) and G3m(21) codons. In the present study, G3m(11) and G3m(21) allotypic analysis revealed frequencies of Gm^{11} & Gm^{21} alleles to be 0.483 & 0.516 amongst the patients and 0.446 & 0.554 amongst the controls. Chi-square analysis, however, revealed statistically non-significant differences between the two series.

Keywords : G3m(11) & G3m(21) allotypes, Rheumatoid Arthritis (RA) Patients, Normal Controls.

Introduction

Immunoglobulins are defined as the most effective and targeted weapons of the immune system against various diseases. Immunoglobulin G (IgG) is the major effector molecule of the humoral immune response in man. It accounts for about 75% of the total immunoglobulins in the plasma of a healthy individual. The other four classes of immunoglobulins IgM, IgA, IgD and IgE constitute the remaining 25% of the immunoglobulins. Basic structure of immunoglobulins reveals that 1,50,000 molecular weight immunoglobulin molecule consists of two 50,000 molecular weight polypeptide chains designated as heavy chains and two 25,000 molecular weight chains designated as light chains.

Both the light and heavy chains have 100 - 110 amino acids variability at the N-terminal end, known as the variable V_H or V_L terminal end. On the other hand, the C-terminal half of the molecule is called the constant region. Two basic amino acid sequences designated as kappa (x) and lambda (λ) are found on the light chains and five basic amino acid sequences mu (μ), gamma (Υ), alpha (α), delta

Address for Correspondence: Dr. Shobha R. Dhiman, 53, Phase II, Urban Estate, Patiala – 147002, Punjab, India.

(δ) & epsilon (ε) corresponding to five different heavy chain constant regions are found which are termed as IgM, IgG, IgA, IgD and IgE respectively. IgG molecule consists of two ϒ heavy chains and two x or λ light chains. The four subclasses IgG1, IgG2, IgG3 and IgG4 are distinguished by differences in ϒ -chain sequences.

Immunoglobulins are structurally highly variable glycoproteins that specifically recognize and bind antigens and are involved in the specific immune response against pathogenic microorganisms. Rheumatoid arthritis is an autoimmune disease in which a person's immune system which normally protects the body from infection and disease attacks joint tissues. It is a multifactorial disease, the pathogenesis of which involves immunological, genetic and environmental factors. The word 'Rheumatism' is derived from the Greek word 'rheuma', which means 'swelling'. It is a chronic disease causing inflammation and deformity of the joints. Sustained inflammation usually results in joint damage and disability. Rheumatoid nodules, arthritis, neuropathy, scleritis, pericarditis and splenomegaly are common to the disease. Patients suffering with this disease have antibodies in their blood which target their own body tissues that are self killing. White blood cells, the soldiers (the agents) of immune system travel to the synovium and cause inflammation (Synovitis) characterized by warmth, redness, swelling and pain. As the disease progresses chronic inflammation leads to destruction of the cartilage, bone and ligaments causing deformity of the joints surrendering muscles, ligaments and tendons become weakened, it may cause osteoporosis.

The disease is three times more common in women as in men. This is due to certain hormonal factors which make women more prone to it. Since it can affect multiple other organs of the body, it is referred to as a systemic illness.

A number of criteria for the classification of rheumatoid arthritis have been listed by the American Rheumatism Association and rheumatoid factor is one of the most important criteria listed by it. Whenever rheumatoid arthritis is suspected the presence of rheumatoid factor is investigated. Although the role of rheumatoid factor in patients with rheumatoid arthritis is unclear, immune complexes that form between rheumatoid factor and IgG can activate the classical complement pathway leading to pathogenic outcomes involving inflammatory events and tissue damage. It has been observed and experimentally established that antibodies precisely detecting Mendelian genetic markers of human Ig in the Gm system are common in rheumatoid arthritis and are rare in other diseases. The Gm system and antibodies against allotypes is of considerable interest in rheumatoid arthritis.

Uhlig *et al.* (1999) reported that smoking tobacco increases the risk of developing rheumatoid arthritis and infectious agents like virus, bacteria and fungi may also be the cause of rheumatoid arthritis.

It has been noted that antiallotypes observed in rheumatoid arthritis are chiefly directed against G3m and G1m allotypes. A large number of allotypes resulted in identification of many different alleles for IgG subclasses. As a result of such studies it has been found that the Gm system is second only to HLA system which shows such a high rate of genetic complexity (Schanfield and Van Loghem, 1986).

Studies employing monoclonal reagents showed that anti Gm's specific for other individuals' genetic markers are significantly more common in rheumatoid arthritis patients lacking that very allotype. Studies of anti Gm's

in rheumatoid arthritis have surprisingly revealed that these anti-Gm's may direct Mendelian allotypes of other individuals. Caucasian rheumatoid arthritis patients may for example have anti-Gm's uniquely specific for Mongolian allotypes. The Gm system and antibodies against allotypes is of considerable interest in rheumatoid arthritis.

IgG subclass levels show differential expression associated with Gm allotypes, as reported by Huizinga *et al.* (1989) and Vlug *et. al.* (1994). They found individuals with G3m(11) allele to have higher levels of IgG3 as compared to G3m(21) individuals.

A number of studies have been carried out in the past between immunoglobulin allotypes and various diseases like myasthenia graves (Nakao *et al.*, 1980), autoimmune chronic active hepatitis (Whittingham *et al.*, 1980), autoimmune hemolytic anemia (Litwin *et al.*, 1973; LePetit *et al.*, 1976), thyroid autoimmunity in graves disease (Farid *et al.*, 1977), diabetes (Gill *et al.*, 1990). The immunoglobulins are also found to play a major role in many diseases like alzheimer's, atherosclerosis, diabetes and rheumatoid arthritis (Nakao and Sasazuki, 1986).

Classically Gm allotypes were determined by hemagglutination-inhibition tests (Steinberg,1967) which require immunological reagents. Although these provide quite reliable results but the longer periods of incubation involved and the difficulty to procure the immunological reagents, some of which are difficult to produce or are not available, this biochemical technology has been replaced by DNA technology especially PCR to study the Gm typing at the genomic level. This technology being cost effective and less time consuming can replace the biochemical technique in clinical labs.

In the case of Caucasians, G3m allotypes can be described simply by two alleles G3m(11) and G3m(21) (Balbin *et al.*, 1994). G3m(11) and G3m(21) allotypes have particularly been studied at DNA level because gene sequence and restriction endonuclease cleavage sites are well known and these have been found to exhibit polymorphism amongst Caucasoid population and since no such study is available on the populations of Indian subcontinent till date, the present study is an endeavor at investigating the G3m(11) and G3m(21) allotypes at genomic level using PCR technology in a group of rheumatoid arthritis patients and normal controls belonging to the population of North Western region of India in the State of Punjab.

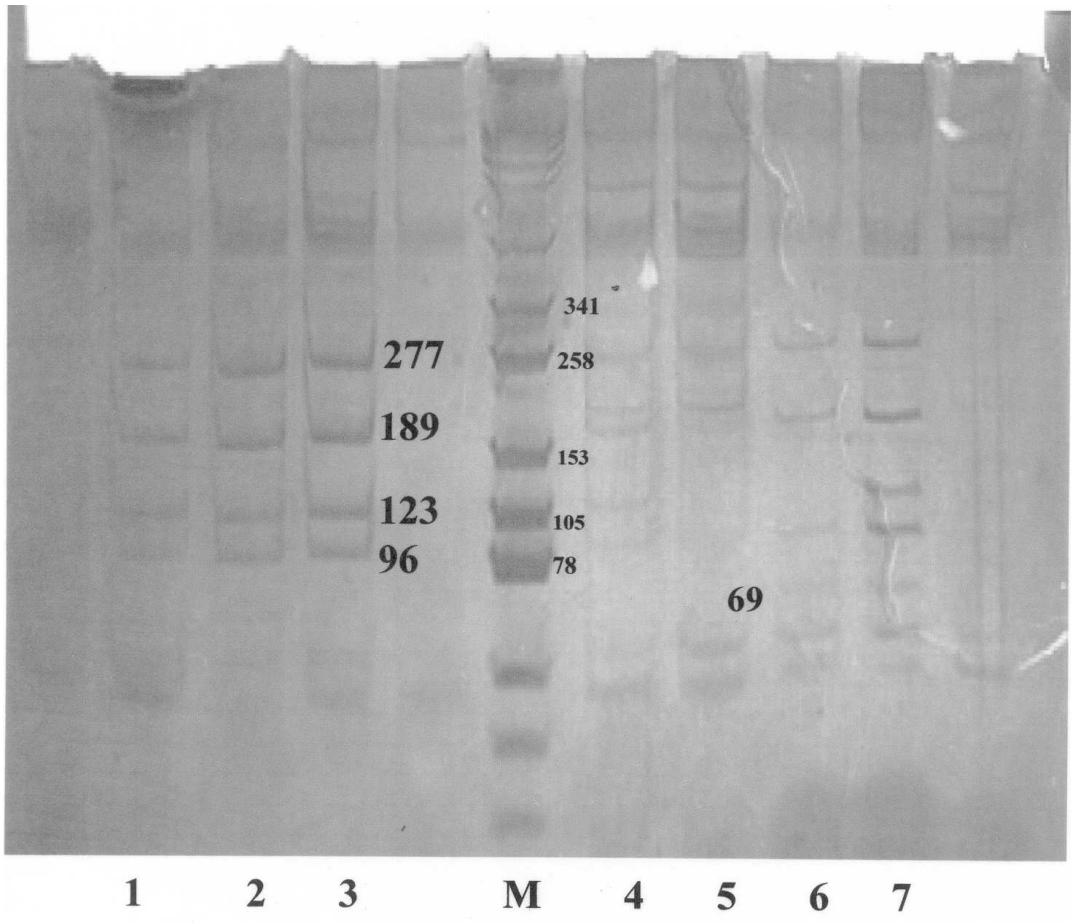
Material and Methods:

2-3ml of fresh blood was collected in EDTA containing vials from 90 rheumatoid arthritis patients with their informed consent, from the local Government Rajindra Hospital/ College, Patiala; Bharat Laboratory, Patiala; Madan's Hospital, Amritsar and Tuli's Diagnostic Lab., Amritsar. The normal control sample for the study comprised of 92 students residing in University hostels. Genomic DNA was isolated from human blood cells using the method of Sambrook *et al.* (1989). The following oligonucleotides primer set:

PR 131 : 5' ACCCAAGGATACCCTTATGATT 3' (22 nucleotides)

PR 132 : 5' AGGCTCTTCTGCGTGAAGC 3' (20 nucleotides)

was used for subclass-specific amplification of a 683 base pair (bp) DNA segment corresponding to a major portion of the second and third constant region domains (C_H^2 and C_H^3) of the human IgG3 heavy chain gene**.** This segment of DNA contains three restriction sites for Rsa1 enzyme in case of G3m(11) allotype and four such sites in case of G3m(21) allotype. The extra site in G3m(21)



Lane1 (11,21) Lane3 (11,11) Lane5 (21,21) Lane7 (11,21)
Lane2 (11,21) Lane4 (11,11) Lane6 (21,21)
M Marker (pUC18/Sau3A1 Digest)

Fig.1 G3m Allotypes Analyzed on 12% PAGE

is generated by the substitution of T by A in codon 296 whereby the TTC encoding phenylalanine becomes TAC coding Tyrosine and generates a GTAC cleavage site for Rsa1. The amplified DNA from the samples was thus digested with restriction enzyme Rsal. This PCR based assay permitted the distinction of G3m(11) and G3m(21) alleles by analyzing the electrophoretic mobility of the DNA fragments generated by digestion of the PCR products with Rsa1 (NEB, England; GT/AC). Digested products were analyzed by electrophoresis in 12% polyacrylamide gels and stained with silver nitrate and photographed (Rosenbaum & Riesner, 1987) (Fig.1).

In the present study, genomic DNA from 90 Rheumatoid Arthritis (RA) patients and 92 Normal Controls was analyzed for G3m(11) and G3m(21) allotypes. The patient series comprised of 26 males (28.89 %) and 64 females (71.11%), whereas, 31 males (33.70%)

TABLE – I. SEX-WISE DISTRIBUTION OF RA PATIENTS & CONTROLS.

S.No.	Sex	RA Patients		Controls	
		No.	%	No.	%
1	Male	26	28.89	31	33.70
2	Female	64	71.11	61	66.30
3	Male & Female	90	100.00	92	100.00

and 61 females (66.33%) comprised of the control series (Table I).Age-wise distribution of patients showed the maximum number of them to be in the 40 - 70 years age group (56.67%) followed by up to 40 years age group (26.67%), least number of the patients being in the above 70 years age group (16.67%), this could be due to lesser number of individuals surviving after 70 years. All the 92 normal controls being students residing in the University Hostels were in the up to 40 years category (Table II).

TABLE –II. AGE-WISE DISTRIBUTION OF RA PATIENTS & CONTROLS.

Age Group	RA Patients		Controls	
	No.	%	No.	%
Up to 40	24	26.67	92	100.00
40-70	51	56.67	-	-
Above70	15	16.67	-	-
Total	90	100.01	92	100.00

Table III depicts the caste wise distribution of rheumatoid arthritis patients and normal controls. The patient group consists of maximum percentage of Brahmins (20.00%) followed by an equal percentage (17.77%) of individuals from the Jat Sikh, Rajput and Baniya communities. The SC Group consists of 14.44% of individuals and the least percentage of rheumatoid arthritis patients belonged to the 'Others' category (12.22%). The 'Others' category includes individuals whose caste group was not specified or not known. Baniyas and Jat Sikhs having an equal percentage (22.82%) formed the major portion of the Control group followed by Brahmins (15.22%), Rajputs (14.13%) and the SC Category (13.04%). Even in the normal controls, the 'Others' Category had the least number of individuals (11.96%).

TABLE III : CASTE-WISE DISTRIBUTION OF RA PATIENTS & CONTROLS.

Caste Group	RA Patients		Controls	
	No.	%	No.	%
Jat Sikh	16	17.77	21	22.82
Rajput	18	20.00	14	15.22
S. C.	13	14.44	12	13.04
Brahmins	16	17.77	13	14.13
Baniyas	16	17.77	21	22.82
Others	11	12.22	11	11.96
Total	90	100.00	92	99.99

Results & Discussion

Table IV shows the distribution of G3m(11) and G3m(21) allotypes and the allele frequency distribution amongst rheumatoid arthritis patients and normal controls series. Both the homozygous G3m(11) and G3m(21) allotypes show a higher percentage frequency amongst the patient series (25% and 28% respectively) compared to the control series, where the percentage frequency of G3m(11) allotype is 14.13% and that of the G3m(21) allotype is 25.00%. The difference between the patient series and control series is much more in case of G3m(11) allotypes, about 11.00% compared to the G3m(21) allotypes, where the difference is only 3.00%. One interesting fact that emerges from the results is that an equal percentage i.e. 25.00% of homozygous G3m(11) allotype is found in the

Table –IV. Distribution of G3m(11) and G3m(21) allotypes and allele frequencies amongst RA Patients & Controls.

Group	G3m Allotypes						Total	Allele Frequency	
	11		11,21		21				
	No.	%	No.	%	No.	%		Gm^{11}	Gm^{21}
RA Patients	22	25.00	43	47.00	25	28.00	90	0.483	0.516
Controls	13	14.13	56	60.87	23	25.00	92	0.446	0.554

x^2 = 4.1139, d.f. = 2, .20 > p > .10.

rheumatoid arthritis patients and of homozygous G3m(21) allotype is found in the normal controls. The percentage frequency of G3m(11, 21) is higher in case of controls (60.87%) as compared to the patient series (47.00%). In the present study G3m(11) and G3m(21) allotypic analysis revealed frequencies of Gm^{11}& Gm^{21} alleles to be 0.483 & 0.516 amongst the patients and 0.446 & 0.554 amongst the controls. However, applying chi-square analysis, the differences in allotype frequencies in the rheumatoid arthritis patient and the control series were found to be statistically non-significant (x^2 = 4.1139, d.f. = 2, .20>P>.10).

The foregoing analysis shows that the frequency of both, G3m(11) and G3m(21) allotypes is higher in the patient series as compared to the control series, the differences between the two series, however, being statistically non-significant. This result is also highlighted by calculating the relative incidence 'x' by Woolf's (1955) method (Table V). The value of 'x' is fairly high in G3m(11) allotype in relation to G3m(21) and G3m(11, 21) allotypes. However, the observed deviations as shown by 'x' values are not statistically significant. For the G3m(21) allotype in relation to G3m(11) and G3m (11,21) allotype combinations, the relative incidence 'x' is either 1 or less than 1 and the computation of chi values for the relative incidence displays statistically non-significant distribution trend of G3m allotypes in the rheumatoid arthritis patients and the controls for the respective combinations for comparison.

Table–V. G3m(11) & G3m(21) Allotypes : Comparison by Woolf's Method

Number		Comparison			
RA Patients	Controls	G3m Allotypes	x	x^2 for x	Probability
90	92	11:21	1.5569	0.9523	.50>P>.30
		11:11,21	2.2039	3.8240	.10>P>.05
		11: 21+11,21	1.9661	3.0551	.10>P>.05
		21:11	0.6423	0.9526	.50>P>.30
		21:11,21	1.4152	0.9686	.50>P>.30
		21: 11+11,21	1.1538	0.1801	.70>P>.50

Level of significance = 0.05, d.f. = 1

A number of studies have been carried out on the association between immunoglobulin allotypes and various diseases, such as coronary heart disease, myasthenia gravis (Nakao *et al.*, 1980), autoimmune chronic active hepatitis (Le Petit *et al.*, 1976), thyroid autoimmunity in Graves disease (Farid *et al.*, 1977) and diabetes (Gill *et al.*, 1990).

Several workers have conducted a number of studies in the past on rheumatoid arthritis patients.Grubb (1958), Podliachouk *et al.* (1958), Harboe (1960), Tiilikainen (1960, 1965), Deicher and Schupp (1963), Strejeek and Herzog (1966) studied the distribution of several Gm factors among patients suffering from various rheumatic diseases. Most failed to find differences with the general population, though some of them reported a higher frequency of G1m(1) and G1m(2) factors in rheumatoid arthritis (Tiilikainen, 1960, 1965).

Archimandritis *et al.* (1975) studied the G1m(1), G1m(2), G3m(4) and G3m(12) in the sera of fifty-six rheumatoid arthritis patients and found that the presence of rheumatoid factor was independent of Gm phenotypes. This finding is in close agreement with the results of other investigations of Grubb, (1958); Podliachouk *et al.*, (1958); Harboe, (1960); Diecher and Schupp, (1963) and differs from that reported by Tiilikainen, (1960, 1965).

Schlesinger *et al.* (1978) found an increase in G1m(1) frequency among patients with rheumatoid arthritis, whereas, Walsh and Cox (1984) did not find any Gm allotype, haplotype (Gm3;11/Gm1;12/Gm1, 2; 11) or phenotype (G1m1,2,3;G3m11, 21,15,16) to be increased or decreased in frequency in two autoimmune diseases – rheumatoid arthritis or chronic active hepatitis in comparison with control frequencies.

Gran *et al.* (1985) did not find any association in Scandinavian patients of Gm allotypes with adult rheumatoid arthritis. Likewise, Sanders *et al.* (1985) analyzed G1m(17,1,2,3), G2m (23) and G3m (11,6, 24, 15,16) and did not find significant differences in allotype frequencies in overall rheumatoid arthritis and control groups.

Truedsson *et al.* (1990) studied the distribution of Gm allotypes, G1m(1), G1m(2), G2m(23) and G3m(11) in seventy-six Swedish patients with juvenile chronic arthritis. No significant correlation between the presence of rheumatoid factor and Gm allotypes was observed, although a tendency to low rheumatoid factor levels in patients with Gm-1, 11 was noted.

Grubb *et al.* (1991) found that the distribution of Gm allotypes G1m(1,2,3) and G3m(11,21) in the Swedish rheumatoid arthritis patients did not deviate from what is expected in the normal Swedish population and is in accordance with earlier observations of Grubb (1970); Grubb and deLange (1989); Gran *et al.* (1985). Eberhardt *et al.* (1993) also found the Gm allotype distribution to be normal and not related to clinical findings.

In the present study, G3m(11) and G3m(21) allotype analysis reveals statistically non-significant differences (x^2 = 4.1139, d.f. = 2, .20>P>.10) in the allotype frequencies amongst the rheumatoid arthritis patient and the control series, which are also supported by applying Woolf's (1955) method. These results do not provide evidence for the assumption of an association between either G3m(11) or G3m(21) allotypes and the occurrence of rheumatoid arthritis. Broadly speaking based on the present investigation it may be inferred that there are no statistically significant differences in the G3m allotypes either in the rheumatoid arthritis patients or the controls.

The relationship between Gm markers and susceptibility to rheumatoid arthritis remains controversial, since there are contradictory reports in the literature regarding the association between immunoglobulin allotypes and this disease. For the present study, since only genomic DNA was tested for allotyping, future study

using other primer sets for detecting minor gene expressions needs to be done. Furthermore, data from other populations of the subcontinent needs to be collected and studies with larger number of Gm allotypes on a larger number of rheumatoid arthritis patients would give a clearer picture.

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