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Original Research Article

Association of Vitamin D Receptor Gene Polymorphisms in Rheumatoid Arthritis Patients from North Coastal Andhra Pradesh

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Abstract

Background: Rheumatoid Arthritis (RA) is a chronic, symmetrical, inflammatory autoimmune disorder that primarily affects small joints, progression to large joints. About 1% of the world's population is affected by rheumatoid arthritis, women three times more frequent than men. The onset is more common between the ages of 40 and 50 years. Other than genetic, environmental factors have been associated with the development of rheumatoid arthritis. Vitamin D deficiency has been associated with several autoimmune disorders especially with RA. One of the candidate gene of RA is vitamin D receptor (VDR). The role of VDR has been well-known in variety of metabolic disorders and in regulation of inflammation. ***Aim:*** The aim of the present study was to investigate the association of VDR Fok1 (rs10735810) and VDR Bsm1 (rs1544410) gene polymorphisms in RA patients and controls from North Coastal Andhra Pradesh. ***Methods:*** A total of 300 samples (150 RA patients and 150 controls) were included in the present study and genotyping was accomplished by using PCR – RFLP technique. ***Results:*** Data was analyzed by SPSS 19 software. Chi square 'p' value revealed that VDR Fok1 (rs10735810) polymorphism has association with RA and VDR Bsm1 (rs1544410) polymorphism does not shows association with RA. ***Conclusions:*** In conclusion, the present study revealed that the VDR FokI (rs10735810) gene polymorphism is associated with RA and VDR Bsm1 (rs1544410) gene polymorphism does not shows association with RA.

Keywords: Rheumatoid Arthritis, Vitamin D Receptor, Gene Polymorphism, Polymerase Chain Reaction, Restriction Fragment Length Polymorphism.

INTRODUCTION

Rheumatoid Arthritis (RA) is a distinguished disorder among the existing eighty different kinds of diseases caused by autoimmunity. It is a systemic collagen vascular disorder characterised by chronic inflammation that can affect many tissues and organs including synovial joints and the cervical spine [1]. Approximately 1% of the world population, including India is burdened with this disorder with women being more traumatised than men [2]. The onset of this progressive disorder is mostly seen between the ages of 40 and 50 but can affect individuals of any age which varies in regard to type, number, and the pattern of joint involvement. Type of arthritis that causes joint inflammation and stiffness for more than six weeks in a children of 16 years or younger is often referred as Juvenile RA (JRA) or Juvenile Idiopathic Arthritis (JIA) which mimics RA except that the absence of rheumatoid factor [3-6]. Many factors including genetic, environmental, nutritional and hormonal, frequency of swollen joints, autoantibody in the serum and the severity of inflammatory process regulates the course of the disease [7, 8].

The underlying cause of the disease is not yet understood but immune system assaulting the body's own organs, tissues and cells paving path to the development of RA. Sequestered antigens or hidden antigens, Neo antigens, cessation of tolerance, cross reacting antigens and loss of immunoregulation are some of the known implications in the immune system. Despite the fact that the reasons for some immune system diseases stay obscure, family history in combination with contaminations and other environmental trigger are probably going to assume a critical part in improvement of RA. Cells of the immune system invade joint tissues (synovium), causing

inflammation and over time damage to the cartilage and bone. A cumulative effect of numerous risk factors and determinants may contribute to the progression of rheumatoid arthritis.

Vitamin D is a steroid and immunoregulatory hormone is central to the control of bone and calcium homeostasis and initiates biological responses by binding to vitamin D receptor (VDR). Vitamin D is a member of the steroid hormone receptor super family located on chromosome 12 (12q12-q14) that regulates gene expression in a ligand dependent manner [9]. Unlike earlier days, deficiency of Vitamin D is a major concern worldwide as it leads to diseases like cancer, osteoporosis and autoimmune disorders. A significant association has been demonstrated between Vitamin D insufficiency and an elevated incidence of autoimmune disorders [10]. In addition, RA patients when administered Vitamin D shown a significant clinical improvement [11]. Vitamin D receptor has been established in the rheumatoid synovium and sites of cartilage erosion in patients with RA [10]. Vitamin D receptor agonists have a critical physiological role in the regulation of the immune system [12, 13]. The impact of VDR gene polymorphism in RA susceptibility has been widely studied. There are controversial and inconclusive results in various reports due to ethnicities, extensive geographic variations and possibly study designs [14-18].

A series of polymorphisms in VDR gene have been reported so far. They include Bsm1, Apa1, Taq1 restriction sites, variable PolyA length and Fok1 restriction site [19]. Of these polymorphic sites Bsm1 are substitutions on intron 8 whereas Fork1 restriction enzyme identifies a polymorphic site in exon 2 at the 50 end of the VDR gene. There are many association International studies that confirm the functional significance of these polymorphisms and their potential effects on RA susceptibility. Very few studies are available from India and none from North Coastal Andhra Pradesh. Thus the study aimed to determine the association of these polymorphisms with RA susceptibility.

METHODOLOGY

The present case control study was carried out with 150 Rheumatoid Arthritis patients (12 males and 138 females) from King George hospital, Visakhapatnam and 150 age and sex matched controls (10 males and 140 females) above 25 years from North Coastal Andhra Pradesh during the period 2017-2019. The study was approved by the institutional ethical committee for blood sample collection from the patients. The informed consent was obtained from each and every participant before collecting blood sample for the evaluation of VDR Fok1 and VDR Bsm1 gene polymorphisms.

Molecular analysis

DNA extraction

The genomic DNA was extracted from peripheral blood by using salting out method. Genotyping was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique.

Genotyping of VDR (Fok1) (rs10735810

One set of forward "5'AGCTGGCCCTGGCACTGACTCTGCTCT3' and reverse: 5'ATGGAAACACCTTGCTTCTTCTCCCTC 3" primers were used for amplification of fragments of 265 base pairs and then amplified fragments was digested with Fok1 fast digestion enzyme. The PCR profile was: initial denaturation at 94°C, 5 minutes, And denaturation: 94°C, 1 minute, annealing: 61°C, 1 minute followed by 35 cycles each of 1 minute and 72°C, then final extension at 72°C for 7 minutes. The amplification product was visualized in a 2% agarose gel under UV light.

The FF (homozygous type allele) genotype produces a single band of 265bp and Ff (heterozygous type allele) genotype produces three bands of 265, 200 and 65bp. The ff (homozygous mutant type allele) genotype produces two bands of 200 and 65 bp.

Genotyping of VDR (Bsm1) (rs1544410)

One set of forward 5'CAACCAAGACTCAAGTACCGCGTCAGTG3'and reverse 5'- AACCAGCGGAAGA GGTCAAGGG -3'primers were used for amplification of fragments of 825 base pairs and then amplified fragments was digested with Mva12691 enzyme. The PCR profile was: initial denaturation at 94°C, 5 minutes, denaturation: 94°C, 1 minute, annealing: 58°C, 1 minute followed by 40 cycles each of 1 minute and 72°C, then final extension at 72°C for 7 minutes. The amplification product was visualized in a 2% agarose gel under UV light.

The BB (homozygous type allele) genotype produces a single band of 825bp and Bb (heterozygous type allele) genotype produces three bands of 825, 650 and 175bp. The bb (homozygous mutant type allele) genotype produces two bands of 650 and 175 bp.

STATISTICAL ANALYSIS

The data was analyzed by using Statistical Package of Social Sciences Software program (SPSS), 19 was used for calculating genotype and allele frequencies. Chi-square analysis was used to test for allele frequencies and odds ratio analysis was used to test for genotype frequencies in comparison of patients and healthy control groups. P value less than 0.05 considered statistically significant.

RESULTS

Table-1: Demographic variable of RA patients and controls

Demographic variables	RA patients (n=150) (%)	Controls (n=150) (%)
Male	12 (8%)	10(6.66%)
Female	138 (92%)	140 (93.3%)
Age years (M ±SD)	45±16.61	51.3±6.2
Disease duration (M ±SD)	12.6±2.1	-
Disease onset (M ±SD)	30±19.25	-

Demographic variables of RA patients and controls were given in table 1. The mean age ±standard deviation (SD) was 45±16.61 in RA patients and 51.3±6.2 in control group. There was 12 (8%) males and 138 (92%) females were in RA patients and there was 10(6.66%) males and 140 (93.3%) females were in control group respectively. The mean value of disease duration was 12.6±2.1 and mean value of disease onset was 30±19.25 in RA patients.

Table-2: Genotype of VDR gene (Fok1 and Bsm1) polymorphism based on Gender in RA patients

VDR gene Polymorphisms and Gender	Genotypes	(n=150)	(%)	X^2 value	P- value
VDR (Fok1)	FF	Ff	ff		
Male	2 (1.33%)	7(4.66%)	3 (2%)	3.514	0.172/NS
Female	61 (40.66%)	51(34%)	26 (17.33%)		
VDR (Bsm1)	BB	Bb	bb		
Male	1(0.66%)	6 (4%)	5 (3.33%)	13.777	0.001**
Female	58(38.66%)	68 (45.33%)	12 (8%)		

**P≤0.01 – Highly Significant; *P≤0.05– Significant; NS – Not Significant

The distribution of VDR gene (Fok1 and Bsm1) polymorphism of genotypes and Gender of RA patients represented in table 2. The chi-square p value reveals that gender was found to be statistically insignificant with VDR Fok1 gene polymorphism. The chi-square p value shows that gender was found to be significantly associated with VDR Bsm1 gene polymorphism.

Table-3: Genotype and allele frequencies of VDR (FokI and Bsm1) gene polymorphisms in RA patients

Gene Polymorphisms	Genotypes and alleles	RA patients (n=150) (%)	Controls (n=150) (%)	Odds ratio	95%CI	P-value
VDR (Fok1)	FF	63(42%)	86 (57.33%)	1	-	-
	Ff	58 (38.66%)	56 (37.33%)	0.285	0.120-0.678	0.004**
	ff	29 (19.33%)	8 (5.33%)	4.948	2.120-11.549	0.000**
	F	184 (61.33%)	228 (76%)	-	15.504	0.000**
	f	116 (61.33%)	72 (24%)			
VDR (Bsm1)	BB	59 (39.3%)	62 (41.3%)	1	-	-
	Bb	74 (49.3%)	65 (43.3%)	1.540	0.757–3.132	0.233/NS
	bb	17 (11.3%)	23 (15.3%)	0.776	0.377-1.597	0.492/NS
	B	192 (64%)	189 (63%)	-	0.065	0.798/NS
	b	108 (36%)	111 (37%)			

**P≤0.01 – Highly Significant; *P≤0.05– Significant; NS – N ot Significant

RA patients and controls

The distribution of VDR gene (Fok1 and Bsm1) polymorphism genotypes and allele frequencies of RA patients and control were represented in table 3. The Fok1 polymorphism of odds ratio p value of genotypes Ff and ff were found to be statistically significance and the chi-square p value reveals that VDR (Fok1) polymorphism shows association with RA. The Bsm1 polymorphism of odds ratio p value and chi-square p value does not shows any statically significance with RA.

Table-4: VDR Fok1 polymorphism and its interaction with VDR Bsm1 gene polymorphism

VDR Fok1 FF v/s VDR Bsm1	RA patients (n=150)	Controls (n=150)	Odds Ratio	95% CI	P-Value
BB	23	47	0.419	0.2088 – 0.8426	0.0146*
Bb	35	30	1.500	0.4985 – 4.5133	0.470/NS
bb	7	9	1.589	0.5256 - 4.8060	0.411/NS
VDR Fok1 Ff + ff v/s VDR Bsm1					
BB	36	15	2.1538	1.0116 – 4.5858	0.046*
Bb	39	35	1.5600	0.6149 – 3.9579	0.349/NS
Bb	10	14	0.2976	0.1083 – 0.8177	0.018*

**P≤0.01 – Highly Significant; *P≤0.05– Significant; NS – Not Significant

The VDR Fok1 polymorphism and its interaction with VDR Bsm1 polymorphism in RA patients and controls represents in table 4. From the above table, it is evident that the FF genotype of VDR Fok1 polymorphism was statistically insignificant with Bb and bb genotypes of VDR Bsm1 polymorphism. The FF and ff genotypes of VDR Fok1 polymorphism were statistically significant with bb genotypes, whereas it is insignificant with Bb genotype of VDR Bsm1 polymorphism.

Table 5 shows Association status of VDR (Fok1 and Bsm1) gene polymorphisms with RA of present study and previous studies.

Table 6 shows the correlation between VDR gene polymorphisms (Fok1 and Bsm1) and Rheumatoid factor in RA patients. The FF and Ff genotypes of VDR Fok1 polymorphism of odds ratio p values shows statistically in significant, whereas ff genotype odds ratio p values shows statistically significant with rheumatoid factor of RA patients. The allelic frequencies of VDR Fok1 polymorphism in RA patients with Rheumatoid factor of the chi-square p value shows that VDR Fok1 gene polymorphism was found to be significantly associated with rheumatoid factor of RA patients. The BB, Bb and bb genotypes of VDR Bsm1 polymorphism of odds ratio p values shows statistically in insignificant with rheumatoid factor of RA patients. The allelic frequencies of VDR Bsm1 polymorphism in RA patients with Rheumatoid factor of the chi-square p value shows that VDR Bsm1 gene polymorphism was found to be significantly associated with rheumatoid factor of RA patients.

Table-5: Association status of VDR (Fok1 and Bsm1) gene polymorphisms with RA of present study and previous studies

Genes	Population	No. of Cases	Genotypes			Alleles		Association with RA	References
			FF	Ff	ff	F	f		
VDR Fok1	French	100	45	43	12	61	36	Susceptible	[18]
	Turkish	98	51 (52%)	40 (41%)	7 (7%)	142 (72%)	54 (28%)	No association	[20]
	Tunisian	108	49 (45.3%)	49 (45.3%)	10 (9.2%)	147 (68%)	69 (31.9%)	Susceptible	[21]
	Egyptian (Mansoura)	128	69 (53.9%)	51 (39.8%)	8.0 (6.2%)	189 (73.8%)	67 (26.2%)	No association	[22]
	Indian	112	58 (51.8%)	50 (44.7%)	4 (3.6%)	166 (74.1%)	58 (25.9%)	No association	[23]
	India (Visakhapatnam)	150	63 (42%)	58 (38.66%)	29 (19.33%)	184 (61.33%)	116 (61.33%)	Susceptible	Present study
VDR Bsm1			**BB**	**Bb**	**bb**	**B**	**b**		
	French	100	19	35	42	50	52	No association	[18]
	Hungarian	64	13 (20.3%)	26 (40.6%)	25 (39.1%)	-	-	No association	[24]
	Turkish	98	12 (12%)	46 (47%)	40 (41%)	70 (36%)	126 (64%)	No association	[20]
	Tunisian	108	21 (19.4%)	47 (43.5%)	40 (37)	89 (41.2%)	127 (58.7%)	No association	[21]
	Egyptian (Mansoura)	128	13 (10.2%)	52 (40.6%)	63 (49.2%)	78 (30.5%)	178 (69.5%)	Protective	[22]
	India (Visakhapatnam)	150	59 (39.3%)	74 (49.3%)	17 (11.3%)	192 (64%)	108 (36%)	No association	Present study

Table-6: Correlation between VDR gene polymorphisms (Fok1 and Bsm1) and Rheumatoid factor in RA patients

VDR gene polymorphisms and Genotypes	Rheumatoid factor positive in RA patients	Rheumatoid factor negative in RA patients	Odds ratio	95% CI	P value
VDR (Fok1)					
FF	41 (27.33%)	22 (14.66%)	1	-	-
Ff	40 (26.66%)	18 (12%)	0.838	0.392-1.793	0.650/NS
ff	26 (17.33%)	3 (2%)	0.256	0.068-0.958	0.043*
F	122 (40.66%)	62 (20.66%)	5.885	-	0.015**
f	92 (30.66%)	24 (8%)			
VDR (Bsm1)					
BB	51 (34%)	8 (5.33%)	1	-	-
Bb	56 (37.33%)	18 (12%)	2.049	0.829-5.117	0.124/NS
bb	12 (8%)	5 (3.33%)	1.296	0.409-4.179	0.663/NS
B	158 (52.66%)	34 (11.33%)	2.847	-	0.091*
b	80 (26.66%)	28 (18.66%)			

**P≤0.01 – Highly Significant; *P≤0.05– Significant; NS – Not Significant

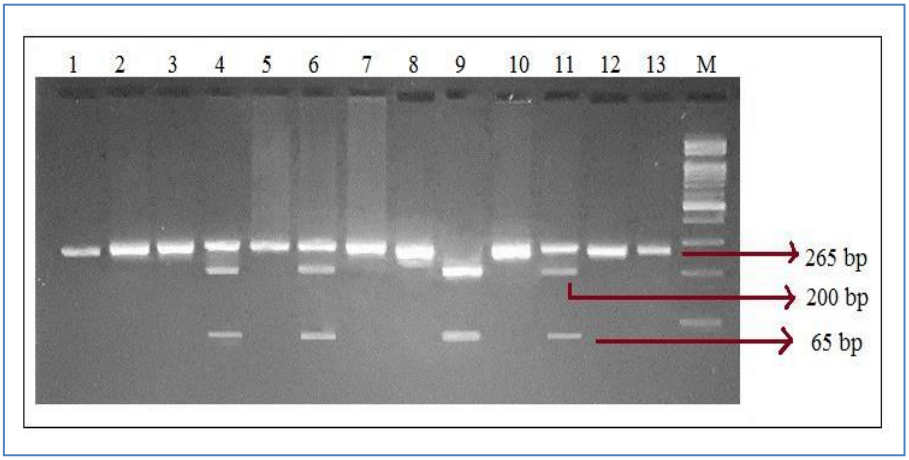

Fig-1: PCR - RFLP of Gel Electrophoresis pattern of VDR Fok1 (rs 10735810) by using restriction enzyme Fok1 fast digestion

Lane M: 100 bp ladder
Lane: 1, 2, 3, 5, 7, 8, 10, 12, 13 showing FF (homozygous wild type) genotype 265bp
Lane: 4, 6, 11 showing Ff (heterozygous) genotype with 265 bp, 200 bp & 65 bp
Lane: 9 showing ff (homozygous mutant) genotype with 200 & 65 bp

Figure 1: For VDR Fok1, the PCR yielded a 265-bp product, which on digestion with enzyme Fok1 fast digestion produced a homozygous, wild type allele (FF) was identified by the presence of one 265-bp band. The heterozygous allele (Ff) was identified by a 265-bp, 200-bp and 65-bp band, while homozygous mutant allele (ff) was identified a 200-bp and 65-bp band.

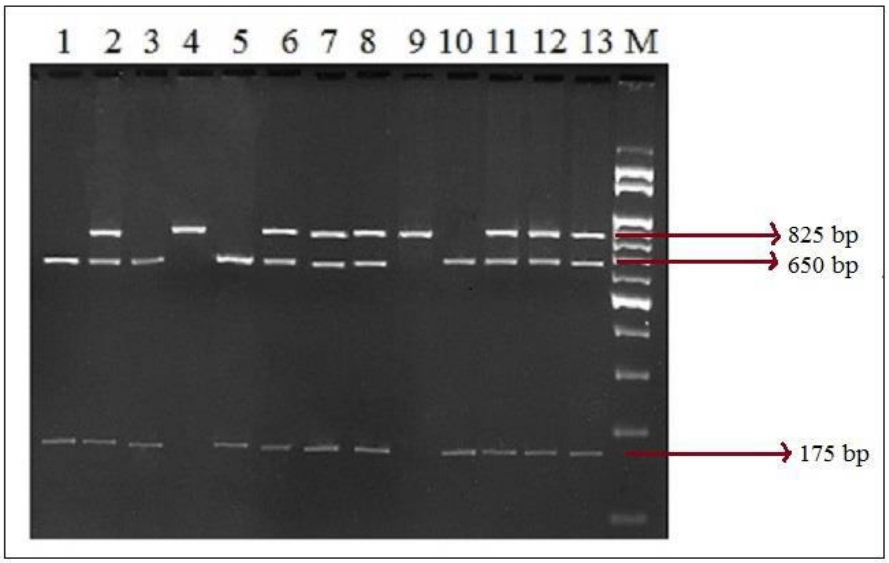

Fig-2: PCR - RFLP of Gel Electrophoresis pattern of Bsm1 (rs 1544410) by using restriction enzyme Mva12691

Lane M: 100 bp ladder
Lane: 4 & 9 showing BB (homozygous wild type) genotype with 825 bp
Lane: 2, 6, 7, 8,11,12,13 showing Bb (heterozygous) genotype with 825 bp, 650 & 175 bp
Lane: 1, 3, 5, 10 showing bb (homozygous mutant) genotype with 650 bp & 175 bp

Figure 2: For VDR Bsm1, the PCR yielded a 825-bp product, which on digestion with enzyme Mva12691 produced a homozygous, wild type allele (BB) was identified by the presence of one 825-bp band. The heterozygous allele (Bb) was identified by a 825-bp, 650-bp and 175-bp band, while homozygous mutant allele (bb) was identified a 650-bp and 175-bp band.

DISCUSSION

RA is an autoimmune inflammatory disease that affects body's joints and bones. The cause of RA is not clear, but both genetic and environmental factors are involved in this condition. RA pathogenesis is an active area of research including several genes. VDR, MTHFR, TGFβ1 and TNFB genes have generated great interest in RA pathogenesis [5, 25, 26].

The FokI polymorphism (an ACG to ATG transition) introduces an initiation codon leading to the addition of three amino acids to the VDR protein. The pattern of genotype and allele distribution in the disease and control groups recommended a lack of association of VDR FokI in RA susceptibility. In the study of [23] the RA group has shown an increase in the FF genotype as compared to the control group. In the case of genotype ff it was not found to be significantly higher in the healthy control group as compared to the RA group. The heterozygous genotype Ff was non significantly distributed in the healthy control group as compared to the RA group. An odds ratio for Ff showed weak or no association with RA susceptibility. The results are consistent with a case control study in the German population which showed no evidence of RA association with VDR [14]**.** The present study not correlates with the above two studies.

A previous study performed in a French population showed significance of the f allele with protection from RA [18] and VDR polymorphisms may contribute to the high incidence of RA in North American native populations [27]**.** The reason behind the inconsistencies may be due to the different origins and populations. In [23] study the allele frequency of the f allele was higher in cases as compared to controls, but not enough to reach the 5% level of significance, unlike the present study which shows significance.

For VDR gene polymorphisms the results of [22, 26] supported the confirmation of the major role of (BsmI, TaqI) polymorphisms in RA susceptibility in the Egyptian population. BsmI results differed between the studies of [26, 28] for the Egyptian population. This variation may be due to the studied gender [26: both females & males; 28: female only**]**, the sample sizes, the age of patients and the disease duration.

In the study of [29] VDR gene Bsm1 (rs1544410) polymorphism investigation shows no statistically significant differences in genotype and allele frequencies of RA patients and healthy controls in Pakistan population. The study of [18] also demonstrated no association of the Bsm1 (rs1544410) polymorphism with the development of RA in French and Tunisian populations [30]. The present study observations are correlate with the above two studies.

In the study of [18] shows the association of 3 VDR polymorphisms (Fok1 and Bsm1) in RA and found that in Fok1 polymorphism the allele F and genotype FF appeared to be associated with

susceptibility to RA. The results obtained in the 'autoimmune' subgroup may outline a specific association of VDR with RA and not with the general process of autoimmune diseases. The present study results are in contrast with a case-control study in German population which showed no evidence of RA association with VDR [14]. This could be due to heterogeneity between populations or lack of power in the German study caused by the small samples used (62 cases and 40 controls).

In summary, in several studies, similar to the present findings, no significant associations have been found between RA and VDR polymorphisms except for FokI polymorphism which was significantly associate with RA in a French population [18]. These results reflect that Fok1 and Bsm1 polymorphisms in the VDR gene may not be responsible for the primary disease association. Because there are controversial results in various reports, additional studies are needed to elucidate the possible association between the VDR gene and clinical features of RA including other polymorphisms in the VDR gene. These controversial results could be due to ethnicities and geographic variations [14-18]. In addition, the age groups of the subjects and the sample sizes of these studies may account for the contradictory results.

CONCLUSIONS

It was concluded from the present study direct association between VDR Fok1 (rs10735810) polymorphism and RA susceptibility have been demonstrated. Another results indicate that VDR Bsm1 gene polymorphism have no role in RA susceptibility. Further studies with extended sample sizes (from the same population) are required to overcome the lack in power results and confirmed these results in the North Coastal Andhra Pradesh population. In addition further investigations of other polymorphisms and its association with RA susceptibility may be helpful to know the pathogenesis of the disease.

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