

Cytokine gene polymorphisms in type I and type II reactions in Hansen's disease

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Abstract

Introduction: Leprosy or Hansen's disease is a chronic debilitating disease caused by *Mycobacterium leprae*. Host genetics are believed to strongly influence the course of the disease. It is known that cytokines play an important role in leprosy and cytokine gene polymorphisms probably influence the course of the disease.

Methods: In the present study, we evaluated 70 patients with leprosy and 243 controls. DNA was extracted from the peripheral blood and genotyping was done for the following polymorphisms: IL-1 RA intron 2, IL-1 β -511 C/T and TNF- α A/G.

Results: A strong association of TNF- α -308 G/A polymorphism with Hansen's disease with both genotypes and alleles was found. However, no correlation was identified between the other two polymorphisms and Hansen's disease. A strong association between the IL-1 β gene polymorphisms and the type of reactions seen in leprosy was found. In contrast, the other two polymorphisms did not show any such association.

Limitations: Genetic polymorphisms are association studies. They are not a direct reflection of the transcriptome or proteome and this is a major limitation of this study.

Conclusion: In conclusion, cytokine gene polymorphisms appear to influence the susceptibility and course of Hansen's disease. An evaluation of the cytokine levels in the skin during lepra reactions would confirm this observation. Possibly, in future, this would be a guide to therapeutic decisions in cases of lepra reactions.

Key words: Cytokine gene polymorphisms, Hansen's disease, lepra reactions

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Introduction

Leprosy or Hansen's disease is a chronic debilitating disease caused by an obligate intracellular pathogen—*Mycobacterium leprae* (*M. leprae*). The clinical spectrum of the disease varies and it depends on the host immunity; one pole is tuberculoid leprosy in which the patients have a strong cellular immune response and the other pole is lepromatous leprosy (LL) where patients have a limited cellular immune response leading to a disseminated disease with diffuse infiltration of skin and nerves and bacilli-loaded foamy macrophages. Between the two poles, the majority of patients

are borderline classified as borderline tuberculoid (BT), mid-borderline (BB) or borderline lepromatous (BL).^{1,2}

Factors influencing the type of leprosy are not well understood. Host genetics are believed to strongly influence the course of the disease. Cytokines play a critical role in triggering host-pathogen interaction in leprosy. The onset of tuberculoid leprosy is correlated with cytokine production by Th1 cells (IFN γ productions) and lepromatous leprosy is correlated with a Th 2 response (IL-4 production). Besides

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How to cite this article: Pragasam V, Vasudevan B, Moorchung N. Cytokine gene polymorphisms in type I and type II reactions in Hansen's disease. Indian J Dermatol Venereol Leprol 2020;86:482-8.

Received: September, 2018. **Accepted:** September, 2019.

Access this article online	
Quick Response Code:	Website: www.ijdvl.com
	DOI: 10.4103/ijdvl.IJDVL_619_18

IFN and IL-4, other cytokines produced by T cells such as tumor necrosis factor- α (TNF- α), IL-6 and IL-10 and by macrophages such as IL-1 family also regulate *M. leprae* cell-mediated and humoral immune responses.³⁻⁷

Leprosy reactions are immune inflammatory-related phenotypes that may occur before diagnosis, during treatment or after Multi Drug Therapy (MDT). Leprosy reactions require immediate treatment to prevent permanent disabilities. There are 2 major types of leprosy reactions. Type I reaction is characterized by acute inflammation of preexisting skin lesions or by the appearance of new lesions and/or neuritis. Systemic symptoms are rare. Type I reactions are seen in patients who have an unstable immune response (BT, BB and BL forms)⁸ or in patients with tuberculoid leprosy.^{9,10} Erythema nodosum leprosum is the main presentation of type II reaction which predominantly affects patients with leprosy showing Th2 immune response and high bacterial load. It is classified more toward the lepromatous pole (BB, BL, and LL) of the disease. Type II reaction is characterized by increased levels of TNF- α and immune complex-associated vasculitis, panniculitis and uveitis.

Cytokine gene polymorphisms have recently attracted considerable interest since it has been discovered that different alleles of cytokine genes are associated with different immunomodulatory diseases.^{11,12} Genes encoding cytokines and related molecules harbor polymorphic regions which are considered to alter gene transcription and thereby influence inflammatory processes in response to infectious disease.^{13,14} Some important cytokines that are known to harbor polymorphic regions are encoded by the genes of the interleukin-1 cluster and TNF- α .

The variability in clinical responses in patients with Hansen's disease is related to the number of cytokines secreted during the disease. Because type I and type II reactions are related to the Th 1 and Th 2 responses, it is logical to assume that cytokine secretion would influence the development of reactions. Because cytokine secretion would be influenced by genetic polymorphisms, it would be possible to predict the clinical response based on the genetic polymorphisms. Sousa *et al.* found an association between IL-6 gene polymorphisms and type II reactions.¹⁵ We were unable to find any previous studies evaluating TNF- α and/or IL-1 β gene polymorphisms and type I and type II reactions.

In this prospective case-control study, we attempted to evaluate the genetic polymorphisms of the TNF- α , IL-1 RA and the IL-1 β genes in patients of Hansen's disease and compare the same with a control population with no evidence of Hansen's disease. We also attempted to evaluate the same genetic polymorphisms in patients showing reactions and compare the same with patients who did not show any reaction.

Methods

In the present study, we evaluated 70 patients with leprosy and 243 controls. The controls were age and sex matched and they were healthy individuals with no history of leprosy. The entire spectrum of Hansen's disease was included including tuberculoid, lepromatous, borderline tuberculoid, borderline lepromatous, borderline leprosy, pure neuritic and histoid leprosy. We also included patients with deformities which included claw hand, lagophthalmos and foot drop. Finally, cases who had undergone a reaction after therapy were also included in the study. These included patients with the type I and II reactions, neuritic and late reversal reactions. Ethical clearance was obtained for the study from the ethical committee of Command Hospital Southern Command.

Two milliliter of blood was collected from all these patients and controls. Genomic DNA was retrieved from the peripheral blood by using a standard kit (Quiagen).

The IL-1 RA intron 2 polymorphism was analyzed by a polymerase chain reaction. The polymerase chain reaction products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide. The variable number of tandem repeats of an 86 bp fragment in the intron 2 was amplified. Five alleles were characterized based upon the number of repeats. Allele 1 (4 repeats) was 410 bp, allele 2 (2 repeats) was 240 bp, allele 3 (5 repeats) was 500 bp, allele 4 (3 repeats) was 325 bp and allele 5 (6 repeats) was 595 bp in length. Allele 2 was the proinflammatory allele.

For restriction fragment length polymorphism analysis of the 511 IL-1 β polymorphism, the polymerase chain reaction products were first amplified. The confirmation of a successful polymerase chain reaction was done by running the products on a 2% agarose gel. If the products were present, they were digested with 10 U of *Ava*I at 37°C for 3 h. Fragments were analyzed by electrophoresis on 3% agarose gel and stained with ethidium bromide. This yielded products of 304 bp (TT allele), 190 bp and 104 bp (CC allele) and 304, 190 and 104 bp (CT allele). The T allele was the proinflammatory allele.

TNF- α gene polymorphisms were genotyped by a polymerase chain reaction and restriction fragment length polymorphism analysis. The polymerase chain reaction products were first amplified. The confirmation of a successful polymerase chain reaction was done by running the products on a 2% agarose gel. The products were digested with 10 U of *Nco*I at 37°C for 15 min. Fragments were analyzed by electrophoresis on 15% polyacrylamide gel and stained with ethidium bromide. This yielded products of 107 bp (AA allele), 87 bp and 20 bp (GG allele) and 107, 87 and 20 bp (AG allele). The A allele was considered proinflammatory.

The primer sequences are given in Table 1. Polymerase chain reaction conditions are noted below. All polymerase chain

Table 1: The primer sequences and the restriction enzymes of the cytokine gene polymorphisms

Target sequence	Primer sequence	Cycling conditions	Site of polymorphism	Restriction enzyme
IL 1 RA	(F) 5' CTC AGC AAC ACT CCT AT 3' (R) 5' TCC TGG TCT GCA GGT AA 3'	Initial denaturation: 95°C × 5 min Denaturation: 95°C × 5 min Annealing: 50°C × 30 s Extension: 72°C × 30 s 35 cycles Final extension 72°C × 5 min	Intron 2 (VNTR)	-
IL 1 β	(F) 5' TGG CAT TGA TCT GGT TCA ATC 3' (R) 5' GTT TAG GAA TCT TCC CAC TT 3'	Initial denaturation: 95°C × 5 min Denaturation: 95°C × 30 s Annealing: 55°C × 30 s Extension: 72°C × 30 s 35 cycles Final extension 72°C × 5 min	C-511T	<i>Ava</i> I
TNF α	(F) 5' GAG GCA ATA GGT TTT GAG GGC CAT 3' (R) 5' GGG ACA CAC AAG CAT CAA G 3'	Initial denaturation: 95°C × 5 min Denaturation: 94°C × 30 s Annealing: 56°C × 30 s Extension: 72°C × 30 s 35 cycles Final Extension 72°C × 5 min	G-308A	<i>Nco</i> I

TNF-α: Tumor necrosis factor-alpha, IL-1 RA: Interleukin 1 receptor antagonist, VNTR: Variable Number of Tandem Repeats

reaction reactions were performed in an Eppendorf Thermal Cycler (MJ Research Inc, Waltham MA).

Polymerase chain reaction conditions

Interleukin-1 receptor antagonist

Initial denaturation	:95°C for 5 min	} 35 cycles
Denaturation	:95°C for 5 min	
Annealing	:50°C for 30 s	
Extension	:72°C for 30 s	
Final extension	:72°C for 5 min	

Interleukin-1β

Initial denaturation	:95°C for 5 min	} 35 cycles
Denaturation	:95°C for 30 s	
Annealing	:55°C for 30 s	
Extension	:72°C for 30 s	
Final Extension	:72°C for 5 min	

Tumor necrosis factor-alpha

Initial denaturation	:95°C for 5 min	} 35 cycles
Denaturation	:94°C for 30 s	
Annealing	:56°C for 30 s	
Extension	:72°C for 30 s	
Final extension	:72°C for 5 min	

Statistical analysis

The sample size was calculated as 31 using the EPI INFO software. The results of the study were statistically analyzed using SPSS 16.0 statistical package program (Statistical Package for Social Sciences, Lead Technologies Inc, USA). The descriptive data were given as mean and standard deviation. The Chi-square test was used to compare the differences between groups. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Demographic profile

A total of 70 patients were evaluated in the study; their age ranged from 20 years to 65 years with an average of 32 years. There were 243 controls recruited in the study with no history of Hansen's disease in the past; their age ranged from 21 years to 45 years with a mean of 34 years. All 70 patients were men. Of the 243 controls, 240 were men and 3 were women. Informed consent was taken from all the patients.

Clinical diagnosis

The clinical diagnosis of the patients is shown in Figure 1. In addition, all the patients were on multidrug therapy and no other therapeutic intervention was performed during the course of the study. The diagnosis of leprosy was confirmed by clinical examination, histopathology and slit-skin smear examination.

Reaction

The types of reactions seen in different patients are shown in Figure 2. In addition, it must be mentioned that all the patients who had reactions were treated by standard protocols.

Deformities

The types of deformities seen in different patients are shown in Figure 3.

Tumor necrosis factor-alpha—308 A/G polymorphism—Association of genetic polymorphisms—Cases versus controls

The G allele was seen in 397 (81.6%) controls and 71 (50.7%) patients. In contrast, the proinflammatory A allele was seen in 89 (18.3%) controls and 69 (49.3%) patients. The GG genotype was seen in 163 (67.1%) controls and 4 (5.7%) patients. In contrast, the AA genotype was seen in only 9 (3.7%) controls and 3 (4.3%) patients. The AG genotype was seen in 71 (29.2%) controls and 63 (90.0%) patients [Table 2].

The A allele was more common in the cases of Hansen's disease as compared to the controls. The AG genotype was more common in the cases as opposed to the controls.

In controls, all the frequencies of tested genotypes and alleles were in Hardy-Weinberg equilibrium. There was a strong association of TNF- α -308 G/A polymorphism with Hansen's disease with both genotypes and allele [Table 2]

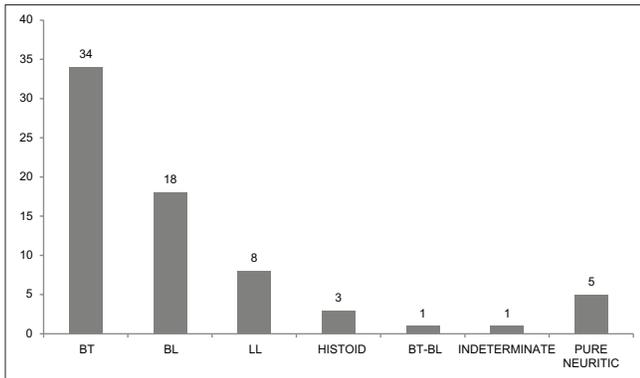


Figure 1: Bar Chart showing the distribution of the patients with the different subtypes of Hansen's disease

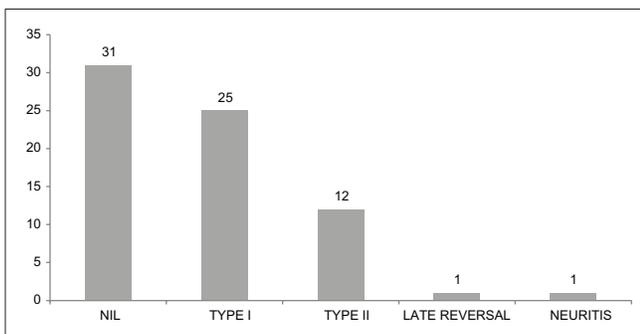


Figure 2: Bar Chart showing the distribution of the different types of reactions in patients with Hansen's disease

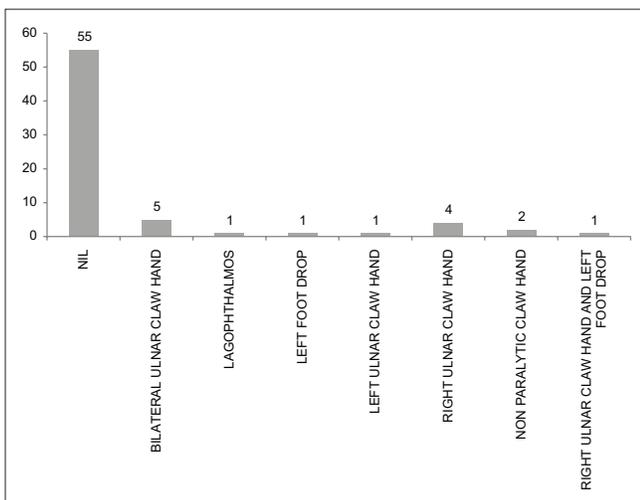


Figure 3: Bar Chart showing the distribution of the different types of deformities in patients with Hansen's disease

as the frequencies of genotypes were significantly different between patients and controls.

Interleukin-1 receptor antagonist polymorphism—Association of genetic polymorphisms—Cases versus controls

The A allele was seen in 368 (75.7%) controls and 100 (71.4%) patients. In contrast, the proinflammatory B allele was seen in 118 (24.3%) controls and 40 (28.6%) patients. The AA genotype was seen in 149 (61.3%) controls and 38 (54.9%) patients. In contrast, the BB genotype was seen in 24 (6.1%) controls and 8 (11.4%) patients. The AB genotype was seen in 70 (28.8%) controls and 24 (34.9%) patients [Table 3].

In controls, all the frequencies of tested genotypes and alleles were in Hardy-Weinberg equilibrium. There was no association between the different IL-1 RA alleles with the cases and the controls ($P = 0.46$).

Interleukin-1 β polymorphism— Association of genetic polymorphisms—Cases versus controls

The C allele was seen in 184 (37.9%) controls and 47 (33.6%) patients. In contrast, the proinflammatory T allele was seen in 302 (62.1%) controls and 93 (66.4%) patients. The CC genotype was seen in 26 (10.7%) controls and 9 (12.9%) patients. The CT genotype was seen in 132 (54.3%) controls and 29 (41.4%) patients. Finally, the TT genotype was seen in 85 (35.0%) controls and 32 (45.7%) patients [Table 4]. There was no correlation between the IL-1 β polymorphisms and cases of Hansen's disease ($P = 0.413$).

In controls, all the frequencies of tested genotypes and alleles were in Hardy-Weinberg equilibrium. There was no

Table 2: Frequency of tumor necrosis factor α genotypes and allelic polymorphisms among Hansen's disease

Cases/ controls	TNF- α genotypes			TNF- α alleles	
	G/G, n (%)	G/A, n (%)	A/A, n (%)	G, n (%)	A, n (%)
Total cases	4 (5.71)	63 (90.0)	3 (4.28)	71 (50.71)	69 (49.28)
Controls	163 (67.07)	71 (29.21)	9 (3.7)	397 (81.68)	89 (18.31)
<i>P</i>	0.0001				

*There were 70 patients with Hansen's disease and 243 controls. The total chromosome number in the patients is 70 with 140 alleles. The total chromosome number in controls is 243 with 486 alleles. TNF- α : Tumor necrosis factor-alpha

Table 3: Frequency of interleukin 1 receptor antagonist genotypes and allelic polymorphisms among Hansen's disease

Cases/ controls	IL-1 RA genotypes (%)			IL-1 RA alleles (%)	
	AA, n (%)	AB, n (%)	BB, n (%)	A, n (%)	B, n (%)
Total cases	38 (54.28)	24 (34.28)	8 (11.42)	100 (71.42)	40 (28.57)
Controls	149 (61.31)	70 (28.8)	24 (6.05)	368 (75.72)	118 (24.27)
<i>P</i>	0.46				

*There were 70 patients with Hansen's disease and 243 controls. The total chromosome number in the patients is 70 with 140 alleles. The total chromosome number in controls is 243 with 486 alleles. IL-1 RA: Interleukin 1 receptor antagonist

association of IL-1β-511 C/T polymorphism with Hansen's disease with both genotypes and alleles [Table 3].

Association of genetic polymorphisms with the presence of a reaction

Association of tumor necrosis factor-alpha genetic polymorphisms with type I and type II reactions

There was no association between the TNF-α gene polymorphisms and the type of reaction ($P = 0.310$). The results are tabulated in Table 5.

Association of interleukin-1 receptor antagonist genetic polymorphisms with type I and type II reactions

There was no association between the IL-1 RA gene polymorphisms and the type of reaction ($P = 0.830$). The results are tabulated in Table 6.

Association of interleukin-1β genetic polymorphisms with type I and type II reactions

There was a strong association between the IL-1β gene polymorphisms and the type of reaction ($P = 0.009$). The results are tabulated in Table 7.

Discussion

It has been reported in various studies¹⁶⁻²⁰ that the TNF-308A allele has a protective effect against the development of the disease. Similar findings have been reported in Nepal.²¹ However, studies conducted in the Indian and Thai populations show a strong association of the A allele with the development of the disease.^{22,23} In the present study, we found a strong association between the A allele of the TNF-308 polymorphism and the susceptibility to leprosy. Our study suggests that the association of the A allele varies between populations and, in the Indian population, there is a strong association with the susceptibility to the development of leprosy.

We did not find an association between the IL-1 RA alleles and the susceptibility to leprosy. In a study involving 47 patients with leprosy, Settin *et al.* found a significant association of the A allele with the susceptibility to leprosy.²⁴ The association of IL-1 RA polymorphisms with inflammatory diseases is well known.^{25,26} However, in the Indian population, the relation between IL-1 RA and inflammatory diseases is less marked.^{27,28} In addition, we did not find an association between the IL-1β-511 C/T genetic polymorphisms and the susceptibility to leprosy. Studies performed in the Indian population have shown a lack of association between the IL-1β-511 C/T genetic polymorphisms and susceptibility to inflammatory diseases involving the skin.²⁹ There are several reasons for this. One hypothesis is that the IL-1β-511 C/T polymorphism is a nonfunctional polymorphism as suggested.^{30,31} However, Reich *et al.* have shown that the IL-1β-511 C/T is functional with the IL-1β-511 C/C genotype producing more IL-1 RA which is an antiinflammatory cytokine.³² It has been shown that for some reason, the IL-1β present in the psoriatic

Table 4: Frequency of interleukin-1β genotypes and allelic polymorphisms among Hansen's disease

Cases/ Controls	IL-1β genotypes			IL-1β alleles	
	CC, n (%)	CT, n (%)	TT, n (%)	C, n (%)	T, n (%)
Total cases	9 (12.85)	29 (41.43)	32 (45.71)	47 (33.57)	93 (66.42)
Controls	26 (10.69)	132 (54.32)	85 (34.97)	184 (37.86)	302 (62.13)
OR (95% CI), P	0.413				

*There were 70 patients with Hansen's disease and 243 controls. The total chromosome number in the patients is 70 with 140 alleles. The total chromosome number in controls is 243 with 486 alleles. IL: Interleukin

Table 5: Frequency of tumor necrosis factor-alpha genotypes and allelic polymorphisms among patients with Hansen's disease showing a type I/type II reaction

Types of Reactions in Hansen's Disease	TNF-α alleles			Total
	AA	AG	GG	
No reaction	1	30	1	32
Type I reaction	2	20	3	25
Type II reaction	0	13	0	13
Total	3	63	4	70
P	0.310			

TNF-α: Tumor necrosis factor-alpha

Table 6: Frequency of interleukin 1 receptor antagonist genotypes and allelic polymorphisms among patients with Hansen's disease showing a type I/type II reaction

Types of Reaction in Hansen's Disease	IL-1 RA alleles			Total
	AA	AB	BB	
No reaction	18	12	2	32
Type I reaction	13	8	4	25
Type II reaction	7	4	2	13
Total	38	24	8	70
P	0.830			

IL-1 RA: Interleukin 1 receptor antagonist

Table 7: Frequency of interleukin-1β genotypes and allelic polymorphisms among patients with Hansen's disease showing a type I/type II reaction

Type of Reaction in Hansen's Disease	IL-1β alleles			Total
	TT	CT	CC	
No reaction	21	7	4	32
Type I reaction	9	12	4	25
Type II reaction	2	10	1	13
Total	32	29	9	70
P	0.009			

IL: Interleukin

skin though increased in amount in experimental models is functionally inactive.³³ This would probably explain the lack of an association between the IL-1 gene polymorphisms and leprosy.

There was a strong correlation between the IL-1β genetic polymorphisms and the lepra reactions. There was no

association between the other two polymorphisms and the lepra reactions. The TT allele is proinflammatory and the CC allele is antiinflammatory. In the present study, it was seen that the TT allele predominated in the cases with both the type I and II lepra reactions. It is likely that an increased amount of IL-1 β is secreted in cases who have lepra reactions. This would account for the exaggerated response which occurs in lepra reactions which is cytokine-mediated. However, it is surprising that the TNF-308 polymorphism was not associated with lepra reactions. TNF- α is believed to be the primary cytokine which regulates the rest of the cytokines in the inflammatory cascade. It is likely that the sample size was not adequate to evaluate this polymorphism.

We had a total of 15 patients who had deformities. However, because the number of cases was small, we could not perform a statistical analysis to evaluate whether the genetic polymorphisms were associated with the deformities.

We believe that this study provides an insight into the pathogenesis of lepra reactions. The polymorphism analysis suggests that there is an increase in the amount of cytokine produced. This finding has been confirmed in a study which measured the levels of the cytokines during reaction states and stated that the levels of TNF- α and IL-1 β are elevated in leprosy reaction states.³⁴ These cytokines are responsible for the aggravation of the lepra reactions. Perhaps, cytokine inhibitors if administered would decrease the severity and duration of the reactions.

The limitation of this study is two. First, any study involving genetic polymorphisms is an association study. Transcriptomics and proteomics give a real-time picture of the changes during the course of the disease. However, in this study, evaluation of the cytokine levels would have been of little or no utility because the levels fluctuate considerably in the body and a single point measurement would not necessarily have given, a true picture. The second limitation was the sample size which appeared small. However, statistical software confirmed that the sample size was adequate considering the rarity of the disease.

Conclusion

In conclusion, an association of cytokine gene polymorphisms with Hansen's disease and the lepra reactions was found. An evaluation of the cytokine levels in the skin during the lepra reactions would confirm this observation. Possibly, in the future, this would be a guide to therapeutic decisions in lepra reactions.

Limitations of the study

Genetic polymorphisms are association studies. The DNA polymorphism may or may not have a relation to the transcriptome or the proteome in the tissue at that particular time. This would be a major limitation of this study.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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