Pityriasis Versicolor: host susceptibility in relation to IL-10 and IFN γ cytokine gene polymorphism

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Abstract

Pityriasis Versicolor is a skin condition caused by the commensal yeast *Malassezia*. Little is known about the pathogenesis of why a commensal only causes symptoms in a subset of infected individuals. Understanding the susceptibility of the host to these commensal-associated diseases may be facilitated by knowledge of genetic polymorphism. The purpose was to investigate the relationship between Single Nucleotide Polymorphism in the IL10 and IFN genes of the host and susceptibility to *Malassezia* infection. There were 38 cases of Pityriasis Versicolor (PV) and 38 healthy controls in the sample. Blood samples were extracted for genomic DNA from all study participants. Amplification refractory mutations system- polymerase chain reaction (ARMS-PCR) with sequence-specific primers was used to genotype cytokines. In all patients and healthy controls, three SNPs (IL10-1082A/G; IL10-819/592C/T; IFN-+874A/T) in two cytokine loci were analyzed. In the PV group, we observed significant differences in allele or genotype distribution for the IL10-819/592C/T and IFN-+874A/T gene polymorphisms. In the present investigation, cytokine gene polymorphism revealed that the host was susceptible to *Malassezia* infection.

Introduction

Malassezia is part of cutaneous commensal flora and is associated with certain superficial cutaneous disorders like Pityriasis Versicolor (PV), atopic dermatitis (AD), and seborrheic dermatitis (SD), etc.[1] *Malassezia* demonstrates two distinct phenotypes: one stimulates the immune system, significantly activating several immunological pathways, while the other greatly restricts immune stimulation, possibly allowing it to coexist as a commensal in the majority of people.[1-3] The immunomodulatory ability of *Malassezia* has been shown to downregulate the production of proinflammatory cytokines which is in marked contrast to the effect of many other organisms.[1]

Pityriasis Versicolor (PV) is a mild, chronic superficial cutaneous condition characterized by hypo or hyper-pigmented plaques that are covered by fine scales, sometimes associated with mild pruritus.[4] PV is mostly distributed in the sebum-rich areas of the skin such as the back, chest, and neck.[4] There is a significant fungal load on the skin but no inflammatory alterations are observed. The excellent adaptive mechanism is attributed to the presence of various metabolites produced by the yeast.[5]

Cytokine gene polymorphism (Single Nucleotide Polymorphism) governing the cytokine production could determine the susceptibility of the host to the disease.[6] The occasional polymorphisms that occur in the normal healthy population are compatible with normal immune function. But when present with certain other susceptibility genes they may contribute to the disease. Cytokine secretion profiles can be considered as promoting Cell-mediated immunity or humoral immunity.

IL10 shifts the balance by down-regulating Th1 response and by suppressing proinflammatory cytokine IFN γ secretion. IL10 is a Th2 anti-inflammatory cytokine and inter-individual variations in IL10 production are genetically determined by polymorphism within the IL10 promoter region -1082 G/A, -819 C/T, and -592 C/A. The polymorphism at -810 C/T and -592 C/A are in linkage disequilibrium with each other.[7]

IFN γ is a Th1 proinflammatory cytokine that can augment the immune response. The functional Single Nucleotide Polymorphism (SNP) +874 T/A is located at the 5' end of a CA repeat at the first intron of the human IFN γ gene. The T allele of IFN γ at +874 provides the binding site for the transcription factor, kB (NF-kB), which in turn leads to high IFN γ production.[8] The goal of the current study is to compare the genetic susceptibility of the host to infections by relating the polymorphism of the cytokine gene to any inherited susceptibility and comparing the polymorphism between the study and control groups. Pityriasis Versicolor aetiologically related to *Malassezia* was chosen to explore this immunological tenet.

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Ethics approval: University College of Medical Sciences Ethical committee approval was taken as per the institutional guidelines before recruiting patients. The study is conformed with the Helsinki Declaration of 1964, as revised in 2013, concerning human and animal rights.

Informed consent: all patients participating in this study signed a written informed consent form for participating in this study.
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Materials and Methods

The study is an Observational prospective laboratory-based study and included 38 consecutive untreated clinically diagnosed cases of PV irrespective of age and sex recruited from the outpatient department of Dermatology of a tertiary care hospital, Delhi over a year, January 2012-January 2013. An equal number of healthy volunteers were also included as controls.

A clearance from the college ethical committee was obtained as per the institu-

tional guidelines before recruiting patients. Informed consent was obtained from the patients. Three mL venous blood sample in an EDTA vial was collected aseptically from all patients and healthy controls for DNA extraction and subsequent study for single nucleotide polymorphism

The diagnosis is based on clinical suspicion, Woods lamp (365 nm) examination showing reddish or yellowish green fluorescence and the so-called evoked scale sign.[9,10] Direct microscopic examinations with 10% KOH were done for numerous budding yeast cells and short hyphae characteristic of the 'Spaghetti and Meatball' appearance. Blood samples were kept at 40°C till further use.

Genomic DNA extraction

Genomic DNA was extracted from blood samples of all study subjects for determining three SNPs in two cytokine genes through Amplification Refractory Mutation system-polymerase chain reaction using sequence-specific primers.[11]

Genomic DNA was extracted from EDTA anticoagulated peripheral blood using a HiPurATM blood genomic DNA extraction kit (Himedia Laboratories, Mumbai, India) following the manufacturer's instructions. 200µl blood sample was collected in a 2.0ml collection tube, and 20 µL of the reconstituted Proteinase K solution (20 mg/mL) was added. The sample was vortexed for 10-15 seconds to ensure thorough mixing. To extract RNA-free genomic DNA, 20 µl of RNase A solution (20 mg/mL) was added, and the mixture vortexed again for 10-15 seconds. The sample was then incubated for 2 minutes at room temperature (15-250°C). Following this, 200 µL of the lysis solution (C1) was added to the sample and vortexed thoroughly for a few seconds to obtain a homogenous mixture. The sample was incubated at 550°C for 10 minutes in a water bath. The lysate for binding to the spin column was prepared as follows; 200 µL ethanol (96-100%) was added to the lysate obtained from the above step and mixed thoroughly by gentle pipetting. Lysate was transferred into the spin column provided with the kit and centrifuged at 10,000 rpm for 1 minute. The flow-through liquid was discarded and the column was placed in a new 2.0 mL collection tube; 500µL of diluted pre-wash solution was added to the column and centrifuged at 10,000 rpm for 1 minute. After discarding the flow-through liquid, 500 µL of diluted wash solution was added to the column and centrifuged at 13,000- 16,000 rpm for 3 minutes to dry the column. Flow-through liquid was discarded and a dry spin was given at the same speed to remove the residual ethanol, if any. The column was put in a new 2.0ml collection tube and 200 µL elution buffer was added without spilling to the sides. The column was incubated at room temperature for 5 minutes for a high yield of DNA and then centrifuged at 10,000 rpm for 1 minute to elute the DNA. The samples were stored at -200C until used. DNA samples were subjected to specific PCR reactions in cytokine genotyping.

Cytokine genotyping by amplification refractory mutations system-polymerase chain reaction (ARMS-PCR)

Amplification Refractory Mutations System-Polymerase Chain Reaction (ARMS-PCR) with sequence-specific primers was used to genotype cytokines from genomic DNA (Sigma Aldrich, Banglore, India).[7,8] All of the patients and healthy controls were tested for three SNPs (IL10-1082A/G, IL10-819/592C/T, and IFN +874A/T) in two cytokine genes. The PCR products were loaded onto a 1 percent agarose gel in a specific order for electrophoresis and run at 150 volts for 20-25 minutes for separating the DNA. Ethidium bromide-stained gel was taken and examined for distinct amplification patterns following electrophoresis. A control band was confirmed to be present in each lane (globulin, 100 bp). The bands in the wells used to detect the cytokines IL10-1082 and IL10-819/592 were 258 bp and 233 bp, respectively.[7] Wells identified the IFNγ +874 cytokines contained a band of 261bp.[12]

The primer sequence is as follows:

IL-10 -1082G/A

Common Primer: 5' – CAGTGCCAACTGAGAATTTGG – 3'
G allele: 5' – CTACTAAGGCTTCTTTGGGAG – 3'
A allele: 5' – ACTACTAAGGCTTCTTTGGGAA – 3'

IL-10 -819C/T / -592C/A
Common Primer : 5' – AGGATGTGTTCCAGGCTCCT – 3'
C allele: 5' – CCCTTGTACAGGTGATGTAAC – 3'
T allele: 5' – ACCCTTGTACAGGTGATGTAAT – 3'

IFN-γ +874T/A
Common Primer: 5' – TCAACAAAGCTGATACTCCA – 3'
A allele: 5' – TTCTTACAACACAAAATCAAATCA – 3'
T allele: 5' – TTCTTACAACACAAAATCAAATCT – 3'

B-globulin (Internal control)
Forward: 5' - ACACAACTGTGTTCACTAGC – 3'
Reverse: 5' – CAACTTCATCCACGTTCACC – 3'

Statistical analysis

Cytokine polymorphisms and genotype frequencies were evaluated by gene counts. The observed and expected genotype frequencies data was analyzed using CHI SQUARE Test. As multiple comparisons were made, Bonferroni's correction was applied to significant *p* values (p<0.02) that were multiplied for the number of genotypes detected.[13] But, as p<0.05 is also considered statistically significant in a small study group, our discussion included all variables considering p<0.05 as significant.

Results

Clinically diagnosed patients with PV (n=38; 23 males, 15 females) were included in the study. Healthy controls (n=38; 21 males and 17 females) were unrelated individuals without a clinical history of any skin disease were also included. Three SNPs in 2 cytokine genes were investigated in all the subjects by cytokine genotyping using sequence-specific primers. In the case-control study, significant differences in allele, or genotype distribution were observed in IL10-819/592C/T (rs1800871: rs1800872) and IFNγ+874T/A (rs2430561) gene polymorphisms (Table 1). IL10-1082 G/A (rs1800896) genotype and allele frequency was not found to be significant.

The distribution of IFN γ+874T/A (p=0.012) and IL10-819/592C/T (p=0.036) alleles were significantly different between patients and healthy control. PV patients were more likely to carry the IL10-819/592 T allele (p=0.036) and it was significantly associated with the disease (OR=0.476, 95% CI 0.236-0.959). IFN γ+874 Allele was significantly associated with PV (OR=0.424, 95% CI 0.216-0.833). In PV patients, the IL10 -819/592 CT genotype frequency was found to be lower (OR 0.260, 95% CI 0.099-0.683; p=*0.005*). The CC genotype frequency was found to be higher (p=*0.05*) in PV patients as compared to healthy controls. Similarly, the IFN γ+874 AA genotype frequency was found to be higher (p=0.037) in PV patients than in controls.

Discussion

Yeasts of the genus *Malassezia* are part of the normal cutaneous commensal microflora and also an etiologic agent of certain diseases.[1] Colonization occurs in infancy and reaches its highest concentration after puberty and in early adulthood. *Malassezia* yeast is found in 75 to 78 percent of healthy adults as normal flora of the skin.[4,14] Malassezia's pathogenic and commensal stages are not easily distinguished from one another.[1] The transition from commensal to pathogenic state is probably a continuum and not an on/off condition. *Malassezia*-associated skin conditions span the whole spectrum between overt inflammatory response (Seborrheic Dermatitis) and a distinct absence of inflammation

(PV). The annual incidence of PV has been reported to range from 5.2 percent to 8.3 percent.[15] Composition of the cell wall lipids and various metabolites produced by *Malassezia* are known to be responsible for altering the host immunological response and thus preventing the killing of the yeast.[3,16]

The role of the host immune system in disease manifestation and severity is critical. Hence, polymorphism in the genes responsible for cytokine production can influence the susceptibility of the host to develop and manifest the disease. Association between specific cytokine gene polymorphism and clinical outcome if found to be significant can determine whether an individual will develop the disorder if he/she carries the particular allele in comparison to the individual without the allele.[6] It is important to determine the allelic frequency of both Th1 and Th2 representative genes as the disease outcome is influenced by their mutual antagonism and therefore individual association may be non-informative.[6]

So far, immunological studies on the association with PV have been scarce. The interaction of *Malassezia* with the dendritic cells, keratinocytes, and PBMC has led to varied results in different human and animal studies.[17,21]

IL10, a Th2 pleiotropic anti-inflammatory cytokine, acts on monocytes and macrophages and downregulates the expression of MHC class II antigens on antigen-presenting cells. IL10 also suppresses the production of nitric oxide and other metabolites responsible for killing pathogens. They also suppress the production of inflammatory mediators e.g. IL1, IL6, IL8, etc. IFN γ is the signature Th1 proinflammatory cytokines, responsible for acute flare-up inflammatory responses. Along with other cytokines of the Th1 subset, it dampens the Th2 response.

In our study, polymorphism in the gene IFN γ at position +874 T/A in the first intron was identified in PV. IFN γ+874 AA genotype frequency was found to be higher in PV than in controls and the T allele was significantly associated with the disease. This finding is in parallel with other studies suggesting that PV patients may produce a lower level of IFN γ.[17-21] We postulate that the time of production and concentration of pro-inflammatory cytokines during the inflammation process may be critical but a dampened T-cell response was observed due to allelic polymorphisms.

The C/T allele of IL10-819 was significantly associated with PV. In PV patients compared to healthy controls, the frequency of the IL10 -819/592 CT genotype was found to be lower and the frequency of the CC genotype to be higher. The reason for the underlying inflammatory response in PV in the presence of gene polymorphism (IL10-819/592 CC) which is associated with high production of IL-10 in our study, is probably suggestive of Th17-induced production of inflammation and hence also explains the neutrophilic infiltration in the PV lesions as documented in studies.[5] Increased IL10 levels have been demonstrated in PBMC challenge studies with *Malassezia* antigens in different patient groups and also in keratinocyte stimulation studies using different species of *Malassezia*.[22]

The finding also suggests the involvement of the regulatory T cell subset in the pathogenesis of the disease since elevated IL10 production as suggested by the genotypic result has been known to be implicated in limiting the development of the inflammatory response towards invading pathogens and allowing its persistence.

Thus, the development of the disease in the host could be explained, in part, by the Th1/ Th2 balance. However, a larger number of subjects need to be studied to understand the development of the disease better. The determination of the genetic profile of the host might allow assessing the susceptibility towards the disease and also explain the differential association of a known commensal to cause symptoms in a selected group of population. The association of certain polymorphisms with a disease phenotype needs to be assessed on a larger scale taking into consideration the role of other cytokine mediators to further expand our knowledge regarding the pathogenesis of infectious diseases. These studies on the host factors could pave the way for determining the changing trend of individual-based diagnosis and future treatment.[23]

The understanding of the disease pathogenesis of PV has been a topic of debate as the *Malassezia* yeasts, a known commensal, is responsible to cause symptoms in only a subset of the population who are colonized with it. Our study was able to provide an understanding of the susceptibility of this subset of the population by comparing their genetic profile with the normal population through a study of the cytokine gene polymorphism in the IL10 and IFN γ SNPs. The results reflected a significant level of polymorphism in all the SNPs. The genotype responsible for higher production of IL10 was found to be significantly higher in the patient group as compared to the healthy. And also the proinflammatory response mediated by the IFN γ, determined by the SNP in its promoter was found to be in favor of a decreased Th1 outcome. In conclusion, the above findings suggest a genetic level of susceptibility in the host toward the development of disease, with the immune response of the host as an important determinant in the host-pathogens' interaction.

Table 1. Allele and genotype frequencies of Cytokine polymorphisms in Pityriasis Versicolor patients and healthy controls.

Cytokine polymorphism			PV, N=38 (%)	HC, N=38 (%)	P value	Odds ratio	95% CI
IL10-1082	Alleles	A	48(63.2)	45(59.2)	0.739	1.181	0.615-2.269
		G	28(36.8)	31(40.8)	0.618	0.847	0.441-1.627
	Genotypes	AA	10(26.3)	7(18.4)	0.409	1.582	0.530-4.717
		AG	28(73.7)	31(81.6)	0.409	0.632	0.212-1.886
		GG	0(0.0)	0(0.0)	-	-	-
IL10-819/592	Alleles	C	58(76.3)	46(63.2)	0.036*	2.101	1.043-4.235
		T	18(23.763.2)	30(63.2)	0.036*	0.476	0.236-0.959
	Genotypes	CC	21(55.3)	9(23.7)	0.05*	3.980	1.488-10.648
		CT	16(42.1)	28(72.7)	0.005*#	0.260	0.099-0.683
		TT	1(2.6)	1(2.6)	1.000	1.000	0.060-16.594
IFN γ +874	Alleles	A	55(72.4)	40(52.6)	0.012*#	2.357	1.200-4.629
		T	21(27.6)	36(47.4)	0.012*#	0.424	0.216-0.833
	Genotypes	AA	21(55.3)	12(31.6)	0.037*	2.676	1.049-6.827
		AT	13(34.2)	16(42.1)	0.479	0.715	0..282-1.811
		TT	4(10.5)	10(26.3)	0.076	0.329	0.093-1.165

PV, Pityriasis Versicolor; HC, healthy control; CI, confidence interval; N, number of subjects. *Significant according to ($p<0.05$); #significant according to Bonferroni correction ($p<0.02$).

Conclusions

Cytokine gene polymorphism data demonstrated the susceptibility of the host to *Malassezia* infections in our study. Data could help to find out who is disease prone, *i.e.*, risk prediction which might influence the use of prophylactic measures, avoid risk factors. This helps in understanding particular pathways used in host resistance to infection and augmenting those using scientific approaches and also devising therapeutic modalities via exogenously supplementing cytokines to balance out the immune response. Vaccines targeting specific genes can be developed to resolve cases of chronic and recurrent lesions. Detailed further studies might direct individual-based treatment depending on the genetic makeup of the patient.

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