Association study of single nucleotide polymorphisms in *IL-10* and *IL-17* genes with the severity of microbial keratitis

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Abstract

Purpose: Exploratory analysis to assess the association of single nucleotide polymorphisms (SNPs) in the interleukin (IL) 10 and IL-17 genes with severity of contact lens keratitis.

Methods: This was a retrospective case control study of 88 contact lens keratitis cases (25 severe) and 185 healthy contact lens wearers recruited from studies conducted at Moorfields Eye Hospital and in Australia-wide during 2003-2005. Buccal swab samples were collected on Whatman FTA cards and mailed by post for DNA extraction and SNP genotyping. IL-10 (rs1800871; rs1800896; rs1800872) and IL-17 (rs1800871; rs1800896; rs1800872) SNPs were screened by pyrosequencing. Genetic association analyses were performed via Cochran-Armitage trend tests and logistic regression models using PLINK software.

Results: None of the SNPs tested showed evidence of association with severity of contact lens keratitis at $P < 0.05$. Nevertheless, minor allele G in SNP rs2397084 of the IL-17F gene was associated with increased risk of severe MK, with OR=2.1 (95% CI=0.9-4.8, P=0.066).

Conclusion: Our study cannot exclude with confidence that genetic variation in the IL-17F proinflammatory cytokine is associated with more severe outcomes of MK. However, there is general body of information that the IL-17 pathway is important in the mechanisms of MK. Studies with larger power and the expanded array of laboratory tools will elucidate the exact role of IL-17 in MK.

Key words:
Keratitis, interleukin, gene, contact lens

Introduction

Microbial keratitis (MK) can be produced by a variety of microbes that infect the cornea and produce an inflammatory response. The main predisposing factors for the disease include contact lens wear, corneal injury and ocular surface disease.[1-3] MK associated with contact lens wear is most often caused by *Pseudomonas aeruginosa*.[3-5]

MK is initiated by interactions of the microbes with resident corneal cells such as epithelial cells and perhaps fibroblasts if injury has exposed the underlying corneal stroma. These cells signal for the recruitment of polymorphonuclear leukocytes (PMNs). Prompt recruitment of PMNs to the cornea is necessary for clearance of infecting bacteria, but their prolonged stay is associated with most of the tissue damage seen during keratitis.[6-8] However, in the absence of PMNs, mice die during the disease initiated by keratitis,[6] emphasizing their importance in preventing the spread of bacteria.

The resident cells and PMNs aid in the innate response to infection and orchestrate the outcome of disease by activating T-helper lymphocytes through antigen presenting cells. ~~The resident cells and PMNs orchestrate the outcome of disease by activating T-helper lymphocytes. These cells aid in the innate response to infection and production of the adaptive immune response~~. There are three types of T-helper cells, Th-1, Th-2 and Th-17, which are all involved in clearance of pathogens from infected tissue.[9] The cytokine milieu dictates whether Th-1, Th-2 or Th-17 cells predominate. If the cytokine milieu is predominated by the interleukins (IL) IL-12 and IL-18 and interferon (IFN)-γ, then a Th-1 response occurs.[9, 10] If the cytokines are predominately IL-4, IL-25, IL,-33 and IL-10, a Th-2 response occurs.[9-11] A cytokine milieu of predominately ~~tissue~~ transforming growth factor (TGF)-β, IL-6 and IL-23 results in a Th-17 response.[9, 10] Neutrophils can produce IL-17 if IL-6 and IL-23 are present, and they respond in an autocrine fashion to IL-17.[12]

We have previously shown that a single nucleotide polymorphism (SNP) rs1800795 in the promotor region of the Th-17-related cytokine IL-6[9] is associated with more severe keratitis.[13] This SNP is associated with reduction in IL-6 protein expression[14] and may be associated therefore with reduced production of Th-17 cells. The SNP rs3212227 in the gene for the Th-17 and Th-1-related cytokine IL-12B (IL-12p40) was associated with a greater likelihood of experiencing so-called sterile keratitis, i.e., lesions outside the central 4mm of the cornea and with no or minimal pain.[13] This SNP increases protein production of IL-12p40.[15]

Keijser et al. found that a haplotype of the Th-2-associated cytokine IL-10, which has been associated with low levels of IL-10 protein production,[16] was protective for MK.[17] However, a haplotype that is associated with high levels of IL-10 protein was associated with less severe disease.[17] Although this study did not measure protein levels it suggests that low IL-10 levels are useful to enhance the corneal defense system to protect against disease onset, whereas during disease, a high level of IL-10 assists in dampening the inflammatory response minimizing tissue destruction and scarring.[17]

Given the role of IL-17 and IL-10 as regulators of the immune response, particularly with IL-17 having links to adaptive immunity, we tested whether SNPs of these genes would be associated with predisposition to more severe cases of MK in an exploratory analysis of a previously recruited case-control cohort.

Materials and Methods

Study participants and DNA samples

The study design and methodology have been described previously.[13, 18] In brief, this retrospective case control study recruited 65 MK cases (25 severe), 23 sterile keratitis and 185 non infected control contact lens wearers of Caucasian ethnicity identified from previous studies of MK nationwide in Australia [19] and in London[20] during 2003-2005.[13, 18] In both countries, local ethics committee approval was obtained and the tenets of the Declaration of Helsinki were observed. Informed consent was obtained for all participants. Buccal swab samples were collected on Whatman FTA cards and sent through the post by participants for DNA extraction and SNP genotyping.

DNA was extracted from the FTA cards using a published pH method.[21] In brief, a 6mm punch of each sample was incubated with 200µl of FTA Purification Agent (Whatman, GE Healthcare Kent, UK) on ice for 30 minutes. The FTA Purification Agent was removed and fresh solution added for a further 15 minutes on ice. The sample was then washed with 150µl of TE buffer for five minutes and subsequently transferred to a clean tube, to which, 200µl of Elution Solution[21] was added for a further five minutes. Three cycles of 5-second vortex mixing in five-minute intervals then took place followed by squeezing and discarding the punch with a sterile pipette tip.

The FTA cards were issued and collected in 2010. DNA was extracted in 2011 and stored at -20°C until genotyping which took place in 2012. Previous genetic studies have shown DNA from FTA cards is stable for short read genotyping, such as SNP analysis, for at least 18 months.[22]

Classification and severity of MK

MK was defined by positive corneal culture or clinical criteria, specifically a corneal infiltrate and overlying epithelial defect with at least one of the following features: lesion within central 4mm, uveitis or significant pain. Corneal infiltrates that did not satisfy the criteria for MK were considered non-infectious. Cases of MK were classified as severe based on the following criteria: if the lesion resulted in the loss of two or more lines of best corrected vision, required surgical intervention, was culture positive, associated with hypopyon, at least 2mm in diameter, or if any part of the lesion was in the central 4mm of the cornea.

Genotyping

Three common (minor allele frequency, MAF > 5%) SNPs in the IL-17 (rs2275913; rs2397084; rs763780) and IL-10 (rs1800871; rs1800896; rs1800872) genes were investigated in this study. The IL-17 SNPs were chosen for their association with severity in rheumatoid arthritis patients.[23] The IL-10 SNPs are in the promotor region of the gene and have been investigated in many disease conditions,[16] including keratitis.[17] Gene sequences were amplified with standard thermal PCR[13] using biotin labeled primers designed from the Pyromark Assay Database (previously available at: http://techsupport.pyrosequencing.com/, last accessed October 19, 2011) or designed with Pyromark ID software. The PCR conditions for IL-10 rs1800871, rs1800896 and rs1800972 were 1 x 95°C for 5 minutes followed by 45 x (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds) and 1 x 72°C for 5 minutes. The PCR conditions for IL-17F rs2397084, rs2275913A and rs763780F were 1 x 95°C for 5 minutes followed by 45 x (95°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds) and 1 x 72°C for 5 minutes.

Genotyping was performed with Pyrosequencing (Biotage, Uppsala, Sweden) following isolation of a short chain of DNA with a sequencing primer. Primer sequences for amplification and sequencing are detailed in Table 1. Genotypes were assigned from the Pyromark ID SNP analysis program (SNP version 1.0.5).

Table 1: Primer sequences used for SNP genotyping.

rsID	Major/Minor Allele	Gene	Sequences (Sense)
rs2397084	A/G	IL-17F	B-CAACAGTCACCAGCACCTTCTC (F)
			GTACAGGCCCAGTGTAGGAACTTG (R)
			CCGTTCCCATCCAGC (S)
rs2275913	G/A	IL-17A	B-CCCTTCCCATTTTCCTTCAG (F)
			CTGGGGATGGATGAGTTTGT (R)
			AATGAGGTCATAGAAGAATC (S)
rs763780	A/G	IL-17F	TTGTTGCAGAGCACTGGGTAAG (F)
			TTGGAGAAGGTGCTGGTGACT-B(R)
			GCACCTCTTACTGCACA (S)
rs1800871	C/T	IL-10	B-TTCTCAGTTGGCACTGGTGT (F)
			AACTGTGCTTGGGGGAAGT (R)
			CAAACTGAGGCACAGAG (S)
rs1800872	C/A	IL10	B-TGGAAACATGTCCCTGAGAA (F)
			CAAGCAGCCCTTCCATTTTA (R)
			CCAGAGACTGGCTTCC (S)
rs1800896	G/A	IL-10	AACCCAACTGGCTCTCCTTA (F)
			GCTGGATAGGAGGTCCCTTA-B (R)
			CTAAGGCTTCTTTGGGA (S)

IL=interleukin; B=Biotin; F=forward; R=reverse; S=sequencing

Statistical analysis

In order to test whether any SNP was associated with severity of MK, we performed a Cochran-Armitage trend test on the variant genotypes coded as 0, 1 and 2 according to the number of minor alleles between severe cases vs 'all others' (controls plus sterile/mild/moderate cases), calculating *P*-values via permutation using PLINK version 1.07 software.[24] Odds ratios (OR) (with 95% confidence intervals, CI) of severity of disease per one copy of minor allele were obtained from logistic regression models.

Results

SNP rs763780 (His161Arg) in the IL-17F gene ~~failed genotyping~~, although present by gel electrophoresis (2%, Sybre Safe DNA gel stain, Life Technologies, Gaithersburg, MD, 80 V, 30 minutes) following PCR~~.~~, was not able to be genotyped using our method of pyrosequencing. Table 2 shows the genotype and allele frequency distribution for the remaining 5 SNPs, and corresponding results for the genetic association testing between severe cases and 'all others' (control/sterile/mild/moderate). No statistically significant difference was found in the allele distribution of the control group *vs* sterile/mild/moderate keratitis for all tested SNPs ($P > 0.30$) so these four categories were combined and tested against severe keratitis. There was no evidence of association with severity of MK for any of the 5 SNPs tested at $P < 0.05$. SNP rs2397084 in the IL-17F gene showed the lowest *P*-value (0.066), with an OR estimate of 2.1 and corresponding 95% CI from 0.9 to 4.8.

Table 2. Genotype distribution of the *IL-10* and *IL-17* SNPs in controls, sterile, mild, moderate and severe cases, and association analysis for severe vs 'all others'.

SNP rsID (Gene)	Genotype counts, N (%)			Genetic association analysis (Severe vs 'all others')		
	Control	Sterile/Mild/ Moderate	Severe	OR	(95% CI)	*P*
rs2397084 (*IL-17F*)				2.08	0.90 – 4.83	0.066
AA	147 (0.79)	50 (0.79)	17 (0.68)			
AG	38 (0.21)	13 (0.21)	7 (0.28)			
GG	0	0	1 (0.04)			
Total	**185**	**63**	**25**			
MAF	*0.10*	*0.10*	*0.18*			
rs2275913 (*IL-17A*)				0.60	0.30 – 1.18	0.144
CC	88 (0.48)	27 (0.43)	16 (0.64)			
CT	76 (0.41)	26 (0.41)	7 (0.28)			
TT	20 (0.11)	10 (0.16)	2 (0.08)			
Total	**184**	**63**	**25**			
MAF	*0.32*	*0.37*	*0.22*			
rs1800871 (*IL-10*)				1.38	0.76 – 2.52	0.295
CC	98 (0.53)	35 (0.56)	14 (0.56)			
CT	72 (0.39)	24 (0.39)	5 (0.20)			
TT	15 (0.08)	3 (0.05)	6 (0.24)			
Total	**185**	**62**	**25**			
MAF	*0.28*	*0.24*	*0.34*			
rs1800872 (*IL-10*)				1.25	0.65 – 2.42	0.524
CC	96 (0.56)	30 (0.49)	12 (0.48)			
CA	66 (0.38)	28 (0.46)	11 (0.44)			
AA	10 (0.06)	3 (0.05)	2 (0.08)			
Total	**172**	**61**	**25**			
MAF	*0.25*	*0.28*	*0.30*			
rs1800896 (*IL-10*)				0.74	0.40 – 1.39	0.342
AA	55 (0.31)	14 (0.23)	12 (0.50)			
AG	94 (0.52)	37 (0.60)	6 (0.25)			
GG	31 (0.17)	11 (0.18)	6 (0.25)			
Total	**180**	**62**	**24**			
MAF	*0.43*	*0.48*	*0.38*			

MAF = Minor Allele Frequency.

Discussion

While organism type, delayed treatment and older age are risk factors for more severe MK in contact lens wearers,[25, 26] a proportion of the severity of the disease risk is still unexplained. In this exploratory analysis, we found no evidence of association with disease severity at a set of candidate

SNPs in the IL-17 and IL-10 genes. Nevertheless, it has to be noted that our analysis had limited power: with 25 (severe) cases and about 10 times controls (N=248, sterile, mild and moderate), if we assume a minor allele frequency (MAF) of 25% and set the odds ratio (OR) at 2, power is 30%; with a MAF of 10% and an OR of 2, the power is reduced to 20%. The association results at SNP rs2397084 in the IL-17F gene (OR=2.1, 95% CI=0.9-4.8) do not rule out with confidence a possible involvement of this variant in disease severity. SNP rs2397084 in exon 3 changes glutamic acid (GAG) to glycine (GGG) at amino acid 126 (Glu126Gly) and could affect the function or expression of IL-17F. Furthermore, tissue specific transcription factors and environment may play roles in protein expression independent of genomic DNA codes.

IL-17F is a proinflammatory cytokine. In a study of rheumatoid arthritis patients[23], SNP rs2397084 was associated with prolonged inflammation and it has been suggested that rs2397084 and neighboring rs763780 (which causes the protein sequence change histidine (CAT) to arginine (CGT) at 161, His161Arg), may act to stabilize IL-17F, thereby increasing the inflammatory response. This may indicate that if rs2397084 is associated with poor outcome of MK it may be due to a proinflammatory action rather than an increased microbial level. In the study of rheumatoid arthritis, His161Arg had greater influence on inflammatory symptoms than Glu126Gly. SNP rs763780 failed to be genotyped by pyrosequencing in our study, and sequencing by an alternative technology in this or a similar population of keratitis patients may provide a more complete picture of the role of IL-17F SNPs in MK severity.

Studies of gene expression and tear cytokine levels in corneal infection patients indicate a role for IL-17 in disease severity. Corneal gene expression studies for early and late presenting cases of filamentary fungal keratitis show IL-17 is upregulated in both early (within 1 week) and late stage (keratoplasty samples) compared to non-infected cadaver corneas.[27]. Chidarambaram et al.[28] recently showed an increase of *in vivo* IL-17 gene expression in early compared to late MK patients. A study of cytokines in tears of Acanthamoeba keratitis cases using a multiplex bead assay found that the proinflammatory Th17 cytokine, IL-22, commonly expressed with IL-17, was associated with increased severity of disease.[29] However, that study did not find detectable levels of IL-17F and IL-17A in either the cases of infection nor controls. In the current study only 9 of the cases were culture positive and no analysis by pathogen type could be performed. This was not unexpected as in tertiary hospitals small early presenting lesions tend to be treated empirically.[30] For lesions that are cultured, around 50% of clinically diagnosed cases are generally culture positive.[31]

In general, murine models of keratitis indicate that the role of IL-17 in disease severity is mediated through a proinflammatory mechanism. In bacterial keratitis, neutralization of IL-17 by antibodies was associated with reduced recruitment of PMNs and corneal pathology, but did not affect removal of infecting *P. aeruginosa*.[32] In Herpes Simplex virus keratitis, IL-17 contributes to the stromal immune response.[33] In contrast, in Acanthamoeba keratitis, IL-17A is protective for severe disease.[34] It is of note that Acanthamoeba keratitis animal models do not display the same severe inflammatory complications, such as scleritis and stromal ring infiltrates seen in around 20% of patients.[35] In the current study, only 9/65 (14%) of the available cases of microbial keratitis had culture positive corneal scrape data available, therefore no analysis by pathogen type could be performed.

The major sources of IL-17A and IL-17F are Th17 cells, although other cell types such as gamma delta T cells, macrophages, natural killer, neutrophils and natural killer T cells can also produce IL-17. There is increasing evidence for different populations of Th17 cells with diverse functions, as well as plasticity of Th17 cells. A study involving IL-17-deficient mice has demonstrated a crucial requirement for IL-17A-producing Th17 cells in the production of sIgA in the gut mucosa.[36] Precisely how these different populations of Th17 cells are regulated and the activity of IL-17 produced by these different populations of cells are yet to be characterized.

In autoimmune disease the damaging role of IL-17 is well established. Secukinumab (Novartis, CA) is a fully human IL-17A monoclonal antibody approved by the Food and Drug Administration for the treatment of psoriasis, ankylosing spondylitis and psoriatic arthritis. In the eye, a multisite Phase 3 study of noninfectious uveitis did not show a significant difference between Secukinumab and

placebo in primary outcomes of reduction of recurrence and vitreous haze during immunosuppressant withdrawal, and improved VA; however, there was a decrease in the use of immunosuppressive medications.[37] A more recent differing dosing regimen randomized controlled trial in non-responsive uveitis patients showed that intravenous Secukinumab was more effective than subcutaneous dosing and high therapeutic concentrations are likely to be required to have an effect in this disease.[38]

In the current study, SNPs in IL-10 were not associated with severity of MK. This confirms the lack of association at IL-10 SNPs previously reported by Keijser et al.[17] Two SNPs were common in both studies, rs1800871 and rs1800896. Indeed, even if we combined the allele frequency data from our study with that of Keijser et al., which gives a control population of 300 and a case population of either 171 if all forms of keratitis were included or 135 if only severe cases were included from the current study, there would be still no association of these IL-10 SNPs with keratitis (Chi Square test, $P > 0.3$ for both SNPs for all keratitis and severe only).

In this exploratory analysis, we did not find statistical evidence for SNPs in IL-10 and IL-17 genes to predispose to more severe MK. Nevertheless, our data cannot reject with confidence the growing body of information that the Th17 pathway plays a role in keratitis outcomes, and larger studies are required to establish whether genetic associations exist. Further investigations are also needed to characterize the distinct roles of the IL-17 family of cytokines in keratitis, including the response to different pathogens and the time course during the infection. While previous studies have shown a genetic predisposition for corneal infection onset and severity associated with other cytokine SNPs (IL-6, IL-12B[13] and IL-10[17]) these studies need to validated with tissue specific studies such as tear protein analysis and gene expression before being clinically relevant. Both animal models and patient studies, using the expanding array of laboratory tools, will be important in this understanding, which will hopefully lead to better outcomes for patients with this disease.

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