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Gene profiling in murine corneas challenged with *Aspergillus fumigatus*

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Purpose: Fungal keratitis (FK) is a blinding infection of the corneas and accounts for a significant portion of all keratitis in the world but little is known about the pathogenesis of FK, especially at the level of molecular biology. This study tried to determine the genes that are modulated in the process and thus deserve further investigation for understanding the pathogenesis of FK.

Methods: Agilent Mouse Oligo Microarray was used to compare the gene profiles in control corneas and in corneas challenged with heat-inactivated *Aspergillus fumigatus* spores. Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry were used to confirm the changes of interested genes.

Results: In the 18,335 genes detected by array, 61 showed an increase of more than one-fold in RNA expression and 48 showed a decrease of over 50%. Among the regulated genes some are known to be related to host defense such as IL3, mannose binding lectin A (MBL-A), and postaglandin D2 synthase (Psgd) while others like Dopachrome tautomerase (Dct) have never been correlated to ocular or host defense. The other 33 changed genes either have unknown functions or encode hypothetical proteins. The changes in the expression of early growth response 4 (Egr4), Dct, Psgd, MBL-A, and hemoglobin α adult chain 1 were confirmed by using RT-PCR. MBL-A expression regulation was further confirmed by using immunohistochemisty in both in vitro and in vivo challenged murine corneas.

Conclusions: MBL-A is among the primary responding genes during the onset of fungal keratitis. Also it was found that the microarray is a useful tool in elucidating the pathogenesis of FK.

Fungal keratitis (FK) is a blinding infection of the cornea, which can lead to the loss of the infected eye if not controlled properly. As a disease caused by infectious microbes, both the incidence and predisposing factors of FK vary greatly in different parts of the world. For example, FK was reported to account for only 2% of all the keratitis cases in New York but for 35% of all keratitis cases in south Florida. This percentage is 44%, 17%, 36%, and 37.6% for south India, Nepal, Bangladesh, and Ghana, respectively [1]. When comparing these figures, we take into consideration that the spectra of causative pathogens of all infectious keratitis or just FK is continously changing due to the shift of dominant infectious agents along the time line [2,3]. For instance, with the treatment and control of viral and bacterial keratitis by using effective antibiotics and anti-viral therapeutics, the percentage of FK among all keratitis has been increasing during the last two decades in China. Other factors such as abuse or misuse of antibiotics, contact lens wearing, popularization of cataract surgeries, and laser in situ keratomileusis also contribute to the increase of FK cases in China. But compared to other infectious keratitis like herpetic simplex keratitis [4], much less is known about the pathogenesis of FK especially at the level of molecular progress [5]. In the current study, we used microarray to profile the genes that might be involved in the response of murine corneal cells to fungal pathogens. The responding genes found in this model can guide which direction the study of the pathogenesis of FK should be taken.

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METHODS

Animals: Eight- to twelve-week old female Balb/c and C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Company (Shanghai, China) and were housed at the Shandong Eye Institute Animal Facility. All animals were maintained and handled according to institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For induction of FK in Balb/c mice, the method described by Wu et al. [6] was used but with some modification. Briefly, about 30 superficial crosses were made with 30-gauge needles on the surface of the left corneas of the mice under a stereoscope. Five μl of *Aspergillus fumigatus* preparations, containing $1x10^6$ viable spores, were applied to the left eye of a mouse in the FK group. Corneas in control mice were similarly sacrificed but received 5 μl saline buffer instead of fungal preparations (sham infected). Over 80% of the fungal-challenged eyes developed typical keratitis in three days and the right eyes of all mice as well as the left eyes in the control group remained unaffected.

Gene profiling using microarray assay: Corneas were removed from Balb/c mice, cut into pieces of about 1 mm and put in RPMI 1640 culture medium with 10% fetal calf serum. Ten million heat-killed *A. fumigatus* spores were added to the culture of five corneas (the FC group), making the final total volume 1.3 ml. The medium control group (the MC group) contained similar amount of cornea tissue but was not treated

with fungal preparation. After 24 h of incubation at 37 °C with 5% CO_2, corneal pieces were picked out, rinsed with PBS, and used for RNA extraction by using EZ Spin Column Total RNA Isolation Kit (BioBasic Inc., Toronto, Canada). The integrity and high quality of RNA samples were confirmed by using agarose electrophoresis and the Lab-on-chip system. Four hundred nanograms of RNA were reverse-transcribed to cDNA during which process a T7 sequences was introduced into cDNA. T7 RNA polymerase-driven RNA synthesis was used for the preparation and labeling of cRNA with Cy3 (the FC sample) and Cy5 (the MC sample), respectively. A Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA) was used to purify fluorescent cRNA probes. An equal amount (1 μg) of Cy3 and Cy5 labeled probes were mixed and used for hybridization on one Agilent Mouse Oligo Microarray (catalog number G4121A, product serial number 5268; Agilent Techonologies Inc., Santa Clara, CA) following the protocol provided by the manufacturer. The hybridization signals were acquired by using Agilent 2100 Bioanalyzer (G2938B) and analyzed using Agilent G2567AA Feature Extraction Software (v7.5). Specifically, the Linear&Lowess method and the rank consistency filter were used for normalization of the features. The rank consistency filter selects features that fall within the central tendency of the data by observing consistent trends between the red and green channels. To be counted as a valid feature, the feature has to pass four criteria that they are positive and significant versus just the background, the signals are uniform in the spot, the signals are not saturated, and they are not population outliers in either channel. If the features failed any one of the criteria, they were flagged and excluded from further analysis.

Reverse transcription-polymerase chain reaction: The fungal challenge of the corneas and subsequent RNA extraction were carried out as described above. Twenty μl of RNA underwent reverse transcription using AMV First Strand cDNA Synthesis Kit (Biobasic Inc., Toronto, Canada). The RNA amount used for cDNA synthesis varied from 100 ng to 1 μg among different batches of experiments but was consistent for all samples in each single experiment. The RT reaction product was diluted ten-fold with water and 1 μl of the diluted mixture was used for the 20 μl PCR reaction. Semi-quantitative PCR was done with primers specific for each interested gene using similar denaturing and extension parameters (30 s at 93 °C, 1 min at 72 °C, respectively) but different annealing temperatures (Table 1). Each target was amplified for increasing the number of cycles of PCR with optimized annealing temperature. For targets expressed at an extremely low level, a second round of PCR with the dilution of the first round PCR product might be necessary. All PCR products were resolved in 1.5% agarose gel. The photograph was obtained with Electrophoresis Documentation and Analysis System 120 (Kodak Digital Science, New Haven, CT) and densitometry of the gel image was done with ImageJ 1.37v (NIH).

Immunohistochemistry: The eyeballs were removed from infected and control mice at different times after application of *A. fumigatus*, fixed with 4% paraformaldehyde in PBS for 2 h, then embedded in OCT compound for cryosecting. Corneas challenged in vitro with heat-killed spores were treated similarly. Conventional immunohistochemistry was done with rat anti-mouse mannose binding lectin-A (MBL-A; HyCult biotechnology, Uden, Netherlands) used as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Golden Bridge Biotechnology, Beijing, China) as the secondary antibody. Samples were mounted with UltraCruz mounting medium (Santa Cruz Biotechnology, Santa Cruz, CA) and were observed using E800 Nikon microscope with Digital Sight DS-U1 CCD cassette (Nikon Ltd. Tokyo, Japan). For background staining monitoring, sections were processed similarly except that the primary antibody was omitted.

RESULTS & DISCUSSION

Validation of the technique: The technique of microarray provides a valuable tool for screening out genes involved in any biological process like FK. As with the G4121A array used in this study, 1,075 internal control probes are loaded to monitor the quality of the whole experimental process. Besides these control probes, 50 biological probes are assigned 10 duplicates that are evenly distributed over the array. The Cy3/Cy5 ratio for each probe spot was obtained for all 10 duplicates and the average and standard deviation (Stdev) of each probe were used for calculating the coefficient variant (CV) of that specific probe according to formula Stdev/mean x100. Among all of the 50 duplicated probes, only five gave CV over 15% and none of the probes that produced Cy3 and Cy5 signals over 300 gave CV over 10% (Figure 1A). The average CV for the 50 biological control probes was 7.33%, indicating the high quality and reliability of the data obtained with this ar-

Table 1. Primers and annealing temperature for reverse transcriptase-polymerase chain reaction

Gene	GenBank ID	Forward primer	Reverse primer	Tm(°C)	Product size
GAPDH	XM_983502	TGGCAAAGTGGAGATTGTTG	AGTCTTCTGGGTGGCAGTGA	55.0	487 bp
Psgd	AB006361	CAGCCCAACTTTCAACAAGAC	TGACTTCTCTCACCTGCGTTT	59.5	500 bp
Hba-a1	NM_008218	TGACAGACTCAGGAAGAAACCA	TGCTCACAGAGGCAAGGAAT	59.5	424 bp
Dct	NM_010024	TACCATCTGTTGTGGCTGGA	GGTCCCATTCAGGAAGGAGT	59.5	474 bp
MBL-A	NM_010775	CAGGGTCACAAACCTGTGAG	CAGAGAGGAGGCAAGGAGAA	57.2	716 bp
Egr4	NM_020596	CTTCAACCTCATGTCTGGCATCT	CTTGGTCCCTACTGCAGAGAAG	59.5	409 bp

Primers were designed by using the online program Primer3 [31] according to the corresponding sequences (GeneBank ID) specified by the array supplier. Annealing temperature for amplification and product size was given for each gene.

ray, especially for those probes that produced high intensity of Cy3 and Cy5 signals (such as over 300).

Changes of gene expression patterns in fungal challenged corneas: Both our own work and the work of others have shown that the corneas are heavily infiltrated with inflammatory cells during fungal keratitis development (Figure 2F inset, and data not shown). Since, in this study, we were more interested in the primary response of the residential corneal cells per se than the response of immigrant inflammatory cells, we used in vitro fungal-challenged corneas for microarray profiling instead of corneas from mice with preset FK, minimizing the possible interference of infiltrating inflammatory or immune cells (Figure 2C,F, upper insets). Among the 20,868 genes or biological probes on the chip, 18,335 were detect-

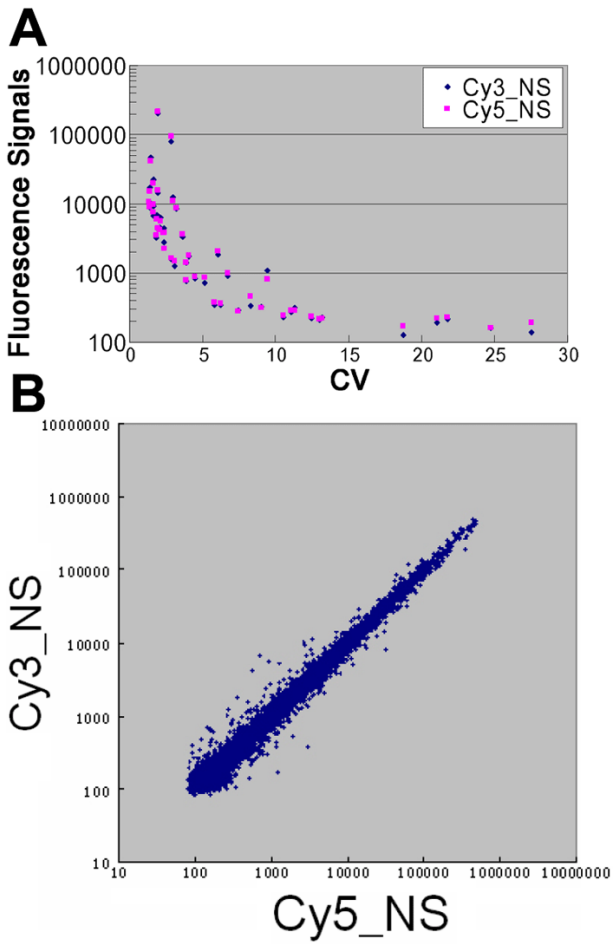

Figure 1. Hybridization signals of duplicates and all probes. **A**: The CV was calculated as described in the text for each of the 50 duplicated probes. In the order of CV from low to high, the name or accession number of the duplicated probes are NM_147068, Acate2, Rab5a, H2-Q7, Il27ra, Prdx2, E030041M21Rik, Tm9sf1, Cav2, Anxa8, Ap3s2, 1110033J19Rik, Nup50, Carhsp1, NM_029366, 5730427N09Rik, Fndc1, Gsta4, Gamt, ENSMUST00000016, Duox2, Tnks1bp1, Lrrk1, NG_001844, 2010004B12Rik, 1810036L03Rik, AK077144, Djc11, Hemt1, Acaa2, AK077144, Taf1a, Cpa1, Cpt1a, Kcnn2, 4930519G04Rik, Bambi, Olfr22-ps1, 2010001M09Rik, Tsga14, Spats1, 2810489O06Rik, Hyal3, 2610016C12Rik, Gabrg1, Sod1, Ntng1, Hspb7, Gcg, and Chgb, respectively. Please note that with some probes (e.g. Gcg) the symbols for Cy3_NS and Cy5_NS overlap due to similar reading. **B**: Normalized signals of control (Cy5_NS) and FC group (Cy3_NS) were given for all detected probes.

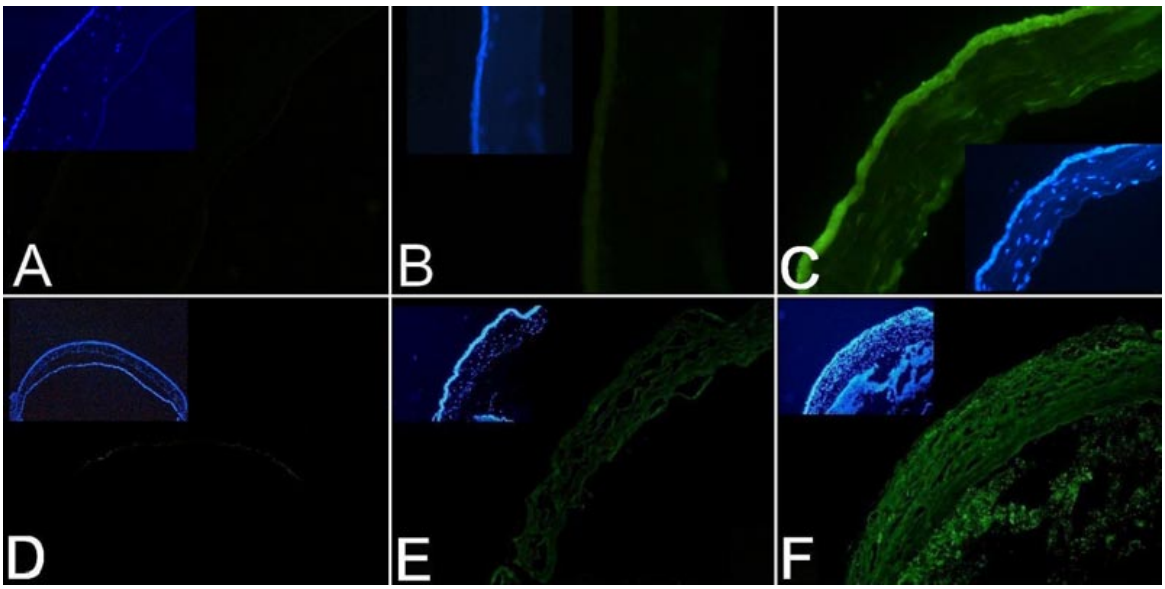

Figure 2. Immunohistochemistry detection of MBL-A in fungi-challenged murine corneas. Immunohistochemistry detection of MBL-A in in vitro-challenged corneas (**A**-**C**) and in corneas from in vivo experimental FK mice (**D**-**F**). For in vitro study, the corneas were either cultured with just the medium (**A**,**B**) or with heat-killed spores (**C**) for 24 h. FK was induced in Balb/c mice as described (**F**) and the corneas were removed three days later for IHC with sham-infected mice as the control (**D**,**E**). The specificity of staining was shown by the negative control (**A**,**D**, and data not shown) where sections from control corneas were handled in exactly the same way except that the primary antibody was omitted in the first staining step. Inlet in each panel shows DAPI staining of nuclei of the same section. Representative results from two (in vitro study) and three (in vivo study) experiments were shown. Capture modes used for pictures: DS-U1 CCD controlled with Nikon ACT-2U software (version 1.40.85) under fluorescence mode. Exposure control: Manual; Exposure time: 1/6 s.

Table 2. Genes that were upregulated in fungus-infected corneas

GenBank ID	Description of gene	Biological Activity	Cy3/Cy5
IMMUNE RESPONSE/DEFENSE			
NM_053251	Proline rich protein MP4	lubrication and defense?	6.30
NM_010775	mannose binding lectin, liver (A)	mannose BIN, complement ACT, heterophilic cell ADH	4.49
NM_021274	chemokine (C-X-C motif) ligand 10	chemotaxis,immune response, inflammatory response	2.16
U18372.1	CD37	lymphocye regulation?	2.14
NM_009776	serine proteinase inhibitor, G-1	complement ACT, blood coagulation	2.13
CYTOKINE/GROWTH FACTOR			
NM_010556	interleukin 3	cytokine, cell-growth	3.95
NM_009263	secreted phosphoprotein 1	ossification,cell ADH,wound healing	2.47
NM_010217	connective tissue growth factor	cell growth/DIF/MIG/ADH,ossification,angiogenesis	2.32
CYTOSKELETON/EXTRACELLULAR MATRIX			
NM_023256	keratin 20	intermediate filament organization	2.39
NM_031170	keratin complex 2, basic, gene 8	cytoskeleton organization and biogenesis	3.32
NM_011595	TIMP3	metalloendopeptidase inhibitor activity	2.19
TRANSPORTER			
AB006361	prostaglandin D2 synthase	transport,prostaglandin MET/biosynthesis	4.24
AF196476	type III Na-dependent phosphate T-ter	phosphate transport	2.96
NM_021544	Na channel, V-gated (V), α polypeptide	voltage-gated ion channel activity	2.85
NM_010001	cytochrome P450, 2c37	electron transport	2.78
NM_008468	karyopherin (importin), α 6	protein-nucleus import	2.45
NM_033145	signal sequence receptor, β	cotranslational protein membrane targeting	2.20
BC016095	kinesin superfamily member 18A	energy conversion,motor activity	2.16
AK048736	ADP-ribosylation related factor protein 2	protein transport	2.08
NM_030696	solute carrier family 16, member 3	monocarboxylate porter,symporter	2.04
METABOLISM/SIGNAL TRANSDUCTION			
NM_010024	dopachrome tautomerase	melanin biosynthesis from tyrosine,MET	10.08
NM_010276	gene overexpressed in skeletal muscle	small GTPase mediated signal transduction	4.88
AK014554	protein phosphatase 2A, 56B,ε-isoform	signal transduction	4.36
NM_021882	Silver	melanin biosynthesis from tyrosine	3.28
NM_053110	glycoprotein (transmembrane) nmb	cell ADH, MET	2.74
AK003078	ubiquitin-conjugating enzyme E2S	ubiquitin cycle,protein modification	2.46
NM_133777	ubiquitin-conjugating enzyme E2S	ubiquitin cycle,protein modification	2.34
NM_009252	Spi2-2, clade A, member 3N	endopeptidase inhibitor,bioprocess unknown	2.21
NM_013509	enolase 2, γ neurol Eno2	glucose MET, glycolysis	2.11
TRANSCRIPTION MODULATOR			
NM_020596	early growth response 4	DNA BIN,regulation of transcription	7.54
NM_173384.1	SRY-box containing gene 30	transcription factor activity	3.03
NEURAL CELL RELATED			
M55181	spermatogenic-specific preproenkephalin 1	behavioral fear response,neuropeptide signaling	3.94
NM_008077	glutamic acid decarboxylase 1	neurotransmitter biosynthesis,synaptic transmission	3.80
AK045348	Glutamate receptor, ionotropic, δ 2	synaptic transmission	3.51
NM_030675	cerebral cavernous malformations 1	small GTPase regulatory activity,protein BIN	2.85
AK053833	gamma-aminobutyric acid T-ter 1	Neurotransmitter	2.69
NM_009115	S100 protein, β polypeptide, neural	memory,regulation of neuronal synaptic plasticity	2.14
OTHER MOLECULES			
NM_009760	BCL2-E1B-interacting protein 1	Pro-apoptosis	2.05
HYPOTHETICAL PROTEIN			
BB754096	S100b homolog	regulation of neuronal synaptic plasticity?	4.23
AK089225	RIKEN cDNA F630045L20 gene	Na+/Ca++ exchange?	2.79
AK041212	tribbles homolog 1	protein phosphorylation?	2.45
AK044506	RIKEN cDNA A930017K13 gene	huCUG-BP and ETR-3 like factor 3 homolog	2.35
AK090124	RIKEN cDNA G431002C21 gene	regulation of transcription?	2.21
AK028591	RIKEN cDNA 4732407F15 gene	weakly similar to CYTOKERATIN 10	2.08
AK045363	RIKEN cDNA B230106I24 gene	catalytic activity,hydrolase activity	2.08
AK020928	weakly similar to melan-A	Unknown	4.87
NM_053216	RIKEN cDNA 1700102P08 gene	Unknown	4.12
AK030224	similar to putative pheromone R V2R2	Unknown	3.49
BF536212	weak similar to Bstni subfamily 2	Unknown	3.37
AK005289	similar to Serine/Theronine protein kise 25	Unknown	2.97
AK014234	Chr 1 segament, ERATO Doi 471	Unknown	2.56
AK021157	RIKEN cDNA C030047K22 gene	Unknown	2.43
BB768593	RIKEN cDNA G370087F11 gene	Unknown	2.42
BU563147	RIKEN cDNA 1110008P14 gene	Unknown	2.40
AK039718	human myopalladin homolog	Unknown	2.40
NM_026459	RIKEN cDNA 1700112P19 gene	Unknown	2.31
AW492251	cDNA clone UI-M-BH3-atl-c-09-0-UI 3'	Unknown	2.21
BC006604	RIKEN cDNA 9130213B05 gene	Unknown	2.05
BC023420	Similar to triuretic peptide receptor 2	Unknown	2.03
NG_001719.1	olfactory receptor pseudogene	Unknown	2.83
NG_001878.1	olfactory receptor pseudogene	Unknown	2.01

GenBank ID and description of each gene were provided by the array supplier. Activities of genes were summarized according to the array supplier in combination with extensive information mainly from NCBI PubMed resources and used for grouping the genes. Cy3/Cy5 ratio represented the proportion of the fluorescence intensity of signals in fungi-challenged corneas to control ones and reflected the change extent of each gene. ACT, activation; ADH, adhesion; BIN, binding; DIF, differentiation; MET, metabolism; MIG, migration; T-ter. transporter.

Table 3. Genes that were downregulated in fungus-infected corneas

GenBank ID	Description of gene	Biological Activity	Cy3/Cy5
IMMUNE RESPONSE/DENFENSE			
BY426609	IG MU chain C region	humoral immune response	0.144
AK003893	polydomain protein	completment control	0.273
NM_010742	Ly6D	defense response	0.435
CYTOKINE/GROWTH FACTOR			
NM_053087	epithelial mitogen	growth factor of epithelial cells	0.474
CYTOSKELETON/EXTRACELLULAR MATIRX			
M12289	perital skeletal myosin, heavy polypeptide 4,	cytoskeleton organization/biogenesis	0.128
NM_009928	procollagen, type XV	cell adhesion	0.419
NM_010808	MMP24	cell growth/differentiation/plasticity	0.443
NM_009394	troponin C2, fast skeletal	muscle contraction/development	0.465
RECEPTOR			
NM_147090.1	olfactory receptor MOR6-1	perception of smell	0.374
NM_146320.1	olfactory receptor 1145	perception of smell	0.499
TRANSPORTER			
NM_008218	hemoglobin α, adult chain 1 (Hba-a1)	oxygen transporter	0.254
NM_008221	hemoglobin Y, β-like embryonic chain	oxygen transporter	0.340
NM_008220	hemoglobin Y, β-like embryonic chain	oxygen transporter	0.431
NM_007474	aquaporin 8 (Aqp8)	water transporter	0.458
METABOLISM/SIGNAL TRANSDUCTION			
NM_008572	mast cell protease 8	proteolysis and peptidolysis	0.293
NM_019489	peptidylprolyl isomerase E	nucleic acid binding,isomerase activity	0.344
NM_013650	S100 calcium binding protein A8	calcium ion binding,cell function modulator	0.420
NM_023043	prion protein dublet	copper ion homeostasis	0.434
NM_021891	fidgetin-like 1	nucleotide binding,ATP binding	0.434
AK018114	ribosomal protein S6 kinase, polypeptide 5	protein amino acid phosphorylation	0.441
NM_011369	Shc SH2-domain binding protein 1	protein binding,SH2-domain binding	0.442
AF194970	leucine, glutamic acid, lysine family 1 protein	cell development	0.449
NM_012012	exonuclease 1	nucleotide and nucleic acid metabolism	0.460
NM_009114	S100 calcium binding protein A9	calcium ion binding,cell function modulator	0.465
BE957099	F-box and leucine-rich repeat protein 20	degradation of cellular regulatory proteins?	0.478
NM_021790	SoxLZ/Sox6 leucine zipper binding protein in testis	unclear	0.493
AK016328	ribosomal protein S6 kinase polypeptide 1	kinase,cell growth control	0.497
BC027557	eosinophil-associated, RNase A family-11	pancreatic RNase activity, RNase activity	0.475
NM_009994	cytochrome P450 family 1, 1b1	monooxygenase,oxidoreductase	0.490
EMBRYONIC DEVELOPMENT			
NM_008818	placentae and embryos oncofetal gene	embryonic development	0.407
NM_010056	distal-less homeobox 5	axonogenesis,embryonic development	0.481
NM_031184	GLIS family zinc finger 2	transactivation/repressor,embryonic development	0.498
CELL CYCLE MODULATOR			
NM_011527	T-cell acute lymphocytic leukemia 1	cell growth/differentiation,hemopoiesis	0.381
BG915576	fibroblast growth factor inducible 16	mitotic chromosome condensation/segregation	0.453
NM_007820	cyclin-dependent kise 8, cytochrome P450	cyclin-dependent protein kinase activity	0.461
NM_021886	centromere autoantigen H	chromosome segregation	0.461
NM_023284	cell division cycle associated 1	spindle microtubules-kinetochore attachment	0.324
OTHER MOLECULES			
NM_008148	glycoprotein 5 (platelet)	blood coagulation,cell adhesion	0.412
HYPOTHETICAL PROTEIN			
AK029570	tripartite motif protein 37	morphogen activity?	0.411
AK029737	phosphatase 1 regulatory subunit 9A homolog	protein phosphatase?	0.473
NM_027178	peptidylprolyl isomerase (cyclophilin) like 5	cell cycle control?	0.491
AK086472	SULFONYLUREA RECEPTOR-1 homolog	glucose-sensitive channel?	0.492
AK015117	RIKEN cDNA 4930412D23 gene	unkonwn	0.439
AK011728	RIKEN cDNA 2410030K01 gene	unkonwn	0.453
BC031396	RIKEN cDNA 1200008O12 gene	unknown	0.461
NM_029434	RIKEN cDNA 4930431B11 gene	unknown	0.474
NM_133775	RIKEN cDNA 9230117N10 gene	unknown	0.477
AK033132	RIKEN cDNA 2310043D08 gene	unkonwn	0.483

GenBank ID and description of each gene were provided by the array supplier. Activities of genes were summarized according to the array supplier in combination with extensive information mainly from NCBI PubMed resources and used for grouping the genes. Cy3/Cy5 ratio represented the proportion of the fluorescence intensity of signals in fungi-challenged corneas to control ones and reflected the change extent of each gene.

able, giving an overall detection rate of 87.86%. Among the detectable genes, most of them (99.41%) remained unchanged in the abundance of mRNA or the change was less than two-fold (Figure 1B). However, 61 genes manifested an expression increase of at least one-fold in the infected group compared to the control (Table 2) while 48 showed a decrease of over 50% of the control after treatment (Table 3).

We divided these genes into different categories according to their main biological properties. Interestingly, about one-third (23/61) of the upregulated genes and one-fifth (10/48) of the downregulated genes are not cloned yet or had only been suggested to encode hypothetical proteins. Involvement of these unknown genes in FK as shown in this study implies that some of them might be related in local or systemic antifungal response of the host. For example, ectopic expression of the olfactory receptor genes/pseudogenes family has been reported in many tissues [7]. The findings that two olfactory receptors (NM_147090, NM_146320) were downregulated while two olfactory receptor pseudogenes (NG_001719, NG_001878) were upregulated in fungi-challenged corneas not only demonstrated their expression in corneas for the first time but also proved that individual members of this family respond differently to the same challenge.

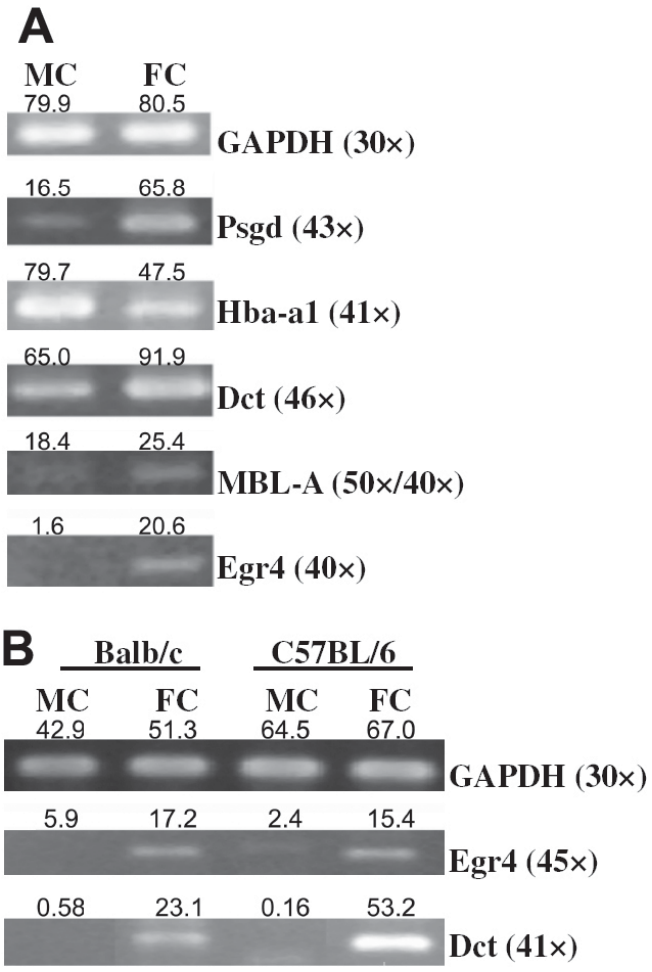

Figure 3. Confirmation of the changes in genes located in heat-killed spore-challenged corneas.. **A**: This figure shows RT-PCR results obtained with the same batch of RNA samples that were used for microarray. MBL-A was amplified for 50 cycles and the product was diluted 1,000 fold. One µl was subjected for another 40-cycle PCR. **B**: This figure shows expression changes of Egr4 and Dct in corneas from Balb/c and C57BL/6 strain mice in one representative of two experiments giving similar results. For densitometry analysis, arbitrary illuminant unit obtained with ImageJ was given above each band. MC: control corneas; FC: fungal spore-challenged corneas.

Upregulated genes: Among the upregulated genes, over one-third of them are functionally involved in biologically fundamental processes like metabolism, signal transduction, mass transportation, and cytoskeleton organization. Only three of the upregulated genes, namely mannose binding lectin (MBL)-A [8,9], CD37 [10], and chemokine ligand 10 (CXCL10) [11], are known to be closely related to immune response to pathogens. MBL is a member of the collectins family. Binding of pathogens to MBL activates a complement pathway or opsonizes the engulfment of pathogens by macrophages [8,12] thus enhancing the antimicrobial reactions of host immune system. MBL-deficient mice were found to be more susceptible to pathogens-induced lethality [13,14], further demonstrating the importance of MBL in controlling infections in affected hosts. Though mainly produced in the liver, MBL-A is also expressed in other tissues [15,16] and the expression of MBL-A increases in response to acute phase stimuli in the liver [17]. For the first time, our array result showed that MBL-A is expressed in corneas and it might be one of the local responders to challenging pathogens. Overexpression of MBL-A was confirmed using immunohistochemisty in both corneas challenged with heat-killed spores in vitro (Figure 2A-C) and corneas from experimental FK mice (Figure 2D-F). It was noteworthy to point out that in corneas cocultured with heat-killed spores, MBL-A expression was restricted to epithelial layers (Figure 2C). In eyes from FK mice, however, MBL-A expression was detected in all layers of the cornea as well as in the infiltrating inflammatory cells located in the anterior chamber of the infected eye (Figure 2F). DAPI staining showed that corneas from FK mice were heavily infiltrated with inflammatory cells (Figure 2F, inset).

Dct and Egr4 are among the genes that responded most strongly to the fungal challenge in our study (Figure 3). Dct has been known as a melanogenic enzyme and its main producers are pigmented cells like melanocytes or retinal pigment epithelial cells both in the human and mice system [18,19]. Very recently, Dct was found to regulate neural progenitor proliferation [20]. Interestingly enough, six other genes that are closely related to neural cell functions were also upregulated in fungal spore-challenged corneas (Table 2). Not yet knowing the biological significance of an increased expression of these genes in FK corneas, we propose that these changes might be the reflection of changes occuring in numerous neural cells in corneas. Egr4 is a member of the Egr family that acts as a nuclear effector of various extracellular signals like tissue injury or apoptotic stimuli. Though no immunomodulating activity has been ascribed to Egr4 by itself, Egr4 may regulate immune response by interacting with proinflammatory cytokine genes [21,22]. Proline rich proteins are a group of molecules that work as lubricants in certain

body parts such as saliva [23] and some of these proteins have been detected in corneal cells [24,25]. As a member of this family, the *B-type proline-rich protein, MP4* gene (NM_053251) was first cloned in 1991 [26] but its function remains unknown.

Overexpression of MP4 in fungi-challenged corneas implied that it might be involved in biological processes that are related to a host's defense against infections. More studies are needed to learn the exact roles of Dct, Egr4, and MP4 in pathogenesis of FK.

Down regulated genes: Of the 48 genes that were downregulated in the experimental fungal keratitic corneas, roughly one-third of them (15/48) are primarily involved in the cellular metabolism process. The many other gene products are proteins with high molecular weights such as IgM chain, polydomain protein, perital skeletal myosin, procollagen, etc. (Table 3). We think this might be a reflection of corneal structure destruction caused by fungi challenge. Only three of the downregulated molecules were related to immunology or host defense but we did not go into details of any other downregulated genes in this study except for confirming the decrease of Hba-a1 (NM_008218) mRNA by RT-PCR.

Though some promising results were obtained, this study bears several limitations that require further studies to clarify. For example, we used heat-killed fungal spores instead of viable ones in the in vitro study to eliminate the potential risk of nutrition deficiency in the culture that would be caused by an overgrowth of live fungi. But the potential difference of the response to viable and heat-killed fungi was not monitored in this study. It has been well-documented that live and killed microbes of the same strain may provoke greatly different responses. Specifically, with *A. fumigatus*, this phenomenon has been observed in both the cells [27,28] and the whole body [29]. Rivera et al. [29] showed that when both are administrated intratracheally, only live *A. fumigatus* spores recruit fungus-specific and IFNγ-producing $CD4^+$ T cells in airways while heat-inactivated spores prime $CD4^+$ cells more efficiently in draining lymph nodes than live ones. Similarly, the possible bias caused by using an in vitro fungal challenge model instead of a real in vivo FK model in gene profiling has to be considered in further study. Furthermore, Huang et al. [30] reported that about 10% of all detected genes are upregulated or downregulated in mouse corneas one day after infection with viable *Pseudomonas aeruginosa* and a significant part of those regulated genes belong to cytokines, chemokines, or inflammatory factor groups. They also showed that many genes showed significantly different or even opposite changes during their response to the same infection in Balb/c and C57BL/6 mice [30]. In our study, however, comparison of gene expression changes in heat-killed spore-challenged corneas from Balb/c or C57BL/6 strain mice was not attempted beyond Egr4 and Dct (Figure 3B). But these limitations do not weaken the significance of this study. On the contrary, it shows the strength of using microarray technology in elucidating the mechanism of fungal keratitis. Further intensive research will be following to solve the present limitations.

In summary, this is the first time the microarray technique was applied in the study of the pathogenesis of fungal keratitis. Our preliminary results revealed some genes that are possibly related to the innate response of corneas during FK initiation such as MBL-A. Besides serving as a good start in a more detailed search of the pathogenesis of FK, this study also gives clues on how to understand the biological functions of those uncloned hypothetical genes that were shown to respond to the fungal challenge in the corneas.

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