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Abstract

We developed an expression profiling array to examine pro-apoptotic and pro-survival genes in dog retinal degeneration models. Gene-specific canine TaqMan assays were developed and included in a custom real-time quantitative reverse transcription-PCR (qRT-PCR) array. Of the 96 selected genes, 93 belonged to known relevant pro-apoptotic and pro-survival pathways, and/or were positive controls expressed in retina, while three were housekeeping genes. Ingenuity Pathway Analysis (IPA) showed that the selected genes belonged to expected biological functions (cell death, cell-mediated immune response, cellular development, function, and maintenance) and pathways (death receptor signaling, apoptosis, TNFR1 signaling, and induction of apoptosis by HIV1). Validation of the profiling array was performed with RNA extracted from cultured MDCK cells in the presence or absence of treatment with 10 μM staurosporin for 5 or 10 h. The vast majority of the genes showed positive amplifications, and a number of them also had fold change (FC) differences > ±3 between control and staurosporin-treated cells. To conclude, we established a profiling array that will be used to identify differentially expressed genes associated with photoreceptor death or survival in canine models of retinal degenerative diseases with mutations in genes that cause human inherited blindness with comparable phenotypes.

Keywords

dog model, retinal degenerative diseases, RVA expression profiling array, real-time quantitative reverse transcription-PCR, pro-apoptosis genes, pro-survival genes, cell death, cell survival, madin-darby canine kidney cells, staurosporin

Disciplines

Disease Modeling | Eye Diseases | Medical Cell Biology | Medical Genetics | Ophthalmology | Veterinary Medicine

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Development and Validation of a Canine-Specific Profiling Array to Examine Expression of Pro-Apoptotic and Pro-Survival Genes in Retinal Degenerative Diseases

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Abstract

We developed an expression profiling array to examine pro-apoptotic and pro-survival genes in dog retinal degeneration models. Gene specific canine TaqMan assays were developed and included in a custom real-time quantitative reverse transcription-PCR (qRT-PCR) array. Of the 96 selected genes, 93 belonged to known relevant pro-apoptotic and pro-survival pathways, and/or were positive controls expressed in retina, while 3 were housekeeping genes. Ingenuity Pathway Analysis (IPA) showed that the selected genes belonged to expected biological functions (cell death, cell-mediated immune response, cellular development, function, and maintenance) and pathways (death receptor signaling, apoptosis, TNFR1 signaling, and induction of apoptosis by HIV1). Validation of the profiling array was performed with RNA extracted from cultured MDCK cells in the presence or absence of treatment with 10 μM staurosporin for 5 or 10 hrs. The vast majority of the genes showed positive amplifications, and a number of them also had fold change (FC) differences > +/−3 between control and staurosporin-treated cells. To conclude, we established a profiling array that will be used to identify differentially expressed genes associated with photoreceptor death or survival in canine models of retinal degenerative diseases with mutations in genes that cause human inherited blindness with comparable phenotypes.

46.1 Introduction

Photoreceptors, like other specialized cells, have the innate ability to die through varied molecular mechanisms, and in response to multiple insults, whether genetic or acquired (Melino et al. 2005). Since the description of apoptosis as one of the final pathways in photoreceptor cell death (Chang et al. 1993; Portera-Cailliau et al. 1994), many studies have examined different molecules and mechanisms involved in the process.

Multiple pathways have been reported to be relevant for both retinal cell death (Cottet and Schorderet 2009; Doonan et al. 2005; Kunchithapautham and Rohrer 2007; Lohr et al. 2006; Rohrer et al. 2004; Sancho-Pelluz et al. 2008; Werdehausen et al. 2007) and survival (Barnstable and Tombran-Tink 2006; Bazan 2006; Jomary et al. 2006; Ueki et al. 2009; Wenzel et al. 2005). These are dependent on the underlying mutation, the model, whether naturally occurring or induced, the speed of the degenerative process, the cell class involved, and other factors. Despite the abundance of such studies, the signaling pathways and molecular mechanisms that link the mutations to the observed phenotypes are still unknown for many of the photoreceptor degenerative diseases. One of these diseases is canine X-

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linked progressive retinal atrophy 2 (XLPRA2), caused by a 2-bp deletion in exon ORF15 of the *RPGR* gene (Zhang et al. 2002). In two recent studies from our group, we characterized by TUNEL labeling the time course of cell death in affected dogs (Beltran et al. 2006), and identified by microarray analysis a number of non-classical apoptosis and mitochondria-related genes that seemed to be involved in the degenerative process (Genini et al. 2010). However, limitations of the latter study included the use of a custom canine cDNA array with a limited number of genes, and without relevant pro- and anti-apoptotic genes. Although for humans and rodent models a comprehensive suite of commercially-available products can be used to analyze RNA and protein expressions of a large panel of genes associated with biological pathways or specific disease states, these tools do not work or are currently not available for the dog.

The aim of this study was to fill this gap by developing and validating a canine specific real-time quantitative reverse transcription-PCR (qRT-PCR) profiling array containing key genes that are directly or indirectly involved in pro-apoptotic and pro-survival processes, autophagy, and/or are related to microglia/macrophages, cells that have been recently associated with retinal disease processes (Langmann 2007; Sasahara et al. 2008).

46.2 Materials and Methods

46.2.1 Development of the Canine-Specific qRT-PCR Array

The canine-specific custom-designed qRT-PCR profiling array (Table 46.1) was developed in conjunction with Applied Biosystems (ABI, Foster City, CA). Canine specific sequences of selected genes, identified from studies in other species, were submitted to ABI to develop gene specific TaqMan assays (http://www3.appliedbiosystems.com/AB_Home/index.htm). These contained unlabeled forward and reverse primers and FAM dye labeled TaqMan MGB probes. The Ingenuity Pathway Analysis (IPA, Ingenuity System Inc., Redwood City, CA) database was interrogated with the 96 genes on the array to better characterize biological functions and pathways involved.

46.2.2 Validation of the qRT-PCR Array Using Madin-Darby Canine Kidney (MDCK) Cells

46.2.2.1 Cell Culture—MDCK cells were grown to 80% confluency in 60-mm Petri dishes in Dulbecco’s modified Eagle’s medium (DMEM, with low glucose and L-glutamine, without sodium bicarbonate) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 1% sodium pyruvate, and 1% MEM non-essential amino acids (Sigma-Aldrich, St. Louis, MO). At 5 and 10 hrs prior cell collection, control cells received fresh supplemented DMEM medium, while treated cells received fresh supplemented DMEM medium containing 10 μM staurosporin (Sigma-Aldrich). For each time point (5 or 10 hrs) and cell type (control or staurosporin-treated), the experiment was done in duplicate, one Petri dish was used to assess cellular viability and the other for qRT-PCR analysis.

46.2.2.2 Assessment of Cellular Viability—Cellular viability of the cultured control and staurosporin-treated MDCK cells was assessed with a LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen-Life Technologies, Carlsbad, CA) following the manufacturer’s recommendation. Petri dishes containing the cells were examined by epifluorescence microscopy (Axioplan, Carl Zeiss Meditech, Oberkochen, Germany). Images were digitally captured (Spot 4.0 camera), and displayed with a graphics program (Photoshop, Adobe, Mountain View, CA).

46.2.2.3 qRT-PCR Analysis—MDCK cells used for qRT-PCR analysis were harvested by adding PBS and removing the cells from the Petri dishes with a plastic 16-cm cell scraper. Total RNA from cell pellets was extracted, DNAse treated, and reverse-transcribed as previously described (Genini et al. 2010). qRT-PCR reactions containing 30 ng of mixed cDNA at a ratio of 2:1 (5 hrs:10 hrs) also were performed as recently described (Genini et al. 2010).

46.3 Results

We developed a profiling array containing 96 canine specific TaqMan probes to test the expression of genes related to pro-apoptotic and anti-apoptotic processes. The selected genes belong to 7 main categories that inform on signaling pathways and disease mechanisms, e.g. 1) pro-death, mitochondria-dependent; 2) pro-death, mitochondria-independent; 3) autophagy; 4) pro-survival; 5) microglia/macrophage related; 6) expressed in retina [rods or cones (*ARR3*, *OPN1SW*, *OPN1MW*, and *RHO*), Müller cells and astrocytes (*GFAP* and *VIM*), bipolar cells (*PKCA*), and in the retinal pigment epithelium (*BEST1*)]; 7) housekeeping genes (*18S*, *ACTB*, and *GAPDH*). Table 46.1 provides a summary of the 96 genes included in the array with their symbols, descriptions, categories, TaqMan assay numbers (ABI), and location on the array.

To evaluate in detail and to confirm the nature of the 96 selected genes, we analyzed them with the IPA program. As expected, the five most relevant IPA molecular and cellular functions identified were cell death, cell-mediated immune response, cellular development, function, and maintenance, as well as DNA replication, recombination, and repair (Table 46.2). Inflammatory disease, immunological disease, neurological disease, cancer, and skeletal and muscular disorders were the five IPA biological functions related to “diseases and disorders” with the highest number of genes (Table 46.2). Furthermore, relevant IPA pathways included death receptor signaling, apoptosis signaling, TNFR1 signaling, induction of apoptosis by HIV1, and tumoricidal function of hepatic natural killer cells (Table 46.2).

The profiling array was validated, and the functionality of the TaqMan assays was tested, with RNA extracted from control and staurosporin-treated MDCK cells. To examine the highest number of genes possible, we mixed with a ratio of 2:1 the cDNAs of the staurosporin-treated cells at 5 hrs (to detect early apoptotic genes) and 10 hrs (to detect late apoptotic genes). The cDNAs from age-matched untreated cells at 5 and 10 hrs post addition of fresh DMEM medium were processed similarly. While the untreated cells were mostly all alive at 5 hrs (Fig. 46.1A) and 10 hrs (Fig. 46.1C), several staurosporin-treated cells were dead at 5 hrs (Fig. 46.1B) and almost all at 10 hrs (Fig. 46.1D) of treatment.

The qRT-PCR results showed that retina-specific control genes did not amplify (*BEST1*, *OPN1LW*, and *OPN1SW*), or had very high CT values between 35 and 38 (*ARR3*, *GFAP*, and *RHO*) in both untreated and treated cells. Furthermore, the additional genes *BNIP3L*, *BID*, *CASP14*, *CD40*, *CD40LG*, *FADD*, *IL10*, *TNFA*, *TNFRSF9*, *TNFSF8*, *TNFRSF21*, and *TYROBP* did not amplify in MDCK cells. For the remaining genes, CT values ranged from 10 (*18S*) to 34 (*FASLG*, *NTF3*, *NTF4*, *PRDX3*, *PTPRC*, and *TP73*). The calculated mean CT values of *GAPDH* and *ACTB* were used for normalization because they did not change between treated and control samples, while *18S* was excluded as it was unstable and highly variable.

A total of 8 genes (*BCL2L1*, *BCL2L11*, *CASP10*, *CCL2*, *DDIT3*, *HSP70*, *NGF*, and *TRAF2*) showed FC differences >3 in control vs. staurosporin-treated cells, while 4 (the retinal genes *ARR3*, *GFAP*, and *RHO*, as well as *IL6*) showed opposite regulation. The expressions of *BNIP3*, *TNFA*, and *GFAP* were also assessed with single assays done separately. The same experimental conditions used for qRT-PCR on the profiling array were applied, with the exception that the quantity of cDNA was augmented to 100 ng per gene and that other primers for *BNIP3* (Genini et al. 2010) were used. The results demonstrated no changes in expression of *BNIP3*, up-regulation of *GFAP* (raw CT values of 34 and 36, respectively) and *TNFA* (raw CT values of 35 and 37, respectively) in staurosporin-treated vs. control cells.

46.4 Discussion and Conclusions

In the present study, we developed a qRT-PCR profiling array containing key genes that are directly or indirectly involved in pro-apoptotic and pro-survival processes, autophagy, and/or are related to microglia/macrophages. For all the selected genes, canine-specific TaqMan assays are now inventoried and available for the research community. The array was validated in canine origin MDCK cell cultures treated with staurosporin, and we identified a number of genes that are important in pro-apoptotic and pro-survival processes, defined as part of signaling pathways that activate apoptosis, attempt to block apoptosis, or attempt to down or up-regulate protective cell functions. A precise and final classification of one gene to one category was a very complex task, because several genes fit into different categories depending on several factors (e.g. cell type, disease, age, interacting molecules) and because this classification is a dynamic process that alters as more information becomes available.

Specific characterization of the selected genes with IPA confirmed their expected biological functions and pathways, including cell death and cell mediated immune response, and provided additional information to better evaluate and dissect the general pattern of the genes on the profiling array.

A few genes, in particular those that are retinal-specific, could not be successfully amplified in MDCK cells with 30 ng of cDNA. This might be due to absence or very low levels of gene expression in MDCK cells, as shown with the single assay for *TNFA* that worked when we used 100 ng of cDNA. Alternatively, this may have been caused by primers not annealing to the sequence of interest. MDCK cells were used for this initial validation step in order to save precious and limited canine retina samples; however additional validation with RNA from retina will clarify the reasons for the failed amplification of certain genes.

This profiling array will be useful in future studies to identify genes, molecular mechanisms, and signaling pathways associated with photoreceptor degeneration in XLPRA2 and also additional canine models, e.g. rcd1, rcd2, XLPRA1, that carry mutations in other genes known to cause retinal degeneration in humans. Inclusion of 3 housekeeping genes used for normalization in the profiling array represents an advantage as it will enable selection of the optimal combination for each different experiment that will be performed.

It is expected that such quantitative analyses of gene expression will be valuable in identifying common, as well as disease-specific pro-death/pro-survival pathways that may represent future novel therapeutic targets.

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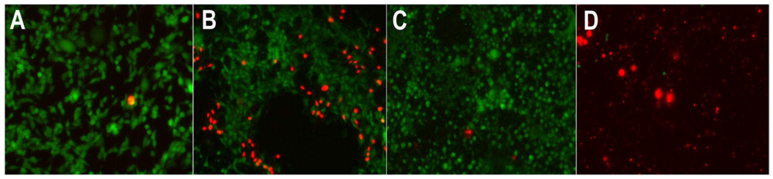

Fig. 46.1.
LIVE/DEAD Viability/Cytotoxicity assay showing live (green) or dead (red) cells in control (**A**, 5 hrs; **C**, 10 hrs) or staurosporin-treated (**B**, 5 hrs; **D**, 10 hrs) MDCK cultures. Note the marked reduction in number of live cells after 10 hrs exposure to staurosporin.

Table 46.1

List of the 96 genes included in the profiling array. Genes are reported with their symbols (in parenthesis the alternative symbols), descriptions, categories, TaqMan assay numbers (ABI), and location on the array. Main categories were: 1) pro-death, mitochondria-dependent; 2) pro-death, mitochondria-independent; 3) autophagy; 4) pro-survival; 5) microglia/macrophage related; 6) positive control expressed in retina; 7) housekeeping.

Gene symbol (alternative symbol)	Gene description	Gene category	TaqMan® assay	Location on array
18S	eukaryotic 18S rRNA	7	Hs99999901_s1	A1
AIFM1 (*AIF*)	apoptosis-inducing factor, mitochondrion-associated 1	1	Cf02636601_m1	A2
SLC25A4 (*ANT-1*)	solute carrier family 25, member 4	1	Cf02730291_g1	A3
APAF1	apoptotic peptidase activating factor 1	1	Cf02695305_m1	A4
ATG3	autophagy related 3 homolog	3	Cf00684119_m1	A5
ATG5	autophagy related 5 homolog	3	Cf02637561_m1	A6
ATG7	autophagy related 7 homolog	3	Cf02656560_m1	A7
ATG12	autophagy related 12 homolog	3	Cf02641158_m1	A8
BAD (*BBC2/BCL2L8*)	BCL2-antagonist of cell death	1	Cf02627333_m1	A9
BAK1	BCL2-antagonist/killer 1	1	Cf02627218_m1	A10
BAX	BCL2-associated X protein	1	Cf02622186_g1	A11
BBC3 (*PUMA*)	BCL2 binding component 3	1	Cf02708330_m1	A12
BCL2	B-cell CLL/lymphoma 2	4	Cf02622425_m1	B1
BCL2L11 (*BAM/BIM*)	BCL2-like 11	1	Cf00708025_s1	B2
PABPN1 (*BCL2L2*)	poly(A) binding protein, nuclear 1	4	Cf02664611_m1	B3
BCL2L1 (*BCL-XL*)	BCL2-like 1	4	Cf02622161_m1	B4
BDNF	brain-derived neurotrophic factor	4, 5	Cf02622349_g1	B5
BECN1 (*ATG6*)	beclin 1	3	Cf02643377_m1	B6
BID	BH3 interacting domain death agonist	1	Cf03460096_m1	B7
GRP78 (*BIP*)	78 kDa glucose-regulated protein	4	Cf02631877_m1	B8
BNIP3 (*NIP3*)	BCL2/adenovirus E1B 19kDa interacting protein 3	1	Cf02654885_m1	B9
BNIP3L (*NIX*)	BCL2/adenovirus E1B 19kDa-interacting protein 3-like	1	Cf03460134_m1	B10
CASP10	caspase 10	1	Cf03460108_m1	B11
CASP14	caspase 14	2	Cf03460139_m1	B12
CASP2	caspase 2	1	Cf02624522_m1	C1
CASP3	caspase 3	1	Cf02622232_m1	C2
CASP4	caspase 4	1	Cf02623472_m1	C3
CASP6	caspase 6	1	Cf02652513_m1	C4
CASP7	caspase 7	1	Cf03460102_m1	C5
CASP8	caspase 8	1	Cf02627553_m1	C6
CASP9 (*APAF3*)	caspase 9	1	Cf02627331_m1	C7
SFRS2IP (*CASP11*)	splicing factor, arginine/serine-rich 2, interacting protein	2	Cf02703447_m1	C8
CAPN1	calpain 1, (mu/I) large subunit	1	Cf02704115_m1	C9

Gene symbol (alternative symbol)	Gene description	Gene category	TaqMan® assay	Location on array
CAPN2	calpain 2, (mu/II) large subunit	1	Cf02645870_m1	C10
CAST	calpastatin	2	Cf02664849_m1	C11
CTSD	cathepsin D	3	Cf02625552_m1	C12
CTSS	cathepsin S	3	Cf02625930_m1	D1
CCL2	chemokine (C-C motif) ligand 2	4, 5	Cf02671955_g1	D2
CD40 (*TNFRSF5*)	TNF receptor superfamily member 5	2, 4, 5	Cf02626290_m1	D3
CD40LG (*CD154*/*TNFSF5*)	CD40 ligand	2, 4, 5	Cf02623314_m1	D4
PTPRC (*CD45*)	protein tyrosine phosphatase, receptor type C	1, 5	Cf02653185_m1	D5
CNTF	ciliary neurotrophic factor	4, 5	Cf03460095_sH	D6
CREB1	cAMP responsive element binding protein 1	4	Cf02667607_m1	D7
CYCS	cytochrome c, somatic	1	Cf02640410_g1	D8
TYROBP (*DAP12/KARAP*)	TYRO protein tyrosine kinase binding protein	5	Cf02642009_m1	D9
DIABLO (*SMAC*/*SMAC3*)	diablo homolog	1	Cf02665346_m1	D10
ENDOG	endonuclease G	1	Cf02703061_u1	D11
FADD (*GIG3*/*MORT1*)	FAS-associating death domain-containing protein	1	Cf03460155_m1	D12
FAS (*TNFRSF6*/*APO-1*/*CD95*)	TNF receptor superfamily, member 6	1	Cf02651136_m1	E1
FASLG (*TNFSF6*/*CD95L*/*CD178*)	FAS ligand	1	Cf02625215_s1	E2
BFGF (*FGF2*)	basic fibroblast growth factor	4, 5	Cf03460065_g1	E3
DDIT3 (*GADD153*/*CHOP10*)	DNA-damage-inducible transcript 3	2	Cf02654858_m1	E4
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	7	Hs02786624_g1	E5
GDNF	glial cell derived neurotrophic factor	4, 5	Cf02691052_s1	E6
HIF1A	hypoxia-inducible factor 1, alpha subunit	4	Cf02741632_m1	E7
HRK (*DP5*/*HARAKIRI*)	BCL2 interacting protein	1	Cf02702255_g1	E8
HSPB1 (*HSP27*)	heat shock 27kDa protein 1	4	Cf02628297_m1	E9
HSPD1 (*HSP60*)	heat shock 60kDa protein 1 (chaperonin)	1, 4	Cf02668830_gH	E10
HSP70 (*HSPA1*)	heat shock protein 70	4	Cf02622418_g1	E11
HSP86 (*HSP90AA1*)	heat shock protein HSP90-alpha	4	Cf03460183_s1	E12
IGF1R (*CD221*)	insulin-like growth factor 1 receptor	4	Cf02625178_m1	F1
IL6 (*IFNB2*)	interleukin 6	2, 5	Cf02624282_m1	F2
IL10	interleukin 10	4, 5	Cf02624265_m1	F3
MAP1LC3A (*LC3*)	microtubule-associated protein 1 light chain 3 alpha	3	Cf02630406_m1	F4
LYZ	lysozyme	3	Cf02642933_m1	F5
PRKCZ (*PKC2*)	protein kinase C, zeta	4	Cf02674616_m1	F6
PRDX3	peroxiredoxin 3	4	Cf03460191_sH	F7
NGF	nerve growth factor	4, 5	Cf02625041_s1	F8
NTF3	neurotrophin 3	4, 5	Cf02700489_s1	F9
NTF4	neurotrophin 4	4	Cf02705704_s1	F10
SOD1	superoxide dismutase 1, soluble	1, 4	Cf02624276_m1	F11
STAT1	signal transducer and activator of transcription 1	1	Cf02662970_m1	F12

Gene symbol (alternative symbol)	Gene description	Gene category	TaqMan® assay	Location on array
STAT3	signal transducer and activator of transcription 3	4, 5	Cf02666647_m1	G1
BIRC5 (*IAP4*)	baculoviral IAP repeat-containing 5 (survivin)	4	Cf02628995_m1	G2
TNFA	tumor necrosis factor alpha	1, 2, 5	Cf02628236_m1	G3
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A	1, 2	Cf02622751_m1	G4
TNFRSF21 (*DR6*)	tumor necrosis factor receptor superfamily, member 21	2	Cf03460083_s1	G5
TNFRSF25 (*APO-3*/*DDR3*)	tumor necrosis factor receptor superfamily, member 25	1, 2	Cf02653814_g1	G6
TNFSF10 (*APO-2L*/*TRAIL*)	tumor necrosis factor (ligand) superfamily, member 10	1, 2	Cf03460069_m1	G7
TNFRSF9 (*4-1BB*/*CD137*)	tumor necrosis factor receptor superfamily, member 9	2	Cf03460132_m1	G8
TNFSF8 (*CD153*/*CD30L*)	tumor necrosis factor (ligand) superfamily, member 8	2	Cf03460158_m1	G9
TP53	tumor protein p53	1	Cf02623148_m1	G10
TP73	tumor protein p73	1, 4	Cf02680478_mH	G11
TRADD	TNFRSF1A-associated via death domain	1, 2	Cf02661903_m1	G12
TRAF2 (*TRAP*)	TNF receptor-associated factor 2	2	Cf02662893_m1	H1
TRAF3	TNF receptor-associated factor 3	2	Cf02659700_m1	H2
XIAP (*AP13*/*BIRC4*)	X-linked inhibitor of apoptosis	4	Cf02625207_m1	H3
ACTB	actin, beta	7	Hs03023880_g1	H4
RHO	rhodopsin	6	Cf02625669_m1	H5
OPN1SW	opsin 1 (cone pigments), short-wave-sensitive, blue opsin	6	Cf03460200_m1	H6
OPN1LW	opsin 1 (cone pigments), long-wave-sensitive, red/green opsin	6	Cf02622926_m1	H7
ARR3 (*CAR*/*ARRX*)	retinal cone arrestin 3	6	Cf03460116_m1	H8
VIM	vimentin	6	Cf02668853_g1	H9
GFAP	glial fibrillary acidic protein	6	Cf02655695_m1	H10
PKCA (*PRKCA*)	protein kinase C, alpha	6	Cf02655322_m1	H11
BEST1 (*VMD2*)	bestrophin 1	6	Cf02697409_gH	H12

Table 46.2

Five most significant IPA biological functions ("molecular and cellular functions" or "disease and disorders") and canonical pathways identified with the 96 genes included in the profiling array.

IPA biological functions

Molecular and cellular functions

- Cell death
- Cell-mediated immune response
- Cellular development
- Cellular function and maintenance
- DNA replication, recombination, and repair

Diseases and disorders

- Inflammatory disease
- Immunological disease
- Cancer
- Neurological disease
- Skeletal and muscular disorders

IPA canonical pathways

- Death receptor signaling
- Apoptosis signaling
- Induction of apoptosis by HIV1
- TNFR1 signaling
- Tumoricidal function of hepatic natural killer cells