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Gene expression analysis of human otosclerotic stapedial footplates

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Abstract

Otosclerosis is a complex disease that results in a common form of conductive hearing loss due to impaired mobility of the stapes. Stapedial motion becomes compromised secondary to invasion of otosclerotic foci into the stapedio-vestibular joint. Although environmental factors and genetic causes have been implicated in this process, the pathogenesis of otosclerosis remains poorly understood. To identify molecular contributors to otosclerosis we completed a microarray study of otosclerotic stapedial footplates. Stapes footplate samples from otosclerosis and control patients were used in the analysis. One-hundred-and-ten genes were found to be differentially expressed in otosclerosis samples. Ontological analysis of differentially expressed genes in otosclerosis provides evidence for the involvement of a number of pathways in the disease process that include interleukin signaling, inflammation and signal transduction, suggesting that aberrant regulation of these pathways leads to abnormal bone remodeling. Functional analyses of genes from this study will enhance our understanding of the pathogenesis of this disease.

Keywords

Otosclerosis; Stapes; Microarray; Gene expression

1. Introduction

Otosclerosis is a common form of conductive hearing loss. It is characterized by sclerosis of the inner ear bony labyrinth due to abnormal bone remodeling in the otic capsule, which normally undergoes very little bone remodeling after development (Frisch et al., 2000). Bone remodeling is a highly regulated process that involves bone resorption by osteoclasts and bone

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formation by osteoblasts (for review of bone remodeling see (Cohen, 2006)). Inappropriate regulation of either of these processes can lead to bony abnormalities. As a result, increased formation of bone at otosclerotic foci in the otic capsule can reflect either over activity of osteoblasts or reduced activity of osteoclasts.

Both clinical and histological forms of otosclerosis are recognized. Clinical otosclerosis is characterized by the encroachment of otosclerotic foci into the stapedio-vestibular joint with subsequent compromise of free motion of the stapes, leading to a conductive hearing loss (Schuknecht et al., 1985). The prevalence of clinical otosclerosis is about 0.2 to 1% in the Caucasian population, with lower prevalences rates reported in black, Asian and Native American populations (Altmann et al., 1967; Gordon, 1989). Age-of-onset varies from 15 to 40 years, with the average age-of-onset falling in the third decade (Menger et al., 2003). Long-term follow up suggests that approximately 10% of clinical otosclerosis patients ultimately develop sensorineural hearing loss (Browning et al., 1984; Ramsay et al., 1994). In contrast to clinical otosclerosis, histological otosclerosis is present in 2.5 to 12% of the Caucasian population. Histological otosclerosis does not have a clinical manifestation and can be diagnosed only by analysis of temporal bone sections (Declau et al., 2007). It is not known whether bone remodeling in clinical and histological otosclerosis is caused by the same trigger.

The cause of otosclerosis remains unknown. Environmental factors like measles virus have been implicated in its pathogenesis (McKenna et al., 1990; Niedermeyer et al., 1994) and genetic factors are supported by the identification of seven discrete genetic loci in families segregating autosomal dominant otosclerosis (Bel Hadj Ali et al., 2008; Brownstein et al., 2006; Chen et al., 2002; Thys et al., 2007a; Tomek et al., 1998; Van Den Bogaert et al., 2004; Van Den Bogaert et al., 2001). However, none of the causative genes at these loci has been identified.

In addition to linkage analyses, association of several genes with otosclerosis has been reported. Association between *COL1A1* and otosclerosis has been reported although with controversial results (Chen et al., 2007; McKenna et al., 1998; Rodriguez et al., 2004). Another more robust study showed that there is an under-representation of an active variant of *TGFBI* in otosclerosis patients, which may inhibit osteoclast differentiation and initiation of aberrant bone remodeling in the otic capsule (Thys et al., 2007b). Association of two bone morphogenetic proteins, BMP2 and BMP4, has also been reported (Schrauwen et al., 2007). In aggregate, these data provide compelling evidence that otosclerosis is a genetically heterogeneous disease, although our understanding of the pathways involved remains speculative; the possibility of environmental triggers remains unanswered.

One approach to identify molecular contributors to otosclerosis is through microarray analyses of gene expression profiles in diseased tissue. In this study, we performed microarray analysis using total RNA from stapes footplates of otosclerosis patients undergoing stapedectomy; control stapes footplates were removed from patients undergoing translabyrinthine surgery for vestibular schwannomas. We report a number of differentially expressed genes in otosclerosis patients that may serve as candidates for further genetic screens and functional analyses to help uncover the genetic components that contribute to this disease.

2. Methods

2.1. Tissue Samples

Stapes tissue samples from nine otosclerosis patients were surgically removed during stapedectomy and immediately placed in liquid nitrogen. This limited number reflects the fact that the preferred surgical treatment of otosclerosis now involves laser-assisted stapedotomy rather than stapedectomy, making the collection of large numbers of pathological specimens

very unlikely. Control stapes were taken from seven patients undergoing translabyrinthine surgery for vestibular schwannomas; harvest conditions were similar and none of these specimens had evidence of otosclerotic foci. All patients gave informed consent prior to collection of samples. The sex, age, ethnicity, or medical history of patients who donated samples for this study was not disclosed.

2.2. RNA Isolation and Preparation

Stapes footplate samples were separated from the stapes suprastructure using a dissecting microscope and then disrupted and homogenized using a tissue-tearer (Biospec Products, Inc.) in 1 ml QIAzol Lysis Reagent. Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) following the manufacturer's protocol. Quality and quantity of total RNA was determined using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

Two rounds of linear RNA amplification were performed on each individual RNA sample using the Applied Biosystems NanoAmpTM RT-IVT Labeling Kit (Applied Biosystems, Inc.). 50 ng of total RNA from each stapes footplate sample were used for the first round of amplification without digoxigenin (DIG)-UTP labeling. After purification and quantification, 120 ng of amplified cRNA was used for a second round of amplification followed by DIG-UTP labeling. Quantity and quality of labeled amplified cRNA was determined prior to array hybridization. RNA amplification and labeling were performed in triplicate for each individual stapes footplate RNA sample.

2.3 Reproducibility of Arrays

To determine the effect of two rounds of RNA amplification between technical replicates, reproducibility of microarray results between replicates was tested. Two arrays from independently amplified and labeled cRNA from the same biological sample were analyzed to determine whether two rounds of amplification of RNA would lead to different results for the same biological sample. This procedure was completed in duplicate using two different biological samples. Analysis of signal intensity between probes of the four arrays showed a correlation coefficient above 97%, indicating high intra-sample reproducibility across arrays after two rounds of RNA amplification.

2.4. Microarray Analysis

Microarray analysis was performed using the Applied Biosystems Human Genome Survey Microarray v2.0 (Applied Biosystems, Inc.). 10 µg of each DIG-UTP labeled cRNA sample from the second round of amplification was hybridized to an Applied Biosystems Human Genome Survey Microarray v2.0 chip at 55°C for 16 hours. All nine otosclerosis samples and all seven control samples were hybridized in triplicate for a total of 48 arrays. Chemiluminescence detection and image acquisition were performed using the Applied Biosystems Chemiluminescence Detection Kit on the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's specifications. Images were auto-gridded and the chemiluminescent signals were quantified, background subtracted, and finally spot- and spatially-normalized using the Applied Biosystems 1700 Chemiluminescence Microarray Analyzer software v1.1.1.

2.5. Statistical Analysis and Gene Ontology Analysis

Array data from each array were quantile normalized and filtered based on the quality flags for the raw data. Detection threshold for probes on the array was set as a signal-to-noise ratio (S/N) > 3 and quality flag <5000. Using this standard, 45% and 47% of the 32,878 probes on the array were detected, representing 47% and 49% of the total 29,098 genes represented on

the array for patient and control samples respectively. After filtering, the statistical significance of the fold change of each transcript between the patient and control data was determined by Student's t-test using log₂ transformed data. For multiple test correction, Storey's q-value false discovery rate (FDR) method was used to control the expected value of the ratio of the number of false-positives and the total number of genes called as significant (Storey et al., 2003). Significantly differentially expressed genes were determined using a false discovery rate (q-value) of 5%, and a fold change greater than 2.0.

To determine the pathways, molecular functions, and biological processes represented in otosclerosis, differentially expressed genes were analyzed using the PANTHER Classification System (Applied Biosystems, <http://www.pantherdb.org/>) (Thomas et al., 2003).

2.6. Sequencing

Significant genes from the microarray analysis located in the *OTSC3* locus were sequenced in the original family used to identify this locus. Sequencing was performed using Big Dye terminator chemistry version 1.1. Sequences of exons and splice site junctions were analyzed using Sequencher 4.5.

2.7. Real-Time PCR

TaqMan® Gene Expression Assays for 7 genes (*CDKN1A*, *LTB*, *ATP1B3*, *PF4*, *IBSP*, *LDLR*, *CCL2*) were used to validate microarray results on first-round amplified RNA from each sample used for microarray analysis. First-round amplified RNA was used due to the limited quantity of original RNA used for the microarray analysis. Real-time PCR was performed on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using standard conditions (95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute). Human beta glucuronidase (*GUSB*) was used as the endogenous control.

3. Results

Comparison of transcript expression in three technical replicates of sample and control stapes footplate RNA samples showed that 110 genes were differentially expressed in patients compared with control samples. Of these 110 genes, 92 were upregulated and 18 were down regulated (Table 1). Of the 92 genes that were upregulated in otosclerosis, 70 were known genes, 1 was a pseudogene, 2 had a predicted protein function assigned by PANTHER, and 19 were genes of unknown function. Of the down-regulated genes, 13 were known genes, 1 was a pseudogene, 1 had a predicted protein function assigned by PANTHER, and 3 were genes of unknown function.

Pathway analysis of the differentially expressed genes using the PANTHER Classification System showed pathways possibly affected in otosclerosis (Figure 1, Table 1). The top pathways include interleukin signaling and inflammation, suggesting an increased inflammatory response in the otosclerosis patients. Other pathways suggested to be involved in otosclerosis in this analysis include p53 signaling, the apoptotic pathway, and two other pathways that have not been implicated in otosclerosis, EGF receptor signaling, and oxidative stress response.

Gene ontology analysis of differentially expressed genes using the PANTHER Classification System showed many genes involved in a number of biological processes and molecular functions (Figure 2). The top biological processes represented were signal transduction, immunity and defense, cell structure and motility, and nucleic acid metabolism. The top molecular functions represented included receptors, transcription factors, and signaling

molecules. Some of the genes are involved in multiple pathways and molecular or biological processes, which are represented in the analysis.

The gene with the largest fold change *PF4* was upregulated 12.16 fold in otosclerosis patients in microarray analysis and upregulated 2.46 fold after analysis by real-time PCR. The gene encoding the bone sialoprotein, *IBSP*, was the most down-regulated gene; it was down-regulated 6.22 fold in microarray analysis and showed a similar fold change after real-time PCR analysis. Five other genes were analyzed by real-time PCR but did not validate the microarray results. These genes include *LDLR*, *CCL2*, and three other genes located in two of the seven previously mapped *OTSC* loci: *CDKN1A* and *LTB* in the *OTSC3* locus, and *ATP1B3* in the *OTSC5* locus. Both *CDKN1A* and *LTB* were sequenced in individuals from the original family mapped to *OTSC3*; however, no mutations were found in the coding region, 5' and 3' untranslated regions, or splice sites.

4. Discussion

Gene ontology analysis of differentially expressed genes in otosclerosis patients provides evidence that a number of pathways may be involved in otosclerosis. The presence of several differentially expressed genes in two pathways, interleukin signaling and inflammation, suggests that an inflammatory response may be a contributing factor in otosclerosis. This association is consistent with data predicting a potential role for measles virus infection and is also consistent with a genetic contribution based on the differential gene expression we observed. For example, gene ontology analysis showed that many of the genes are involved in signal transduction, suggesting that aberrant regulation of different signal transduction pathways may lead to abnormal bone remodeling in otosclerosis.

Genes that have been previously described as differentially expressed in otosclerosis were not detected in this study. In the study by Lehnerdt et al. otospongiotic stage stapes footplates showed increased expression of BMP-2, -4, and -7 (Lehnerdt et al., 2007). In studies by Karosi et al. TNF-alpha has been noted as expressed in otosclerotic stapes; expression was associated with detection of measles virus RNA (Karosi et al., 2005). This group has also shown that osteoprotegerin expression is decreased in otosclerotic stapes footplates that are positive for measles virus and TNF-alpha expression (Karosi et al., 2006). Decreased parathyroid hormone-parathyroid hormone related peptide receptor expression has also been documented in otosclerotic stapes samples (Grayeli et al., 1999). Failure to identify these genes in this current study could be due to the limited amount of samples available for this study or to the complex nature of the disease.

Microarray analysis identified *CDKN1A*, which encodes the cyclin dependent kinase inhibitor p21, and *LTB*, which encodes the TNF superfamily member lymphotoxin beta, located in the *OTSC3* interval and *ATP1B3*, which encodes the beta subunit of the Na/K ATPase, located in the *OTSC5* interval. However, these candidate genes could not be validated via real-time PCR analysis on the same samples used for the initial microarray analysis. This suggests that nonlinear amplification during RNA amplification may have occurred. Unexpectedly, two pseudogenes were identified as differentially expressed between otosclerosis patients and controls in the microarray analysis (Table 1). Real-time PCR was not performed on these two transcripts to determine whether this result is valid and whether these transcripts are specific to the otic capsule.

It is noteworthy that two genes found to be differentially expressed and validated by real-time PCR relate to transforming growth factor beta (TGF- β) signaling. TGF- β signaling plays a role in the regulation of bone remodeling by stimulating osteoblast chemotaxis, proliferation and differentiation. The presence of TGF- β 1 at low levels primes osteoclast precursors for

osteoclast differentiation, and at higher levels inhibits osteoclastogenesis (Karst et al., 2004). A recent case/control association study has shown that there is a variant of *TGFB1* that is under-represented in patients with otosclerosis as compared to control subjects in Belgian-Dutch and French populations (Thys et al., 2007b). Identification of an association of two bone morphogenetic proteins, BMP2 and BMP4, with otosclerosis lends additional support to a role for TGF- β signaling in the disease process (Schrauwen et al., 2007).

Two genes that were differentially expressed in both microarray and real-time PCR analysis have been shown to be affected by TGF- β 1 signaling. Platelet factor 4 (*PF4*), selectively inhibits binding of TGF- β 1 to the type I TGF- β 1 receptor (Whitson et al., 1991). Increased expression of PF4 may be inhibiting bone resorption by down-regulating signaling through the TGF- β pathway, which primes osteoclast precursor cells to undergo osteoclast differentiation. TGF- β has also been shown to regulate expression of the *IBSP* homolog in rat (Ogata et al., 1997). Increased TGF- β signaling results in increased expression of *IBSP*. Down-regulation of *IBSP* in otosclerosis patients could be due to decreased TGF- β 1 signaling. However, *IBSP* can also be negatively regulated by the transcription factor AP-1, which is composed of jun and fos heterodimers (Yamauchi et al., 1996). A member of the fos family of AP-1 transcription factors, *FOSB*, was also upregulated in otosclerosis patients, suggesting increased expression of *FOSB* in otosclerotic lesions may be responsible for the decrease in expression of *IBSP*.

In summary, microarray analysis of stapes footplate samples from otosclerosis patients has identified differentially expressed genes and defined a number of biological pathways that are predicted to play a role in the pathogenesis of otosclerosis. These data provide a rational basis for the in depth study of specific genes and in complement with additional genetic association studies will lead to a better understanding of this disease.

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Abbreviations

OTSC	otosclerosis
PF4	platelet factor 4
IBSP	bone sialoprotein

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Figure 1. Pathways represented by differentially expressed genes. Differentially expressed genes were parsed through the PANTHER Classification System to determine pathways over-represented in otosclerosis patients. Values represent the number of genes present in each pathway.

Figure 2.

A. Molecular functions represented by differentially expressed genes in otosclerosis. B. Biological processes represented by differentially expressed genes in otosclerosis. Values represent the number of genes in each group. Genes assigned multiple molecular functions or biological processes by PANTHER are included.

Table 1

Up- and down-regulated genes in otosclerosis patients. Fold change and q-value are based on microarray analysis.

<i>Upregulated Genes</i>				
Gene	Gene Function	Panther Pathway	Fold Change	q-value
<i>ADAMTS4</i>	disintegrin-like and metalloprotease with thrombospondin type 1 motif, 4		9.249	0.0029
<i>APOLD1</i>	apolipoprotein L domain containing 1		2.832	0.0395
<i>ARG2</i>	arginase, type II		2.930	0.0095
<i>ARL11</i>	ADP-ribosylation factor-like 11		4.653	0.0213
<i>ARMC5</i>	armadillo repeat containing 5		2.203	0.0249
<i>ATP1B3</i> [†]	ATPase, Na ⁺ /K ⁺ transporting, beta 3		2.320	0.0094
	polypeptide			
<i>ATXN7L2</i>	ataxin 7-like 2		2.773	0.0024
<i>BC070289</i>	hypothetical protein		3.245	0.0029
<i>BTNL8</i>	butyrophilin-like 8		6.409	0.0081
<i>C11orf75</i>	chromosome 11 open reading frame 75		2.078	0.0249
<i>C13orf33</i>	chromosome 13 open reading frame 33		2.473	0.0009
<i>C1orf21</i>	chromosome 1 open reading frame 21		2.168	0.0126
<i>CCL2</i> [†]	chemokine (C-C motif) ligand 2	Inflammation	6.157	0.0024
<i>CCLA</i>	chemokine (C-C motif) ligand 4	Inflammation	3.651	0.0029
<i>CDKN1A</i> [†]	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Interleukin signaling, p53 signaling	4.252	0.0117
<i>CHST11</i>	carbohydrate (chondroitin 4) sulfotransferase 11		2.227	0.0117
<i>CLEC1B</i>	C-type lectin domain family 1, member B		3.243	0.0129
<i>CLEC4E</i>	C-type lectin domain family 4, member E		3.490	0.0182
<i>CRISPLD2</i>	cysteine-rich secretory protein LCCL domain containing 2		2.348	0.0351
<i>DKFZp451A211</i>	Homo sapiens DKFZp451A211 protein		7.286	0.0095
<i>EDN2</i>	endothelin 2	Endothelin signaling	3.267	0.0423
<i>FAM129C</i>	family with sequence similarity 129, member C		2.036	0.0259
<i>FLJ16165</i>	hypothetical protein		2.001	0.0117
<i>FOSB</i>	FBJ murine osteosarcoma viral oncogene homolog B		7.941	0.0178
<i>FPR1</i>	formyl peptide receptor 1	Inflammation	2.572	0.0095
<i>GIYD2, GIYD1, SULT1A3</i>	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3		2.628	0.0156
<i>GPR158</i>	G protein-coupled receptor 158		3.962	0.0095
<i>HBA2, HBA1</i>	hemoglobin, alpha 2; hemoglobin, alpha 1		2.460	0.0029
<i>HBD</i>	hemoglobin, delta		4.088	0.0057
<i>HBEGF</i>	heparin-binding EGF-like growth factor	EGF receptor signaling	3.200	0.0204
<i>HBG1, HBG2</i>	hemoglobin, gamma		3.507	0.0092
<i>HYAL1</i>	hyaluronoglucosaminidase 1		3.095	0.0095
<i>IGSF9B</i>	immunoglobulin superfamily, member 9B		2.468	0.0259
<i>IL1R2</i>	interleukin 1 receptor, type II		3.942	0.0019
<i>IL6</i>	interleukin 6 (interferon, beta2)	Interleukin signaling	6.088	0.0474
<i>IL8RA</i>	interleukin 8 receptor, alpha	Inflammation, Interleukin signaling	2.457	0.0009
<i>IL8RB</i>	interleukin 8 receptor, beta	Inflammation, Interleukin signaling	7.904	0.0009
<i>ITGAX</i>	integrin, alpha X	Integrin signaling	2.010	0.0098
<i>ITPKC</i>	inositol 1,4,5-trisphosphate 3-kinase C		3.381	0.0201
<i>KCNJ15</i>	potassium inwardly-rectifying channel, subfamily J, member 15		3.991	0.0374
<i>KIAA1539</i>	hypothetical protein		2.365	0.0117
<i>KIAA1622</i>	HEAT-like repeat-containing protein		2.948	0.0432
<i>LDLR</i> [†]	low density lipoprotein receptor	Alzheimer disease-presenilin pathway	4.031	0.0305
<i>LILRA1, LILRB1</i>	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 1		3.734	0.0223
<i>LILRB3, LILRB2</i>	leukocyte immunoglobulin-like receptor, subfamily B		3.651	0.0095
<i>LOC387763</i>	hypothetical protein		5.873	0.0043
<i>LTB</i> [†]	lymphotoxin beta (TNF superfamily, member 3)	Apoptosis	2.785	0.0162
<i>MT1A</i>	metallothionein 1A		2.610	0.0095
<i>MT1B</i>	metallothionein 1B		2.290	0.0104
<i>MT1E</i>	metallothionein 1E		2.386	0.0098
<i>MT1J</i>	metallothionein 1J		3.801	0.0060
<i>MT1L</i>	metallothionein 1L		2.289	0.0121
<i>MT1M</i>	metallothionein 1M		2.743	0.0273
<i>MT2A</i>	metallothionein 2A		2.642	0.0024
<i>MTHFD2</i>	methylene tetrahydrofolate dehydrogenase (NAD ⁺ dependent), methenyltetrahydrofolate cyclohydrolase		2.747	0.0474
<i>MYC</i>	v-myc myelocytomatosis viral oncogene homolog	Interleukin signaling, Oxidative stress response, p53 signaling, WNT signaling, PDGF signaling	3.128	0.0110
<i>MYEOV</i>	myeloma overexpressed gene		4.286	0.0076

Upregulated Genes

Gene	Gene Function	Panther Pathway	Fold Change	q-value
<i>NFIL3</i>	nuclear factor, interleukin 3 regulated		2.331	0.0095
<i>NPBWR2</i>	neuropeptides B/W receptor 2		2.338	0.0320
<i>NRN1</i>	neuritin 1		2.121	0.0206
<i>OVOL1</i>	ovo-like 1 (<i>Drosophila</i>)		2.185	0.0314
<i>OR7E47P</i>	olfactory receptor, family 7, subfamily E, member 47 pseudogene		9.883	0.0420
<i>PF4^{†,§}</i>	platelet factor 4 (chemokine (C-X-C motif) ligand 4)	Inflammation	12.163	0.0117
<i>PLEC1</i>	plectin 1, intermediate filament binding protein		3.390	0.0009
<i>PPBP</i>	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)		3.972	0.0001
<i>PRPH2</i>	peripherin 2		2.271	0.0249
<i>PSMD9</i>	proteasome (prosome, macropain) 26S subunit, non-ATPase, 9	Ubiquitin proteasome pathway	2.055	0.0098
<i>RGC32</i>	response gene to complement 32		2.966	0.0161
<i>S100A8</i>	S100 calcium binding protein A8 (calgranulin A)		2.938	0.0425
<i>SERTAD1</i>	SERTA domain containing 1		2.175	0.0242
<i>SLA</i>	Src-like-adaptor		2.045	0.0347
<i>SLC39A14</i>	solute carrier family 39 (zinc transporter), member 14		5.292	0.0264
<i>STATH</i>	statherin		6.455	0.0095
<i>TBC1D10B</i>	TBC1 domain family, member 10B		2.082	0.0156
<i>TIPARP</i>	TCDD-inducible poly(ADP-ribose) polymerase		2.341	0.0089
<i>TNFAIP8L3</i>	tumor necrosis factor, alpha-induced protein 8-like 3		2.520	0.0003
<i>TNFRSF10C</i>	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	Apoptosis, p53 signaling	2.314	0.0020
<i>TREML1</i>	triggering receptor expressed on myeloid cells-like 1		2.515	0.0460
<i>TSHR</i>	thyroid stimulating hormone receptor		2.767	0.0223
<i>TUBB6</i>	tubulin, beta 6	Huntington disease, Cytoskeletal regulation by Rho GTPase, Hedgehog signaling pathway	2.143	0.0273
<i>ZKSCAN1</i>	zinc finger with KRAB and SCAN domains 1		3.601	0.0497
unassigned	chr6: 4083845-4105692		2.099	0.0447
unassigned	chr22:23689553-23690241		2.216	0.0447
unassigned	chr19:33316188-33318797		2.227	0.0264
unassigned	chr3:9362200-9366861		2.239	0.0035
unassigned	chr6: 170835774-170849263		2.351	0.0474
unassigned	chr13:23061887-23063001		2.381	0.0490
unassigned	chr4:185449989-185450691		2.487	0.0060
unassigned	chr4:72073747-72074717		2.945	0.0104
unassigned	chr1:210416660-210417060		3.012	0.0026
unassigned	chr5:74422818-74423714		3.248	0.0117
unassigned	chr16:41179572-41181020		5.996	0.0038

Down-regulated Genes

Gene	Gene Function	Panther Pathway	Fold Change	q-value
<i>CASR</i>	calcium-sensing receptor		2.556	0.0447
<i>CHST9</i>	carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9		2.550	0.0223
<i>DUSP16</i>	dual specificity phosphatase 16	Oxidative stress response	3.733	0.0440
<i>FLJ4394</i>	hypothetical protein		8.370	0.0387
<i>FOXE1</i>	forkhead box E1 (thyroid transcription factor 2)	Interleukin signaling, Insulin/IGF pathway, PI3 kinase pathway, TGF- β signaling	2.062	0.0009
<i>IBSP^{†,§}</i>	integrin-binding sialoprotein (bone sialoprotein)		6.221	0.0319
<i>IFIT1</i>	interferon-induced protein with tetratricopeptide repeats 1		2.112	0.0095
<i>KBTD10</i>	kelch repeat and BTB (POZ) domain containing 10		2.666	0.0262
<i>KRT19</i>	keratin 19		2.485	0.0110
<i>LOC124220</i>	similar to common salivary protein		3.761	0.0009
<i>LOC160313</i>	keratin 19 pseudogene		2.655	0.0117
<i>NDP</i>	Norrie disease (pseudoglioma)		3.031	0.0294
<i>OTX1</i>	orthodenticle homolog 1 (<i>Drosophila</i>)		3.560	0.0232
<i>SOX2</i>	SRY (sex determining region Y)-box 2		2.433	0.0009
<i>SPRY1</i>	sprouty homolog 1, antagonist of FGF signaling (<i>Drosophila</i>)	EGF receptor signaling, FGF signaling	2.781	0.0365

Upregulated Genes

Gene	Gene Function	Panther Pathway	Fold Change	q-value
<i>TAF2</i>	TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor	Transcription regulation by bZIP transcription factor, General transcription regulation	2.039	0.0327
unassigned	chr11:69182960-69190468		3.955	0.0209
unassigned	chr16:18863672-18878591		2.016	0.0162

[†] Tested via real-time PCR

[§] Validated via real-time PCR