Immune Dysregulation and Tumor-Associated Gene Changes in Recurrent Respiratory Papillomatosis: A Paired Microarray Analysis

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Recurrent respiratory papillomas (RRP) are benign airway tumors, caused primarily by human papillomaviruses (HPV) types 6 and 11. The disease is characterized by multiple recurrences after surgical removal, with limited effective therapy. To identify novel targets for future therapy, we established transcriptional profiles for actively growing papillomas compared with autologous, clinically normal, laryngeal epithelia (adjacent tissue). Total ribonucleic acid (RNA) from 12 papillomas and 12 adjacent tissues were analyzed by microarray, and the matched sets of tissues compared by paired *t* test, to identify differentially expressed genes in papilloma tissues while minimizing variations intrinsic to individual patients. Quantitative polymerase chain reaction (PCR) was used to confirm the relative expression levels for a subset of genes. Within the 109 differentially expressed transcripts whose expression varied at least three-fold were two large groups of genes with related functions. The first group consisted of 18 genes related to host defense, including both innate and adaptive immunity. The second group contained 37 genes that likely contribute to growth of papillomas as benign tumors, since the altered pattern of expression also had been reported previously in many cancers. Our results support our previous studies that document a systemic T_H2-like adaptive immune response in RRP, and suggest that there is a role for altered innate immunity in RRP as well. We propose that HPV 6 and 11 infection establishes a tumorigenic microenvironment characterized by alteration of both innate inflammatory signals and adaptive immune responses that prevent effective T_H1-like response, in conjunction with altered expression of numerous genes that regulate cellular growth and differentiation.

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INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is caused primarily by human papillomavirus (HPV) types 6 and 11, with all other HPV types causing less than 2% of disease (1,2). These viruses induce the growth of benign tumors in the larynx, and less frequently, in the lower respiratory tract. Standard treatment is repeated surgery to remove papillomas that, because of their location in the airway, cause significant morbidity, and on occasion mortality (3,4). The interval between surgical intervention varies between patients, ranging from 3 wks to several years (3). Differences in host immune responses to HPV infection may explain this variability.

An effective immune response to viral infection involves activation of both innate and adaptive immunity, with a balance between T_H1-, T_H2-like, and T_H17-derived chemokines and cytokines, and appropriate signaling through the receptors they bind (5). We previously reported differences in HPV-specific immune responses by RRP patients and controls that predict disease susceptibility and severity (6–9). Peripheral blood mononuclear cells (PBMC) from these patients respond to HPV 6/11 E6 protein by expressing T_H2-like cytokines and interleukin (IL)-10 (8,9). Recently, we also identified increased levels of the T_H2-like chemokine CC chemokine ligand (CCL)18 (10) in patient serum. Select class I major histocompatibility complex (MHC) and class II MHC genes are enriched in RRP (6,7,11,12), and are associated with disease predisposition, and/or disease severity (7,12), and correlate with PBMC expression of IFN-γ when challenged with E6 protein (6). Thus, we pro-

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posed that the inability of RRP patients to eliminate HPV-infection is likely due to an HPV-specific, T_H2-like/IL-10-biased microenvironment within papillomas that suppresses effective T_H1-like responses, and thereby favors recurrent disease.

The immune system also plays a complex role in regulating the growth and metastasis of malignancies (13,14); however, its role in the development of benign lesions is less well understood. To better understand the expression of specific immune response genes within papilloma tissues, and to identify host genes that are important in the pathophysiology of RRP, we compared the gene expression profiles of paired laryngeal papilloma tissues and autologous adjacent epithelia. We found differences in expression of both innate and adaptive immune response genes, and in many genes associated with a variety of malignancies.

MATERIALS AND METHODS

Patients

Biopsies of papilloma and adjacent epithelia were obtained from patients with RRP undergoing surgery at Long Island Jewish Medical Center following informed consent as approved by the North-Shore Long Island Jewish Health System Institutional Review Board. None of the patients included in our study had high-grade dysplasia in their papillomas. Surgical pathologic studies of these papillomas were performed by the Pathology Department at the Long Island Jewish Medical Center.

RNA Isolation and cRNA Synthesis

Total RNA was extracted immediately (RNeasy spin columns, Qiagen, Valencia, CA, USA), and stored at –70° C. Matched sets yielding 2 μg total RNA from both tissues (n = 12 pairs) were studied. Double stranded cDNA was synthesized from 2 μg of total RNA (Superscript Double-Stranded cDNA Synthesis Kit, Invitrogen, Carlsbad, CA, USA). Total cDNA was used to generate biotinylated cRNA (BioArray High Yield RNA Transcript Labeling Kit, Enzo Life Sciences Inc., Farmingdale, NY, USA).

Microarray and Data Analysis

Twenty μg of fragmented cRNA was hybridized to Human U133A (n = 2) or U133A2.0 (n = 10) microarray chips (Affymetrix, Santa Clara, CA, USA). Samples were processed on a Gene Chip 450 fluidics station (Affymetrix), scanned (Gene Chip 3000 scanner), and analyzed (Affymetrix MAS 5.0 software) according to the manufacturer. Raw data from the arrays is available from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/projects/geo) as series GSE10935. Data mining was performed using Genesifter software (VizX Labs, Seattle, WA, USA). Log transformed data sets were normalized using gas chromatography robust multi-chip average (GC-RMA). Both "pair wise" analysis between autologous specimens, and group analysis (adjacent tissue versus papilloma) using the false discovery rate algorithm, the Benjamini and Hochberg correction, a fold change of three, and a $P < 0.05$ were employed. A subsequent filtering step excluded candidate genes that either failed to show a change for half of the matched data sets, or if both normal and papilloma groups were classified as "absent call." Two subsets of biologically relevant genes were further analyzed, namely, immune response genes, and genes associated with malignant transformation. Hierarchical clustering was performed only on the ten paired data sets obtained using the U133A2.0 arrays on all 22,000 probe sets with GeneSpring GX 7.3 software (Silicon Genetics). Briefly, .cel files were transformed using RMA, normalized by setting values below 0.01 to 0.01, normalized to 50th percentile per chip and to median by gene. Genes with significant differences ($P < 0.04$) were used to create a condition tree and a relevant gene tree.

Quantitative PCR

To validate microarray results, 16 representative genes from both the immune and tumor-associated groups were examined by quantitative reverse transcriptase PCR (Q-RT-PCR) with gene specific primers (Table 1) and a probe from the Universal Probe Library Set (Roche, Mannheim, Germany). *IL-1F9* was measured using a Taqman probe. Samples were amplified with an Applied Biosystems 7900 HT thermocycler and results analyzed using the delta-delta C_t method.

Association between Gene Expression Fold Change and Disease Severity

Disease severity criteria (3,8,9) were used to classify the RRP patients into two separate disease categories: severe, or mild/moderate. These disease severity groups were treated as ordinal variables when calculating disease associations. A quantitative measure of disease severity can be calculated by determining the extent, location, number, and size of the lesions, and dividing that value by the time between surgical interventions measured in d. Individual severity scores range from 0.001 to greater than 0.8. We have empirically established a cut-off at 0.06, with scores exceeding that value being classified as severe, and those below that value as mild/moderate. Our study contained equal numbers of severe and mild/moderate patients. The differences between groups for select genes (upregulated immune and angiogenic genes with fold change ≥ 3.7) was compared using SAS software V9.1.3 (SAS Institute, Cary, NC, USA) and a two-tailed, unpaired t test.

Ingenuity Analysis

Data were analyzed by the Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). A dataset containing gene identifiers was mapped to its corresponding gene object in the Ingenuity knowledge base using a fold change of > 3.0. These genes (Supplementary Table 1) were overlaid onto a global molecular network developed by Ingenuity. Networks were then algorithmically generated based on their connectivity. Pathways were constructed using both

Table 1. Confirmation of Gene Expression by Q-PCR

Gene Descriptions	Gene Name	ARRAY	Q-PCR	Oligonucleotide Primer Sequences[a]	Probe Sequence
C-C Ligand 5	*CCL5*	–3.7	–4.7	TTGTCAAAAGGAAGTCTCTAGGTTC CTTGTCACAGAGCCCTTGC	AGCCAGAG
C-C Ligand 14	*CCL14*	–6.4	–99.7[b]	GCTTCCCACAGCATGAAGA CCCTAGGGCGATGGTGAT	CTTCCTCC
C-C Ligand 19	*CCL19*	–3.8	–28.9	AGTGGCACCAATGATGCTG GTACCCAGGGATGGGTTTCT	CTGCTGCC
C-C Ligand 20	*CCL20*	5.1	11.1	GTGGCTTTTCTGGAATGGAA CAACCCCAGCAAGGTTCTT	AGCCCAAG
C-C Ligand 21	*CCL21*	–4.3	–4.4	AGAAAGGAAAGGGCTCCAAA AGGCTTCAAGCGTTGGTG	CCTGGAGC
C-X-C Ligand 1	*CXCL1*	3.7	4.7	AAGCAAATGGCCAATGAGAT ATCTAAACAGTTACAAAACAGATGTGC	GAAGGCAG
C-X-C Ligand 6	*CXCL6*	3.3	10.9	TGACACTTGTGAAAAGGCTTGTA AGCAAAAATAGAAATTCACAACCA	CTCCTCCC
Interleukin-1 family member 9	*IL1F9*	6.9	13.1	TTCAGAGCTCATGCGCGTTA GGAATAAAGCAAAACAGAAACAGAGA	CCACGATGGCATGACTAGCACAGAGC
Plasminogen Activator, tissue	*PLAT*	3.6	7.8	TCCTCAAAAGCACCCTTGAC CCTTCTGAGAGCCAGGGAGT	CTCCTTCC
Parathyroid Hormone-like-Hormone	*PTHLH*	9.3	15.2	TCCAAGGACATATTGCAGGA CAATGTGCAGTTTCATAGAGCAA	GGAGACAG
Inhibitor of DNA binding 1	*ID1*	5.4	2.7	CCAGAACCGCAAGGTGAG GGTCCCTGATGTAGTCGATGA	AGGTGGAG
Inhibitor of DNA binding 2	*ID2*	3.1	2.1	AGGTCTTTTCAGAGCGTGGA GCCTTGGCATAGTTTGGAGA	GGAAGGAG
Vascular Endothelial Growth Factor A	*VEGFA*	4.8	5.3	TTTTGCTAACACTCAGCTCTGC CCCTCTTTCAAAGGAATGTGTG	CTGGCTCC
S100 calcium binding protein A7	*S100A7*	8.6	11.8	CACCAGACGTGATGACAAGATT GTTGGGGAAGTTCTCCTTCA	GCCTGCTG
S100 calcium binding protein A12	*S100A12*	3.3	11.2	TCATATCCCTGGTAGCCATTG ACCTACTCTTTGTGGGTGTGGT	GCTGCCCA
Tenascin XB	*TNXB*	–5.8	–22.3	GGCAGGTGACTACTCCATCC GTCGTACTGGGCGAACACA	GGGCTGGG

[a]Top sequence is forward primer, bottom is reverse. All primers are displayed 5′ → 3′.
[b]Fold change may be greater than calculated (40 cycle cut-off).

direct and indirect relationships. Gene products are represented by shapes, with the biological relationship between two genes represented as a line which is supported by at least one reference from the literature.

All supplementary materials are available online at molmed.org.

RESULTS

Differential Gene Expression Arrays

The use of "paired, autologous tissue sets" helped identify genes that might be obscured in an analysis using non-autologous control tissue, because of intrinsic patient-to-patient variability. The initial pair-wise comparison of papillomas and adjacent tissue identified 364 candidate genes. A filtering step eliminated genes with marginal or absent expression, resulting in 134 unique identifiers comprised of 109 individual candidate genes. Seventy-three genes were upregulated, and 36 others downregulated in papillomas (Supplementary Table 1).

Hierarchical clustering revealed a clear discrimination between papillomas and adjacent tissues (Figure 1). There was no correlation between the overall transcriptional profile of papillomas from patients with severe disease, in comparison to those from patients with mild/moderate disease, with respect to age, gender, or disease severity. Differentially expressed genes could be divided into three groups: 1) immune response genes; 2) genes that likely play a role in papilloma formation since they also are associated with malignant tumors; and 3) genes whose role in RRP is not yet apparent. There were significant differences between papilloma and adjacent tissue in expression of multiple immune response genes as seen in Figure 2 and the associated Table 2. The expression pattern of

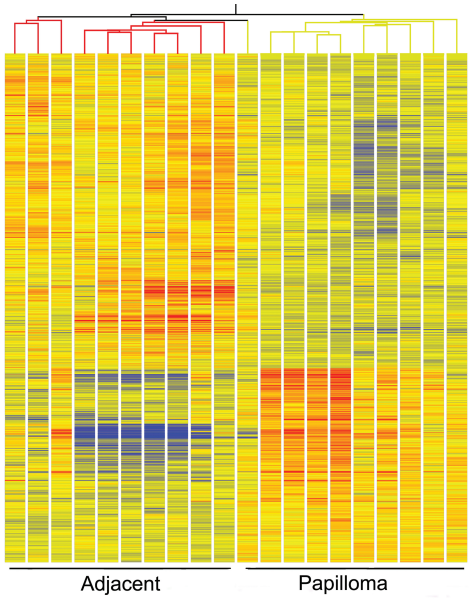

Figure 1. Unsupervised hierarchical clustering. The comparison of overall gene expression patterns of 20 tissue samples, 10 papilloma, and 10 adjacent clinically normal tissues from patients with RRP. The dendrogram was obtained by unsupervised hierarchical cluster analysis using Gene Spring software. The analysis included all genes contained on the Human Affymetrix GeneChip U133A 2.0. A primary branching pattern reveals two distinct expression patterns showing segregation of all papilloma samples (right) from adjacent tissue (left). The color codes are shown in the color bar where blue represents transcripts below the median, and red represents transcripts above the median.

specific genes suggested a local tissue bias away from a robust T_H1 response. The T_H1-like chemokines *CCL19* and *CCL21*, and the chemo attractants *CCL5*, *CCL14*, and *CXCL12*, which would promote T_H1-like immune infiltrates, all were decreased in papillomas. In contrast, the T_H2-like chemokine *CCL20*, and the interleukin *IL-23*, which maintains T_H17 cells, both were increased.

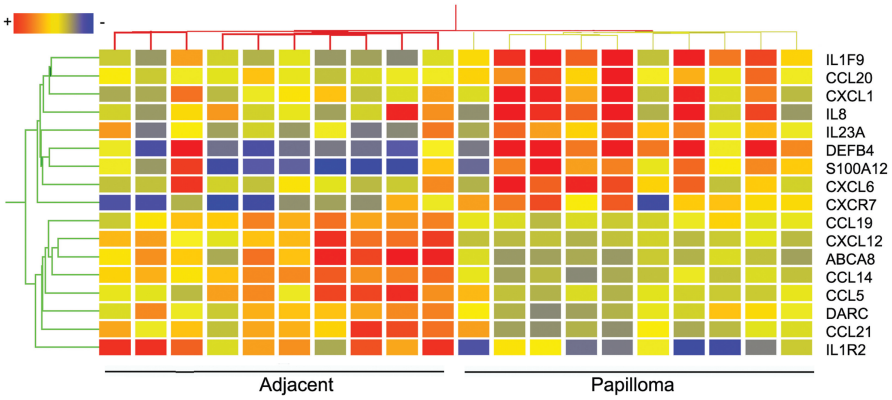

Figure 2. Genes associated with immune response. Immune response genes identified in Table 2 show a bias of chemokine and interleukin gene expression in all samples (n = 10). The expression of 9 genes (top), were increased in papillomas, and 8 genes (bottom) were decreased in papillomas relative to the corresponding autologous adjacent tissue. Highly expressed genes are shown as red boxes, low expressed genes are shown as blue boxes, and intermediately expressed genes are shown as yellow boxes.

Papillomas also showed altered expression of many innate immune genes that could affect the balance between alternative adaptive immune responses. Of these, *hBD4*, *S100A2*, *S100A7*, and *S100A12* all were elevated, while *ABCA8*, required for release of IL-1β, was decreased. Intriguingly, *IL-1F9* was elevated markedly in papillomas. This interleukin is an agonist for *IL-1Rrp2*, a newly described member of the IL-1 receptor family that likely induces an alternative to classical IL-1β signaling. This suggests that the innate immune response is also altered in RRP.

Several other differentially expressed immune response genes appear inconsistent with suppression of a T_H1-like response. While *CXCR7*, the receptor for *CXCL12* was elevated, *CXCL12* itself was downregulated. *IL-1R2*, a decoy receptor that suppresses IL-1β signaling was decreased, but the simultaneous reduction of *ABCA8* would limit IL-1β signaling. The pro-inflammatory *CXCL1*, *CXCL6*, and *CXCL8* (*IL-8*) all were elevated in papillomas, however these chemokines also have angiogenic functions, that apparently supersede their immunoregulatory function in the pathogenesis of HPV-induced respiratory papillomas. In addition, a number of the innate immune response genes altered in papillomas also are associated with malignancy. These include *HSPA8*, *DARC*, *S100A2*, and *S100A7*. In every case, the direction of gene expression in papillomas was the same as that reported in malignant tumors.

A large number of non-immune response genes whose expression was altered in the papillomas also have been associated with malignant tumors. These tumor-associated genes included angiogenesis and growth factors, matrix-associated proteins, cell cycle regulators, and tumor suppressors, all of which affect cell growth, differentiation, or survival (Table 3 and Figure 3). Changes in expression of other genes, for example, keratins, most likely reflect abnormali-

Table 2. Immune Response Genes

Gene Name	Gene	NCBI Accession	Fold Change
Decreased Expression			
Chemokine (C-C motif) ligand 5	*CCL5*	NM_002985	–3.6
Chemokine (C-C motif) ligand 14	*CCL14*	NM_004166	–6.4
Chemokine (C-C motif) ligand 19	*CCL19*	U88321	–3.8
Chemokine (C-C motif) ligand 21	*CCL21*	NM_002989	–4.3
Chemokine (C-X-C motif) ligand 12	*CXCL12*	U19495	–6.1
ATP-binding cassette, sub-family A, member 8	*ABCA8*	NM_007168	–14.1
Duffy blood group, chemokine receptor	*DARC*	NM_002036	–3.1
Interleukin 1 receptor, type 2	*IL1R2*	NM_004633	–5.1
Increased Expression			
Chemokine (C-C motif) ligand 20	*CCL20*	NM_004591	5.1
Chemokine (C-X-C motif) ligand 1	*CXCL1*	NM_001511	3.7
Chemokine (C-X-C motif) ligand 6	*CXCL6*	NM_002993	3.3
Chemokine (C-X-C motif) ligand 8	*CXCL8*	NM_000584	4.8
Chemokine (C-X-C motif) receptor 7	*CXCR7*	AI817041	3.7
Defensin β 4	*DEFβ4*	NM_004942	16.1
Heat shock protein A8	*HSPA8*	AB034951	5.6
Interleukin 1 F9	*IL1F9*	NM_019618	6.9
Interleukin 23 A	*IL23A*	NM_016584	3.5
S100 calcium binding protein A2	*S100A2*	NM_005978	3.4
S100 calcium binding protein A7	*S100A7*	NM_002963	8.6
S100 calcium binding protein A12	*S100A12*	NM_005621	3.3

ties in keratinocyte growth and differentiation, but are not important in pathogenesis. In all but three genes in Table 3, the direction of change in the papillomas was the same as reported by others for malignancies. Thus, a subset of these genes is likely required for growth of both benign and malignant tumors.

The direction and magnitude of the gene fold changes identified by microarray were confirmed by Q-PCR for a subgroup of immune response and tumor-associated genes (Table 1). The direction of change in papillomas was the same as identified by microarray, however, the magnitude of differential expression was greater in general.

Association of Gene Expression and Disease Severity

Since the expression of many immune response and angiogenesis-related genes were altered in papillomas, we asked whether the expression of these genes varied between patients with severe disease as compared with those with mild/moderate disease. Four genes, *IL-1F9* which may determine the type of innate inflammatory response initiated by the host, chemokines *CXCL1* and *CXCL8*, and the growth factor *VEGFA* all showed more significant elevations in patients with severe disease (Table 4). Taken together, elevation in expression of these particular genes in patients with severe RRP suggests that angiogenesis and the regulation of innate inflammatory responses are key factors in RRP pathogenesis. In contrast, the only transcripts which were differentially regulated to a greater degree in patients with mild/moderate disease severity were those for hemoglobin alpha, and hemoglobin beta, which were decreased in the papillomas from patients with less severe disease. The disparity in hemoglobin transcripts might reflect larger numbers of erythrocytes resulting from increased vascularity in papillomas from patients with severe disease.

Pathway Analysis

We utilized the Ingenuity Pathways Analysis to establish relationships between the full set of differentially expressed genes (Supplementary Table 1). The top scoring network (z = 51) was cellular movement/immunological disease/cellular growth and proliferation with a P value of 10^{-15} (Figure 4A). This network included 25 of the genes we identified with a central role for VEGFA linked to several growth factors, and NF-κB linked to many cytokines. Moreover, there were associations between VEGFA and NF-κB. The pathway with the second highest significance (z-score) was cancer/cellular movement/reproductive system disease (Figure 4B), which revealed prominent involvement of HIF1α, IL-8, and CXCL12, with central importance of p38 MAPK, PI3K, AP1, and Akt consistent with our previous reports of PI3K and p38 pathway activation in papillomas (15,16). The identification of these pathways underscores the relationship between gene expression in papillomas induced by HPVs with low oncogenic potential, and polarization of cellular pathways reminiscent of malignancy.

DISCUSSION

We have found altered expression of many immune response genes in papillomas compared with autologous laryngeal epithelium. These differences could contribute to the persistence of infection and recurrence of disease by biasing the papilloma micro-environment away from effective T_H1-like responses. Previously, we reported that PBMCs from RRP patients respond to HPV proteins with increased expression of T_H2-like and regulatory cytokines without adequate expression of *IFN-γ* (8). We now have evidence that there is a T_H2-like chemokine bias in papillomas (increased expression of *CCL20*), and concomitant downregulation of T_H1-like chemokines (*CCL19* and *CCL21*). CCL19 and CCL21 are ligands for CCR7, are required for recruitment of naïve $CCR7^+$ T cells that become T_H1-like memory cells, (17) and direct activated $CCR7^+$ antigen-presenting cells into inflamed tissues (18). Mice with a genomic deletion of both *CCL19* and *CCL21* have increased numbers of T_H2-inducer-type myeloid dendritic cells ($CD8\alpha^-$ $CD11b^+$) (19), and defective $CD8^+$ T-cell responses

Table 3. Non-Immune Genes Associated with Malignancy

Gene Name	Gene	NCBI Accession	Fold Change in RRP	Direction of Change in Malignancy[a]
Increased Expression				
Angiogenesis				
Fibroblast growth factor bp 1	*FGFBP1*	NM_005130	5.3	↑
Phosphoglycerate kinase 1	*PGK1*	S81916	4.5	↑
Placental growth factor	*PGF*	BC001422	3.1	↑/↓
Vascular endothelial growth factor	*VEGFA*	AF022375	4.8	↑
Hypoxia-induced				
Carbonic anhydrase II	*CAII*	M36532	8.3	↑
Carbonic anhydrase XII	*CAXII*	BC000278	4.0	↑
Hypoxia-inducible factor 1_	*HIF1A*	NM_001530	3.1	↑
Hypoxia-inducible protein 2	*HIG2*	NM_013332	5.5	↑/↓
Growth, Differentiation and Apoptosis				
Cyclin-dependent kinase inhibitor 1A	*CDKN1A*	NM_000389	3.7	↓
Inhibitor of DNA binding 1	*ID1*	D13889	5.4	↑
Inhibitor of DNA binding 2	*ID2*	NM_002166	3.1	↑
Insulin-like growth factor bp 3	*IGFBP3*	BF340228	3.4	↑/↓
Parathyroid hormone-like hormone	*PTHLH*	BC005961	9.3	↑
TP53 apoptosis effector	*PERP*	NM_022121	5.8	↑
Membrane, Adhesion and Extracellular Matrix-Associated				
Calcium chloride channel activated 2	*CLCA2*	NM_006536	4.4	↑/↓
Fascin 1	*FSCN1*	NM_003088	4.2	↑
Kallikrein-related peptidase 12	*KLK12*	NM_019598	4.5	↑
Lectin, galactoside-binding, soluble, 7	*LGALS7*	NM_002307	7.7	↑
Plakophilin 1	*PKP1*	AI378979	5.4	↓
Enzymes and Enzyme Inhibitors				
Aldo-keto reductase family 1 B10	*AKR1B10*	NM_020299	3.6	↑
Cathepsin L2	*CTSL2*	AF070448	3.7	↑
Serine Protease Inhibitor B3	*SERPINB3*	AB046400	4.8	↑
Serine Protease Inhibitor B4	*SERPINB4*	U19557	3.3	↑
Serine Protease Inhibitor B13	*SERPINB13*	AF169949	4.1	↑
Decreased Expression				
Extracellular Matrix Associated				
Dermatopontin	*DPT*	AI146848	–6.8	↑
Mucin 5AC	*MUC5AC*	AW192795	–11.7	↓
Tenascin XB	*TNXB*	M25813	–5.8	↓
Tumor Suppressors				
Apolipoprotein D	*APOD*	NM_001647	–4.1	↓
Four and a half LIM domains 1	*FHL1*	NM_001449	–3.7	↓
Insulin-like growth factor bp 5	*IGFBP5*	L27560	–4.0	↓
SPARC-like 1 (mast9, hevin)	*SPARCL1*	NM_004684	–3.7	↓
Other				
Apolipoprotein J	*APOJ*	AI982754	–7.0	↓
Glutathione S-transferase A2	*GSTA2*	NM_000846	–5.6	↓

[a]The direction of change in malignancy is based on an extensive literature review of the specific gene alternations in multiple tumors including breast, colon, lung, cervical, and brain tumors.

to influenza virus (17). Additionally, CCL19 and CCL21 have been used as adjuvants to enhance CTL responses to tumors (20). Consistent with this, papillomas do not contain significant numbers of CTLs (9). In addition, both *CCL5* and *CCL14* were downregulated in papillomas. CCL14 is a chemo-attractant for both T cells and monocytes (21), while CCL5 attracts monocytes, memory T-helper cells, and eosinophils (22). Taken together, changes in these chemokines would result in a relative absence of effector T cells, especially CTL and T_H1-like T cells, in papillomas.

T_H17-like T cells are a new addition to the classical T_H1/T_H2 paradigm (23). T_H17-like T cells selectively inhibit T_H1-like cells by their expression of *IL-17* and *IL-23* (24). Conversely, T_H1-like T cells inhibit T_H17-like T cells by their expression of *IFN-γ* and *IL-12*, neither of which were expressed in papillomas. *CXCL1*, *CXCL6*, *hBD4*, and *CCL20* were all upregulated in papillomas and, interestingly, all are expressed by human bronchial epithelial cells when treated with IL-17A (25). *IL-23* was elevated in papillomas and is required for maintenance of T_H17-like T cells *in vivo* (26). Thus, polarization of T_H0-like T cells toward the T_H17-like lineage may occur in RRP. However, expression of *IL-6* and *TGFβ1* by most papillomas in the absence of *IL-21* and *IL-22* suggests that T-regulatory cells (T-regs), but not T_H17-cells, are induced preferentially in papillomas (27). We have detected increased numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ CD127low T-regs in papilloma tissue as compared with autologous blood, (28) supporting the possibility that functional T-regs in RRP may be responsible for the absence of inflammation caused by T_H1-, and T_H17-like cells in this RRP.

Expression of multiple innate immune response genes also were altered in papillomas. Of particular interest was the elevated expression of *IL-1F9* mRNA levels that were significantly higher in patients with severe disease ($P = 0.03$), suggesting a central role for IL-1F9 in predisposing to severe disease. IL-1F9 binds to IL-1Rrp2 (29), and is thought to alter innate immune response signaling. Subsequent polarization of the adaptive immune response remains unknown (30), however, several lines of evidence (31, 32) suggest that IL-1F9 likely induces an alternative to IL-1β signaling. IL-1F9 has been implicated recently in allergen-

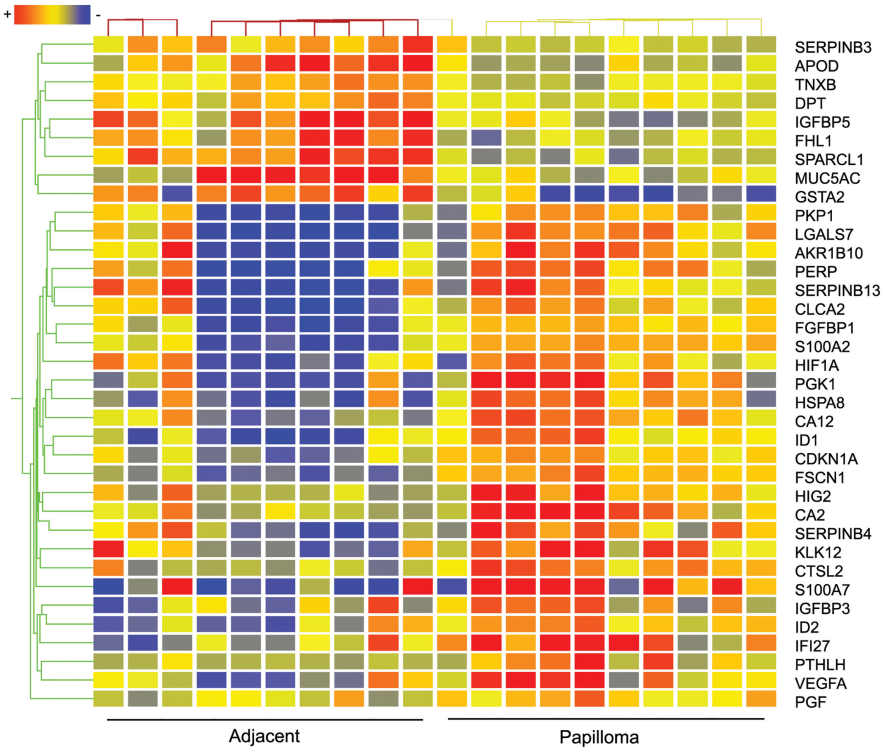

Figure 3. Genes associated with malignancy. Genes that are deregulated in various malignancies as identified in Table 3. Twenty-five of 27 genes that are increased in malignancy (tumor promoters/growth factors) also are increased in papillomas (bottom). Eight of nine genes which are decreased in malignancy (tumor suppressors) also were decreased in papillomas (top). Highly expressed genes are shown as red boxes, low expressed genes are shown as blue boxes, and intermediately expressed genes are shown as yellow boxes.

induced T_H2-like bronchial hyperresponsiveness (Abhr1) in mice (33). Furthermore, stimulation of human bronchial epithelial cells with *Pseudomonas aeuruginosa* induced the expression of *IL-1F9*, suggesting that IL-1F9 regulates T_H2-like innate responses that normally occur following bacterial exposure. This suggests that there may be a distinct, non IL-1β-inducible innate pathway stimulated by this interleukin in humans. We speculate that *IL-1F9* expression induces a yet-to-be characterized innate response in papillomas that polarizes adaptive T cells away from a T_H1-like response.

Altered expression of a small number of genes in papilloma tissues have been reported using qRT-PCR, *in situ* hybridization, or RNase protection, and many of those changes including *hBD4*, *CXCL8* (34), and *VEGFA* (35) also were identified in our analysis, further validating our findings. However, we did not detect elevated levels of survivin mRNA (36), or transcripts for *p16INK4A* and *p53* (37). These discrepancies may reflect, in part, our use of matched papilloma and autologous, epithelia pairs, rather than other control tissues.

A number of genes differentially expressed in papillomas have been associated with malignancies, affecting both tumor growth and immune responses. These include cytokines CXCL1, CXCL6, and CXCL8, and VEGFA that can all function as growth and angiogenic factors (38,39). Furthermore, increased expression of *CXCL1*, *CXCL8*, and *VEGFA* correlated significantly with severe disease (Table 4), suggesting that angiogenesis, a histological hallmark of RRP, is central to the pathology of this disease. Also evident were reductions in expression of three tumor suppressors (*IGFbp5*, *FHL1*, and *SPARCL1*) and elevated expression of several growth factors (*PGF*, *IGFbp3*, and *PTHLH*). Three members of the S100 family of proteins also were altered in papillomas. These proteins are involved in regulation of numerous cellular processes, including cell growth, differentiation, and progression toward cancer (40). They all likely play a role in regulating the innate immune response to pathogens. S100 proteins are damage-associated molecular pattern molecules which can function as pro-inflammatory factors of innate immunity (41). S100A2 has been reported to both promote tumor growth (42) and function as a tumor suppressor gene (40,43). S100A7 is overexpressed in breast cancer (44), epithelial skin tumors (45), bladder cancer (46), and is markedly elevated in lesions from psoriasis patients (47), suggesting a role in keratinocyte differentiation, and in regulating the innate immune response associated with epithelial inflammation (46). S100A12, a potent monocyte chemoattractant (48) that mediates allergic inflammation by

Table 4. Genes that Show Significant Correlation between Differential Expression and Disease Severity

Gene Name	Fold Change		*P*
	Mild/Moderate ($n = 6$)[a]	Severe ($n = 6$)[a]	
IL-1F9	4.6 ± 4.3	26.0 ± 2.6	0.04
CXCL1	1.9 ± 3.3	11.3 ± 2.8	0.02
IL-8	1.1 ± 2.6	11.3 ± 1.5	0.0003
VEGFA	2.46 ± 2.0	7.0 ± 1.7	0.02

[a]Mean ± SD, two-tailed *t* test.

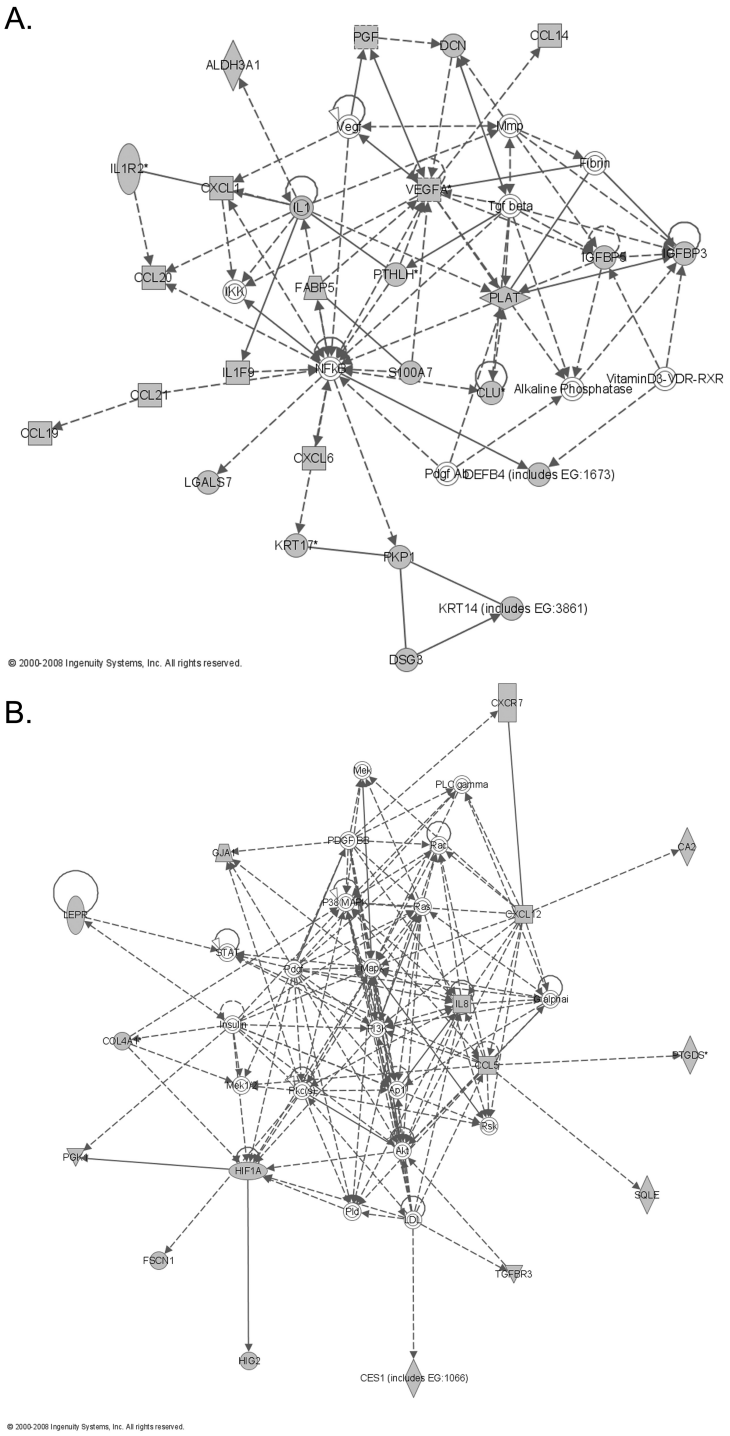

Figure 4. Ingenuity pathway analysis. Multiple pathway interactions showing both direct (solid line) and indirect (dashed line) associations between multiple dysregulated genes, having a fold change greater than 3.0, including: *VEGF, NFKB, PLAT,* and various chemokines (4A) having a significance score of 51, and *MAPK, PI3K, AkT, Ap1*, Pkc, and HIF1α, and IL8 (4B) having a significance score of 28. This analysis was performed on the 109 genes listed in Supplementary Table 5. Square: cytokine/growth factor; vertical diamond: enzyme; horizontal diamond: peptidase; circle: other; parallelogram: transporter; circle-in-circle: complex; oval: trans-membrane receptor; shaded circle-in-circle: group.

activating mast cells (49), also was increased in expression in papillomas. These observations suggest that the less oncogenic HPVs can reprogram cellular pathways similar to that described in some malignancies. Studies are underway to compare HPV 6/11 induced changes with those induced by the oncogenic HPV 16, to identify key cellular processes that distinguish their differential expression in benign versus malignant tumorogenesis.

In summary, we have used microarray analysis to identify changes in the transcriptional profiles of papilloma tissue from patients with RRP, as compared with autologous, laryngeal epithelium. We identified several groups of genes that may contribute to the disease process and disease severity. However, genes that are comparably expressed in both tissues would not be detected, even though some also may be important to disease susceptibility and/or severity. Our results support our previous contention that RRP is a disease characterized by a defective T_H1-like response in adaptive immunity. In this communication, we now suggest that altered innate responses to HPV also are present. Our findings may be relevant to other HPV-induced diseases, such as cervical cancer, where oncogenesis complicates and overshadows the inherent immunologic responses made to the more oncogenic HPVs. Our results provide new insight into the disease process associated with RRP, identify for the first time that papillomaviruses with low oncogenic potential can induce gene expression changes characteristically found in malignancies, and identify novel targets for future therapeutic interventions in RRP.

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