Gene Expression Screening of Salivary Gland Neoplasms

Molecular Markers of Potential Histogenetic and Clinical Significance

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Salivary gland neoplasms comprise phenotypically and biologically diverse lesions of uncertain histogenesis. The molecular events associated with their development and clinicopathological heterogeneity remain unknown. To reveal these events, we performed microarray expression analysis using a nylon-filter membrane platform on 18 primary lesions representing the most common benign and malignant types. Our study identified a small set of genes that are differentially altered between normal salivary gland tissues and benign and malignant tumors. Of the 5000 genes arrayed, 136 genes were differentially expressed by normal tissue, benign tumors, and various malignant neoplasms. Hierarchical clustering analysis differentiated between adenoid cystic carcinomas (ACCs) and other malignant subtypes. Non-ACC specimens manifested overlapping patterns of gene expression within and between tumors. Most of the differentially expressed genes share functional similarities with members of the adhesion, proliferation, and signal transduction pathways. Our study identified: 1) a set of genes that differentiate normal tissue from tumor specimens, 2) genes that differentiate pleomorphic adenoma and ACCs from other malignant salivary gland neoplasms, and 3) different patterns of expression between ACCs arising from major and minor salivary gland sites. The differentially expressed genes provide new information on potential genetic events of biological significance in future studies of salivary gland tumorigenesis. (*J Mol Diagn* 2004, 6:180–190)

Salivary gland neoplasms are uncommon lesions^{1–3} characterized by widely varied phenotypic features and unpredictable clinical outcomes.^{4–9} Such overlapping histologies and variable biological progression pose clinical and differential diagnostic challenges. Despite efforts to identify new parameters to improve their diagnosis and therapy, little progress in the management of patients with these tumors has been achieved in the last 3 decades.

Previous cytogenetic and molecular genetic analyses of these tumors have been limited in scope and size and did not account for their inherent morphological and biological heterogeneity.^{10–16} Large-scale gene expression analysis offers a broad approach to exploring the genetic alterations of functional significance in tumors and identifying potential diagnostic and prognostic markers. Recent studies using these technologies have identified different profiles in gene expression among histological phenotypes in several tumor types,^{17–20} including a recent analysis of adenoid cystic carcinomas (ACCs).²⁰

To identify new genetic markers of clinical relevance, we analyzed the gene expression profile of 18 primary tumors representing the major benign and malignant subtypes of these tumors using a membrane-based DNA microarray platform.

Materials and Methods

Tissue Samples

Fresh frozen tissues from 18 primary salivary gland tumors [three each pleomorphic adenoma (PMA), mucoepidermoid carcinoma (MEC), acinic cell carcinoma (ACI), salivary duct carcinoma (SDC), and 6 ACCs] and matched normal tissues excised at the Department of Head and Neck Surgery and Pathology at the University

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Figure 1. Example of comparative data analysis using the *t*-statistic and the mean of the gene expression for salivary gland tumors relative to normal tissue.

of Texas M.D. Anderson Cancer Center between 1995 and 2000 formed the materials of this study. Tissue samples were immediately taken from surgical specimens, frozen in liquid nitrogen, and stored at -80°C until used.

RNA Extraction

Total RNA was extracted from normal salivary gland tissues and tumors (1.5 mg) using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's protocol with some modifications. The quality of RNA samples was verified by electrophoresis on 2% agarose gel.

cDNA Microarray Analysis

The gene filter used in this study was composed of a nylon filter array GF200 (Research Genetics, Huntsville, AL) containing ~ 5000 genes, including function-known genes and unknown expressed sequence tags. Probe preparation and hybridization were performed as previously described.²¹ The filter was wrapped in 3M Whatman paper and scanned by the Cyclone Phosphor Im-

aging System (Packard Bioscience Company, Meriden, CT).

Statistical Considerations

Our main objectives were to identify differentially expressed genes in tumor and normal tissue and to perform molecular classification using the bidirectional cluster analysis algorithm. A total of 5453 genes were spotted on GF200 with 192 control-positive spots containing the total genomic DNA (tgDNA) and 192 housekeeping genes (HKGs). A logarithm 10-based transformation was applied to all raw intensity data. The hexbin scatter plot and scatter plot matrix using the S-PLUS (S-PLUS 200 Guide, 2000) were used to display the log intensity of gene expression of tumor *versus* normal tissue for each matched-pair experiment. Brushing technique was applied to highlight selected genes such as tgDNA or HKG. From the scatter plots, we found that tgDNA clustered by itself with moderate intensity (indicating adequate hybridization) in both normal and tumor samples. Because tgDNA is used for the purpose of providing positive controls and it contains both human and nonhuman genes, we removed the tgDNA genes from further analysis. There were a total of 8055 unique genes included in the analysis.

Normalization of the gene expression intensity in the normal and tumor tissues for each pair was performed by plotting the logarithm-transformed intensity in tumor *versus* normal tissue; fitting a nonparametric regression line using the loess (locally weighted regression scatter plot smoothing) method; and assuming that most genes do not vary greatly in their expression levels (the goal was to move the loess line to superimpose with the 45° line where *x* and *y* coordinates are the same). This was achieved by projecting each point to the 45° line along the *x* axis and the *y* axis and then taking the average of the two. The differential expression was computed by forming the difference between normalized, log-transformed expressions in tumor and normal tissue and then taking the arithmetic average in replicated experiments. To identify the expressed genes between normal and

Table 1. Highly Expressed Genes in PMA Compared to Normal Salivary Gland

Cluster ID	GB no.	Gene	<i>t</i> -Stat	T/N ratio
90283	AA490172	Collagen, type I, alpha 2	5.5	6.7
119498	AA485677	Zyxin related protein ZRP-1	10.9	4.9
44	AA001449	Pleiotrophin	6.8	4.2
83574	W81191	Nucleolar autoantigen No55	5.8	3.8
79624	T51689	Human hybrid receptor gp250 precursor	5.3	2.8
62313	T60235	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	4.4	2.7
2062	AA485226	Vitamin D (1,25-dihydroxyvitamin D3) receptor	4.7	2.6
10461	T58932	Fos-related antigen 2	8.3	2.5
21223	AA399519	Calponin	11.9	2.4
1092	T81033	Human CACC box-binding protein	18.3	2.3
79059	H62473	Transforming growth factor, beta receptor III	5.6	2.2
37045	W37306	Parathyroid hormone	4.5	2.2
7594	AA406551	Glucose transporter type 3	11.7	2.1
20894	AA463635	Heparan sulfate- <i>N</i> -deacetylase/ <i>N</i> -sulfotransferase	4.6	2.1
83790	R26672	KIAA0305 gene	5.5	2.1

GB no., Gene Bank number; T/N, tumor to normal ratio.

Table 2. Highly Expressed Genes in Normal Salivary Gland Compared to PMA

Cluster ID	GB no.	Gene	t-Stat	N/T ratio
81337	AA434102	Galectin-9 isoform	4.8	158.0
75367	AA485141	Putative src-like adapter protein (SLAP)	6.0	64.0
76494	AA434342	Proline arginine-rich end leucine-rich repeat protein	19.2	11.8
85050	AA427940	Phospholamban	4.4	4.3
4	N93428	Alcohol dehydrogenase 2 (class I)	4.7	4.1
24030	R76281	Human putative cooper uptake protein (hCTR2)	8.4	3.5
108660	N80617	Human multidrug resistance protein 5 (MDR5)	10.4	2.5
19944	AA427490	Long (electrocardiographic) QT syndrome 2	7.1	2.4
78765	T47229	Zinc finger protein	9.8	2.1

GB no., Gene Bank number; N/T, normal to tumor ratio.

tumor tissues for each of the two subtypes we applied the *t*-test with 5 degrees of freedom and select genes with $P < 0.01$. Method 5 takes the variability among patients into consideration by dividing the mean average by its SE. The Q-Q plots show that the statistics follow *t*-distribution with 5 degrees of freedom.

Hierarchical clustering analysis was performed on both GF200 differentially expressed genes of the 12 cases as previously described.²¹ Bidirectional clustering was performed to cluster both patients and genes. The Pearson correlation coefficient was used as the similarity metric, and the average-linkage method was applied to assemble all elements into a clustering tree.

The magnitude of gene was initially analyzed by Pathways Software (Research Genetics) and normalized using the standardization and normalization of microarray data method.²² A total of 5069 genes were normalized after excluding 192 positive control spots and 192 HKGs. Hierarchical clustering analysis and computation of *t*-statistics of the normalized log ratios were done as previously reported.²¹ Genes that exhibited an average intensity ratio more than twice as much as tumor or normal tissue in each histological subtype were considered to be differently expressed.

Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

To assess the gene expression level of telencephalin (ICAM5), which was expressed differently in ACCs than in major salivary gland tissue in the cDNA microarray analysis, we performed a real-time quantitative RT-PCR under the endogenous control of the β -actin gene using the

ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Mixtures of primer and FAM-labeled TaqMan probe of ICAM5 and β -actin were obtained from Assays-on-Demand gene expression products (Applied Biosystems). Five samples of normal salivary gland, five cases of PMA, five cases of ACC from major salivary gland (ACC-major), and six cases of ACC from minor salivary gland (ACC-minor) were used in this assay. PCRs were performed in duplicate in 25- μ l samples containing 100 ng of total RNA. The ABI TaqMan one-step RT-PCR master mix reagents kit was used for specimen analysis. The reaction conditions were 30 minutes at 48°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

A relative quantification method ($\Delta\Delta C_T$ method) was used for the quantification of gene amplification recognized as cycle numbers. The quantity of target genes, normalized by the HKG (β -actin), was evaluated as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is $\Delta C_T^{\text{target}} - \Delta C_T^{\beta\text{-actin}}$. Higher $2^{-\Delta\Delta C_T}$ values reflect a relatively higher amount of ICAM5 transcript.

Immunohistochemistry

Paraffin-embedded tissue blocks from all tumors were available and a review of the corresponding hematoxylin and eosin-stained sections was performed. Selected blocks were sectioned at 4 μ m for immunostaining monoclonal antibody for von Hippel-Lindau protein Ab-1 (VHL) (clone Ig22; Lab Vision/Neomarkers, Fremont, CA). Immunohistochemistry was performed using the avidin-biotin complex (ABC) method.

Table 3. Genes that Classify Carcinoma and Pleomorphic Adenoma

Cluster ID	GB no.	Average T/N ratio		Gene
		Carcinoma	Adenoma	
83384	AA424045	1.4370	20.5484	S-100 PROTEIN, BETA CHAIN
73952	AA488051	0.2780	0.0008	Proline-rich protein <i>HaeIII</i> subfamily 1
81337	AA434102	0.2333	0.0063	Galectin-9 isoform
71891	AA243828	0.8992	7.8461	Receptor protein tyrosine kinase
75367	AA485141	0.2847	0.0156	Human putative src-like adapter protein (SLAP)
79000	H05445	0.5358	18.5497	Growth-associated protein 43
2064	AA487812	3.9577	60.0593	Vimentin
23199	H64324	0.9222	0.0861	Guanine nucleotide exchange factor

GB no., Gene Bank number; T/N, tumor to normal ratio.

Figure 2. Heat map of 49 genes selected to classify ACCs and other carcinomas. Expression levels are relative to matched normal salivary gland tissue. The colors ranging from red to green correspond to increased and decreased gene expression. P11 to P16: ACC; P21.1 to P23.1: MEC; P31.1 to P33.1: ACI; P41.1 to P43.1: SDC.

Results

The patients included nine men and nine women between 28 and 89 years of age. Tumor sites included 12 in parotid glands, 2 in submandibular glands, and 4 in oral minor glands. Histologically, there were six ACCs, and three each of SDC, MEC, ACI, and PMA.

Differential Gene Expression

Figure 1 shows the plotted data based on the *t*-statistic and the simple average value of tumor to normal tissue. As depicted diagonally, the expression of most genes did not differ between normal salivary tissues and tumors, and only small number of overexpressed and

Table 4. Highly Expressed Genes in MEC Compared to Normal Salivary Gland

Cluster ID	GB no.	Gene	t-Stat	T/N ratio
76325	T70057	Immunoglobulin J chain	5.8	14.7
20144	R96668	Chemokine HCC-1	5.5	6.5
78223	AA456408	N-acylaminoacyl-peptide hydrolase	4.5	4.4
75337	AA488526	KIAA0035 gene	4.5	2.6
77066	T80232	Autotaxin	4.8	2.4
28988	AA291163	Glutaredoxin (thioltransferase)	8.1	2.4
77606	H91456	Farnesol receptor HRR-1 (HRR-1)	6.0	2.0

GB no., Gene Bank number; T/N, tumor to normal ratio.

underexpressed genes by both simple average and *t*-test methods are shown in the upper right and lower left corners of the graph.

PMA

Tables 1 and 2 list the genes that were differentially expressed between PMA and normal salivary tissues. Fifteen known genes were overexpression and nine were underexpressed in PMA including type I collagen, calponin, galectin-9 isoform, transforming growth factor- β , and MDR-5. Genes that differentiated between PMA and carcinomas are listed in Table 3. Among these were S-100 β , galectin-9 isoform, vimentin, and tyrosine kinase recep-

tor, which were more highly expressed in adenomas than in carcinomas.

Salivary Gland Carcinomas

The analysis of these tumors showed differences between ACCs and other types of malignant tumors (Figure 2). The hierarchical clustering analysis showed that the latter group, including SDC, MEC, and ACI, shares overlapping gene expression patterns. The known differentially expressed genes are listed in Tables 4 to 13. Thirteen known genes were differentially expressed in MEC and ACI, 37 in SDC, and 59 in ACCs. Highly expressed genes included the genes for immunoglobulin J chain

Table 5. Highly Expressed Genes in Normal Salivary Gland Compared to MEC

Cluster ID	GB no.	Gene	t-Stat	N/T ratio
79877	N50247	D13S824E locus	4.9	3.3
68783	N80741	ATP-binding protein	9.1	3.0
21413	AA167670	Human clone 23586 mRNA sequence	8.6	3.0
74576	AA488681	Human rab GDI alpha	14.7	2.8
89695	AA001614	Insulin receptor	6.4	2.5
83849	AA521453	Human homolog of <i>D. melanogaster</i> flightless-I gene	6.6	2.1

GB no., Gene Bank number; N/T: normal to tumor ratio.

Table 6. Highly Expressed Genes in ACI Compared to Normal Salivary Gland

Cluster ID	GB no.	Gene	t-Stat	T/N ratio
68257	AA282092	General transcription factor IIF, polypeptide 1	11.4	4.0
92909	AA431848	SON DNA-binding protein	6.7	2.9
82128	AA428514	5T4 gene for 5T4 oncofetal antigen	17.9	2.9
93649	AA489017	USF2a and USF2b, clone P9DH	7.1	2.4
54576	AA449459	Estrogen sulfotransferase	8.9	2.3
80248	W67323	RBP-MS/type 1	4.6	2.1
83086	H82977	GT212	4.5	2.1

GB no., Gene Bank number; T/N, tumor to normal ratio.

Table 7. Highly Expressed Genes in Normal Salivary Gland Compared to ACI

Cluster ID	GB no.	Gene	t-Stat	N/T ratio
19126	R01281	SKAP55 protein	6.5	5.8
738	AA486628	Early growth response protein 1	5.5	2.9
35379	AA410591	Met proto-oncogene	10.2	2.3
81134	T72877	Interleukin 1 receptor antagonist	39.0	2.3
77617	AA447481	Nuclear antigen Sp100	5.8	2.2
3085	95553	Hypothetical myeloid cell line protein 5	4.5	2.1

GB no., Gene Bank number; N/T, normal to tumor ratio.

Table 8. Highly Expressed Genes in SDC Compared to Normal Salivary Gland

Cluster ID	GB no.	Gene	t-Stat	T/N ratio
78160	H73054	Von Hippel-Lindau syndrome	8.1	24.0
23590	R73003	Human putative monocarboxylate transporte (MCT)	13.2	17.2
74645	AA458661	Short-chain alcohol dehydrogenase (XH98G2)	9.1	4.9
61840	AA054287	Human putative RNA binding protein RNPL	5.1	4.2
1028	AA477165	Radixin	94.3	3.6
78361	AA284329	Neuronal membrane glycoprotein M6b	5.1	3.6
79026	AA480835	Myelodysplasia/myeloid leukemia factor 2	4.5	3.1
1034	R75819	FK506-binding protein precursor	10.3	3.0
27747	R52852	Human putative endothelin receptor type B-like protein	7.1	2.6
4788	R96527	KIAA0253 gene	56.1	2.6
79162	R14855	High mobility group box	4.5	2.5
2726	H98218	High-mobility group phosphoprotein insform I	7.9	2.4
3610	R91264	KIAA0205 gene	8.5	2.3
74276	AA486518	Nuclear chloride ion channel protein (NC27)	19.9	2.1
2494	H06675	NAD-dependent succinate-semialdehyde dehydrogenase	6.3	2.1
75349	H23459	TUP1-like enhancer of split gene 1	5.0	2.1
56845	R92806	Human rab GDI	6.4	2.0
1424	AA047666	Flavin-containing monooxygenase 1	4.5	2.0

GB no., Gene Bank number; T/N, tumor to normal ratio.

Table 9. Highly Expressed Genes in Normal Salivary Gland Compared to SDC

Cluster ID	GB no.	Gene	t-Stat	N/T ratio
1192	N54344	KIAA0074 gene	5.5	3.3
4082	R97340	Prostate carcinoma tumor antigen (pcta-1)	12.0	3.2
11951	T70503	Plasma-cell membrane blycoprotein (PC-1)	4.7	3.1
101580	AA252318	HIV1 tataelement modulatory factor	6.2	3.1
32974	H14383	K+ channel beta2 subunit	5.9	2.8
2490	T95052	Interleukin-1 beta convertase precursor	6.0	2.7
78829	AA465611	KIAA190 gene	5.9	2.7
68257	AA282092	General transcription factor IIF, polypeptide 1	7.9	2.6
77501	R55105	Beta-sarcoglycan A3b	5.3	2.6
83634	R69885	HCF1 gene-related sequence	7.1	2.5
82408	H65034	cAMP phosphodiesterase	8.4	2.4
75649	AA401035	Extracellular signal-regulated kinase 3	15.5	2.4
93121	H51262	LGN protein	21.2	2.3
75297	AA015793	Fibroblast growth factor 1	7.4	2.3
24194	AA453816	Folate receptor beta precursor	6.7	2.2
2969	W69471	V-ski avian sarcoma viral oncogene homolog	23.4	2.2
75576	T73187	Plasminogen-like protein	4.3	2.1
80620	AA451891	KIAA0277 gene	10.6	2.1
77666	AA598887	KIAA0178 gene	8.2	2.0

GB no., Gene Bank number; N/T, normal to tumor ratio.

Table 10. Highly Expressed Genes in ACC-Major Compared to Normal Salivary Gland

Cluster ID	GB no.	Gene	t-Stat	T/N ratio
31544	R87840	Human telencephalin precursor	4.6	3.3
83252	R02346	U1 snRNP 70K protein	6.9	3.0
28491	A011215	Spermidine/spermine N1-acetyltransferase	17.7	3.0
115279	H65526	H. sapiens YPT3	4.7	2.8
23517	AA449762	Human nuclear factor NF90	4.9	2.7
83855	AA521067	Inositol polyphosphate 5 phosphatase	6.8	2.7
4788	R96527	KIAA0253 gene	8.1	2.6
75544	N90630	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase	4.5	2.4
75929	H96738	Cadherin 11 (OB-cadherin)	5.3	2.4
101337	AA192553	Uncoupling protein 3	4.3	2.3
79385	AA113331	Human clone 23574 mRNA sequence	5.0	2.1
11711	H75699	KIAA0297 gene	4.7	2.1
76253	R10604	Spinocerebellar ataxia 2	4.7	2.1
75365	H45967	CDC2-related kinase (CDK9)	9.7	2.0

GB no., Gene Bank number; T/N, tumor to normal ratio.

Table 11. Highly Expressed Genes in Normal Salivary Gland Compared to ACC-Major

Cluster ID	GB no.	Gene	t-Stat	N/T ratio
80395	AA227594	Mal, T-cell differentiation protein	6.0	408.4
74070	W23757	Keratin 13	6.7	171.5
89591	H17882	Kallmann syndrome 1 sequence	5.4	41.9
12337	AA026831	Kinase insert domain receptor (type III receptor tyrosine kinase)	5.1	36.2
76667	H82706	Inhibitor of DNA binding 2	4.3	19.6
83968	W68291	Integrin, beta 2	10.5	18.3
81071	N79484	Extracellular matrix protein 1 (ECM1)	5.0	18.0
78225	H63077	Annexin I (lipocortin I)	4.6	9.1
1940	AA504943	Crystallin, alpha B	14.1	4.5
79769	AA44357	Protocadherin 42	8.0	3.8
1795	AA486280	Tissue inhibitor of metalloproteinase 2 (TIMP2)	7.0	2.7
90875	AA012984	Human guanine nucleotide exchange factor mss4	4.8	2.3
111611	AA598582	Ribosomal protein L27	5.3	2.3
923	R05693	Single-stranded DNA-binding protein	5.5	2.1
34	T73556	Long chain fatty acid acyl-coA ligase	4.4	2.1
82032	AA485911	ER lumen protein retaining receptor 2	5.6	2.0

GB no., Gene Bank number; N/T, normal to tumor ratio.

and chemokine HCC-1 in MEC, transcriptional factor IIF and SON DNA-binding protein in ACI, and von Hippel Lindau gene (VHL) in SDC. Underexpressed genes included the genes for the D13S824E locus in MEC, SKAP 55 protein in ACI, and KIAA0074 and prostate carcinoma tumor antigen in SDC.

Interestingly, a distinctively different pattern within the ACCs was noted based on their site of origin (Figure 3).

According to the heat map of clustering within ACC cases, 11 known genes were found to discriminate between ACC cases from major salivary gland (ACC-major) and ACC from minor salivary gland (ACC-minor). Tables 10 to 13 list the known genes that are differentially expressed in ACC-major and ACC-minor. The expression of keratin 5 and keratin 13 was markedly lower in ACC-major than in ACC-minor.

Figure 3. Heat map of 17 genes selected to classify ACC-major and ACC-minor. Expression levels are relative to matched normal salivary gland tissue. The colors ranging from red to green correspond to increased and decreased gene expression. P11 to P13: ACC-major; P14 to P16: ACC-minor.

Table 12. Highly Expressed Genes in ACC-Minor Compared to Normal Salivary Gland

Cluster ID	GB no.	Gene	t-Stat	T/N ratio
75216	AA598513	Protein tyrosine phosphate, receptor type	5.7	14.3
75967	T71757	Heme oxygenase (decycling) 1	4.4	12.2
2064	AA487812	Vimentin	16.0	11.7
75813	AA448998	Polycystic kidney disease protein 1	9.8	10.6
55514	W32660	Human rap mRNA for ras-related protein	7.7	9.0
242	T98886	Glucose-6-phosphate	4.6	8.9
38481	H73724	Cyclin-dependent kinase 6	5.4	8.9
75309	R20379	Eukaryotic translation elongation factor 2	6.0	8.1
46468	N57964	G protein-coupled receptor (STRL22)	7.3	7.0
77526	AA465479	KIAA0040 gene	4.5	6.8
5022	H93815	IPW mRNA sequence	4.7	6.5
18288	AA432030	Interferon alpha-inducible protein (clone IFI-6-1)	5.2	6.4
82719	R99733	Human clone 23720 mRNA sequence	5.2	6.1
19582	AA417665	hTCF-4	5.7	5.2
2379	AA477893	Cytochrome P450, 51	7.6	5.1
102331	H94944	Ras-related protein RAL-1	7.1	5.0

GB no., Gene Bank number; T/N, tumor to normal ratio.

Quantitative RT-PCR Analysis of ICAM5

To confirm the microarray results real-time quantitative RT-PCR was performed for the ICAM5 gene. Comparative levels of each gene were normalized using the cycle number of β -actin amplification. As shown in Figure 4, the average expression levels of ICAM5 gene estimated as $2^{-\Delta\Delta C_T}$ value were elevated by 1.2-fold in PMA, 1.0-fold in ACC-minor, and 8.1-fold in ACC-major compared with the average value in normal salivary gland tissue (Figures 4 and 5). Comparative value was significantly higher in ACC-major than other groups ($P < 0.01$).

Immunohistochemistry for VHL

Immunostaining of archival tissue from tumor used in array analysis showed restricted cytoplasmic positivity of the VHL protein in only SDCs (Figure 6) and negative staining in other benign and malignant salivary gland tumors (Figure 6, C and D).

Discussion

Our study identified a small set of genes that are differentially expressed between normal salivary tissues and tumor specimens. These genes included several known and yet to be identified expressed sequence tags. Among the highly expressed genes in PMA are the genes for S-100, vimentin, type-1 collagen, and calponin gene markers that have been previously reported in PMA.²³⁻²⁶ Similarly, extracellular matrix and basement membrane-related genes including the genes for laminin and type IV collagen have been found to be widely expressed in myoepithelium or terminal duct origin salivary gland tumors.²⁷ We also noted that the β -chain isoform of S-100 was up-regulated only in PMA, not in other salivary gland carcinomas (Table 3). S-100 protein isoforms have previously been shown to differentiate myoepithelial cells in certain salivary gland neoplasms.²⁴

Hierarchical cluster analysis delineated ACCs from other types of salivary carcinomas on the basis of differentially expressed genes of factors mostly related to the

Table 13. Highly Expressed Genes in Normal Salivary Gland Compared to ACC-Minor

Cluster ID	GB no.	Gene	t-Stat	N/T ratio
111572	T67053	Rearranged immunoglobulin lambda light chain	6.2	39.8
81564	T97181	Platelet factor 4	16.8	5.4
90370	AA490209	Sop2p-like protein	5.0	4.7
100533	T67549	Plasminogen-like protein	5.1	3.5
76669	T72235	Nicotinamide N-methyltransferase (NNMT)	4.7	3.4
43697	AA463830	ETS-related protein erm	6.1	3.3
79351	N62620	Human two P-domain K ⁺ channel TWIK-1	9.1	3.2
79158	N70794	Acyl-coenzyme A dehydrogenase	6.8	3.1
77328	AA464711	Human putative G protein-coupled receptor (AZ3B)	7.6	2.9
57431	H93087	Human non-histone chromosomal protein HMG-17	5.7	2.9
38483	R67042	KIAA0265 gene	6.1	2.8
73956	AA491124	Quinone oxidoreductase (NQO2)	6.3	2.7
9908	T55639	Human NifU-like protein (hNifU)	6.2	2.6

GB no., Gene Bank number; N/T, normal to tumor.

Figure 4. Results of relative gene expression of ICAM5 under the endogenous control of β -actin in normal salivary gland tissue, PMA, and ACC derived from major salivary gland (ACC-major) and minor salivary gland (ACC-minor).

cell cycle, cell matrix, and extracellular adhesions. Of the down-regulated genes identified is the T-cell differentiation protein MAL, a putative cell-cycle tumor suppressor associated with several human cancers.^{21,28,29} *In vitro* induction of the MAL transcript was achieved by treat-

ment with 5-aza-2'-deoxycytidine in some esophageal cancer cell lines,²⁹ supporting a major role for methylation in the down-regulation of this gene. Interestingly, the overexpression of plasminogen activator and integrin family members seen in our study has been previously reported in ACC.^{20,30}

We, however, identified novel up-regulated genes in ACC, including the telencephalin (ICAM5) precursor-associated gene, a neural adhesion factor molecule,³¹ and the spermidine/spermine N1-acetyltransferase gene, a polyamine metabolism-associated gene.³² Our quantitative RT-PCR analysis validated the overexpression of ICAM5 mRNA in ACC in the microarray analysis. To our knowledge, this is a first report of up-regulated ICAM5 in any human cancers. ICAM5 is a cell surface glycoprotein molecule that is mapped at chromosome 19p13.2 and possesses structural and functional similarities with ICAM1. However, its expression has been limited to the telencephalic neurons.^{31,33} Previous studies have shown that overexpression of ICAM1 is associated with distant metastasis in gastric cancer, lung cancer, and melanoma.^{34–36} The ICAM5 gene may also induce factors such as BDNF and NCAM adhesion molecules, which have been associated with perineural invasion in ACC.^{37,38}

We also observed unique gene expression patterns within and between different carcinoma subtypes. Of these, the mRNA of the sequence located at the D13S824E locus on chromosome 13q12 was found to be markedly underexpressed in MEC. This sequence shares marked homology to the sequence encoding the myotubularin-related protein-6 (MTMR6), which possess a structural resemblance to the PTEN tumor suppressor gene.³⁹ Significantly, underexpression and overexpressed genes in ACL include SKAP 55, the enhancer of T-cell adhesion to antigen-presenting cells through fibronectin and ICAM1,⁴⁰ and the transcription factor IIF (TFIIF), respectively. The latter is recognized as a positive regulator of androgen receptor-mediated transcription.⁴¹

Moreover, we observed the high expression of VHL tumor suppressor gene in SDC. The inactivation of this gene by genetic and epigenetic alterations is associated with familial and sporadic renal cell carcinoma.^{42,43} However, high expression of VHL protein was previously reported in carcinomas of the lung, prostate, colon, breast, bladder, and thyroid in addition to renal cell carcinoma.^{44,45} In addition, it has also been reported that VHL-overexpressing renal cell carcinoma cell lines resist chemically induced apoptosis through bcl-2 activation *in vitro*.⁴⁶ These findings suggest that the VHL gene may play different oncogenic roles in tumorigenesis depending on the tumor type.

In general, our findings indicate that the functional properties of the genes identified appear to be linked to their putative origin from different salivary duct anatomical segments. This is supported by the genetic differences in PMA and ACC from those of acinar- and major duct-derived malignant tumors. Further studies of the genes associated with these tumors may lead to the identification of prognostic and therapeutic targets of these tumors.

Figure 5. Representative graph of quantitative RT-PCR for ICAM5 and β -actin as an endogenous control in normal salivary gland (A) and ACC (B).

Figure 6. Photomicrograph of VHL-immunostaining in normal parotid gland with faint cytoplasmic staining in small duct (A), negative staining in ACI (B), and strong cytoplasmic staining in two different SDCs (C and D).

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