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Large Scale Molecular Analysis Identifies Genes with Altered Expression in Salivary Adenoid Cystic Carcinoma

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Salivary gland cancers comprise a heterogeneous group of neoplasms whose biological and clinical characteristics differ considerably from those of mucosal squamous cell carcinomas of the head and neck. One of the most common subtypes, adenoid cystic carcinoma (ACC), is notable for its myoepithelial differentiation, proclivity for hematogenous spread, and slow but progressive clinical course. The molecular alterations that underlie its development and progression are poorly characterized. Here we used oligonucleotide microarray analysis to survey the expression of 8920 different human genes in 15 ACCs, one ACC cell line, and five normal major salivary glands. We observed expression of genes indicative of myoepithelial differentiation, as expected, including those whose protein products are components of basement membranes and extracellular matrix. Other genes that were highly ranked for their expression in ACC were those encoding the transcription factors SOX4 and AP-2γ, the latter of which also was overexpressed in ACC relative to 175 other carcinomas from 10 anatomical sites that we had previously profiled. Additional genes, which were highly expressed in ACC compared to the other carcinomas, included *casein kinase 1, epsilon* and *frizzled*-7, both members of the Wnt/β-catenin signaling pathway. Our study documents for the first time the diverse spectrum of genes overexpressed in ACC and highlights gene products and pathways that in the future might be exploited as therapeutic targets for this cancer, which up until now, has shown limited response to chemotherapeutic approaches. ***(Am J Pathol 2002, 161:1315–1323)***

Unlike mucosal squamous cell carcinomas of the head and neck, carcinomas of the salivary glands consist of a diverse histopathological spectrum of neoplasms. Adenoid cystic carcinoma (ACC) is one of the most common subtypes of salivary gland cancer, and in several series is the most frequent malignant tumor of the submandibular and minor salivary glands.[1,2] Histopathologically, ACC often forms both true lumens as well as pseudolumens in varying proportions. It characteristically shows myoepithelial differentiation and produces in large amounts specific proteins that comprise basement membranes and extracellular matrix.[3] The neoplasm has a proclivity for invading nerves, but it infrequently spreads via the lymphatic system. ACC has a protracted clinical course with local recurrences, hematogenous metastases, and poor response to classical chemotherapeutic approaches. After surgery and radiation therapy for patients with ACC, the disease-specific survival at 15 years is ~40%.[4]

The constellation of genes that are critical for the development and progression of ACC are not known. Furthermore, genes whose expression in ACC is altered relative to those in normal salivary glands and other carcinomas have not yet been compiled. In this study, we used large-scale microarray analysis in an effort to characterize the expression profiles in this salivary gland malignancy, and to specifically identify those genes differentially expressed between ACC and normal salivary gland epithelium. Importantly, we have also compared the compendium of expressed genes in ACC with that for 175 other carcinomas representing the 10 most common types of fatal carcinoma in the United States to determine which genes are uniquely expressed in ACC, and which specific pathways might be exploited for novel therapeutic approaches in this malignancy.

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Materials and Methods

Tissue Specimens, cRNA Synthesis, Oligonucleotide Array Analysis

The use of human tissue samples from the University of Virginia was approved by the UVA Human Investigation Committee, whereas those from M.D. Anderson Cancer Center were approved by the M.D. Anderson Institutional Review Board. The histological distinction of ACC from other salivary gland tumors was made using established criteria,[5] whereas the grade of ACC was determined using the criteria of Szanto and colleagues[6] in which grade I tumors had no solid component, grade II ACC had <30% solid areas, and grade III cancers contained >30% solid components. Hematoxylin and eosin (H&E)-stained frozen sections of portions of normal submandibular gland from two patients and normal parotid gland from three patients were examined to assess the relative amount of normal epithelium *versus* stroma and lymphocytes; frozen tissue blocks were then trimmed to enrich for normal salivary gland epithelium, while stroma and lymphocytes were avoided as much as possible. Frozen tumor samples from 15 ACCs that consisted predominantly of neoplastic cells were also selected. It is estimated that trimmed frozen tissues consisted of at least 75% neoplastic cells. Of the 15 patients with ACC, the age range was 26 to 74 years (median, 48 years). Ten patients were men and five were women. Five ACCs arose from a major salivary gland, and 10 arose in minor salivary glands. Seven ACCs were grade I, five were grade II, and three were grade III.

Frozen specimens were stored at −80°C before processing for microarray analysis and uniformly provided high-quality RNA. In duplicate analysis, one normal submandibular gland sample and one ACC specimen were each divided and processed independently. Several milligrams of each sample were sharply dissected and homogenized with a rotary homogenizer in RNeasy lysis buffer (Qiagen, Valencia, CA). RNA was prepared using the RNeasy Mini Kit (Qiagen). Labeled cRNA was then prepared and hybridized to Hu95a Affymetrix oligonucleotide GeneChips (Affymetrix, Santa Clara, CA) as described previously[7] and references cited therein.

Cell Culture

ACC3 cells, procured from a human ACC,[8] were cultured in RPMI 1640 with 10% fetal calf serum, 1% glutamine, 50 μg/ml streptomycin, and 50 IU/ml penicillin. RNA was extracted and prepared for microarray analysis as above.

Data Analysis

Scanned image files were inspected visually for artifacts and analyzed with GeneChip 3.1 (Affymetrix). Each GeneChip was scaled to an average hybridization intensity of 200, which corresponds to ~3 to 5 transcripts per cell.[9] Differential expression of genes in normal salivary and ACC specimens was estimated using a hybrid metric[10,11] based on equally weighted contributions from the difference of hybridization intensities, the quotient of hybridization intensities, and the result of an unpaired *t*-test between expression levels in tumor and normal tissues. The genes were scored with respect to each of the three metrics, and then ranked according to the sum of the three scores. Analysis of variance was used to determine whether there were differences in gene expression according to grade of ACC (I, II, or III) or site (major or minor salivary glands). Genes that were differentially expressed in ACC relative to the 10 most common types of fatal carcinoma previously analyzed were determined using recently described molecular classification methods.[7] For each gene, a Wilcoxon rank score was successfully calculated for samples in each carcinoma class with the highest mean expression *versus* samples from all of the other carcinoma classes (implemented in Matlab v6.0). The genes with the lowest *P* values in each carcinoma class were then ranked based on their predictive accuracy for discriminating one carcinoma class *versus* all other carcinoma classes by leave-out-one cross-validation using a support vector machine classifier.[7] Expression of the ACC classifier genes was compared in ACC samples and normal salivary gland samples using an unpaired *t*-test to identify those genes with elevated expression in ACC.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

The ACC3 cell line and frozen tissue specimens from five normal major salivary glands and five ACCs were used for RT-PCR analysis. Three of the ACCs had undergone gene expression profiling, while two had not. H&E-stained frozen sections of the ACCs and normal salivary glands were microdissected to obtain target epithelial populations of ~1000 cells each with at least 95% purity for tumor and, also, at least 95% purity for normal gland using a Leica AS LMD (laser microdissection system) (Leica Microsystems Inc., Bannockburn, IL). Each microdissected sample was subjected to RNA isolation by guanidine isothiocyanate buffer extraction, phenol/chloroform extraction, and alcohol precipitation (Micro RNA isolation kit; Stratagene, La Jolla, CA). The RNA samples were treated with 20 U of RNase-free DNase (Stratagene) for 1 hour at 37°C, followed by phenol/chloroform extraction and alcohol precipitation. First strand cDNA was synthesized in 20-μl reactions using random hexamer primers and Superscript II (Life Technologies, Inc., Gaithersburg, MD). Reactions were incubated on ice for 5 minutes, then at ambient temperature for 5 minutes, then at 42°C for 2 hours. Ten μL of the cDNA solutions were placed in 50-μL PCR containing 2 U of *Taq* polymerase. Sox 4 primers (5′-GCGGCGGGAGCAGCAAC-3′, 5′-GGAGCCGCAGCTCTTTTTC-3′, 92-bp product) were at 1 μmol/L final concentration and β2-microglobulin primers (5′-ATTCACCCCCACTGAAAAAG-3′, 5′-TCCATGATGCTGCTTACATG-3′, 106-bp product) were at 0.1 μmol/L final concentration. Cycling conditions were: 40

cycles of 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 30 seconds. PCR products were visualized by agarose gel electrophoresis, ethidium bromide staining, and UV light illumination. Relative RNA concentration was determined by image capture and analysis on an Alpha-Imager workstation (α Innotech, San Leandro, CA).

Immunohistochemistry

Deparaffinized zinc formalin-fixed, paraffin-embedded tissue sections of normal parotid and submandibular glands, ACC3, and three ACCs that were analyzed for global gene expression were immunostained using antibodies to keratin 17, cyclin D1, collagen IV, laminin, and β-catenin. An independent set of 10 ACCs whose transcripts were not profiled was also immunostained with antibodies to cyclin D1, collagen IV, laminin, and β-catenin to assess the frequency of protein expression in these tumors. Slides were placed in citrate buffer and heated in a microwave oven for 20 minutes before staining for keratin 17, cyclin D1, and β-catenin. Predigestion with protease was used for anti-collagen IV, while trypsin was used for anti-laminin. The primary antibodies selected included a mouse monoclonal antibody to keratin 17 (clone Ks17.E3, 1:50 dilution; Research Diagnostics, Flanders, NJ), a rabbit polyclonal antibody to cyclin D1 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal antibody to collagen IV (clone CIV22, 1:50 dilution; DAKO, Glostrup, Denmark), a mouse monoclonal antibody to β-catenin (clone 14, 1:200 dilution; BD Biosciences, San Diego, CA), and a mouse monoclonal antibody to laminin (clone LAM-89; 1:50 dilution; Novocastra, Newcastle, UK). After incubation with the primary antibody and the addition of the biotinylated secondary antibody, avidin-biotin immunoperoxidase was applied. Diaminobenzidine was used as the chromogen. Sections were then counterstained with hematoxylin. For the antibodies to keratin 17, cyclin D1, and laminin, immunoreactivity was scored semiquantitatively as negative, 1+ (<1% cells positive), 2+ (1 to 10% cells), 3+ (11 to 25% cells), or 4+ (>25% cells).

Results

Profiles of Gene Expression Distinguish Samples of Normal Salivary Glands and ACC

The expression levels of genes in normal and ACC tissue samples and one ACC-derived cell line (ACC3) were determined by hybridization of RNA to oligonucleotide microarrays comprising 12,533 probe sets representing 8920 unique human genes (listed by RefSeq numbers and Unigene ID). In total we hybridized 23 RNA samples from five normal major salivary glands, 15 ACCs, and one ACC cell line (ACC3). One of the normal salivary gland samples and one ACC sample were divided into approximately equal proportions, processed, and hybridized in duplicate to assess reproducibility of the experiments. Genes that showed the largest variation across the samples were selected to group them based on their similarities in expression levels.[12] With a SD cut-off of 500, 1038 genes were clustered. Using this approach, a dendrogram was constructed, and showed that the samples of normal salivary gland in duplicate were highly correlated, as were the duplicate samples of one ACC (Figure 1). Within the dendrogram, the normal submandibular and parotid glands clustered near each other but separately, whereas the 15 ACCs clustered into three groups. No overt differences were seen for patient age, sex, or site of tumor origin between the three clustered groups of tumors. All three grade III ACCs clustered in the largest group of tumors, whereas all ACCs in the smallest group ($n = 3$) were grade I; three other grade I ACCs were in the largest clustered group, however, and one other was in the third group. Analysis of the ACC3 cell line revealed that it clustered with the largest group of ACCs from patient samples.

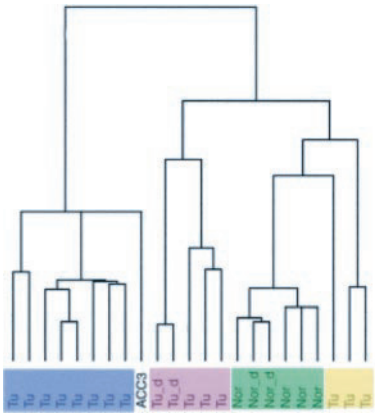

Figure 1. Dendrogram of samples showing overall similarity in gene expression profiles across all samples. Tu, each human ACC sample; Tu_d, duplicate ACC sample; ACC3, cell line; Nor, normal human major salivary gland; Nor_d, duplicate normal salivary gland sample.

Differentially Expressed Genes in ACC and Normal Salivary Glands

As normal major salivary glands and ACCs could be distinguished by their gene expression profile, we used a metric method to rank genes with the largest and most uniform differences in expression between the two sets of tissue samples (Table 1). For the 30 genes with the highest ranked differential over-expression in ACL, the fold level ranged from 3.5 to 78 (mean, 13.9; median, 9.2).

According to our ranking metric, the most significantly overexpressed gene in ACC was *Sox4*, which encodes a transcription factor. We also observed overexpression of other genes encoding transcription factors among the 30 most highly ranked genes, such as *AP-2*α and γ, and the *NGFI-A binding protein 1*. Functionally, a group of genes encoding extracellular matrix proteins and basement membrane components such as *versican*, *biglycan*, *laminin-*β*1*, and *type IV collagen-*α*1* were also identified. Indeed, the highest fold differences between ACC and normal major salivary glands were seen for *laminin* (78-fold) and *versican* (29-fold). Overexpressed genes in ACC encoding cytoskeletal or associated proteins in-

Table 1. List of Highest Ranked Overexpressed Genes in ACC Compared with Normal Salivary Glands

Affy ID	Symbol	Annotation*	RefSeq number	Unigene ID	*P* value†	Fold change
33131_at	SOX4	SRY (sex determining region Y)-box 4	NM_003107	Hs.83484	6.92E-07	14.9
40567_at	none	X01703:Human gene for alpha-tubulin (bα 1)/cds = (213 GenBank = X01703	X01703 (ACC#)	None	6.94E-07	14.8
38111_at	CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	NM_004385	Hs.81800	1.72E-06	29.5
33878_at	FLJ13612	Hypothetical protein FLJ13612	NM_025202	Hs.24391	1.53E-07	9.1
40454_at	FAT	FAT tumor suppressor homolog 1 (Drosophila)	NM_005245	Hs.166994	6.90E-08	6.9
37678_at	NMA	Putative transmembrane protein	NM_012342	Hs.78776	2.28E-07	11.7
41531_at	TM4SF1	Transmembrane 4 superfamily member 1	None	Hs.351316	2.19E-07	6.3
2020_at	CCND1	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	NM_053056	Hs.82932	9.27E-07	9.7
581_at	LAMB1	Laminin β 1	NM_002291	Hs.82124	5.88E-06	77.7
32832_at	MAEA	Macrophage erythroblast attacher	NM_005882	Hs.20815	1.45E-07	7.8
34301_r_at	KRT17	Keratin 17	NM_000422	Hs.2785	7.16E-06	13.8
38086_at	IGSF3	Immunoglobulin superfamily, member 3	NM_001542	Hs.81234	4.96E-08	28.3
34320_at	PTRF	Polymerase I and transcript release factor	None	Hs.29759	8.82E-08	5.3
33178_at	JAG1	Jagged 1 (Alagille syndrome)	NM_000214	Hs.91143	2.79E-07	14.9
38126_at	BGN	Biglycan	NM_001711	Hs.821	1.40E-07	4.8
34677_f_at	None	AJ012755:Homo sapiens mRNA for TL132/ cds = (1241 GenBank = AJ012755	AJ012755 (ACC#)	None	2.60E-07	9
38750_at	NOTCH3	Notch homolog 3 (Drosophila)	NM_000435	Hs.8546	3.03E-07	5.9
40303_at	TFAP2C	Transcription factor AP-2 γ(activating enhancer binding protein 2 gamma)	NM_003222	Hs.61796	1.82E-08	5.2
32749_s_at	FLNA	Filamin A, α(actin-binding protein 280)	NM_001456	Hs.195464	2.20E-07	4.6
32623_at	GABBR1	γ-Aminobutyric acid (GABA) B receptor, 1	NM_001470	Hs.167017	2.15E-06	14.2
38692_at	NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)	NM_005966	Hs.107474	5.14E-11	9.3
40339_at	GABRP	γ-Aminobutyric acid (GABA) A receptor, π	NM_014211	Hs.70725	2.72E-05	42.7
36186_at	RNPS1	RNA binding protein S1, serine-rich domain	NM_006711	Hs.75104	1.13E-08	4.5
39333_at	COL4A1	Collagen, type IV, alpha 1	NM_001845	Hs.119129	7.38E-06	9.4
38233_at	HOMER-3	Homer, neuronal immediate early gene, 3	NM_004838	Hs.166146	2.35E-08	8
36224_g_at	SFPQ	Splicing factor proline/glutamine rich (polypyrimidine tract-binding protein associated)	NM_005066	Hs.180610	9.44E-07	5.3
34296_at	None	ESTs	None	Hs.355863	6.54E-07	19.7
34304_s_at	SAT	Spermidine/spermine N1-acetyltransferase	NM_002970	Hs.28491	1.06E-09	3.5
32154_at	TFAP2A	Transcription factor AP-2 α (activating enhancer binding protein 2 α)	NM_003220	Hs.334334	1.42E-07	3.8
37333_at	DNMT1	DNA (cytosine-5-)-methyltransferase 1	NM_001379	Hs.77462	3.96E-08	17.6

*Genes 1, 7, 9, 10, 11, 12, 14, 19, 20, 21, 23, 26, 27, and 30 were overexpressed >3 fold in the ACC3 cell line compared with normal major salivary glands.
†Calculated by unpaired t-test.

cluded *keratin 17*, *α-tubulin (bα1)*, and *filamin A.* Genes encoding membrane proteins were observed at elevated levels in ACC, and included *macrophage erythroblast attacher*, *FAT tumor suppressor*, *transmembrane 4 superfamily member 1*, *Notch 3*, *Homer neuronal immediate early gene 3*, *GABA A* and *B receptors*, and *immunoglobulin superfamily member 3*.

Overexpression of *Sox4* transcripts in ACC relative to normal parotid and submandibular glands was validated by RT-PCR using laser-microdissected cells (Figure 2).

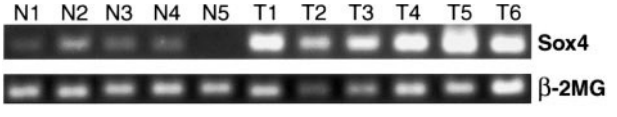

Figure 2. RT-PCR showing amplification of *Sox4* transcripts (**top row**) in laser microdissected cells from frozen sections of five separate normal major salivary glands (N1 to N5) and five different human ACCs (T1 to T5). T6, ACC3 cell line. Amplification of *b-2-microglobulin* transcripts (**bottom row**).

This was observed for each of the five ACCs as well as for ACC3.

We also sought to compare specifically overexpressed transcripts to protein expression using immunohistochemistry and antibodies to keratin 17, cyclin D1, collagen IV, and laminin. For keratin 17, two ACCs and ACC3 showed 3+ immunoreactivity, one ACC was 2+, while the normal major salivary glands had 2+ staining. The three ACCs whose transcripts were profiled showed 2+ to 3+ immunopositivity for cyclin D1, whereas the epithelial cells from the normal salivary glands stained 1+ (<1% positive cells) (Figure 3). The 10 ACCs not profiled showed 2+ to 4+ immunoreactivity for cyclin D1. Immunostaining with anti-collagen IV for the three ACCs that were profiled revealed very thick bands of positivity around nests, ducts, and the inner lining of pseudolumens as well as some conspicuous (often intense) immunoreactivity within the hyaline material of the pseudolumens (Figure 3). Each of the additional 10 ACCs also

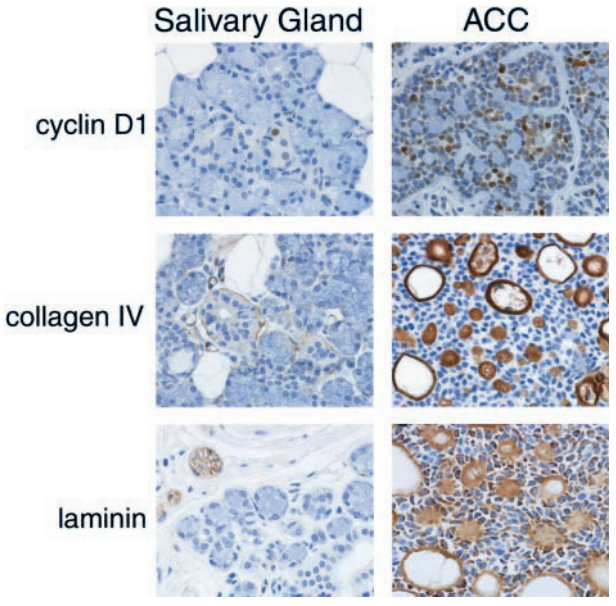

Figure 3. Immunohistochemical staining of normal major salivary gland (**left**) and ACC (**right**) showing differential immunoreactivity for cyclin D1, collagen IV, and laminin. Original magnifications, ×400.

showed strong immunopositivity for collagen IV in the same distribution. Collagen IV was inconspicuous or, at most, appeared as a very thin line around normal salivary ducts and, less often, around acini. Similarly, staining for laminin was inconspicuous or sometimes focal around normal salivary ducts and acini. In the three ACCs whose transcripts were profiled, there was 4+ immunoreactivity in tumor cells as well as staining immediately surrounding nests, lining pseudocystic spaces, and sometimes within hyaline material (Figure 3). Seven of the nine additional ACCs also showed 4+ immunopositivity. Approximately 10% of the ACC3 cells were immunopositive for laminin.

Some differences in gene expression in ACC were found relative to tumor grade and anatomical site of occurrence. Approximately 100 genes showed differences in expression according to tumor grade ($P < 0.01$), while ~40 had variable differences relative to tumor location in major or minor salivary glands ($P < 0.01$). (These lists of genes will be made available on request.)

Approximately 60% of the 100 genes that showed the highest levels of expression in the 15 ACCs relative to the normal salivary glands also showed high levels of expression in the ACC3 cell line. Among these genes were *Sox4*, *transmembrane 4 superfamily member 1*, *keratin 17*, *jagged 1*, *filamin A*, *GABA-B receptor 1*, and *NGFI-A binding protein 1*.

We also identified many genes that were significantly down-regulated in ACC relative to normal salivary glands (Table 2). Of the most highly ranked down-regulated genes were those encoding secretory proteins such as *histatin 3*, *amylase*, *fucosyltransferase 6*, *carbonic anhydrase VI*, *lactoperoxidase*, *statherin*, and *salivary proline-rich proteins*.

Identification of Genes Uniquely Overexpressed in ACC Relative to Other Common Carcinomas

To identify genes whose overexpression may be characteristic of ACC relative to other more common carcinomas, we used a recently described molecular classification method that seeks classifier genes that can predict the anatomical origin or tumor type of a blinded tumor sample by cross-validation or independent testing.[7] Genes expressed in ACC were compared with those from 175 other carcinomas from 10 different anatomical sites (lung, breast, prostate, bladder/ureter, liver, kidney, esophagus and stomach, colorectum, ovary, and pancreas).[7] Using this approach, we identified 21 genes that could accurately classify 13 of the 15 ACC samples with high confidence (Figure 4A). We have previously shown that such classifier genes typically represent features of the tissues from which the tumors arise, as well as features of the carcinomas from these tissues. Thus, we compared the expression of the 21 classifier genes in ACC with the normal major salivary glands (Figure 4B). The differential expression of these genes was generally highly significant (*t*-test; 8.4×10^{-3} to 1.8×10^{-8} with the exception for *Sox10*), implying that most of these classifiers represent genes expressed at high levels in ACC rather than the normal salivary glands from which they arose. Four of these genes (*AP-2γ*, *macrophage erythroblast attacher*, *versican*, and *laminin-β1*) were among the most highly ranked genes in ACC compared with normal major salivary glands. Known genes that were the most specific for ACC relative to the other carcinomas included *versican*, *TGF-β type III receptor*, and *c-myb*. Two other highly expressed genes included *casein kinase 1*, *epsilon* and *frizzled-7*, members of the Wnt/β-catenin signal transduction pathway. Immunostaining for β-catenin in 13 ACCs (3 whose transcripts were profiled and 10 others) revealed variable immunopositivity limited to the cytoplasmic membrane; no nuclear or cytoplasmic immunoreactivity was observed.

The two outlier ACCs that we could not readily classify by leave-out-one cross-validation had profiles similar to pulmonary squamous cell carcinomas, although they lacked histological evidence of squamous differentiation. These two ACCs had relatively low levels of expressed transcripts for *AP-2γ*, *versican*, *frizzled-7*, and *laminin-β1*, genes that showed important differential expression for the other ACCs compared with the cancers from the 10 other anatomical sites.

Discussion

Salivary gland neoplasms consist of a diverse category of tumors with a spectrum of histological appearances, various degrees of differentiation, and differing clinical behaviors. ACC is one of several salivary gland neoplasms that characteristically shows dual myoepithelial and ductal epithelial differentiation.[13] More than many others, these tumors frequently secrete abundant extracellular matrix proteins (such as chondroitin sulfate) and basement membrane components. This is accurately re-

Table 2. List of Highest Ranked Overexpressed Genes in Normal Major Salivary Glands

Affy ID	Symbol	Annotation	RefSeq number	Unigene ID	*P* value*	Fold change
35051_at	CA6	Carbonic anhydrase VI	NM_001215	Hs.100322	3.36E-08	23.7
32006_r_at	PROL3	Proline rich 3	NM_006685	Hs.2207	1.08E-07	18
39680_at	STATH	Statherin	NM_003154	Hs.37048	2.90E-09	11.7
36680_at	AMY2B	Amylase, alpha 2B; pancreatic	NM_020978	Hs.335493	9.29E-13	10.4
36290_s_at	FUT6	Fucosyltransferase 6 (alpha (1,3) fucosyltransferase)	NM_000150	Hs.32956	1.21E-07	11.0
41148_at	HTN3	Histatin 3	NM_000200	Hs.177888	7.71E-11	8
37006_at	None	Homo sapiens, clone MGC:24130 IMAGE:4692359, mRNA, complete cds	None	Hs.76325	1.57E-08	9.4
35691_r_at	NFIX	Nuclear factor I/X (CCAAT-binding transcription factor)	NM_002501	Hs.35841	4.00E-04	18.0
31977_at	GUCY2D	Guanylate cyclase 2D, membrane (retina-specific)	NM_000180	Hs.1974	5.87E-07	11.0
34161_at	LPO	Lactoperoxidase	None	Hs.234742	4.62E-06	26.8
31446_s_at	PBI	Protein homologous to salivary proline-rich protein P-B	NM_012390	Hs.166099	2.98E-06	11.9
41846_at	CRX	Cone-rod homeobox	NM_000554	Hs.249186	4.44E-06	8.3
31635_g_at	PRH1	Proline-rich protein HaeIII subfamily 1	NM_006250	Hs.278469	7.48E-08	5.9
33613_at	none	Homo sapiens SNC73 protein (SNC73) mRNA, complete cds	None	Hs.293441	4.17E-06	10
34823_at	DPP4	Dipeptidylpeptidase IV (CD26, adenosine deaminase-complexing protein 2)	NM_001935	Hs.44926	4.28E-06	35.0
41770_at	MAOA	Monoamine oxidase A	NM_000240	Hs.183109	6.96E-08	7.1
41832_s_at	JTB	Jumping translocation breakpoint	NM_006694	Hs.6396	1.22E-11	5.2
39756_g_at	XBP1	X-box binding protein 1	NM_005080	Hs.149923	4.56E-06	9.8
31459_i_at	IGLα	Immunoglobulin λ locus	None	Hs.181125	7.38E-06	11.3
34213_at	KIAA0869	KIAA0869 protein	None	Hs.21543	1.54E-06	10.4
40456_at	LOC64116	Up-regulated by BCG-CWS	NM_022154	Hs.284205	3.89E-06	12.5
41144_g_at	CALM1	Calmodulin 1 (phosphorylase kinase, δ)	NM_006888	Hs.177656	7.40E-07	6.5
39755_at	XBP1	X-box-binding protein 1	NM_005080	Hs.149923	1.96E-06	6.7
41388_at	MEIS2	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)	NM_020149	Hs.104105	3.65E-07	11.4
33274_f_at	IGLJ3	Immunoglobulin lambda joining-3	None	Hs.336946	4.15E-06	6.6
33501_r_at	none	Homo sapiens SNC73 protein (SNC73) mRNA, complete cds	None	Hs.293441	3.73E-06	6.4
38966_at	GPSN2	Glycoprotein, synaptic 2	NM_004868	Hs.306122	8.81E-07	5.1
33442_at	KIAA0367	KIAA0367 protein	None	Hs.23311	1.50E-09	47.5
34674_at	S100A1	S100 calcium-binding protein A1	NM_006271	Hs.292707	9.88E-06	17.6

*Calculated by unpaired t-test

flected in our immunohistochemical analyses by the presence of the myoepithelial/basal keratin 17,[14] laminin, and collagen IV. Previous immunohistochemical studies have also documented the presence of laminin and type IV collagen in ACC.[3,15–19] We found highly overexpressed transcripts for versican, biglycan, laminin-β1, and collagen IV α1 in ACC relative to normal major salivary glands.

The most significantly overexpressed gene in ACC relative to the normal major salivary glands was *SOX4*, which encodes a transcription factor expressed in a wide variety of tissues in mice, and has functional importance in heart, brain, the reproductive system, and B-cell development.[20–23] The *SOX* gene family members have a highly conserved HMG-domain responsible for sequence-specific DNA binding.[24] In serial analysis of gene expression, *SOX4* was overexpressed in normal mammary epithelium relative to ductal carcinoma *in situ* and invasive carcinoma of the breast.[25] Some breast cancer cell lines, however, have been shown to highly express SOX4, which is increased by progestins.[26] Although elevated expression of *SOX4* was not unique to ACC, the genes that it transcribes and its role in tumor development or progression requires additional study.

The genes encoding transcription factors AP-2α and AP-2γ were also overexpressed in ACC. The AP-2 family members have been reported to regulate the expression of genes required for murine development of several tissues such as neural crest and skin.[27,28] Genes regulated by AP-2 are important in numerous biological functions and include, among others, *estrogen receptor*, *keratinocyte-specific genes*, and *c-kit*.[29–32] KIT protein has been found to be overexpressed in many ACCs.[33] Interestingly, *versican*[34] and *biglycan*[35] have binding sites for AP-2 suggesting a direct correlation between expression levels of these genes and AP-2. Both AP-2α and AP-2γ are reported to be overexpressed in breast carcinoma.[36] Although we noted that *AP-2γ* was modestly expressed in our breast cancers, there was on average a threefold increase in expression in ACC compared with the mammary tumors.

Genes encoding transmembrane proteins highly expressed in ACC included *FAT tumor suppressor* and

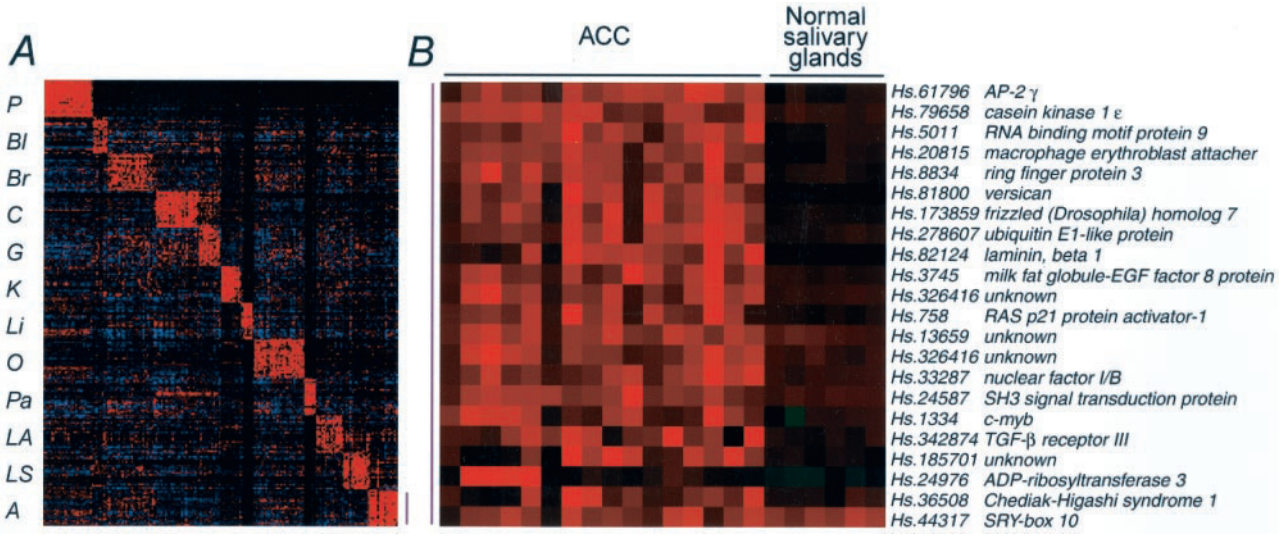

Figure 4. A: Tumor-specific genes for class prediction of ACC. Using ~20 genes/class, 13 of 15 ACCs could be easily classified with high confidence, having gene expression profiles different from those of 175 examples from 10 other anatomical sites. P, prostate; Bl, bladder/ureter; Br, breast; C, colorectum; G, stomach/esophagus; K, kidney; Li, liver; O, ovary; Pa, pancreas; LA, lung adenocarcinoma; LS, lung, squamous cell carcinoma; A, ACC. **B:** List of classifier genes for adenoid cystic carcinoma.

transmembrane 4 superfamily member 1. The former contains 34 cadherin repeats, is expressed in many types of epithelium, and encodes a putative tumor suppressor protein.[37–39] *FAT tumor suppressor* has been shown to be down-regulated in metastatic prostate cancer.[40] The importance of its overexpression in ACC, therefore, is interesting and the functional consequences of its overexpression require additional study. *Transmembrane 4 superfamily member 1*, also known as *tumor-associated antigen L6*, encodes a cell surface protein implicated in cell growth. It is highly expressed in several carcinomas including those of the lung, breast, ovary, and colon,[41] and has been suggested as a target for monoclonal antibody therapy.

Genes encoding secreted proteins highly expressed in normal parotid and submandibular glands relative to ACC are those indicative of acinar cell differentiation. These genes such as *amylase*, *carbonic anhydrase VI*, and *salivary proline-rich proteins*, among others, would be predicted to be expressed in salivary gland neoplasms having acinar differentiation such as acinic cell carcinoma.

Approximately 60% of the 100 genes overexpressed in ACC relative to normal major salivary glands were also overexpressed in the ACC3 cell line. These genes included *Sox 4*, *keratin 17*, *transmembrane 4 superfamily member 1*, and *laminin*. ACC3 has been shown to secrete large quantities of basement membrane proteins including laminin and type 4 collagen *in vitro* as well as in mice.[42,43] The overlap in overexpressed genes in human ACC and ACC3 would suggest that ACC3 is a valid model system for the study of molecular alterations that might be important in human ACC carcinogenesis.

Thirteen of 15 ACCs had profiles distinctly different from 175 other carcinomas from 10 anatomical sites, enabling their classification by cross-validation with high confidence. Both *versican* and *AP-2γ* were among the top ranked classifier genes in ACC. The former was either absent or lowly expressed in carcinomas other than ACC, whereas *AP-2γ* was consistently expressed at modest levels among many of the 175 other tumors, but showed the highest statistical difference in ACC relative to the other tumors and the normal major salivary glands.

Two of the most highly ranked classifier genes in ACC included components of the Wnt/β-catenin signal transduction pathway. *Frizzled-7*, expressed normally in various adult and fetal tissues, encodes a receptor for WNT proteins and may down-regulate the function of APC and therefore promote β-catenin signaling.[44] The protein product of *casein kinase 1*, ε,[45] expressed in multiple human cell lines, complexes with axin and perhaps other components of the Wnt system; its overexpression stabilizes β-catenin, thereby promoting the transcription of β-catenin-dependent genes.[46] Using immunohistochemistry, we noted variable cytoplasmic membrane immunopositivity for β-catenin in ACC, but no nuclear or cytoplasmic staining. This finding would suggest that the Wnt/β-catenin pathway is not overtly dysregulated in ACC, as it is, for example, in colorectal carcinomas in which a pathway member is typically mutated leading to translocation of β-catenin to the nucleus.

Another classifier gene in ACC, *nuclear factor I/B*, encodes a transcription factor that is important in tissue-specific gene expression during growth and differentiation.[47,48] *Nuclear factor I/B*, located at 9p24, has been found to be involved in a hybrid transcript with *HMGIC* in two pleomorphic adenomas of salivary gland,[49] perhaps extending the importance of this gene in ACC. In that regard, it is of note that ~30% of ACCs have cytogenetic evidence of translocations involving 9p13-23.[50] Whether *nuclear factor I/B* is a target of this translocation or is dysregulated by other means in ACC is currently unknown. In addition, *c-myb*, located at 6q22, was also overexpressd in ACC. Interestingly, 6q is usually the translocation partner with 9p in ACC.

Our study has elucidated those genes that are overexpressed in ACC relative to normal major salivary

glands. We have also identified certain genes that are selectively overexpressed in ACC relative to 10 other types of carcinoma. Among the overexpressed genes are several that encode transcription factors, extracellular matrix components, and signal transduction pathway members. The information derived from such global transcription profiling offers clues as to which regulatory pathways should be first targeted for further study regarding their effects on tumor growth and behavior. The identification of genes overexpressed in ACC may suggest new therapeutic targets for this carcinoma, which up until now, has primarily been attacked by the surgical approach, and which when it becomes metastatic cannot be treated with complete success.

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