[CANCER RESEARCH 64, 55–63, January 1, 2004]

Identification of a Gene Expression Signature Associated with Recurrent Disease in Squamous Cell Carcinoma of the Head and Neck

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ABSTRACT

Molecular studies of squamous cell carcinoma of the head and neck (HNSCC) have demonstrated multiple genetic abnormalities such as activation of various oncogenes (*Ras*, *Myc*, epidermal growth factor receptor, and cyclin D1), tumor suppressor gene inactivation (*TP53* and *p16*), and loss of heterozygosity at numerous chromosomal locations. Despite these observations, accurate and reliable biomarkers that predict patients at highest risk for local recurrence have yet to be defined. In an effort to identify gene expression signatures that may serve as biomarkers, we studied 41 squamous cell carcinoma tumors (25 primary and 16 locally recurrent) from various anatomical sites and 13 normal oral mucosal biopsy samples from healthy volunteers with microarray analysis using Affymetrix U133A GeneChip arrays. Differentially expressed genes were identified by calculating generalized *t* tests ($P < 0.001$) and applying a series of filtering criteria to yield a highly discriminant list of 2890 genes. Hierarchical clustering and image generation using standard software were used to visualize gene expression signatures. Several gene expression signatures were readily identifiable in the HNSCC tumors, including signatures associated with proliferation, extracellular matrix production, cytokine/chemokine expression, and immune response. Of particular interest was the association of a gene expression signature enriched for genes involved in tumor invasion and metastasis with patients experiencing locally recurrent disease. Notably, these tumors also demonstrated a marked absence of an immune response signature suggesting that modulation of tumor-specific immune responses may play a role in local treatment failure. These data provide evidence for a new gene expression-based biomarker of local treatment failure in HNSCC.

INTRODUCTION

Head and neck malignancies account for 6% of all cancers diagnosed in the United States and result in an estimated 14,000 deaths annually (1). Although improvements in local control and survival have been achieved with the use of combined modality therapies, 5-year survival rates for head and neck cancers have not improved significantly over the past 20 years (2, 3). Local-regional relapse after definitive therapy is a major cause of morbidity and mortality in patients with head and neck squamous cell carcinoma (HNSCC) and has prompted substantial efforts in identifying molecular biomarkers that accurately predict patients at risk for disease recurrence (4). Identification of biomarkers that signal increased risk of treatment failure in HNSCC would have a major impact on treatment planning decisions. For instance, biomarkers could be used to identify surgically treated patients at highest risk for local regional recurrence, thus allowing improved accuracy in selecting patients most likely to benefit from postoperative radiation therapy. In addition, biomarkers that predict local recurrence may be useful in identifying patients who may be spared postradiation neck dissection or those likely to benefit from concomitant chemoradiation approaches, altered fractionation radiation schedules, or new molecular-targeted therapies.

Many clinical and pathological prognostic factors have been described in HNSCC (tumor stage, lymph node involvement, postsurgical margin, and histological grade). However, as prognostic biomarkers, clinical or pathological factors lack sensitivity and accuracy in the clinical setting and, with the exception of disease stage, are infrequently used to guide treatment decisions. More recent work has focused on defining tumor-specific molecular abnormalities that predict patient outcome (4, 5). These studies have revealed numerous molecular abnormalities in HNSCC, including activation of various oncogenes (*Ras*, *Myc*, epidermal growth factor receptor, and cyclin D1; Refs. 6–14), tumor suppressor gene inactivation (*TP53* and *p16*; Refs. 15–19), expression of angiogenic factors (20–22), DNAploidy (23, 24), and loss of heterozygosity at numerous chromosomal locations (25–28). Despite these molecular-based observations, biomarkers that precisely identify patients at highest risk for local recurrence have yet to be defined. This may be due, in part, to the molecular heterogeneity of HNSCC and the limited capacity of any single molecular biomarker to accurately and reliably predict outcome in individual patients. A more comprehensive screen of the molecular defects in HNSCC such as that obtained through microarray analysis may reveal biomarkers with enhanced sensitivity and specificity in the clinical setting.

High-throughput gene expression profiling techniques offer a unique mechanism for interrogating transcriptome-wide levels of gene expression and have proven value in defining gene expression signatures for clinically important subsets of patients who would otherwise be undetected by conventional prognostication schemes (29, 30). In addition, microarray approaches have been very successful in identifying subsets of tumors that correlate with clinical parameters such as survival, histological grade, and response (31–35). In this study, we applied gene expression microarray technology to a group of 41 HNSCC tumors resected at surgery and compared the gene expression to 13 normal oral mucosal biopsies from healthy volunteers. Our aims were to obtain a comprehensive view of gene expression differences in HNSCC and normal oral mucosa (NOM) and to identify gene expression signatures that correlate with defined clinical parameters with the hope of identifying new prognostic gene expression-based biomarkers in HNSCC.

MATERIALS AND METHODS

Patient Characteristics and Biopsy Samples

Consent from patients and volunteer control subjects was obtained in accord with guidelines set forth by the Institutional Review Board of the Human Subjects Protection Committee at the University of Minnesota. The University of Minnesota Cancer Center Tissue Procurement Facility obtained tumor samples from surgical resection specimens from patients undergoing surgery for HNSCC using standardized procedures. All samples were immediately

Received 7/18/03; revised 9/23/03; accepted 10/22/03.

Grant support: American Cancer Society Institutional Research Grant IRG-58-001-40IRG44.

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placed on ice and within 30 min of devascularization frozen in liquid nitrogen after removal of portions needed for pathological diagnosis. Histological analyses were performed to ensure that each specimen contained >50% tumor tissue and <10% necrotic debris, and those samples not meeting these criteria were rejected. Healthy control subjects without a history of oral cancer, premalignant lesions, or periodontal disease were recruited through the University of Minnesota School of Dentistry. After administration of a local anesthetic, a 6-mm punch biopsy of tissue was obtained from the buccal mucosa in the region adjacent to the third molar. Tissue specimens were flash frozen in liquid nitrogen and stored until extraction of mRNA. On average these tissues contained 40% epithelial mucosa and 60% submucosa tissue.

Extraction of Total RNA and Probe Preparation

Fifty to 100 mg of tissue were submerged in 1 ml of Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) and immediately homogenized using a rotor-stator homogenizer (Powergen 700; Fischer Scientific) under RNase-free conditions. Total RNA was extracted from the samples using the Trizol extraction protocol after a 1-min spin at 12,000 × *g* to pellet particulate matter. Total RNA was precipitated by incubating with 0.5 ml of isopropyl alcohol for 10 min followed by centrifugation at 12,000 × *g* for 10 min at 4°C. The pellet was washed twice with 75% ethanol, dissolved in RNase-free water, and stored at −80°C until further use. Agarose gel electrophoresis was performed on each sample before additional analysis to confirm the presence of nondegraded RNA.

Five to 10 μg of total RNA were used to prepare biotinylated cRNAs for hybridization using the standard Affymetrix protocol (Affymetrix, Santa Clara, CA). Briefly, RNA was converted to first strand cDNA using a T7-linked oligodeoxythymidylic acid primer (Genset, La Jolla, CA), followed by second strand synthesis (Invitrogen Corporation, Carlsbad, CA). The double-stranded cDNA was then used as template for labeled *in vitro* transcription reactions using biotinylated ribonucleotides (Enzo, Farmingdale, NY). Fifteen μg of each labeled cRNA were hybridized to Affymetrix U133A GeneChips (Affymetrix, Santa Clara, CA) using standard conditions in an Affymetrix fluidics station.

Analysis of Microarray Data

Preprocessing of Microarray Data. Scanned Affymetrix array data were uploaded into the GeneData Cobi 4.0[6] database maintained by the Supercomputing Institute's Computational Genomics Laboratory at the University of Minnesota. Preprocessing of the Affymetrix arrays was carried out using GeneData Refiner 3.0[6] software to correct for variations in hybridization intensity because of gradient effects, dust specks, or scratches. Gene expression intensity for each array was scaled to an arbitrary value of 1500 intensity units to allow comparisons across all arrays. Expression intensity values for each gene were derived using Refiner by applying the Microarray Suite 5.0 algorithm.

Statistical Analysis. Genes differentially expressed between the 41 HNSCC and 13 normal oral mucosal biopsies were identified using a Satterthwaite *t* test (36) to robustly estimate significance despite unequal variance among groups. Genes that merited additional investigation met the following criteria: $P < 0.001$, absolute value of the difference in mean expression between the two groups of samples (Δ) > 100 intensity units, and fold difference in mean expression ≥ 2.0 or ≤ 0.5. Unsupervised hierarchical clustering was performed with Cluster (37) using Pearson's correlation distance metric and average linkage followed by visualization in Treeview (37). Because of the large number of statistical tests performed we estimated the Bayesian posterior true positive, posterior true negative, and posterior false negative rates for each of the genes using the mix-*o*-matic method of Allison *et al.* (38) and to illustrate that more genes were observed to be differentially expressed than expected (supplemental data Figure 1; http://www.gaffney.umn.edu./suppl).

Immunohistochemistry

Immunohistochemical analysis was performed to validate the differential expression of selected genes in tissue sections and to localize the expression of the genes. On the basis of their gene expression profiles, 4 of the HNSCC (2 samples with high expression and 2 with low expression of inflammation and immune response-related genes) were selected. Sections of HNSCC were formalin-fixed and paraffin-embedded, sectioned at 4-μm and immunostained according to standard protocols. Immunostaining with antibodies against CD3 (PS1, prediluted; Ventana Medical Systems, Inc., Oro Valley, AZ) and cytokeratin (AE1 AE3, prediluted; Dako Corporation, Carpinteria, CA) was performed on the automated immunostainer NexES IHC (Ventana Medical Systems, Inc.).

Real-Time PCR Analysis

Changes in mRNA levels were compared by quantitative real-time PCR analysis, using the Light Cycler (Roche Diagnostics Corp). One μg of total RNA was converted to cDNA using Superscript II (Invitrogen Corporation) according to manufacturers specifications. PCR reaction mixtures consisted of 2 μl of Faststart DNA Master SYBR Green I mixture [containing TaqDNA polymerase, reaction buffer, deoxynucleotide triphosphate mix (with dUTP instead of dTTP), SYBR Green I dye, and 10 mM $MgCl_2$], 0.5 μM of each target primer stock, 2 or 4 mM $MgCl_2$ [glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *PTHLH*, 4 mM *SNAI2*, and 2 mM *MET*] in a final reaction volume of 20 μl. Cumulative fluorescence was measured at the end of the extension phase of each cycle. Product-specific amplification was confirmed by a melting curve analysis and agarose gel electrophoresis analysis. Quantification was performed at the log-linear phase of the reaction and cycle numbers obtained at this point were plotted against a standard curve prepared with serially diluted samples. Results were normalized to *GAPDH*. Primer sequences and reaction parameters are as follows: *GAPDH*, forward, 5′-ACCACAGTCCATGCCATCAC-3′ and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′; *MET*, forward, 5′-GAAGGAGGGACAAGGCTGAC-3′ and reverse, 5′-ATGGCAGGACCAACTGTGC-3′; *PTHLH*, forward, 5′-GCTCAAGACACCTGGGAAGA-3′ and reverse, 5′-AAGCCTGTTACCGTGAATCG-3′; and *SNAI2*, forward, 5′-CATCTTTGGGGCGAGTGAGTCC-3′ and reverse, 5′-GGCCAGCCCAGAAAAAGTTGAAT-3′.

RESULTS

To identify differentially expressed genes present in HNSCC and NOM, we obtained specimens from 41 patients undergoing surgical resection for HNSCC. Clinical characteristics of the 41 patients are shown in Table 1 and supplemental data Table 1. This study population closely parallels the general population of patients with HNSCC having a median age of 64 years and greater percentage of males with disease. The majority of cancers were located in the oral cavity (44%)

Table 1 *Clinical features of 41 head and neck squamous cell carcinoma patients and 13 normal control subjects*

	Head and neck squamous cell carcinoma patients		Normal subjects	
Clinical feature	*n*	%	*n*	%
Sample size, *n*	41		13	
Male gender, *n* (%)	29	71%	6	46%
Median age (yr)	64		60	
Tobacco use[a], *n* (%)	37	90%	5	38%
Alcohol use[b], *n* (%)	14	32%	0	
Anatomic location, *n* (%)				
Oral cavity	18	44%		
Oropharynx	4	10%		
Hypopharynx	1	2%		
Larynx	15	37%		
Sinus	3	7%		
Clinical stage, *n* (%)				
Stage I–II	13	32%		
Stage III–IV	28	68%		
Tumor differentiation, *n* (%)				
Well	6	15%		
Moderate	20	48%		
Poor[c]	15	37%		

[a] Past or present use.
[b] ≥4 drinks/day.
[c] Includes samples with mixed moderate/poor histology.

[6] Internet address: http://www.genedata.com.

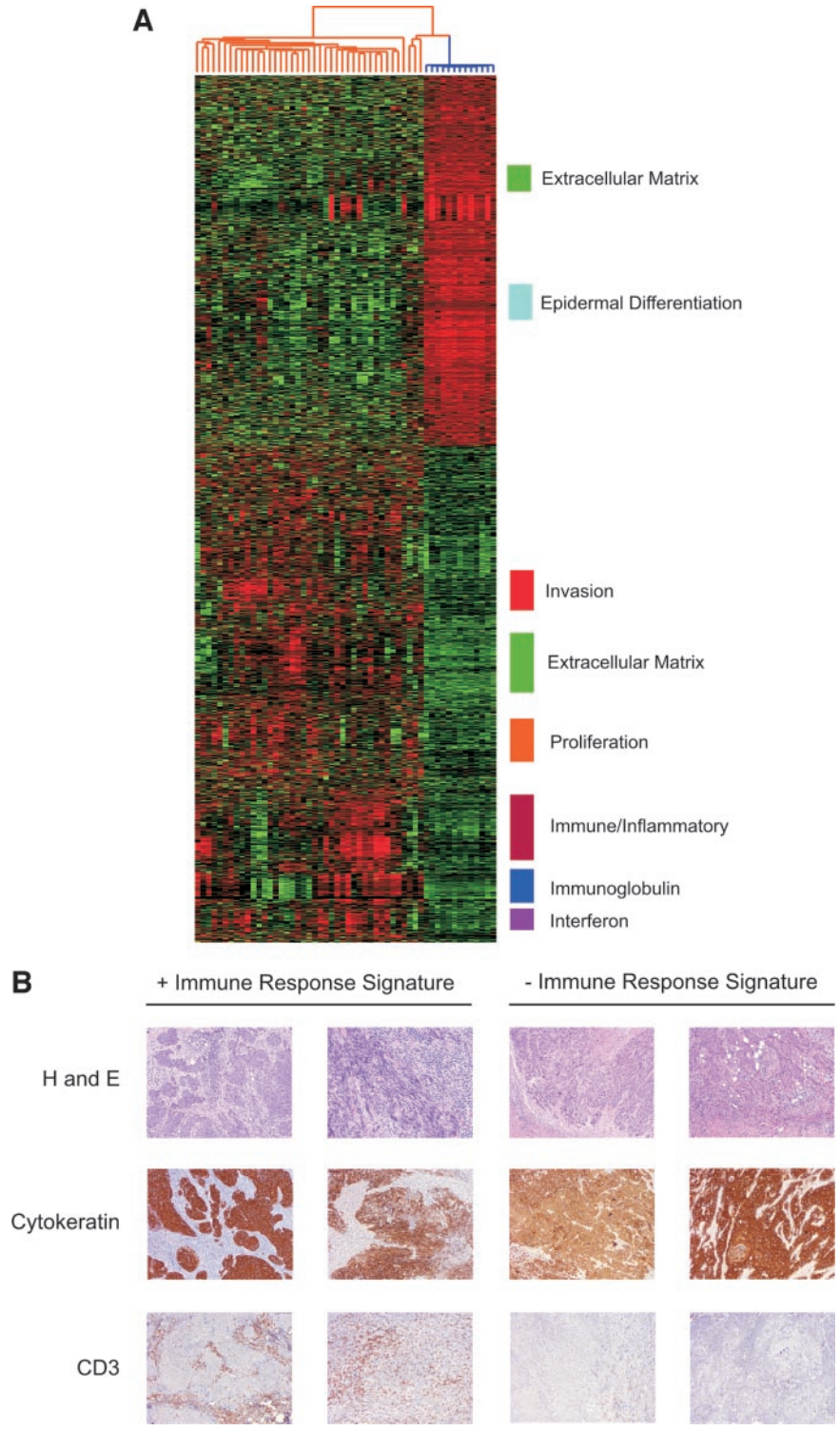

Fig. 1. Gene expression profiles of 41 HNSCC and 13 normal oral mucosal biopsy samples. Differentially expressed genes were identified by parametric T tests with correction for unequal sample variance. A total of 2890 genes were identified using the following filter criteria: *t* test $P < 0.001$, difference in mean intensity ≥ 100, fold change in mean intensity ≥ 2.0 or ≤ 0.5. To visualize the data, genes and samples were grouped using Cluster[7] and graphically represented in Treeview.[8] Prominent gene expression signatures are highlighted with *colored bars* to the right of the figure. *B*, immunohistochemistry staining of two representative tumors with and without the immune response signature using antibodies against cytokeratin and CD3, markers for squamous carcinoma and T lymphocytes, respectively.

and larynx (37%) and over two-thirds of the cancers were advanced stage (stage III/IV) consistent with referral patterns to the University of Minnesota. Ninety percent of patients smoked tobacco and one-third drank alcohol in excess of 4 drinks/day. We chose to compare gene expression in HNSCC to that of NOM from healthy volunteers because these tissues would likely provide the widest spectrum of gene expression differences and would not be subject to the field cancerization effect observed in this malignancy when using normal mucosa adjacent to resected tumor. No attempt was made to control for tobacco or alcohol consumption in the control group assuming that the potential influence of these factors on gene expression would likely be overshadowed by the differences between normal and neoplastic tissue. Of the 13 normal subjects, 1 was currently a smoker, 5 had smoked at some time in the past, whereas the remaining 7 had never smoked. None of the 13 normal subjects reported excessive alcohol use defined as ≥4 drinks/day.

To visualize the gene expression data, hierarchical clustering was performed using genes that satisfied stringent filtering criteria

[7] Internet address: http://rana.lbl.gov.

Table 2 *Functional gene classes demonstrating significant differential expression between HNSCC and NOM*

Function Name	Total[a]	P[b]	FDR[c]	Bonferroni[d]
Biological process				
Inflammatory response	71	1.11E-16	4.72E-14	4.72E-14
Immune response	95	8.44E-15	1.79E-12	3.59E-12
Epidermal differentiation	38	1.65E-11	2.34E-09	7.02E-09
Cell-cell signaling	100	3.14E-10	3.34E-08	1.34E-07
Cell adhesion	77	5.72E-09	4.86E-07	2.43E-06
Chemotaxis	43	8.73E-09	6.18E-07	3.71E-06
Cellular defense response	40	1.74E-08	1.06E-06	7.39E-06
Development	80	3.44E-08	1.83E-06	1.46E-05
Antimicrobial humoral response (sensu Invertebrata)	45	9.90E-08	4.68E-06	4.21E-05
Response to viruses	18	7.16E-07	3.04E-05	3.04E-04
Cell surface receptor linked signal transduction	54	3.29E-06	1.27E-04	1.40E-03
Cell motility	47	3.55E-06	1.26E-04	1.51E-03
Cell proliferation	79	1.81E-05	5.90E-04	7.67E-03
Protein biosynthesis	6	1.81E-05	5.49E-04	7.69E-03
Skeletal development	36	2.59E-05	7.34E-04	1.10E-02
Signal transduction	221	6.40E-05	1.70E-03	2.72E-02
Cellular component				
Extracellular matrix	42	1.21E-10	1.59E-08	1.59E-08
Extracellular space	105	1.70E-09	1.12E-07	2.23E-07
Extracellular	40	5.69E-08	2.49E-06	7.46E-06
Integrin complex	13	3.22E-05	1.06E-03	4.22E-03
Intermediate filament	18	4.22E-05	1.11E-03	5.53E-03
Plasma membrane	135	8.73E-05	1.91E-03	1.14E-02
Collagen type IV	8	9.89E-05	1.85E-03	1.30E-02
Cytosol	30	1.96E-04	3.21E-03	2.57E-02
Molecular function				
RNA binding	9	6.38E-12	3.33E-09	3.33E-09
Chemokine	29	3.23E-11	8.43E-09	1.69E-08
Collagen	29	1.30E-10	2.26E-08	6.79E-08
Cell adhesion molecule	59	1.43E-09	1.86E-07	7.44E-07
Structural constituent of muscle	24	4.48E-08	4.68E-06	2.34E-05
Cytokine	18	9.40E-06	7.01E-04	4.91E-03
Hyaluronic acid binding	8	1.34E-05	8.74E-04	6.99E-03
Cell adhesion receptor	21	1.69E-05	9.79E-04	8.81E-03
Proteoglycan	9	1.72E-05	8.98E-04	8.98E-03
Structural constituent of cytoskeleton	37	1.90E-05	9.00E-04	9.89E-03
Endopeptidase inhibitor	17	2.34E-05	1.02E-03	1.22E-02
Tropomyosin binding	8	2.80E-05	1.12E-03	1.46E-02
Extracellular matrix glycoprotein	12	5.91E-05	2.20E-03	3.08E-02
Collagenase	5	7.87E-05	2.74E-03	4.11E-02

[a] Total number of genes identified in list of 2890 differentially expressed genes corresponding to specific Gene Ontology classification.
[b] Binomial distribution *P*-value.
[c] Adjusted *P*-value using False Discovery Rate (FDR) correction (Ref. 47).
[d] Adjusted *P*-value using Bonferonni correction (*P*-value $\times$ total genes for that function).

($P < 0.001$, $\Delta > 100$, fold difference ≥ 2.0 or ≤ 0.5) yielding a highly discriminant set of 2890 genes (Fig. 1*A* and supplemental data Table 2). A number of gene expression signatures are apparent by visual inspection mostly associated with genes over expressed in HNSCC. The expression pattern of genes down-regulated in HNSCC compared with NOM is relatively uniform, although a few clusters of functionally relevant genes can be defined. A cluster of genes mapping to a region of chromosome 1q21 (involucrin, *SPRR 1A*, *1B*, *2B*, *3*, *NICE-1*, *S100 A1*, *6*, *8*, *9*, *14*), termed the epidermal differentiation complex, is down-regulated in the majority of HNSCC samples. Genes located in this region are highly expressed in terminally differentiated squamous epithelium and have been shown to play an important role in maintenance of the epithelial phenotype (39). Down-regulation of these genes in HNSCC is consistent with dedifferentiation of squamous epithelial cells associated with malignant transformation. Another cluster of genes down-regulated in HNSCC includes several extracellular matrix proteins regulating epithelial adhesion and collagen fibril formation [tenascin X, dermatopontin, and collagen 14 type, α 1 (undulin)]. No specific role in cancer progression has been ascribed to dermatopontin or collagen 14. However, tenascin X knockout mice demonstrate increased susceptibility to tumor invasion and metastasis after inoculation of footpads with B16-BL6 melanoma cells (40).

Genes up-regulated in the HNSCC samples formed several signatures enriched for genes associated with an array of biological processes, including extracellular matrix functions, interferon response, proliferation, and immune response. The proliferation signature included many genes involved in cell cycle control and chromosome maintenance and has been identified in transcriptional profiling studies of other cancers and likely represents a common cancer-specific gene expression program (41). A prominent signature associated with genes expressed in inflammation and immune response was present in over half of the squamous carcinomas and likely represents a robust tumor immune response. To investigate whether this signature was in part because of tumor infiltrating immune cells and not because of aberrant expression of these genes by HNSCC, we selected two tumors expressing and not expressing this signature and performed immunohistochemistry staining using antibodies that identify squamous carcinoma (cytokeratin) and T lymphocytes (CD3). Although the percentage of squamous carcinoma in each specimen is roughly equivalent, tumor-infiltrating T lymphocytes are clearly more abundant in tumors expressing the immune response signature, thus verifying the immune response signature is due, in part, to tumor-infiltrating T lymphocytes (Fig. 1*B*).

Another prominent gene expression signature highly expressed in a subset of HNSCC includes genes implicated in invasion (snail homologue 2, met proto-oncogene; Refs. 42, 43), *RAS* signaling, (r-ras homologue 2), cell surface/extracellular matrix interactions (collagen type IV α_5 and α_6, laminin γ_2, α_3, β_3; integrin α_3, α_6, β_4, β_6), and angiogenesis (vascular endothelial growth factor C, placental growth factor). The high expression of these genes in this subset of tumors

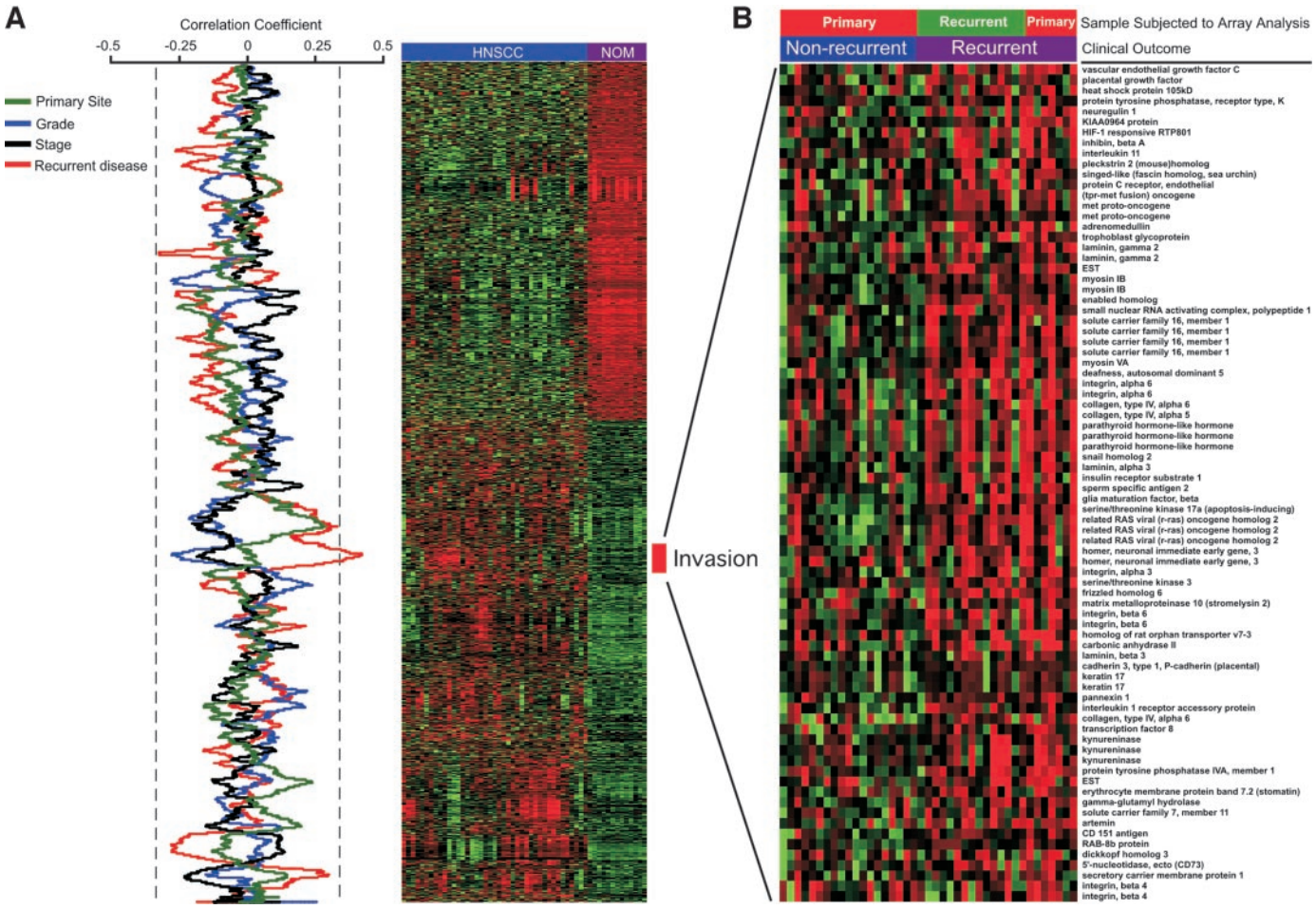

Fig. 2. Correlation of gene expression signatures with clinical parameters. *A*, correlation coefficients were calculated for each gene and clinical parameters corresponding to site of primary disease, stage of disease, tumor grade, and presence of recurrent disease after definitive treatment. Correlation coefficients are plotted as a moving window average of 30 genes to the left of the cluster diagram. The level at which there is a $\leq$1% chance that the correlation is spurious was determined by 5000 permutations of the clinical parameter classifications and is depicted by the *vertical dashed lines*. *B*, the genes that comprise the gene expression signature correlating with recurrent disease were identified by grouping samples by the presence or absence of recurrent disease and performing one-dimensional clustering of genes only. The label at the top of the figure designates the type of sample that was applied to the microarray (cRNA from recurrent or primary tumor).

suggests a potentially aggressive phenotype prone to invasion and metastasis.

To more thoroughly characterize sets of functionally related genes differentially expressed between HNSCC and NOM, we used OntoExpress (44, 45) to classify genes according to the following Gene Ontology (GO) (46) categories: biological process; cellular role; and molecular function. The numbers of genes corresponding to each GO category among the 2890 differentially expressed genes was tallied and compared with the number of genes expected for each GO category based on their representation on the Affymetrix U133A array. Significant differences from the expected were calculated with a two-sided binomial distribution. False discovery rates (47) and Bonferroni adjustments were also calculated based upon the number of GO categories having at least 1 gene in the list of 2890 differentially expressed genes. Table 2 shows all GO functional classes with a Bonferroni-corrected significance of $P < 0.05$, the significance of each class, the false discovery rate for that class, as well as the number of genes corresponding to each GO functional class identified in our differentially expressed gene list. The functional gene groups demonstrating the most significant representation in our set of differentially expressed genes appear under the biological process ontology and map to the inflammatory response, immune response, and epidermal differentiation categories. Genes involved in inflammatory and immune response are highly expressed in subsets of HNSCC and correlate with the presence of tumor infiltrating immune cells (Fig. 1, *A* and *B*). In contrast, genes corresponding to the epidermal differentiation category are highly expressed in NOM compared with HNSCC, reflecting the loss of normal epithelial architecture associated with malignant transformation (Fig. 1*A*). Functional categories significantly represented under the cellular component and molecular function ontologies include genes involved in extracellular matrix functions, integrin complexes, RNA binding, chemokine signaling, and cell adhesion.

A major aim of this study was to identify correlations between gene expression signatures and clinical parameters with the goal of identifying new gene expression-based biomarkers of HNSCC. To identify associations between genes and clinical parameters, we calculated correlation coefficients for each gene across all 41 HNSCC tumor samples with stage of disease, tumor grade (well, moderate, or poorly differentiated), primary tumor site, and presence of locally recurrent disease. The significance of the correlation was determined by permuting the class labels (5000 permutations) pertaining to each clinical parameter and calculating a level at which the correlation would possess a type 1 error of $\leq$1%. The resulting correlation curves are plotted alongside the corresponding gene as a moving average (window size = 30 genes; Fig. 2*A*). Interestingly, no correlations exceeding the 1% type one error threshold were seen between gene expression signatures and

tumor stage, tumor grade, or anatomical location of the primary tumor. However, a correlation (correlation coefficient = 0.42, $P < 0.01$) exceeding this threshold was detected between patients who experienced recurrent disease and the signature previously identified as enriched for genes associated with tumor invasion and metastasis (Fig. 1*A*). Importantly, the signature associated with recurrent disease was present in 7 primary tumors procured before administration of definitive therapy (Fig. 2*B*). Therefore, it is quite likely that the recurrent disease signature is an intrinsic feature of a subset of HNSCC tumors and not simply a biased result secondary to analyzing a collection of recurrent tumors. Furthermore, all 7 patients whose tumors expressed the recurrent disease signature recurred within 12 months (median time to recurrence = 8 months) of completing therapy, indicating that this signature may function as a predictor of early local treatment failure in HNSCC.

To confirm the findings of the microarray analysis we performed real-time PCR (quantitative-PCR) analysis using primers specific for *MET*, *SNAI2*, and *PTHLH* and compared the fold differences in expression predicted by microarray to that obtained with quantitative-PCR. Twelve tumor samples demonstrating a wide range of expression values for *MET*, *SNAI2*, and *PTHLH* by microarray were selected for analysis. These genes were selected for confirmation because of their prominent overexpression in the recurrent disease signature. For expression values from microarray, the mean intensity for each probe set [two for *MET* (Affymetrix probe IDs: 203510_at, 213807_x_at), one for *SNAI2* (213139_at), and three for *PTHLH* (206300_s_at, 210355_at, 211756_at)] was normalized to the mean *GAPDH* intensity [three probe sets (M33197_5_at, M33197_M_at, and M33197_3_at)] for each of the 12 tumor samples. We selected *GAPDH* as the normalization standard because it was uniformly expressed across all tumor samples by microarray. Normalized expression values from microarrays were then rank ordered from highest to lowest for each of the transcripts and fold changes calculated by dividing the sample with the highest expression by the sample with the lowest expression. This was repeated sequentially using the next highest and lowest values until all fold change calculations were made. The procedure was then repeated using the corresponding normalized expression levels for each transcript for all 12 samples derived by quantitative-PCR. Fold change concordance for each transcript is shown in Table 3. Quantitative-PCR confirmed the direction of fold change for nearly all of the samples where the microarray fold change was >2.0 with one exception. Overall, these results confirm our findings of differential gene expression by microarray analysis.

Table 3 *Concordance in fold change direction between microarray and real-time PCR (QPCR) expression levels*

MET		PTHLH		SNAI2	
Microarray	QPCR	Microarray	QPCR	Microarray	QPCR
37.7	8.2	172.2	837.9	7.3	30.4
8.7	14.9	115.3	41.4	5.1	13.8
1.9	0.3	36.3	124.8	4.5	10.6
1.5	2.2	10.6	6.3	2.8	0.5
1.4	1.3	7.3	15.5	2.1	2.1
1.1	0.7	1.7	0.6	1.0	0.7

DISCUSSION

The objectives of this study were to investigate the range of gene expression differences between HNSCC and NOM to identify gene expression programs disregulated in HNSCC and to correlate gene expression signatures with clinical parameters to identify potentially new biomarkers of HNSCC. Using statistical and data filtering criteria, we identified 2890 genes differentially expressed between HNSCC and NOM. Functional gene classes (defined by GO annotation) highly represented in HNSCC include those involved in inflammatory response, immune response, epidermal differentiation, cell adhesion, and extracellular matrix functions.

The presence of a robust immune response in a subset of HNSCC tumors is associated with infiltrating T lymphocytes within the tumor (Fig. 1*B*), a phenomenon well documented in the literature for HNSCC as well as other cancers (48). Earlier studies on HNSCC reported that a peritumoral lymphocyte infiltrate was associated with a better prognosis when compared with tumors not demonstrating immune infiltrate (49–53). In addition, greater peritumoral lymphocyte infiltrate correlated with lower tumor stage and less invasive growth (51–53). More recent work has shown that tumor-infiltrating lymphocytes in HNSCC acquire defects in T-cell receptor signaling and undergo apoptotic death as a result of tumors that express *FAS* ligand on their cell surface (54, 55). Interestingly, circulating T lymphocytes from patients with HNSCC demonstrate defects in tumor directed cell killing suggesting that events that modulate immune function in the tumor bed may extend to systemic immune system dysfunction in patients with HNSCC (56). Other work has demonstrated that primary tumors from patients with HNSCC and HNSCC cell lines express a variety of proinflammatory cytokines, including interleukin 1a, interleukin 6, interleukin 8, and granulocyte macrophage colony-stimulating factor that may attract immune effector cells to the tumor microenvironment (57). It is interesting to speculate from our data that the presence of a robust tumor immune/inflammatory response may be associated with decreased risk for local treatment failure. The data presented in Fig. 1*A* demonstrate that tumors highly expressing the recurrent disease signature lack a substantial immune/inflammatory response signature. Although we cannot rule out the possibility that some of this difference may be attributable to variations in the cellular components of the tissue samples, this may represent a potentially important interaction between the immune system and the tumor where genes expressed in the recurrent disease signature modulate antitumor immune system responses.

The correlation of a gene expression signature with risk for local treatment failure in HNSCC identifies a potential new gene expression-based biomarker for HNSCC. The genes that comprise this signature are interesting from an oncological perspective and many have been identified as important in HNSCC as well as other cancers. In particular, the met proto-oncogene and its cognate ligand, hepatocyte growth factor/scatter factor display a diverse range of functions in carcinogenesis, including modulation of cell proliferation, migration, survival, angiogenesis, and invasion (58). *MET* has been shown to be overexpressed in a wide variety of epithelial based cancers through gene amplification events, increased transcription, or constitutively activated via mutation (58). In HNSCC, overexpression of met has been demonstrated in primary tumors and lymph node metastasis by immunohistochemistry (59, 60) and is inversely correlated with survival in patients with nasopharyngeal cancer (61). Recent work has demonstrated hepatocyte growth factor/met pathway important for inhibition of apoptosis upon loss of extracellular matrix contact in HNSCC cell lines (62).

A second interesting gene identified in the recurrent disease signature is snail homologue 2 (*SNAI2/SLUG*). *SNAI2* belongs to a family of transcriptional repressors that include Snail (*SNAI1*), *E47*, *ZEB-1*, and *SIP-1* implicated in initiating epithelial/mesenchymal transitions through repression of E-cadherin gene transcription (63). *SNAI2* represses E-cadherin gene expression and triggers a complete epithelial/mesenchymal transition in MDCK cell lines (42). In breast cancer cell lines, both Snail and *SNAI2* repress E-cadherin gene expression, however, *SNAI2* demonstrates a much stronger correlation with E-cadherin transcriptional repression than Snail (64). *SNAI2* also ap-

pears to modulate sensitivity to apoptotic stimuli because it is able to protect hematopoietic progenitor cells from radiation-induced apoptosis *in vivo* (65). To our knowledge, this is the first evidence demonstrating overexpression of *SNAI2* in HNSCC.

The recurrent disease signature also contains several genes important in tumor cell/extracellular matrix interactions. For instance, laminin α_3, β_3, and γ_2 and integrin α_6 and β_4 are all present in this signature. The three laminin subunits together comprise laminin 5, which serves at the ligand for the $\alpha_6\beta_4$ integrin. Laminin 5 and integrin $\alpha_6\beta_4$ are up-regulated in squamous carcinoma of the skin, colon, esophagus, and larynx, and higher expression is correlated with increased invasiveness (66). The importance of laminin 5-$\alpha_6\beta_4$ integrin interactions in promoting invasive squamous carcinoma was recently demonstrated using a model in which human epidermal keratinocytes stably transfected with oncogenic RAS and IκBα were transplanted into *scid/scid* mice (67). The identification of laminin 5 subunits and integrin $\alpha_6\beta_4$ expressed in the recurrent disease signature from our study provides additional evidence that this signature is associated with a highly invasive subset of HNSCC tumors.

Several gene expression profiling studies in head and neck cancer have been performed recently (68–79). These studies have used a variety of array platforms, tissue types, and experimental designs, making direct comparisons with our data difficult. However, the results of two studies, performed on primary tumor tissues with reasonably large sample sizes, can be compared with our study. The study by Belbin *et al.* (68) evaluated gene expression signatures in 17 patients with HNSCC. Their study was a two-color cDNA array analysis using mRNA from normal human epithelial keratinocytes as a control. Two groups of squamous cell carcinomas were identified based on supervised clustering with confirmation of the clustering results performed using a bootstrap resampling method. Modest association was seen with poorer cause-specific survival at 2 years in tumors expressing a group 1 signature.

In other work, Mendez *et al.* (69) evaluated 26 oral squamous carcinomas, 2 premalignant lesions, and 18 normal mucosal samples, 9 of which were adjacent to oral cancers. They used the Affymetrix HuGene FL platform for their analyses and a series of regression modeling techniques and unsupervised clustering methodologies to determine differentially expressed genes. The aim of this study was to identify gene expression signatures that differentiate early-stage from late-stage disease or metastatic from nonmetastatic cancers. No significant differences in gene expression were seen that defined early- and late-stage disease or metastatic and nonmetastatic cancers. Our data concurs with their finding that gene expression signatures do not correlate well with stage of disease. However, our data set is unable to assess the relative contribution of metastatic *versus* nonmetastatic tumors because no metastatic tumors were included in our study.

The data presented in this article identify distinct gene expression signatures that differentiate HNSCC from NOM. The complex gene expression patterns identified in our study are due in part to our choice to use bulk tumor tissues for the analysis. As such, this study is limited in the ability to differentiate tumor-specific gene expression, and additional studies using microdissected tissues will be needed to address this issue. The use of bulk tumor tissue, however, does provide substantial amounts of information related not only to the tumor itself but also to the molecular events ongoing in the tumor microenvironment. We provide evidence for the association of a gene expression signature enriched for genes associated with invasion and metastasis with the incidence of locally recurrent disease. The identification of this signature in 7 primary tumors suggests that this signature is intrinsic to the tumorigenic process and may serve as an important biomarker of local treatment failure in HNSCC. Future studies will focus on confirming these findings in an independent, prospectively collected cohort of HNSCC patients with the goal of implementing gene expression-based biomarkers into the clinical management of patients with HNSCC.

ACKNOWLEDGMENTS

We thank Drs. Timothy Behrens, Kathy Moser, Erik Peterson, Brian Van Ness, and Jennifer Hall for critical review and helpful discussions during the preparation of this article. We also thank Sarah Bowell and Diane Rauch of the University of Minnesota Cancer Center Tissue Procurement Facility, Aaron Becker and Jill Plumb-Smith of the University of Minnesota Affymetrix Core Facility, and SeonaCherie Hanson of the Department of Laboratory Medicine and Pathology Immunohistochemistry Laboratory for their expert technical assistance.

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