Background: Current treatment modalities in squamous cell carcinoma of the head and neck have failed to improve survival. Advances in the discovery of novel biomarkers and targets for therapy are necessary.

Design: Differential display and microarray analysis were used to identify differences in gene expression between squamous carcinoma and matched nonmalignant biopsy specimens. Differences in gene expression found in vivo were also tested in vitro by comparing primary cultured normal oral epithelium with head and neck squamous cell carcinoma (HNSCC) cell lines. Results were confirmed by relative reverse transcriptase–polymerase chain reaction and immunohistochemical analysis.

Results: In tumors, microarray analysis showed down-regulation of calgranulin B (CAGB), CD24, lymphoepithelial Kazal-type–related inhibitor (LEKTI), zinc finger protein (ZNF-185), transglutaminase-3 (TGM3), and the ETS homologous factor (EHF). In addition, differential display revealed down-regulation of headpin. In contrast, periostin and the human homologue of the Drosophila white gene (ABCG1) were found to be up-regulated by microarray analysis and differential display, respectively. In HNSCC cell lines, LEKTI, ZNF-185, TGM3, headpin, and ABCG1 showed an expression pattern similar to that observed in tumor specimens. Periostin showed an opposite expression pattern in cell lines compared with that of tumor specimens. No consistent pattern of expression was found for CAGB, CD24, and EHF in cell lines. Immunohistochemical analysis revealed that the expression of headpin in nonmalignant mucosa was undetectable in tumors.

Conclusion: Using differential display and microarray analysis, we have identified and confirmed the differential expression of 9 genes in HNSCC. Work is in progress to determine the biological significance of these genes and their potential as biomarkers or targets for therapy.

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cell lines and identified several differentially expressed genes, one of which was shown to be EGFR. Simultaneously, Patel et al compared cell lines derived from primary tumors and matched metastases and identified 6 novel genes that may be related to metastatic potential. More recently, a study using both DD and MA in murine cell lines has shown differential expression of a number of genes involved in growth, apoptosis, angiogenesis, and the nuclear factor-κB signal pathway. Further investigations by Villaret et al in HNSCC tissue samples using a subtractive hybridization library found 13 independent genes to be overexpressed, 4 of which were previously unidentified. Other studies using MA have compared HNSCC tissue biopsy specimens, showing differential expression of a wide range of genes known to be related to different processes in cancer, including apoptosis, angiogenesis, and cell cycle. The most recent work has proposed gene profiling by MA for the prediction of patient outcome.

In the present study, using both DD and MA, we identified 9 genes with significant differential expression in HNSCC tissue and for which the biological implications in head and neck cancer have yet to be characterized. Given that cancer represents complex alterations in the expression of numerous genes, comprehensive fingerprinting of these transcriptional differences in both well-studied and uncharacterized genes is paramount to our understanding of potential molecular therapeutic targets.

Consent was obtained from all patients for tissue procurement by a nonsurgical team research member under an institutional surveillance committee–approved protocol for tumor banking. Tumor samples and adjacent nonmalignant mucosa were obtained from 3 patients with SCCs of the oropharynx. For each patient, comparisons were made between the tumor biopsy specimen and the corresponding matched nonmalignant mucosa from the same patient. All samples were pathologically confirmed and snap-frozen in liquid nitrogen. Additional specimens were maintained in optimal cutting temperature medium at −80°C, as well as by formalin fixation and paraffin embedding.

All oligonucleotide primers were synthesized and cartridge purified by Genosys Biotechnologies, Inc (The Woodlands, Tex). Superscript II (GibcoBRL, Gaithersburg, Md) reverse transcriptase was used in all reverse transcription reactions. Sequencing of DD products was performed by using the Sequenase Kit (Amersham, Piscataway, NJ). Sequencing of complementary DNA (cDNA) clones for sequence establishment and confirmation were performed by the M. D. Anderson Cancer Center DNA core facility (Houston, Tex). Stoffel Taq Polymerase (Perkin-Elmer, Norwalk, Conn) was used for the relative reverse transcriptase–polymerase chain reaction (RT–PCR). A monoclonal antibody for headpin was generously provided by Transduction Laboratories (Lexington, Ky).

DIFFERENTIAL DISPLAY RT-PCR

Total RNA was isolated from tissue specimens using the Trizol Reagent (GibcoBRL) and contaminating DNA removed by DNase I treatment. Differential display RT-PCR was performed using a kit (Display Systems, Los Angeles, Calif) that uses 24 arbitrary upstream primers and 9 downstream primers (dT11VN). Polymerase chain reaction products labeled with [α-33P]dATP were resolved by nondenaturing polyacrylamide gels and excised following autoradiography. Recovered fragments were reamplified, cloned into the pCR-TRAP plasmid (GenHunter, Nashville, Tenn), and sequenced.

MICROARRAY ANALYSIS

Probe preparation and microarray hybridization were performed by Incyte Genomics (Palo Alto, Calif). Poly A+ RNA was isolated from tissue specimens, and labeled cDNA was prepared using nucleotides labeled with the fluorescent dyes Cy3 or Cy5. Labeled cDNAs were then subjected to competitive hybridization to UnIGEM V microarrays, which contained spotted cDNAs corresponding to 9330 expressed sequence tags. Primary quantitative data, spot geometry, and background fluorescence analysis were performed by Incyte. Defective cDNA spots (ie, those with irregular geometry, scratched, or containing less than 40% of the area compared with average) were eliminated from the data set. All data sets were normalized by using a balancing coefficient of the median of all Cy5 channel measurements divided by the median of all Cy3 channel measurements, to give balanced differential expression ratios. Statistical methods were used to determine which of the more than 9000 genes examined by MA should be studied further (ie, confirmation by relative RT–PCR). Essentially, probability density functions were estimated from the log of the balanced differential expression ratios of all the genes analyzed and a set of control genes included on the MA. Only genes with ratios within the upper and lower 2.5 percentiles for all 3 experiments were considered for further analysis. The probability for a balanced differential expression ratio to fall in the 2.5 percentile for all 3 experiments by chance was calculated (P<.001).

The sequences of clones that showed differential hybridization were analyzed by basic local alignment search tool (BLAST) homology search against all available sequence databases. In addition, MA was performed on a GF-204 filter obtained from Research Genetics (Huntsville, Ala) following the manufacturer’s instructions. The gene filters were read using a Storm-860 PhosphoImager (Molecular Dynamics, Sunnyvale, Calif) and analyzed using ImageQuant 5.1 software (Molecular Dynamics). Statistical analysis identical to that used on the Incyte microarrays was performed on this data set as well.

RELATIVE RT-PCR

Total RNA was isolated from normal and tumor site–matched biopsy tissue and also extracted from 7 established head and neck tumor cell lines and primary cultures of normal epithelial cells. Using 2 μg of total RNA, reverse transcriptase reactions of the biopsy specimens were performed in duplicate, while those of cell lines were performed in single reactions.

Probes specific for genes were chosen with the least nucleotide identity to their respective homologous genes. Prior to relative RT-PCR, a PCR titration using 2 dilutions (1:20 and 1:200) of the highest expression biopsy specimen was performed to establish the PCR conditions that were in linear range for detecting the genes of interest. The selected primer sequences are given in Table 1. After the appropriate PCR cycle number was established (Table 1), relative RT-PCR was performed under the following PCR conditions: a hot start at 94°C for 30 seconds, followed by the appropriate number of cycles of 94°C×30 seconds, annealing×30 seconds, and 72°C×1 minute, followed by a 7-minute extension at 72°C. Annealing temperatures were set at 5°C below the lower melting temperature of a given primer pair. The 18S competitor primer system (Ambion, Austin, Tex) designed to PCR amplify a fragment of the 18S RNA was used as an internal control for normalization of PCRs. At a competitor-primer ratio of 8:2, the 18S product
Table 1. Primers Used for Relative RT-PCR Amplification

| Gene  | Primers (5'-3')   | Cycles |
|-------|-------------------|--------|
| CAGB  | S-ACC AGG GGG AAT TCA AAG AG | 24     |
|       | AS-ATG ACC CTC TGT CAT CTT CT |        |
| CD24  | S-AAC TAA TGC CAC CAC CAA GC | 25     |
|       | AS-CTT TTT CCA TGG CAC AT |        |
| LEKT1 | S-TGT GCC ATG TGT AAG CAG GT | 23     |
|       | AS-TCA TGG TCA CTC GCT ACA GG |        |
| EHF   | S-AGG AGG TGG TGA GAT TGG AG | 26     |
|       | AS-AAA CCG ACT GGA AAC ATT GC |        |
| Peristin | S-GTC TTT GAG ACG CTG GAA GG | 25     |
|       | AS-AGA TCC GTG AAG GTG TTG TG |        |
| ABCG1 | S-CTC CGG TCT CTT CTT CT | 32     |
|       | AS-TAC ACG ATG CAG TAG GC |        |

Abbreviations: AS, antisense; RT-PCR, reverse transcriptase–polymerase chain reaction; S, sense.

Table 2. Relative Expression of Various Genes in HNSCC Tissue Specimens Compared With Matched Nonmalignant Tissue

| Gene   | Expression (Fold) | Method | Predicted Function |
|--------|-------------------|--------|--------------------|
| CAGB   | ↓ (14.2)          | MA     | Proliferation/differentiation |
| CD24   | ↓ (8.7)           | MA     | Cell adhesion       |
| LEKT1  | ↓ (6.6)           | MA     | Protease inhibitor  |
| ZNF-185| ↓ (5.4)           | MA     | Proliferation/differentiation |
| TG3M3  | ↓ (32.1)          | MA     | Differentiation     |
| EHF    | ↑ (7.3)           | MA     | Transcription factor |
| Headpin| ↓ (NA)            | DD     | Protease inhibitor  |
| Peristin| ↑ (29.7)         | MA     | Cell adhesion       |
| ABCG1  | ↑ (NA)            | DD     | ABC transporter     |

Abbreviations: ABC, adenosine triphosphate-binding cassette; DD, differential display; HNSCC, head and neck squamous cell carcinoma; MA, microarray analysis; NA, not applicable.

was also in linear range. Relative RT-PCR products were resolved on a 6% polyacrylamide gel in 0.5% Tris/acetate acid/EDTA buffer run at 18 W.

**IMMUNOHISTOCHEMISTRY**

Paraffin-embedded sections (7 μm) were obtained from the same patients examined by DD and MA. Sections were deparaffinized and heated at 65°C, followed by rehydration through successive washes in xylene and decreasing concentrations of ethanol in water. Antigens were retrieved by incubating the sections with Unmasking solution (Vector Laboratories, Burlingame, Calif) in a steamer apparatus for 20 minutes, followed by two 5-minute washes in phosphate-buffered saline (PBS) and blocked in Biotin Block solution (Vector Laboratories) for 30 minutes at room temperature. After two 5-minute washes in PBS, sections were subsequently incubated with a protein blocking solution (Dako Corp, Carpinteria, Calif) for 30 minutes at room temperature and then incubated overnight with the primary antibody or appropriate control IgG at 4°C at the appropriate dilutions (headpin 1:150 dilution with 0.1% bovine serum albumin in PBS). After two 5-minute washes, sections were incubated with the secondary antibody provided in the appropriate Vectastain Kit (Vector Laboratories) for 30 minutes at room temperature, washed twice for 5 minutes in PBS, incubated with the avidin-biotin complex reagent for 30 minutes at room temperature, and washed twice for 5 minutes in PBS. Antigen-antibody complexes were visualized using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate (Vector Laboratories) for 30 minutes at room temperature, and washed twice for 5 minutes in PBS. Dotted and solid vertical lines are the lower and upper 2.5 percentiles of log ratios for the control and detectable gene data sets, respectively. All genes listed in Table 2 (by microarray analysis) are outside the 2.5 percentiles, indicated by solid circles. Identical results were obtained for all 3 patients (P < .001).

**RESULTS**

**DIFFERENTIAL DISPLAY AND MICROARRAY ANALYSIS**

To identify changes in the expression of various genes in HNSCC, DD and MA were performed on tumor specimens of the oropharynx and patient-matched nonmalignant mucosa obtained from 3 individuals. For MA, a probability density function was estimated using a log transformation of the balanced differential expression ratios. Genes that fell outside the lower and upper 2.5 percentiles of the distribution (Figure 1) for all 3 patients and were potentially involved in processes linked to cancer were selected for further analysis (Table 2). Microarray analysis showed reduced expression of calgranulin B (CAGB), CD24, EHF, lymphoepithelial Kazal-type–related inhibitor (LEKT1), zinc finger protein (ZNF-185), and transglutaminase-3 (TGM3) and an increased expression of peristin (Table 2). Alternatively, DD showed a reduced expression of headpin in tumors (Table 2) and an increased expression of ABCG1 (Figure 2).

**RELATIVE RT-PCR**

To confirm differences in expression of genes identified by DD and MA, relative RT-PCR of the same HNSCC specimens and matched nonmalignant tissue was performed. Results for 6 such genes are shown in Figure 3. Consistent with DD and MA, the strong bands representing the

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**Figure 1.** Probability density function of gene expression levels. This graph was obtained from a microarray comparing normal and tumor tissue from 1 patient. A log transformation was performed on the ratios of balanced differential expression to estimate a probability density function. Dashed curve was estimated using data from normalization controls included on the microarray. Solid line was estimated from all detectable genes included on the array. Dotted and solid vertical lines are the lower and upper 2.5 percentiles of log ratios for the control and detectable gene data sets, respectively. All genes listed in Table 2 (by microarray analysis) are outside the 2.5 percentiles, indicated by solid circles. Identical results were obtained for all 3 patients (P < .001).

**Figure 2.** (REPRINTED) ARCH OTOLARYNGOL HEAD NECK SURG/VOL 129, JULY 2003 WWW.ARCHOTO.COM

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expression of CAGB, CD24, LEKTI, and EHF in nonmalignant tissue were dramatically decreased in all respective matched tumor samples (Figure 3). Relative RT-PCR also confirmed the down-regulation of ZNF-185, TGM3, and headpin in tumor specimens (not shown). On the contrary, an increased expression of peristin and ABCG1 was detected in tumors (Figure 3), which was consistent with the respective MA (Table 2) and DD (Figure 2).

Since there is some heterogeneity in the origin of cells present in biopsy specimens, it is helpful to examine the expression of identified genes in homogeneous cell cultures as well. This can provide clues regarding the cellular origin of gene expression detected in biopsy specimens. We examined the relative expression of genes listed in Table 2 in cultured normal oral epithelium (NOE) and established HNSCC lines. CAGB, which was downregulated in tumor biopsy specimens, was not expressed in 3 of 7 established HNSCC lines. The expression of CD24 and EHF in all HNSCC cell lines was at the same or higher level as that of NOE cells, with the exception of the MDA183 cell line (Figure 4). LEKTI was undetectable in all HNSCC cell lines compared with NOE cells (Figure 4), which was consistent with its downregulation in tumors. Also, the expression of TGM3, headpin, and ZNF-185 in HNSCC cell lines was lower than that found in NOE cells (not shown). ABCG1 was upregulated in almost all HNSCC cell lines (Figure 4), as was found in tumor specimens. Interestingly, the low expression of peristin in HNSCC cell lines (Figure 4) was opposite to the increased levels found in tumor specimens (Figure 3).

Levels of gene expression were compared between cultured NOE cells and snap-frozen normal oral mucosa. All genes studied were detected in NOE cells; however, CAGB, CD24, and EHF were detected at a lower level than in nonmalignant oral mucosa (Figure 4).

**IMMUNOHISTOCHEMICAL STAINING**

To characterize the expression of some of the identified genes at the cellular and protein level, immunohistochemical staining was performed in tissue sections obtained from the same matched samples used in relative RT-PCR. The immunohistochemistry for headpin is represented in Figure 4, where the strong immunoreactivity for headpin observed in squamous epithelium of nonmalignant mucosa (Figure 5A) is shown to be clearly absent in the section of matched tumor (Figure 5B). Negative immunohistochemical staining with control isotype mouse IgG ruled out nonspecific staining (Figure 5C and D). Identically designed experiments were performed for TGM3 and EHF, showing that the immunoreactivity of these proteins in the squamous epithelium of nonmalignant mucosa was absent in areas of SCC (data not shown).

**COMMENT**

The identification of genetic alterations associated with cancer has been the basis of our understanding of the processes by which normal cells acquire malignant behavior. Many of these genetic alterations have been discovered by comparing the profile of relative gene expression between tumor cells and nonmalignant cells. Our laboratory has undertaken a search for genes that could serve as biomarkers or targets for biological therapy in head and neck cancer. Initially, DD was chosen as a screening tool because it allows for the identification of previously unknown genes, but was limited by the fact that it is not a high throughput technique. Subsequently, following the introduction of MA, we were able to rapidly extend the screening to a large number of genes. Presently, we communicate the identification of 9 genes differentially expressed in HNSCC by comparing oropharyngeal cancer biopsy specimens with site-matched nonmalignant tissue. The advantage gained through the use of biopsy specimens is the preclusion of potential artifactual changes in gene expression, which may be acquired during the pro-
and peristin and CD24 have been involved in cell adhesion.17,18 It has been well established that proteinases and cell adhesion molecules play pivotal roles in the biological processes of cancer including cell invasion19,20 and metastasis.20,21 Of particular interest is the absence of headpin in tumor specimens, since other serpins have been shown to be tumor suppressors.22 On the other hand, the down-regulation of EHF (transcription factor family) and TGM3 (transglutaminase family), which are normally seen in late differentiating squamous epithelium,23,24 is consistent with the loss of differentiation features in tumor cells. The specific functions of EHF are as of yet unknown; however, other ETS transcription factors have been involved in cellular proliferation, differentiation, and apoptosis.25 Moreover, TGM3, which has also been shown to be down-regulated in esophageal cancer,26 is involved in stabilizing the cell cornified envelope. This process precedes the transition of keratinocytes to become corneocytes by apoptosis. Therefore, down-regulation of TGM3 in HNSCC could be interrupting a potentially critical step to apoptosis, thereby favoring tumor cell survival.

ABCG1 belongs to the family of adenosine triphosphatase–binding cassette transporters. The up-regulation of this recently described gene in HNSCC is interesting because related genes have been associated with resistance to chemotherapy.27 To our knowledge, this is the first time that ABCG1 has been shown to be overexpressed in cancer cells, therefore constituting a potential novel marker for the prediction of chemotherapy resistance. Of all the genes identified, ZNF-185 is of interest owing to the lack of knowledge regarding its function. However, based on its gene structure, it would be predicted to belong to a family of LIM domain–containing proteins involved in regulating cellular proliferation and differentiation.28

Analyzing gene expression in cell cultures is useful because it allows for further study of the mechanisms that alter gene expression profiles between normal and malignant cells. Differences in gene expression of LEKTI, ZNF-185, TGM3, headpin, and ABCG1 in cell lines were consistent with that found in biopsy specimens. However, the decreased expression of CAGB, CD24, and EHF found in tissue specimens was not observed in HNSCC cell lines. Peristin had an unusual pattern of expression in that the differential expression in vivo and in vitro were actually opposite. The complete absence of peristin from all of the HNSCC lines suggests that the high levels observed in HNSCC tumor biopsy specimens may have been due to its expression by stromal cells inhabiting the tumor microenvironment. We observed an identical phenomenon for another differentially expressed gene, BRAK, which was shown to be abundantly expressed in both normal mucosa and stromal cells found specifically in tumors, but virtually absent from the cancerous cells.29 The ability to detect differential expression of genes in stromal elements may actually be advantageous because it reveals potentially important tumor-host interactions.

In some cases, there was a lack of concordance between expression of genes in uncultivated nonmalignant mucosa vs primary cultures of NOE. Although cultured NOE cells display phenotypic characteristics of keratinocytes from the oral mucosa, the profile of gene expression can change when tissue is adapted for in vitro
growth due to a variety of reasons including alterations in growth factors and other extracellular molecules, loss of stromal components, and clonal selection. The changes in the microenvironment may modify the level of expression of a subset of genes, including some of those studied here. This may be related to the relative lack of differentiation present during culturing. Indeed, Redondo et al. have shown that the expression of CD24 increases when primary cultures of keratinocytes are induced to differentiate.

The cellular heterogeneity of tumors can be accounted for and examined by immunohistochemical analysis, which allows for a detailed discrimination of the cells responsible for the expression of a specific protein. In an initial approach to characterize the expression of some of the identified genes at the cellular and protein levels, immunohistochemical analysis was performed for headpin. Results confirmed that the differences in headpin expression occurred between normal squamous epithelium and squamous carcinoma cells. In similar studies, the immunoreactivity of TGM3 and EHF was also found to be reduced in tumors, confirming our initial findings by DD and MA.

In summary, MA and DD are shown to be powerful tools in gene discovery. Owing to the use of different technologies, the 2 methods, when combined, allow for a more complete analysis of gene expression profiles. The genes discovered by DD (headpin and ABCG1) were not part of the expressed sequence tags included on the microarray and therefore could not have been found by this method. Conversely, the genes detected by MA were not isolated by DD. This may be due to the fact that not every quantitative difference will be detectable in DD because of the limitations of multiplex PCR. Nonetheless, those differences in gene expression that were detected by DD proved to be valid by relative RT-PCR. Furthermore, many legitimate differences in gene expression have been described using DD. The genes identified herein have biological functions related to several processes that are known to be involved in tumor progression and metastasis. Further work is under way to understand the biological implications of these findings and the potential of these genes as biomarkers or targets for therapy.

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Corresponding author and reprints: Gary L. Clayman, DDS, MD, Department of Head and Neck Surgery, University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030 (e-mail: gclayman@mdanderson.org).

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