**Plasminogen Activator Inhibitor-2: A Molecular Biomarker for Head and Neck Cancer Progression**

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**Abstract**

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy in which the early diagnosis of premalignant lesions is known to directly correlate with increased survival. However, only a portion of biopsies showing dysplasia will progress to cancer, and there are no currently accepted criteria for predicting which lesions will progress. Therefore, diagnostic protocols that can identify the lesions that are likely to become HNSCC are required. RNA was isolated from normal keratinocytes, the immortalized but nontumorigenic HaCat cell line, and the tumor cell lines SCC-4, SCC-9, SCC-25, and OSCC-3. The RNA was then labeled and used to probe nylon microarray filters that contained a total of 9184 genes (5295 named and 3889 Expression Sequence Tags). Genes whose expression demonstrated a 3-fold or greater change were considered significant. Comparison of expression profiles from normal, HaCat, and four tumor cell lines revealed changes in gene expression in a total of 508 genes. Of these, 16 genes showed a consistent loss of expression when comparing normal to immortalized keratinocytes. In addition, 10 genes demonstrated a consistent loss of expression in the tumor cell lines only. In this latter group of genes, plasminogen activator inhibitor-2 (PAI-2), a gene whose expression has been linked to cell invasion, was additionally investigated. Altered expression of PAI-2 in the different cultured cells was validated via real-time quantitative-PCR. In addition, immunohistochemical evaluation of biopsy samples revealed a high expression of PAI-2 in both normal and dysplastic epithelium with a marked decrease of expression in areas of the biopsies containing HNSCC. These data demonstrate that genomic profiling can then be used to identify potential genotypic/phenotypic biomarkers that may predict which dysplastic lesions are most likely to progress to HNSCC.

**Introduction**

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy that is the sixth most common neoplasm in the world today. At current rates, ~50,000 cases will be diagnosed in the United States in the year 2001, of which >30,000 derive from the oral cavity and pharynx (1). Screening and early detection in populations at risk have been proposed to decrease both the morbidity and mortality associated with oral cancer (2). However, the histological definition of premalignancy is problematic. Lesions currently considered precancerous based upon cytological changes consistent with dysplasia. The criteria for diagnosing and grading dysplasia are subjective and open to a wide range of interpretation, even among highly qualified pathologists (3). In addition, no genotypic/phenotypic based criteria currently exist for predicting the risk of cancerous transformation of a given dysplastic lesion. Therefore, the histological findings can only be used to indicate that a given lesion has a malignant potential and cannot be used for the prediction of malignant change. Two studies underscore this concept. Mincer et al. (4) evaluated 45 patients with oral dysplastic lesions followed for up to 8 years. Only 11% of lesions underwent malignant change during the period of observation, although a higher percentage regressed or spontaneously disappeared (4). Similarly, Evenson (5) found that dysplastic lesions appeared to regress more frequently than to undergo malignant change. These findings emphasize the fact that, at present, we are unable to accurately prognosticate on the basis of histological change, which underscores the need to develop molecularly based protocols that can help refine our skills and help address these diagnostic dilemmas.

Epithelial carcinogenesis is thought to be a multistep process involving sequential activation of oncogenes as well as the inactivation of tumor suppressor genes in a clonal population of cells (6–9). These genetic changes generate concomitant phenotypic changes in the tumor cells that promote survival and proliferation. A number of genetic alterations in oncogenes and tumor suppressor genes, some definitively identified and some inferred from tumor-specific chromosomal alterations, have been found in HNSCC. However, the specific pattern of genetic alterations required for progressive transformation in human HNSCC has not been delineated. Because conventional histology is unable to predict which lesions are likely to progress to cancer, the development of molecularly based approaches to identify predictive genetic changes would greatly improve the potential for early detection, prognostication, and intervention. Here, using genomic profiling and a two-step validation process, we describe the identification of PAI-2 as one such molecular biomarker for HNSCC that may be useful specifically for predicting which premalignant lesions will progress to invasive HNSCC.

**Materials and Methods**

**Cell Culture and RNA Preparation.** Human squamous cell carcinoma cell lines SCC-4, SCC-9, and SCC-25, were purchased from the American Type Culture Collection. These cells were grown in DMEM/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum, hydrocortisone (0.4 μg/ml), penicillin (100 units/ml), and streptomycin (50 μg/ml). The OSCC-3 cell line was grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (50 μg/ml). Normal human keratinocytes were purchased from Clonetics/Cambrex (San Diego, CA) and cultured in KGM-2. HaCat, an immortal human keratinocyte cell line, (kindly provided by Brian Nickoloff) was grown in DMEM/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (50 μg/ml). Cultures were incubated in 5% CO2, 95% air environment in humidified incubators.

**RNA Extraction.** Total RNA was isolated from each cell line or cell strain using Trizol solution (Invitrogen, Carlsbad, CA). Briefly, 70–80% confluent cells were washed twice in PBS and incubated with Trizol for 5 min at room temperature. RNA was extracted with 200 μl of chloroform/ml Trizol and reverse transcription.
precipitated with 100% isopropanol at −20°C. The RNA pellet was resuspended in DEPC-treated water and stored at −80°C until additional use.

**Labeling, Hybridization, and Scanning of Microarray.** The labeling and hybridization procedures were conducted as specified by the manufacturer of the microarray filters (Research Genetics/Invitrogen). cDNA probes were made from 5 μg of total RNA with [3H]dATP (Amersham Biosciences, Piscataway, NJ) by oligodeoxynucleotidyl acid-primer polymerization using SuperScript II Reverse Transcriptase (Invitrogen). Probes were purified by gel chromatography (BioSpin 6; Bio-Rad Laboratories, Hercules, CA), boiled for about 3 min and allowed to cool to room temperature. ResGen GeneFilters microarrays GF205 (Human GeneFilters Microarrays) and GF211 (Human ‘Named Genes’ GeneFilters Microarrays) from Research Genetics (Invitrogen) were used. Each microarray filter consists of thousands of distinct sequence-verified genes spotted onto a 5 × 7 cm positively charged nylon membrane. Each spot contains at least 0.5 ng of DNA representing −1 kb from the 3’ end. Genes are selected based on sequence verification and UniGene clusters. Genomic DNA and housekeeping genes provide controls to ensure quality and probe labeling and hybridization. The microarrays were prehybridized for no less than 2 h and then hybridized with the denatured probe overnight at 42°C. Filters were washed with 2× SSC and 1% SDS for 15 min at 50°C, followed by two changes of 0.5× SSC and 1% SDS for a total of 30 min at room temperature. Washed filters were then exposed to phosphorimager screens, which were then scanned by a Molecular Dynamics Storm Imager (Packard, Meriden, CT) at 600-dpi resolution. Scanned files containing the microarray were analyzed with Pathways software (Research Genetics/Invitrogen).

**Data Analysis.** Raw image files have been analyzed with Pathways software (Research Genetics/Invitrogen), and Matlab functions have been used to quantify the background intensity and to determine which genes are actually expressed. To compare the different experiments, the raw expression data have been rescaled to account for different array intensities. This standardization has been based on the intensity of internal control spots. After preprocessing, gene expression values were subjected to a variation filter, which excluded genes with minimal variation across the samples being analyzed. The variation filter tests for a fold-change over samples, thus eliminating genes that did not show a relative change of at least 3-fold in at least one experiment. To group transcripts sharing similar expression patterns, genes have been clustered using hierarchical agglomerative clustering. Hierarchical clustering has been performed using Cluster software on standardized data and expression maps of genes. Each spot contains at least 0.5 ng of DNA representing −1 kb from the 3’ end. Genes are selected based on sequence verification and UniGene clusters. Genomic DNA and housekeeping genes provide controls to ensure quality and probe labeling and hybridization. The microarrays were prehybridized for no less than 2 h and then hybridized with the denatured probe overnight at 42°C. Filters were washed with 2× SSC and 1% SDS for 15 min at 50°C, followed by two changes of 0.5× SSC and 1% SDS for a total of 30 min at room temperature. Washed filters were then exposed to phosphorimager screens, which were then scanned by a Molecular Dynamics Storm Imager (Packard, Meriden, CT) at 600-dpi resolution. Scanned files containing the microarray were analyzed with Pathways software (Research Genetics/Invitrogen).

**RTQ-PCR.** For quantification of RNA, Platinum Quantitative PCR SuperMix-UDG (Invitrogen) was used in a two-step RT-PCR procedure after cDNA synthesis with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The same RNA samples collected for microarray experiments were used. Briefly, 2.5 μg of total RNA was reverse-transcribed using 50 ng of Random Hexamers, 10 mm deoxynucleotide triphosphate mix, and 50 units of SuperScript II at 42°C for 50 min. The resulting first strand-cDNA was used as template for the RTQ-PCR analysis. The 5’ nuclease activity of TaqDNA Polymerase generates a real-time quantitative DNA assay in the presence of oligonucleotide hybridization probes with 5’ fluorescent dye and 3’ quencher. A relative standard curve representing five 10-fold dilutions of stock cDNA (10000–0.1 pg) was used for linear regression analysis of unknown samples. The sequences of selected genes were confirmed using National Center for Biotechnology Information GenBank and Unigene databases, and appropriate clones were selected for linear regression analysis. The primers and probe were designed with Oligo Analyzer 2.5 software available online. The specificity of the primers and probe sequences was confirmed by National Center for Biotechnology Information blast module and by gel analysis of the amplicons generated by PCR. The iCycler IQ Multicolor Real-Time PCR Detecting System (Bio-Rad Laboratories) was used to determine gene expression levels. The sequences of the PCR primer pairs and fluorogenic probe (5’-3’), respectively, were: PAI-2: GTTACCCCCATGACTCCAGA, CGCAGACTTTC-CACCAAAAC, and CGCTTCAGGCAACAGCTCG-BHQ-1.

**Immunohistochemistry.** The avidin-biotin immunoperoxidase method was performed on deparaffinized formalin-fixed, paraffin-embedded sections. Briefly, deparaffinized slides were hydrated and then placed in citrate buffer (pH 6.0) and heated with a microwave for 20 min. The slides were then washed and incubated with primary anti-PAI-2 antibody (American Diagnostica, Greenwich, CT), 1:10,000 dilution, at room temperature for 12 h. Secondary antirabbit antibody was applied for 30 min at room temperature. Slides were counterstained with hematoxylin.

**Scoring of Immunohistochemistry.** The combined scoring method that accounts for intensity of staining as well as percentage of cells stained was used for this study (11). Strong, moderate, weak, and negative staining intensities were scored as 3, 2, 1, and 0, respectively. For each of the intensity scores, the percentage of cells that stained at that level was estimated visually. The resulting combined score consisted of the sum of the percentage of stained cells multiplied by the intensity scores. For example, a case with 10% weak staining, 10% moderate staining, and 80% strong staining would be assigned a score of 270 (10 × 1 + 10 × 2 + 80 × 3 = 270) of a possible score of 300.

**Results**

**Analysis of Gene Expression Profiles of Normal, Immortal, and Malignant Keratinocytes.** Comparison of expression profiles from normal, HaCat, and four tumor cell lines revealed changes in gene expression in a total of 508 genes that demonstrated a 3-fold or greater change in expression. Using hierarchical agglomerative clustering we identified a total of 26 genes whose expression was consistently up or down-regulated in such a fashion that the altered expression could potentially be linked to HNSCC progression (Fig. 1A). Sixteen of these genes demonstrated loss of expression when comparing normal to the HaCat cell line (Fig. 1B). We chose to use the HaCat, a spontaneously immortalized keratinocyte cell line to represent close as one could get to a dysplastic cell line. The HaCat has a mutant p53 tumor suppressor gene and does not form tumor in nude mice. Thus, at least some of its genotypic and phenotypic characteristics are similar to dysplastic cells. An additional 10 had high levels of expression in both the normal and HaCat cells with consistent loss of expression in the tumor cell lines. Among this set of genes, PAI-2 expression was consistently low in all four tumor cell lines when compared with the HaCat cell line and the normal keratinocytes (Fig. 1C). To validate the genomic profiling results for PAI-2, RTQ-PCR was performed using the same RNA samples that were collected for the microarray experiments. Normal keratinocytes and the HaCat cell line contained high numbers of PAI-2 transcripts, whereas all four cancer cell lines demonstrated markedly reduced copy numbers (Fig. 2).

**Immunohistochemical Analysis of PAI-2 Expression in HNSCC Progression.** To determine the in vivo relevancy of the microarray and RTQ-PCR results from cells grown in culture, we examined biopsy specimens using representative slides that contained areas of normal, dysplastic, and malignant mucosa. Normal and dysplastic oral mucosa were strongly positive for expression of PAI-2, whereas the underlying connective tissue stroma and inflammatory cells were strongly positive (Fig. 3). Conversely, areas containing invasive HNSCC demonstrate marked reduction of PAI-2 expression. Interestingly, salivary gland ductal epithelium also showed strong staining. It was also interesting to note that there was an extremely low level of PAI-2 expression in the basal cell layer of normal mucosa. However, with maturation, markedly increased amounts of PAI-2 could be observed in the stratum spinosum, granulosum, and corneum.

*We used a scoring method that accounts for the relative intensity of staining as well as the percentage of cells stained to quantify the*
difference in PAI-2 expression (11). In general, normal (n = 8, mean score = 260, SD = 60) and dysplastic (n = 10, mean score = 80, SD = 80) mucosa were strongly positive for expression of PAI-2 (Table 1). Conversely, areas containing tumor cell invasion were either negative or weakly staining (n = 12, mean score = 12, SD = 18). Therefore, the results of the immunohistochemical analyses using archival tissue validates the findings of the genomic profiling and RTQ-PCR of the cultured cells. Specifically, they confirm that PAI-2 expression is present in both normal and dysplastic keratinocytes and its expression is markedly decreased when invasion into the underlying connective tissue stroma is observed. These findings support the hypothesis that loss of PAI-2 expression may be a useful biomarker for prediction of which dysplastic lesions will undergo progression to HNSCC.

**Discussion**

Head and neck cancer is often heralded by the development of premalignant lesions within the oral mucosa. Surgical excision of histologically dysplastic areas is unlikely to decrease the risk of subsequent invasive cancer because of the widespread distribution of genetically abnormal cells within normal appearing mucosa. Furthermore, we are unable to predict which lesions are likely to be biologically more aggressive than others. To improve diagnostics and to better target chemopreventive therapy only to that population with lesions highly likely to progress to malignancy, better predictors of clinical progression are needed. Current diagnostic techniques for premalignant disease, based on recognition of nuclear and cellular atypia, are relatively poor predictors of ultimate clinical outcome.
Because morphological or cytological changes may occur late in the process of transformation, histologically benign-appearing lesions may have malignant potential. Conversely, even severely dysplastic oral lesions can undergo spontaneous regression. Thus, histological findings cannot clearly predict malignant change. Additional methods such as the elucidation of specific biomarkers for detecting premalignant lesions at an early stage and for predicting the likelihood of malignant progression are required.

There are many reports concerning the identification of biomarkers that may be predictive of the clinical progression of HNSCC. In general terms, these have included genomic aberrations, loss of heterozygosity, aneuploidy, microsatellite DNA, gene mutations/deletions, proliferation and differentiation markers, and chromosomal instability (12–14). For example, the ploidy status of potential premalignant lesions has recently been shown to predict progression of HNSCC (15). However, the limitation of many of these biomarkers is that they are rather nonspecific. Their ultimate usefulness as biomarkers will be limited in predicting a tumor’s biological behavior and responsiveness to treatment because few can be definitively linked to specific phenotypes required for cancer progression. The phenotype of each individual lesion is thought to be largely determined by the specific genotypic alterations that have taken place in that particular clone of cells. Therefore, the identification of genes that can be tied to specific phenotypes required for carcinogenic progression would represent the ideal type of biomarker. The data presented here represents one such step in this process. Using genomic profiling, we have...
identified PAI-2 as a potential biomarker that may predict the progression of dysplastic lesions to HNSCC. PAI-2 expression was seen in normal and immortalized cultured keratinocytes as well as histologically normal and dysplastic oral mucosa. However, its expression at both mRNA and protein levels dropped dramatically in cultured HNSCC cells as well as in HNSCC biopsy specimens when invasion into the underlying connective stroma had occurred. The loss of PAI-2 expression in concurrence with invasion of the underlying connective tissue stroma is consistent with the biology of this gene.

In addition to identifying a potentially novel predictor of HNSCC progression, the identification of PAI-2 as a gene whose expression is lost at the time of invasion provides new insights into the biology of this cancer’s development. PAI-1 and PAI-2 are members of the serine protease inhibitor super-family and are involved in the regulation and inhibition of binding between urokinase-type plasminogen activator and its receptor. These proteins are involved in physiological and pathologic proteolysis and extracellular matrix degradation, and many lines of evidence suggest an important and causal role for urokinase-type plasminogen activator-catalyzed plasmin generation in cancer cell invasion through the extracellular matrix (16). A significant decrease in PAI-2 expression has also been associated with increased aggressiveness and progression in certain epithelial cancers (17–19). Therefore, the loss of PAI-2 expression may represent one of the final critical genetic alterations required for invasion of dysplastic oral mucosa.

In conclusion, our ability to predict the biology of premalignant lesions has been limited by controversial clinical terms, inaccurate and subjective assessments because of lack of well-defined criteria for grading, interexaminer variability, and most importantly, the lack of genotypic/phenotypic-based biomarkers. It is our hope that PAI-2 will be one of a number of such specific molecular biomarkers that will improve our ability to diagnose, prevent, and treat this aggressive disease. In addition, further inquiry of this gene as it relates to HNSCC may provide new insights with regard to the biology of this and other neoplasms.

References

Table 1 PAI-2 immunohistochemical score from tissue analysis for patients with histologically normal, dysplastic, and malignant lesions of the oral cavity.

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