EXPRESSION OF THE SERINE PROTEASE DESC1 CORRELATES DIRECTLY WITH NORMAL KERATINOCYTE DIFFERENTIATION AND INVERSELY WITH HEAD AND NECK SQUAMOUS CELL CARCINOMA PROGRESSION

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Abstract: Background. As part of ongoing studies aimed at identifying the molecular events involved in head and neck squamous cell carcinoma progression, we recently isolated a novel serine protease, DESC1. This study was conducted to further characterize DESC1.

Methods. Specimens of normal, dysplastic, and carcinoma-tous oropharyngeal mucosa (n = 31) were evaluated for DESC1 immunoreactivity using standard streptavidin-biotin immunoperoxidase techniques. Between-lesion stain intensity values were analyzed using multiple Wilcoxon tests. DESC1 expression was also evaluated in cultured human keratinocytes after induction of differentiation by calcium challenge, with subsequent real-time reverse transcriptase-polymerase chain reaction quantification.

Results. DESC1 immunoreactivity decreased as lesions approached a malignant phenotype. Post hoc testing comparing the different lesion types and DESC1 staining values showed significance between “normal” and “carcinoma” (p = .0017) groups. Induction of normal keratinocyte differentiation by calcium challenge was accompanied by an increase in DESC1 expression (p = .002).


Keywords: DESC1; head and neck squamous cell carcinoma (HNSCC); serine protease
Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial cancer with an overall relatively poor prognosis. Most cases of HNSCC are related to known and preventable risk factors such as chronic cigarette, cigar, or pipe smoking; excessive alcohol or mate’ consumption; and betel quid (paan) chewing habits.1 As the eighth leading cancer among men, and the 14th most common among women in the United States, HNSCC results in approximately 8000 deaths every year.2 Worldwide, more than a half million cases of HNSCC are diagnosed each year. Currently, HNSCC is associated with a higher mortality rate than either breast, prostate, lung, or colorectal cancer.3,4

Over the last several decades, clinical and histopathologic features that characterize precancerous and cancerous (HNSCC) change have been well delineated.5,6 However, with the exception of more recent reports,7,8 relatively less is known with respect to the early molecular and genetic events involved in the evolution of HNSCC. Identifying early genotypic alterations may provide a molecular fingerprint for premalignant lesions, which could be predictive of clinical behavior or aid in the detection of early-stage disease. Studies are ongoing in our laboratories to identify the molecular events involved in the evolution and progression of HNSCC. As part of these studies, we recently applied representational difference analysis (RDA) to tissue samples from patients with HNSCC, and were able to identify a novel gene, differentially expressed in squamous cell carcinoma (DESC1).9

DESC1 is expressed in normal mucosal epithelium from patients with HNSCC but is absent from their cancerous epithelium and metastatically involved lymph nodes. However, the conditions leading to loss of DESC1 expression during carcinogenesis and metastasis, and the precise function and localization of the gene product, are unknown. Furthermore, because DESC1 was isolated from normal epithelium in patients with HNSCC, it is important to determine whether DESC1 is expressed in normal epithelium from patients without HNSCC to control for ‘field cancerization’ effects, possible contamination of normal tissue by adjacent malignancy, or other confounding variables that may exist in a patient with HNSCC.10,11

Therefore, this study was conducted with the following specific aims: (1) to characterize for the first time the immunohistochemical expression profile of DESC1 in normal, precancerous, and cancerous (HNSCC) mucosal epithelium of the head and neck, (2) to use reverse transcriptase-polymerase chain reaction (RT-PCR) to evaluate DESC1 expression in normal oropharyngeal mucosa obtained from patients without HNSCC and compare this to previous RT-PCR results characterizing DESC1 expression in mucosa obtained from individuals with HNSCC, and (3) to determine whether calcium challenge–induced normal keratinocyte differentiation, or terminal epithelial differentiation, is accompanied by increased DESC1 expression.

MATERIALS AND METHODS

Before initiation of this study, appropriate institutional review board approval was obtained (Protocol No. 02E0297).

Specimen Collection. Tissue specimens for PCR and immunohistochemical analysis were obtained from healthy individuals (n = 19) with no history of cancer who were undergoing elective oral surgery procedures. All specimens were harvested from clinically normal tissue, which was confirmed by subsequent histopathologic evaluation. Each participant completed a detailed medical history, social history, and consent form. At the time of surgery, each specimen obtained from a study patient was cut into two pieces. The first half of the specimen was placed in formalin fixative (10%) for 2 hours and then routinely processed and cut into multiple sections for histopathologic and immunohistochemical evaluation. The remaining fresh portion was placed in RNA preserving solution (RNA Later; Ambion Inc., Austin, TX) for 3 hours at room temperature and then stored in a –80°C freezer for subsequent PCR analysis.

DESC1 Antibody Development and Western Blot Analysis. The DESC1 antibody (polyclonal) was affinity purified from rabbit antiserum generated by immunization with a 13-residue peptide-keyhole limpet hemocyanin (peptide-KLH) conjugate obtained from the catalytic domain of the DESC1 gene. Western blot analysis was performed to validate the specificity of the DESC1 antibody before immunohistochemical evaluation. Pathologically confirmed normal human oral tissue was lysed in mammalian protein extraction reagent (M-PER) protein extraction reagent (Pierce, Rockford, IL) and 50 μg of lysate/lane run on a 10% nupolycylamide gel electrophoresis (NuPAGE) gel and blotted using an XCell II Blot Module (Invitrogen,
Presence of DESC1 was confirmed by WesternBreeze (Invitrogen) chemi-luminescence detection, using a 1:1000 dilution of antibody Ab 5-2 and a second antibody, Ab 5-1, raised against the amino-terminal 14 amino acids of the DESC1 protein. Negative control is pooled preimmune serum from the rabbits used to generate DESC1 Ab 5-1 and Ab 5-2. The peptide used to generate DESC1 antibody Ab 5-2 was chosen from a region of DESC1 that contains little sequence identity with other serine protease family members, having only two amino acids identical to that of its nearest neighbor, the serine protease human airway trypsin-like protease (HAT).

Immunohistochemical Analysis. Formalin fixed, paraffin-embedded tissue specimens of premalignant (n = 11) and malignant (n = 10) oropharyngeal mucosa were retrieved from our pathology tissue registry. The premalignant lesions included various grades of dysplastic epithelium, ranging from mild to moderate to severe. The malignant lesions included superficially invasive and well-differentiated to poorly differentiated invasive squamous cell carcinomas. For normal tissue, we used specimens obtained from healthy individuals as described earlier under ‘Specimen Collection.’ Seven samples yielded adequate tissue for both histopathologic and immunohistochemical evaluation. To this group, we added three reactive lesions: two cases of acanthosis and keratosis and one case of necrotizing sialometaplasia, a condition that is occasionally mistaken histopathologically and clinically for squamous cell carcinoma.

One section of each specimen underwent routine hematoxylin-eosin (H&E) processing and was reviewed independently by two pathologists to verify accuracy of histopathologic diagnosis for study inclusion. Subsequent sections were stained using standard streptavidin-biotin immunoperoxidase techniques with diaminobenzidine as the substrate (DAB kit; Vector, Burlingame, CA). A high-temperature antigen retrieval method was applied to these sections by placing them in a Coplin jar with 0.01 M buffered citrate solution (pH = 6.0) and microwaving them for 2 minutes at a high power setting (level 9) and 12 minutes at a medium power setting (level 4) in a conventional microwave oven (Amana Radarange; Maytag, Newton, IA). To inhibit endogenous peroxidase and reduce nonspecific background staining, blocking reagent (PowerBlock; BioGenex, San Ramon, CA) was applied to sections in accordance with the manufacturer’s instructions. Sections were subsequently incubated for 1 hour at 25°C with antibodies against proteins already known to be expressed in terminally differentiating and normally proliferating epithelium. These antibodies would serve as known epithelial standards that could be compared with and used to help characterize DESC1 expression with respect to staining pattern and localization. These antibodies included the epithelial differentiation markers involucrin (Clone SY5; Novocastra, UK), cytokeratin (CK10/11-Clone LHP1; Novocastra, UK) and filaggrin (Clone 15C10; Novocastra, UK), the proliferation marker Ki-67 (Clone MM1; Novocastra, UK), and the tumor suppressor gene retinoblastoma (Rb-Clone 13A10; Novocastra, UK).

The DESC1 antibody used for immunohistochemical analysis was Ab 5-2. To establish a working dilution for the previously uncharacterized DESC1 antibody, serial dilutions were performed on sections of epithelial tissue at antibody to diluent (DAKO Antibody Diluent S0809; Carpinteria, CA) concentrations of 1:50, 1:100, 1:150, 1:200, and 1:300. Positive controls included prostatic and testicular epithelium, which are known to express DESC1; negative controls consisted of sections of human lung, spleen, brain, placenta, and kidney, which fail to demonstrate DESC1 expression. Final DESC1 staining on all cases was performed at a 1:100 working dilution. The staining pattern, localization, and intensity of DESC1 expression were evaluated with a bright-field microscope interfaced with a high-resolution spot camera and computer-assisted image analysis software (Image PCI; Compix Inc., Cranberry Township, PA). Intensity scores for DESC1 immunoreactivity, ranging from 0 to 3, were assigned to each case by two pathologists working independently. A score of 0 was assigned to cases showing no evidence of immunoreactivity, a score of 1 to cases with focal or weak staining, a score of 2 to cases with moderate staining intensity, and a score of 3 to cases with diffuse and intense immunoreactivity. Interrater reliability for intensity scores was assessed using the Fleiss–Cohen weighted kappa statistic with a 95% confidence interval. Lesions were divided into three groups for comparison: normal, dysplasia, and carcinoma. Between-lesion (group) stain intensity values were analyzed with multiple exact Wilcoxon tests with p values adjusted using the stepdown Bonferroni method of Holm.
confirmed by gel electrophoresis. After RNA isolation, 1.0 µL of total RNA was used for first-strand cDNA synthesis in a total volume of 25 µL and reactions otherwise performed according to manufacturer’s instructions (ProSTAR, Stratagene, La Jolla, CA). PCR amplification was performed as previously described. Primers used in PCR reactions were housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) primers HPRT1, 5'-GTAATGACCAGTCACAGGGGAC-3' and HPRT2, 5'-CCAGCAAGCTTGCACACCA-3' and DESC1 primers D10, 5'-CCTGTCACAAATGCAGTAC-3' and D11, 5'-TGACGTTTTTATTGAG-3'. PCR amplification of the HPRT (housekeeping) gene was performed as a control to demonstrate equal loading, for normalization and to determine integrity of RNA. Primers were designed by computer analysis (Oligo 4.0; NBI, Hamel, MN) of available DNA sequence for each gene and are intron-spanning, precluding PCR amplification of any residual DNA present in RNA samples. Optimum cycle number for PCR amplification was predetermined for each primer set using a mixture of RT reactions from 10 random normal and tumor samples (data not shown). This step is necessary to ensure that PCR amplification remains in the linear range and that production of the PCR product does not plateau. Under reaction conditions used, quantity of PCR product is, therefore, directly proportional to the amount of radioactivity incorporated into the DNA.

Keratinocyte Culture and the Induction of Differentiation. Normal human keratinocytes (15 mean population doublings), which are known to be capable of malignant conversion, were cultured in a modified version of keratinocyte growth medium (MDCB153; Clonetics Corporation, San Diego, CA) supplemented with 0.15 mM calcium chloride. When the cells were around 50% confluent, the medium was changed, and the cells were cultured for a further 48 hours in either 0.15 mM calcium chloride or 1.0 mM calcium chloride before the isolation of total RNA for real-time RT-PCR analysis. Results were evaluated for statistical significance using a paired t test, with significance accepted at p < .05.

Real-Time RT-PCR Analysis. Quantitative real-time RT-PCR was performed with use of the ΔΔCT (Applied Biosystems, Foster City, CA) comparative method using HPRT as the normalization control gene and normal human skin RNA (Invirogen, Carlsbad, CA) as the calibrator. Reverse transcription was performed using Multiscribe Reverse Transcriptase (Applied Biosystems; Foster City, CA) using 100 ng of total RNA. Real-time PCR amplification was subsequently performed using the TaqMan PCR Kit (Applied Biosystems; Foster City, CA). Primers used for PCR amplification and fluorescent probes were designed using Primer Express software (Applied Biosystems; Foster City, CA). DESC1 primer D10: 5'-AGA-ATGTTATGTGCTGGCTCCTTA-3', lower: 5'-GA-ACTAAC-CAGTGTCCTCTCAAGA-3' and probe: 5'-AGGAA-AAACAGATGCAATGCCAGGTG-3', HPRT upper: 5'-CGGCTCCGTATATGGGGCGTG-3', lower: 5'-GGTCATACCTGTTTCACTACAC-3' and probe: 5'-CGCAGCCCTGGCGTCGTGA-3'. All RNA samples were subject to DNase treatment before PCR amplification; however, primers were additionally selected to produce an intronspanning PCR product, precluding amplification of contaminating DNA. Real-time PCR amplification was performed in a Perkin-Elmer/Applied Biosystems 5700 machine. Method for study comprised use of fluorogenic 5' nuclease chemistry, utilizing an oligonucleotide probe 5' labeled with fluorochrome 6-carboxyfluorescein (6-FAM) and a quencher fluorochrome, 6-carboxy-tetramethylrhodamine (TAMRA).

RESULTS

Immunohistochemical Analysis. Patients in this study population (n = 31) ranged in age from 21 to 86 years old (mean age, 57 years) as shown in Table 1. Most patients with histopathologic evi-
idence of precancerous and cancerous change had a significant smoking history. Various anatomic sites in the oropharyngeal cavity were represented, but most dysplastic and cancerous lesions arose from high-risk sites such as the ventrolateral tongue and floor of mouth. The pattern of DESC1 expression in normal epithelium was similar to its expression in reactive epithelium. This pattern of immunoreactivity was diffuse and finely granular, often extending the full thickness of the epithelial strata. DESC1 (Ab 5-2) was predominantly localized to the cytoplasm of epithelial keratinocytes, as shown in Figure 1A, analogous to a cytokeratin pattern of immunoreactivity. Occasionally, areas of cytoplasmic and membranous staining were observed, similar to volucrin or filaggrin staining patterns. These areas were usually in well-differentiated regions of carcinoma where squamous eddies or keratin pearl formation could be observed, as seen in Figure 1B. Finally, basal nuclear positivity, in addition to cytoplasmic positivity, was observed in all of the reactive cases (cases 8, 9, and 10). This nuclear positivity was reminiscent of Rb or Ki-67 staining patterns in basal keratinocytes. Interestingly, the reactive case representing necrotizing sialometaplasia showed strong positivity not only in the superficial epithelium but also in the underlying meta-plastic squamous epithelium, which is occasionally mistaken histopathologically for squamous cell carcinoma. This staining pattern, both nuclear and cytoplasmic as shown in Figure 1C, was not observed in any of the dysplasias or HNSCCs but was observed in all of the reactive lesions. In addition, salivary acini and ductal epithelium, which have not been investigated previously for DESC1 expression, were immunoreactive in this case.

In terms of staining intensity, DESC1 immunoreactivity was generally strong in normal and reactive epithelium and in most mild to moderately dysplastic epithelium. In some cases of mild to moderate dysplasia, staining was greater in the suprabasal layers, as illustrated in Figure 1D. In cases of moderate to severe dysplasia, little or no immunoreactivity was observed, as shown in Figure 1E. A lack of DESC1 expression or weak staining intensity was also observed in most cases of HNSCC. When tumors did demonstrate immunoreactivity for DESC1, it was usually in more well-differentiated carcinomas, where squamous eddies or keratin pearl formation could be observed, or in dysplastic epithelium adjacent to overt carcinoma as illustrated in Figure 1F. Poorly differentiated carcinomas, or poorly differentiated regions of more well-differentiated carcinomas, showed little or no immunoreactivity for DESC1. Statistical analysis of the correlation between DESC1 staining and lesion type is shown in Table 2. Interrater reliability for staining values was excellent (kappa = 0.94; 95% confidence interval, 0.88–1.0). Post hoc testing comparing the different lesion types and DESC1 intensity scores showed significance between ‘normal’ and ‘carcinoma’ groups (p = .0017).

**Western Blot Analysis.** Evaluation of DESC1 antibodies Ab 5-1 and Ab 5-2 by Western blot analysis allowed detection of a single band corresponding to DESC1, which was absent from the pre-immune lane (control) as shown in Figure 2. Markers are colorimetric MultiMark (Invitrogen, Carlsbad, CA). These results validate the specificity of the DESC1 antibody used in immunohistochemical analysis as described previously.

**RT-PCR Analysis.** DESC1 expression in normal oropharyngeal mucosal epithelium obtained from clinically healthy patients without HNSCC is shown in Figure 3. DESC1 expression was identified in 17 of 18 cases, yet the relative level of expression varied between specimens, including two specimens from the same individual (M-166A and M-166B), whereas the expression level of the control gene HPRT remained constant. These findings are comparable to previously characterized DESC1 expression patterns in matched normal mucosa obtained from individuals with HNSCC, indicating that previously observed variability in DESC1 RNA expression from matched normal epithelium is unlikely a result of ‘field cancerization.’

**Induction of Differentiation.** Immunohistochemical studies suggest that the expression level of DESC1 increases as epithelial cells migrate through the spinous layer and undergo terminal differentiation. To determine whether DESC1 expression is induced by the process of differentiation, normal human keratinocytes (15 mean population doublings) were cultured, as described in the “Materials and Methods,” for 48 hours in the presence of either 0.15 mM calcium chloride or 1.0 mM calcium chloride, and the expression levels of DESC1 RNA measured. Raising the calcium concentration to 1.0 mM has been shown to induce many markers of normal keratinocyte differentiation, such as involucrin, transglutaminase, and filaggrin (and unpublished data). After calcium
treatment, RNA was isolated from the cells and subject to quantitative real-time RT-PCR analysis to measure changes in the expression level of DESC1. The data are shown in Figure 4 and represent the relative expression level of DESC1 (normalized to the housekeeping gene HPRT) in keratinocytes cultured in low (L) or high concentration (H) calcium chloride compared in each case with the level of expression present in normal human epithelium. The results show that the level of

FIGURE 1. (A) The finely granular pattern of DESC1 immunoreactivity in oropharyngeal mucosal epithelium is evident in this medium-power photomicrograph. Staining is predominantly localized to the cytoplasm of keratinocytes, extending the full thickness of the epithelial strata. (B) High-power photomicrograph demonstrating the lace-like membranous DESC1 staining pattern observed in cases of well-differentiated squamous cell carcinoma. This pattern was usually identified in areas where squamous eddies or keratin pearl formation could be identified. (C) A nuclear and cytoplasmic pattern of DESC1 immunoreactivity can be seen in this high-power photomicrograph of the reactive condition representing necrotizing sialometaplasia. Salivary acini demonstrate positive staining even before the transition to a metaplastic squamous epithelium. (D) DESC1 staining is greater in the suprabasal layers in some cases of mild to moderate dysplasia, as illustrated in this high-power photomicrograph. (E) In more advanced cases of dysplasia or in overt carcinoma, DESC1 staining intensity was weak or equivocal, as illustrated in this high-power photomicrograph of a severely dysplastic epithelial lesion. (F) Immunoreactivity for DESC1 in carcinoma was usually found in well-differentiated regions or in dysplastic epithelium in proximity to overt carcinoma as illustrated in this high-power photomicrograph. The larger arrow shows DESC1 immunoreactivity in dysplastic epithelium overlying invasive carcinoma, whereas the smaller arrow shows diminished DESC1 immunoreactivity in the subjacent carcinoma. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
DESC1 RNA expression increases significantly \((p = .002)\) when the cells are induced to differentiate.

**DISCUSSION**

As part of ongoing studies aimed at identifying the molecular events involved in the evolution and progression of HNSCC, we recently isolated a novel human gene, DESC1. DESC1 has been mapped to chromosome 4q13.2 (GenBank; NCBI Accession No. NP_054777), and the gene product putatively functions as a serine protease. DESC1 shares homology with two other known genes in the human genome that also function as serine proteases: (1) normal epithelial cell specific gene (NES1), and (2) human airway trypsin-like protease (HAT). Serine proteases have diverse enzymatic functions and have been identified in inflammatory processes, developmental processes, and tumorigenesis. NES1 was originally identified in breast cancer cell lines, where it was expressed in normal mammary epithelial cells but downregulated or absent in breast carcinoma, suggesting a possible differentiation or tumor suppressor role for the gene. Further studies confirmed this, and investigators have recommended the use of NES1 as a unique biomarker for breast cancer. Similarly, it is likely that DESC1 plays a role in regulating normal cellular proliferation and differentiation, because it too is downregulated during head and neck squamous cell carcinogenesis and upregu-
lated after induction of normal keratinocyte differentiation as shown in this study. Therefore, DESC1 may ultimately serve as a unique biomarker for head and neck cancer progression. To date, many gene-specific abnormalities consistently identified in HNSCC have been implicated in disease development, including prognosis, recurrence, or metastatic potential and comprise the tumor suppressor genes p53, p16, p21, p27, Rb, and the oncogenes cyclinD1, mdm2, and epidermal growth factor receptor (EGFR).21–25

In this study, we were able to further characterize the novel gene DESC1. Immunohistochemical results indicate that the DESC1 gene product is consistently identified in normal epithelium yet downregulated during carcinoma progression. This substantiates previous findings using RDA and PCR methods to evaluate DESC1 expression. By use of PCR methods, we were able to demonstrate in this study that DESC1 is consistently expressed in normal mucosal epithelium from patients without HNSCC. However, the relative level of expression varied between different individuals and, in one case, between different tissue samples from the same individual. This could be attributable to genetic differences, the type of epithelial tissue studied (keratinized/non-keratinized), environmental influences, or other as yet undetermined factors. Finally, data from our calcium-induced differentiation study imply that DESC1 is upregulated during induction of terminal keratinocyte differentiation, supporting a role in normal epithelial turnover. These results suggest that DESC1 may function in regular epithelial differentiation under normal conditions or function in circumventing tumorigenesis under cancer-promoting conditions. In contrast to the many published studies describing protease upregulation in cancer, the preceding data, in concert with the recent report by Yamashita et al.,26 suggest the possibility that downregulation of a family of serine trypsin-like proteases may also be a common event in carcinoma development. Continued research with larger study populations and further investigation of the molecular and biochemical nature of DESC1 activity will help clarify the precise function of DESC1 in health and disease.

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REFERENCES