Decreased plasma DEK Oncogene Levels Correlate with p16-Negative Disease and Advanced Tumor Stage in a Case–Control Study of Patients with Head and Neck Squamous Cell Carcinoma

Abstract
Head and neck cancer (HNC) remains the sixth most common malignancy worldwide and survival upon recurrence and/or metastasis remains poor. HNSCC has traditionally been associated with alcohol and nicotine use, but more recently the Human Papilloma Virus (HPV) has emerged as a favorable prognostic factor for oropharyngeal HNSCC. However, further stratification with additional biomarkers to predict patient outcome continues to be essential. One candidate biomarker is the DEK oncogenic protein, which was previously detected in the urine of patients with bladder cancer and is known to be secreted by immune cells such as macrophages. Here, we investigated if DEK could be detected in human plasma and if DEK levels correlated with clinical and pathological variables of HNSCC. Plasma was separated from the peripheral blood of newly diagnosed, untreated HNSCC patients or age-matched normal healthy controls and analyzed for DEK protein using ELISA. Plasma concentrations of DEK protein were lower in p16-negative tumors compared to both normal controls and patients with p16-positive tumors. Patients with lower plasma concentrations of DEK were also more likely to have late stage tumors and a lower white blood cell count. Contrary to previously published work demonstrating a poor prognosis with high intratumoral DEK levels, we show for the first time that decreased concentrations of DEK in patient plasma correlates with poor prognostic factors, including HPV-negative status as determined by negative p16 expression and advanced tumor stage.

Introduction
Head and neck cancer (HNC) remains a global health concern accounting for 650,000 new cases each year, resulting in 350,000 deaths worldwide. The identification of human papillomavirus (HPV) infection as a favorable prognostic factor has fueled clinical studies investigating de-intensification strategies in this HPV positive disease subset. However, the overall survival of HPV
negative tumors as well as recurrent tumors remains poor despite intensive therapy. Early stage localized tumors are often cured with a single modality including surgical resection or radiation therapy; however, locally advanced HNC often requires a multimodality approach. Despite primary intensive treatment of locally advanced disease, relapse free survival at 3 years remain at 30–50% in primary surgically resected disease [1]. Overall survival remains poor in the recurrent and metastatic setting despite aggressive treatment with median overall survival being 7–10.5 months [2]. Even though the addition of targeted agents such as the EGFR inhibitor, cetuximab, has resulted in an incremental rise in overall survival both in locally advanced and metastatic disease, the overall impact has been minimal [2–4].

Patients with HPV positive HNC often have an excellent prognosis and, if patients have less than a 10 pack-year smoking history, greater than 90% chance of cure in the locally advanced setting [5]. Importantly, HPV E7 induces expression of the human DEK oncogene [6]. DEK is primarily a chromatin structural and remodeling protein and has been shown to be an important driver of tumorigenesis in vitro and in vivo [7–13]. DEK over-expression in tumors, compared to adjacent normal tissue, has been observed in all cases of HNSCC tested to date and has also been observed with high frequency in breast cancer, melanoma, and many other solid tumors [8,14–16]. DEK over-expression induces cellular proliferation and invasion/metastasis both in cell culture and mouse models [13,17,18]. Furthermore, recent work has demonstrated that DEK over-expression is an independent prognostic factor predicting poor outcome in multiple solid tumors, including pancreatic ductal adenocarcinoma, gastric adenocarcinoma, breast cancer, melanoma, neuroendocrine prostate cancer, and non-small cell lung carcinoma [16,19–22].

Interestingly, despite largely being a chromatin-bound protein, DEK has been shown to be secreted by IL-8 treated macrophages in vitro and subsequently can function as a chemotactic factor for neutrophils, CD8+ T cells, and natural killer (NK) cells [23]. Extracellular DEK protein and auto-antibodies also have been detected in patients with several types of autoimmune diseases, including in the synovial fluid of patients with juvenile idiopathic arthritis [24–28]. Furthermore, extracellular DEK protein was also detected in the urine of bladder carcinoma patients [29]. Thus, we hypothesized that DEK would be present at different concentrations in HNC patient plasma compared to normal healthy controls. In this study, peripheral blood was collected from newly diagnosed, treatment naive HNSCC patients or age-matched normal healthy controls. Plasma was separated from the samples and subjected to DEK specific ELISA. Plasma DEK concentration levels were compared to normal controls, and to clinical and pathological variables. Interestingly, despite elevated intratumoral levels of DEK protein compared to healthy tissue, there was a trend to lower levels of DEK in HNC patient plasma compared to healthy controls in those patients that were HPV-negative, as determined by immunohistochemical staining for p16. Additionally, low DEK plasma levels correlate with advanced stage, and hence a likely poor prognosis, as well as a lower white blood cell count. Together our observations suggest plasma DEK levels correlate with important prognostic factors for HNSCC. Further characterization may aid in predicting patient outcome to various treatment modalities and may give insight to novel treatment strategies.

Methods

Patient Selection

Study participants included patients with a known or suspected diagnosis of HNSCC or normal healthy controls. Key inclusion criteria included the ability to understand and sign informed consent, collection of samples during routine procedures (except for normal healthy patients), and patients must have had adequate bone marrow and overall systemic function to withstand an extra blood draw. Key exclusion were patients with prior treatment, or any patients who did not meet the above and for healthy controls, patients with any history of cancer or auto-immune disease were excluded. Key demographics of the population tested are presented in Table 1. Median age of healthy controls (“NML”) was 51.94 while median age of HNSCC patients was 56.67. Two HNSCC patients presented with multiple malignancies and were not included in analysis beyond detecting DEK concentrations. Not all control participants answered each question regarding demographics and lifestyle habits. Complete blood counts and tumor p16 status were obtained from chart review and were performed as part of standard care by the University of Cincinnati Department of Pathology and Laboratory Medicine. Written informed consent was obtained from all subjects. All patients and healthy controls were informed of the purpose of this research program and provided their written, informed consent. The study protocol was approved by the University of Cincinnati Institutional Review Board and is study protocol numbers 2014–6326 and 2014–4755.

Blood Collection and Analysis

Plasma was isolated from whole blood collected in EDTA tubes. Plasma was transferred to cryovials in 1 ml aliquots and stored at −80°C until used for analysis. Additional blood samples from patients were collected simultaneously and analyzed by University of Cincinnati Hospital for complete blood counts with differential, as part of standard care. Patient and control plasma was diluted 2-fold in sample buffer prior to analysis with a DEK-specific ELISA performed according to manufacturer’s directions (Cusabio, Wuhan, China) and measured with a Molecular Diagnostics microplate reader and Softmax Pro 3.1 software.

Immunohistochemistry

Archived biopsy or resection human head and neck squamous cell carcinoma tissue were obtained with IRB approval. Tissues were fixed in formalin, embedded in paraffin, sectioned at 5 μm thickness, and fixed on to slides. Fixed paraffin sections were deparaffinized in xylene

Table 1. Demographics of Study Participants

| Variable               | Controls | HNC Patients | P     |
|------------------------|----------|--------------|-------|
| **Race**               |          |              |       |
| Caucasian (n = 27)     | Caucasian (n = 35) | 0.0066 |
| Non-Caucasian (n = 10) | Non-Caucasian (n = 1) |
| **Sex**                |          |              |       |
| M (n = 10)             | M (n = 30) | <0.0001       |
| F (n = 27)             | F (n = 6)  |
| **Diabetes Mellitus**  |          |              |       |
| Y (n = 6)              | Y (n = 4)  | 0.7361        |
| N (n = 31)             | N (n = 31) |
| **Auto-Immune**        |          |              |       |
| Y (n = 0)              | Y (n = 2)  | 0.2328        |
| N (n = 37)             | N (n = 33) |
| **Smoke**              |          |              |       |
| Any History (n = 17)   | Any History (n = 28) | 0.0119 |
| No History (n = 17)    | No History (n = 7)  |
| **Alcohol**            |          |              |       |
| Y (n = 15)             | Y (n = 17) | 0.6356        |
| N (n = 22)             | N (n = 18) |
and incubated in sodium citrate solution for antigen retrieval. Sections were treated with the anti-mouse IgG Elite Vectastain ABC kit (Vector Labs, Burlingame, CA, USA) for detection of DEK (BD Biosciences) and Ki67 (ThermoScientific), which were diluted 1:500 and 1:1000, respectively. Sections were stained with Vector Laboratories 3,3′-diaminobenzidine, counterstained with hematoxylin and 0.2% ammonia hydroxide, and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA). Additional staining for p16 was performed using a mouse monoclonal antibody to p16 (Roche) on a Ventana Benchmark LT automated immunostainer according to standard protocol. Positive and negative controls were included routinely. Scoring criteria for p16 positive tumor status was ≥70% tumor cells staining positive. Ki67 scoring was determined as percent of tumor cells staining positive, described in increments of 10%. DEK scoring was determined by multiplying an intensity score (0/negative, 1/weak, 2/moderate, 3/strong) by the distribution score (0/<33% positive, 2/33–66% positive, 3/>66% positive) to achieve a total score from a range between 0–9.

Statistics

Descriptive statistics were reported for continuous and categorical variables as median (range), and frequency, respectively. DEK levels were compared across groups with Wilcoxon rank sum tests. Fisher exact tests were used to compare two categorical variables. Correlation was found using Pearson’s method, except in the instance of comparing histological staining for Ki67 and DEK, in which case Spearman correlation (ρ) was used. To adjust for p16 status, a multiple linear regression model was used. All computations were done in R version 3.2.4 (Vienna, Austria). All comparisons were two-sided, statistical significance was set at .05.

Results

Common Patient Risk Factors and Characteristics Do Not Influence DEK Plasma Protein Levels

Between December 2014 and December 2015, 38 newly diagnosed HNSCC patients and 37 age-matched normal healthy control patients were consented for blood collection. Comparing control and HNSCC patients, there were significant differences in sex and race distribution and smoking status, all of which are known risk factors for HNSCC (Table 1), thus, our study participants are representative of the HNSCC patient population.[30] Blood was collected prior to treatment, processed, and plasma was separated for analysis. Using DEK specific ELISA, DEK protein was detected in both normal healthy control and HNSCC patients. There was not a significant difference between plasma DEK concentrations in healthy controls and the collective HNSCC patient population (Figure 1A). Many other common patient characteristics and risk factors are known to be associated with HNSCC as well as prognosis. Therefore, DEK plasma levels were compared with these confounding factors such as smoking status, alcohol use, race and sex. DEK plasma levels did not correlate with any of these common clinical and pathological variables tested in either normal healthy controls or HNSCC patients (Table 2).

Plasma DEK Levels Correlate with HPV Status in HNSCC Patients

Although we noticed no difference in plasma DEK levels comparing normal and HNSCC patients, we did observe a larger range of values in patient samples. Importantly, HNSCC comprises a heterogenous group of cancers. Therefore, patient samples were separated into clinical anatomical sites. Patients with oropharyngeal tumors had statistically significant higher levels of DEK plasma protein compared to laryngeal carcinomas (Figure 1B).
Oropharyngeal tumors are often HPV positive, which carry a favorable prognosis, and HPV E7 protein has previously been shown to up-regulate DEK expression [6,31]. Given the latter, as well as that HPV positive tumors have been found to harbor different mutations based on TCGA analyses [32], samples were stratified for HPV status based on TCGA analyses [32], samples were stratified for HPV status.

Plasma DEK levels were lowest in patients with p16 negative disease (390.4 pg/ml), and were significantly lower than those found in controls (473.2 pg/ml; \(P = .009\)) (Table 3). These findings were similar whether p16 status was compared in HPV-negative HNSCC patients with p16-negative disease (\(P = .0062\)). Furthermore, plasma DEK protein levels were lowest in patients with p16 negative HNSCC (390.4 pg/ml), and were significantly lower than concentrations found in controls (473.2 pg/ml; \(P = .009\)) (Table 3). These findings are consistent with previous studies showing decreased DEK expression in HPV-negative tumors compared to HPV-positive tumors [6,31].

### Table 3. Plasma DEK Levels Correlate with p16 Status

| Variable | All Patients | HNC Patients |
|----------|--------------|--------------|
| Race     |              |              |
| Caucasian (n = 36) | 430.7 (226.1–1252.1) | 409.5 (172.2–1252.2) |
| Non-Caucasian (n = 31) | 473.2 (226.1–1292.1) | 566.4 (249.0–714.5) |
| Sex      |              |              |
| M (n = 10) | 440.5 (226.1–1292.4) | 384.2 (267.2–714.5) |
| F (n = 27) | 473.3 (347.5–973.2) | 454.1 (199.2–1252.1) |
| Diabetes Mellitus |              |              |
| Y (n = 6) | 429.7 (248.6–1292.7) | 454.1 (199.2–1252.1) |
| N (n = 31) | 530.5 (226.1–973.2) | 430.7 (199.2–1252.1) |
| Auto-Immune |              |              |
| Y (n = 0) | No comparison possible | |
| N (n = 37) | No comparison possible | |
| Smoke    |              |              |
| Any History (n = 17) | 440 (226.1–1292.4) | 713.2 (199.2–775.6) |
| No History (n = 17) | 473.3 (347.5–973.2) | 409.5 (199.2–1252.1) |
| Alcohol  |              |              |
| Y (n = 15) | 429.7 (248.6–1292.4) | 454.1 (199.2–1252.1) |
| N (n = 22) | 530.5 (226.1–973.2) | 430.7 (199.2–1252.1) |

**DEK Plasma Levels Decrease with Larger Tumor Size**

Advanced stage in HNSCC, especially advanced T stage, is correlated with worse patient overall survival. When samples were analyzed by tumor stage, plasma DEK concentrations were significantly lower in patients with T3 and T4 stage disease compared to early stages T1 or T2 (Figure 1C). Given that DEK levels are decreased in HPV/p16-negative patients and in patients with advanced disease, low plasma DEK concentrations appear to correlate with variables associated with poor disease outcomes. Due to this difference in tumor T stage and DEK concentrations, we next assessed patient survival. We did not detect a difference in survival based on DEK plasma concentrations (\(P = .42\)); however, analysis comparing plasma DEK levels with treatment response is limited here by a small sample size and short time for follow-up. We were able to confirm in our cohort that HPV negative HNSCC has a poor clinical outcome compared to HPV positive disease (\(P = .0012\), data not shown).

### Table 4. Correlation of Plasma DEK Concentrations with Complete Blood Cell Counts

| Cells | Median (Range) | Univariate Coefficient | Adjusted for p16 Status Coefficient | \(P\) Value | \(P\) Value |
|-------|----------------|------------------------|------------------------------------|-------------|-------------|
| WBC   | 7.8 (4.5–18.3) | 0.26                   | 0.15                               | 29.1        | 0.029       |
| RBC   | 4.7 (3.1–5.7)  | 0.15                   | 0.49                               | 0.28        | 0.997       |
| Platelet | 239 (112–653) | 0.37                   | 0.82                               | 0.04        | 0.64        |
| ANC   | 5255 (2214–8749) | 0.13             | 0.02                               | 0.53        | 0.03        |
| ALC   | 1630 (233–3510) | 0.43             | 0.12                               | 0.03        | 0.10        |
| AMC   | 675 (50–1245)  | 0.14                   | 0.12                               | 0.49        | 0.17        |
| Eosino | 144 (0–676)    | 0.50                   | 0.77                               | 0.01        | 0.66        |
| Basophil | 49.6 (12–131) | 0.27                   | 0.22                               | 0.19        | 3.10        |

WBC: white blood cell; RBC: red blood cell; ANC: absolute neutrophil count; ALC: absolute lymphocyte count; AMC: absolute monocyte count; Eosino: eosinophil count. Correlation determined by linear regression.
Low DEK Plasma Levels Correlate with Decreased Pretreatment White Blood Cell Counts

*In vitro* studies have shown that DEK can be a chemoattractant for neutrophils, cytotoxic CD8+ T cells, and NK cells, and is released by macrophages treated with the pro-inflammatory cytokine IL-8 [23]. Furthermore, elevated extracellular levels of DEK are observed in biofluids collected locally from affected tissues in patients with inflammatory autoimmune diseases (*i.e.* synovial fluid from patients with juvenile idiopathic arthritis) [24,27]. This suggests that extracellular DEK may also be associated with differences in tumor immune responses. Indeed, after adjustment for p16 status, patients with low DEK levels correlated with a lower white blood cell (WBC) count compared to patients with higher DEK plasma concentrations, with lymphocytes (ALC) and eosinophils comprising the majority of this difference (Figure 3 and Table 4). Interestingly, there was also a direct correlation between plasma DEK concentrations and platelet counts and a trend towards a direct correlation with basophil counts (Table 4).

**Intratumoral versus Plasma DEK Levels**

Previously published work has found a significant positive correlation between intratumoral DEK and p16 expression by immunohistochemistry [33]. We next analyzed intratumoral DEK expression by immunohistochemistry and compared it to other histological markers of disease and plasma DEK concentrations. We did not observe a difference between intratumoral DEK immunohistochemical staining and p16 status; which may be due to the small sample size tested here. There also was no correlation between intratumoral DEK staining scores and plasma DEK concentrations (\(\rho = 0.22, P = .31, N = 25\)), suggesting that the tumor cells may not be the source of DEK protein detected in plasma. However, we did observe a strong positive correlation between intratumoral DEK immunohistochemistry score and the percentage of tumor cells staining positive for proliferation marker Ki67 (Figure 2), a finding which replicates previous work associating DEK with cell proliferation (Spearman correlation \(\rho = 0.77, P = .0098, N = 10\)) [15,17,18,20,34].

**Discussion**

Previous work has demonstrated that DEK could be detected in the urine of bladder cancer patients and, thus, may be useful as a disease biomarker [29]. Given this, and that activated macrophages can secrete DEK, we hypothesized that DEK may be measured in patient plasma and used as a biomarker for other solid tumors. We are the first to report that, indeed, DEK can be detected in human plasma at significant concentrations ranging from 172–2192 pg/ml. Although DEK levels were not predictive of the presence of HNSCC in general, further analysis demonstrated that DEK concentrations correlated with several important pathological characteristics and prognostic factors of HNSCC tumors, including tumor site, HPV status as determined by p16 staining, and tumor size. Although we did not find an association with patient outcome, our study was limited by a small patient size and a short time-to-follow-up of only 1–2 years. Additional studies with larger sample sizes and longer follow-up times are warranted.

Future work will be needed to determine why p16+ cancers correlate with increased plasma DEK levels. Several possibilities exist to potentially explain this phenomenon. First, given the role of extracellular DEK in inflammatory and anti-pathogenic immune responses such as neutrophil extracellular traps [42], elevated DEK plasma concentrations may be due to an immune response to the HPV antigens associated with p16+ disease. Alternatively, it may be that excess DEK protein produced as a result of the HPV E7 oncoprotein [6,31] is secreted by the HPV-transformed cancer cells. This hypothesis is not likely, as we did not observe a correlation between plasma and intratumoral levels of DEK. Finally, work in

![Figure 2](image-url). Intratumoral levels of DEK correlate with Ki67 staining as a marker of cancer cell proliferation. Representative examples of patient samples demonstrating the positive correlation of intratumoral DEK and Ki67 protein levels by immunohistochemistry. The patient on the left demonstrates a tumor with both high DEK and Ki67 protein levels while the patient on the right is an example of low DEK and Ki67 staining (Spearman correlation \(\rho = 0.77, P = .0098, N = 10\)).
vitro has demonstrated that DEK is poly(ADP-ribosyl)ated and released into the extracellular space by apoptotic cells. Therefore, apoptotic cancer cells may explain the increased plasma levels of DEK and its association with favorable prognostic markers.

Interestingly, we observed a direct correlation between plasma DEK concentrations and white blood cell counts, largely due to differences in lymphocytes and eosinophils. Importantly, this was independent of the p16 status, as a marker of HPV infection, of the tumors. Exogenous recombinant human DEK protein has been shown to suppress the proliferation of hematopoietic progenitor cells isolated from human cord blood in vitro, which could have an impact on hematopoiesis in vivo [35]. Thus, secreted DEK levels may be directly influencing hematopoiesis in patients, resulting in different CBC results. However, in our studies we observe a positive correlation between plasma DEK levels and elevated blood cell counts, whereas in vitro studies demonstrated that exogenous DEK was inhibitory to hematopoiesis. This suggests that the observations reported here, directly correlating DEK plasma levels and CBC results, are likely not due to the influence of DEK on hematopoiesis. We hypothesize that this correlation may be indicative of an anti-tumor immune response. Tumor associated eosinophils recently were shown to facilitate the anti-tumor immune response by enhancing CD8+ T cell infiltration and have been linked to favorable prognosis in oral squamous cell carcinomas [36,37]. Several studies analyzing absolute lymphocyte count (ALC) in pre-operative patients with solid tumors have demonstrated that lower lymphocyte counts, as we observed with low DEK plasma concentrations, are associated with decreased survival rates [38–41]. Additional studies are needed in order to determine if the lower eosinophil and lymphocyte counts on CBC from patients with low plasma DEK concentrations correlate with limited intratumoral immune responses and poor survival.

The finding that low plasma DEK concentrations correlate with factors associated with poor outcome is the opposite of numerous reports demonstrating that high intratumoral staining for DEK is an independent prognostic factor of poor outcome. However, this is not entirely surprising, given the previous reports linking extracellular DEK protein with activation of the immune response. Thus, we hypothesize that extracellular DEK protein, as found in patient plasma, may be an indicator of an inflammatory, anti-tumor immune response and, thus, is temporarily beneficial to limit tumorigenesis and disease progression. Therefore, future efforts to therapeutically target DEK in malignancies should be approached with caution as it may negatively impact the anti-tumor immune response.

Conclusions

Low plasma DEK concentrations were associated with p16-negative disease, which is typically found in sites other than the oropharynx, and has a poor prognosis. In addition, low plasma DEK levels correlated with larger tumor size, a measurement used during TNM staging. Finally, we also noted a direct correlation between plasma DEK levels and pre-operative ALC. Combined, the results indicate that lower amounts of DEK in patient plasma may be predictive of advanced disease status and poor treatment outcomes. Importantly, these findings should also be taken into consideration when developing DEK-targeting therapeutics. Further studies are needed to investigate the prognostic value of plasma DEK levels and the interplay between extracellular DEK in patient biofluids and the anti-tumor immune response.

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