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In vitro cytokines release profile: Predictive value for metastatic potential in head and neck squamous cell carcinomas

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Abstract

Background—Head and neck squamous carcinomas (HNSCC) have devastating morbidity rates with mortality mainly because of metastasis.

Methods—Multiplex Enzyme-linked-immunosorbent-assay (ELISA) to assay a variety of cytokine levels secreted by a panel of stage- and anatomic site-specific primary, recurrent and metastatic University of Michigan-HNSCC cell lines over a 72-hour time-course.

Results—Conditioned medium from metastatic or recurrent HNSCC showed significantly higher amounts of interleukin (IL)-6, IL-6 receptor, Tumor Growth Factor-beta (TGF- β) and Vascular Endothelial Growth Factor (VEGF) than nonmetastatic cells or normal oral keratinocytes. Tumor Necrosis Factor was only secreted by the stage IV, metastatic, or recurrence-derived cell lines.

Conclusion—The cytokine profile of cultured HNSCC cells suggests that high levels of IL-6 and IL-6R, TGF- β , and VEGF are significantly related with their metastatogenic potential and provide rationale for determining if serum testing for a combination of these four soluble factors could be of predictive value for the HNSCC tumor progression and clinical outcome.

Keywords

Head and neck squamous carcinoma (HNSCC); cell lines; cytokines profiling; multiplex analysis; clinical outcome; metastasis

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Introduction

Head and neck squamous cell carcinomas (HNSCC) are associated with poor clinical outcome and mortality rates among the highest of all carcinomas.⁽¹⁾ Among the HNSCC, oral cancer is statistically shown to be the most devastating with high mortality rates because of their high metastatic potential.⁽¹⁾ The extent of metastatic potential is dependent on the tumor extracellular environment with intercellular signaling by cytokines thought to be one of the critical components. Cytokines can have autocrine effects promoting tumor cell growth as well as paracrine effects on myeloid, lymphoid and endothelial cells enhancing metastatogenesis.⁽²⁻⁷⁾ Thus, certain carcinomas recruit vascular endothelial growth factor (VEGF) receptor 1-positive haematopoietic progenitors to the pre-metastatic niche by secreting VEGF-1.⁽²⁻⁵⁾ Tumor growth factor-beta (TGF- β) can promote tumor cell proliferation through an autocrine effect.⁽²⁻⁵⁾ Elevated serum levels of cytokines like interleukin (IL)-6 that are involved in inflammatory processes are often indicative of tumor proliferation and a poor clinical prognosis and outcome.⁽²⁻⁸⁾ In the current study, we compared the levels of cytokines secreted by a panel of cell lines established by our group (University of Michigan-squamous cell carcinomas [UM-SCC] 10A, -10B, 11A, 11B, 22A, 22B, 12, 6, 103, 14A, 14B, and 47).^(9, 10) (See Table 1 in Lin et al⁽¹⁰⁾) with the American Type Culture Collection (ATCC) clones SCC4 (nonmetastatic) and Cal27 (metastatic), and Normal Oral Keratinocytes (NOK) over a time course of 72 hours. Our goal was to investigate the cytokine signature of cultured HNSCC and to assess the plausibility of using cytokine profiles as possible diagnostic and prognostic tool.

The cytokines tested were Interleukins 2, 3, 4, 7, 6 and its receptor, Tumor Necrosis Factor (TNF) and its receptor, Interferon (IFN) alpha and gamma, VEGF, and TGF- β . We postulate that differences in cytokine profiles that are associated with aggressive tumor characteristics could be used to our advantage clinically. Cytokine concentration assays are easy and fairly inexpensive to perform, and could be the basis of a quick test of predictive value for the tumor progression and clinical outcome in patients who present symptoms of head and neck cancer.

Materials and Methods

Cell lines and Cell Culture

The cell lines used in this study were established by our group.⁽⁹⁾ Table 1 summarizes their origin, staging, and treatment subjected to prior to derivation. We also used American Type Culture Collection (Manassas, VA) clones SCC4 (CRL-1624) and Cal 27 (CRL-2095). All carcinoma cell lines were cultured in complete Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad CA), 1% nonessential amino acids 100X (Invitrogen), 1% Penicillin-Streptomycin, 100X (Invitrogen). The flasks were placed in 37° Celsius incubators with 5% CO₂. They were passaged at a split ratio of 1:3 – 1:6. The cell lines were periodically monitored for mycoplasma contamination and tested negative. The cell lines were genotyped to rule out cross-contamination and to confirm their identity, and their morphologies were regularly examined. Human NOKs were obtained from the ScienCell Research Laboratories (Carlsbad, CA; catalog number : #2610) and grown in basal keratinocytes growth media for the routine serum-free culture of normal human

keratinocytes in accord with the manufacturer's instructions (Lonza, Walkersville, MD) supplemented with hydrocortisone, insulin (bovine), human epithelial growth factor, and bovine pituitary extract.

Supernatants Collection

Cells were passaged at different densities to achieve 70% confluence at varying time periods (4, 8, 24, 48, and 72 hours). Supernatants were collected in each of these time points and stored at -80°C until they could all be processed together. Protease inhibitors (Complete Mini Kit with EDTA, Roche, San Francisco, CA) were added to the thawed supernatants which were then concentrated by centrifugation in a Beckman swing-bucket table-top centrifuge at 3750 rpm in Amicon Ultra-15 filter tubes (Millipore, Billerica MA) that had been passivated overnight with 0.1% Tween-20 in accord with the manufacturer's instructions. Concentration coefficients were recorded and all cytokine values are adjusted according to concentration factors. Protease Inhibitors (SIGMA) leupeptin, pepstatin, and aprotinin, as well as phenylmethylsulfonyl fluoride (PMSF) and Ethylenediaminetetraacetic acid (EDTA) were then added to the concentrates.

Multiplex enzyme-linked immunosorbent assay

Multiplex ELISA was outsourced to the University of Maryland Cytokine Core and was carried out using a Millipore Kit (Upstate Biotechnologies, New York, NY). Appropriate reagents were used and all the procedures were followed in accord with the manufacturer's instructions. The cytokines tested were ILs 2, 3, 4, 7, and 6, and its receptor, TNF and its receptor, IFN alpha and gamma, VEGF, and TGF- β . Standard curves were used and controls were run for each cytokine tested. Triplicates tests were carried out for each sample. The plates were read using Luminex technology and IS. software. The final concentrations (expressed in pg/mL) were calculated using Upstate multiplexing software and were adjusted so they are statistically comparable.

Statistical analysis

Triplicate tests were carried out for each sample. Values for repeats have been averaged and standard deviation (SD) has been added to the reported means. We performed a statistical analysis of all the samples including all time points using PASW Statistics 17 software. We have used the two most popular nonparametric tests of central tendency, the Mann-Whitney and Wilcoxon tests; Table 2 summarizes the means and SDs for all the time points tested. Statistical values are listed for each comparison and p values $< .05$ were considered significant.

Results

Head and neck carcinoma versus normal oral keratinocyte – overview

HNSCC cell lines (their complete information is summarized in Table 1) and NOK cytokine concentrations have been measured (pg/mL) for various time periods of incubation (4, 8, 24, 48, and 72 hours). The cytokine expression of the HNSCC cell lines was compared to NOK. The results of this comparison were consistent for all the time points analyzed; Figures 1-4 highlight the conclusions by analysis of cytokine values corresponding to the 48-hour time

point for each of the cell lines shown. Despite the various growth factors contained in the keratinocytes growth media used for the routine serum-free culture of normal human keratinocytes, the values measured in NOK for all the cytokines tested were undetectable or low.

The Mann-Whitney and Wilcoxon test results as an average for all the time points tested are summarized in Table 2 (gray highlights the results of the statistically significant). Within the tested panel, only IL-6, IL-6 receptor, VEGF, and TGF- β showed significant differences in expression between HNSCC and NOK. By normalizing the levels of cytokine expression of each cell line versus the NOK we found that there are also differences in the expression of these cytokines between oral cancer originated cell lines (UM-SCC 14A, 14B, 6, 47, 103, SCC4, and Cal 27) and non-oral cancer cell lines (UM-SCC 10A, 10B, 11A, 11B, 12, 22A, and 22B). Finally, a difference was found between cell lines established from a primary tumor site and its metastatic relative; conditioned medium of metastatic cell lines showed significantly higher amounts of IL-6, IL-6 receptor, TGF- β , and VEGF than conditioned medium from nonmetastatic cells or oral keratinocytes. No significant levels of IFN-gamma or IL-2, 3, 4, and 7 were measured. Finally, TNF-alpha was only secreted by stage IV metastatic and recurrence-derived cells.

Briefly, the statistical analysis shows that VEGF levels were the best indicators for all comparisons run. VEGF values were higher for non-oral as opposed to oral cancers, for cell lines derived from advanced as opposed to early stage cancers, and for lines from cancers with positive as opposed to negative lymph node metastases (Table 2). VEGF, IL-6 and IL-6R activity was very high in head and neck cancer cell lines as opposed to normal keratinocytes (Figure 1A) and were detectable in cell lines from even early stage oral and non-oral primary cancer sites. VEGF values seemed to differentiate between cell lines from early or late stage tumors in both oral and non-oral cancers.

TGF- β expression for the cancer cell lines was over three times higher than NOK. A maximum threshold has been detected in the highest expressing stage IV tumor-derived cells, UMSCC11B, in which TGF- β activity was about ten times that of NOK. Interestingly, TGF- β expression was significantly higher in supernatants from late-stage carcinoma cell lines, particularly in those that originated from the oral cavity. Values increased between stage IV primary oral cavity cell lines with distant metastasis and the stage IV primary oral cavity cell lines without distant metastasis. A schematic view of the data summarized in Table 2 and discussed above is presented in Figure 1A to D ((values are expressed in picograms/mL).

Early stage versus late stage

Our analysis indicated that supernatants from HNSCC cell lines established from tumors at a late stage (III-IV) have levels of IL-6, IL-6R, and VEGF that are significantly higher than the levels of the same cytokines in supernatants from cell lines of tumors at early stages (I-II) (Figure 1B). TGF- β was also higher in late-stage tumor cell lines, although the difference was not significant (Table 2 and Figure 1B).

Non-oral versus oral carcinomas

The mean expression of IL-6R, VEGF, and TGF- β was significantly higher in non-oral than in oral SCC (OSCC) cell lines, whereas IL-6 levels, although not statistically significant, were found to be twice as high in non-oral cell lines as the levels in OSCC (Table 2 and Figure 1C). Although this is consistent for all time points analyzed, only the differences at the 48-hour time period are illustrated for simplicity.

Metastatic versus nonmetastatic head and neck squamous cell carcinoma

Our data indicated that IL-6, IL-6R and VEGF were significantly higher in supernatants from HNSCC cell lines derived from patients with lymph node metastasis than in those with negative lymph node metastasis at all the analyzed time points. In contrast, there was no significant difference for TGF- β (Table 2). Our analysis also found a difference between metastatic (UM-SCC 10B, 11B, 14B, and 22B) and non-metastatic cell lines (UM-SCC 10A, 11A, 22A, 47, Cal 27, and SCC4), which is illustrated in Figure 1D.

IL-6 expression was higher for metastatic cell lines than for the primary site cell lines. VEGF was also significantly higher for metastatic HNSCC. IL-6R expression in metastatic HNSCC was lower than the expression observed in primary site cell lines, whereas TGF- β expression was significantly higher in stage IV primary oral cavity cell lines with distant metastasis than the stage IV primary oral cavity cell lines without distant metastasis.

Interleukin-6/Interleukin-6R ratio

IL-6R levels were many folds higher when compared to the control NOK levels (Figure 2). It is notable, however, that the non-oral cell lines with the latest staging and the highest IL-6 (Figure 2C and D) concentration also had greatly reduced levels of IL-6R (Figure 2B and E). Overall, non-oral cell lines of stage III and oral cancer-derived cell lines of Stage IV show a ratio of IL-6/IL-6R that is lower than that of NOK, which was exactly equal to 1 (Figure 2C and F). In contrast, Stage IV non-oral cancer cell lines 11A and 11B had 3 orders of magnitude higher IL-6R expression than NOK (Figure 2C). With the exception of values for stage IV cell lines, IL-6 values and IL-6/IL-6R ratios tend to increase during the progression of the tumor from primary to recurrence/metastasis, as is demonstrated by the rising slope of the line connecting the value of primary versus recurrent tumor/metastasis illustrated in Figure 2A, C, D, and F. The maintenance of the physiologic phenotype may be dependent on the maintenance of the ratio between the cytokine and its receptor.

Head and neck squamous cell carcinomas versus normal oral keratinocytes – interleukin-6, interleukin-6R, vascular endothelial growth factor, and transforming growth factor-beta

When compared to NOK, both non-oral-derived and oral-origin-derived cell lines had concentrations of IL-6, IL-6R (Figures 2A, B, D, and E) and VEGF (Figures 3B and D) that were orders of magnitude higher than the values for NOK. TGF- β , though high in absolute value (expressed in picograms/mL), was slightly higher in HNSCC when compared to NOK (Figure 3A and C).

Head and neck squamous cell carcinomas versus normal oral keratinocytes – tumor necrosis factor

An interesting trend was the higher expression of TNF in cell lines of late stage in both non-oral and oral-derived cell lines (Figure 4a and B). No TNF was detectable in NOK cells, therefore the results shown in Figure 4 could not be normalized for NOK expression.

Discussion

The goal of the current study was to investigate the cytokine signature in cell line supernatants from HNSCC and to provide rationale for use of cytokine profiles as possible diagnostic and prognostic clinical tools. Head and neck cancers are very immunogenic cancers, and are often heavily infiltrated by lymphocytes, macrophages, neutrophils and dendritic cells.⁽¹⁾ Chronic inflammatory processes including cancer have been associated with deregulated expressions of cytokines.⁽¹¹⁾ Elevated serum levels of cytokines that assist those inflammatory processes like IL-6 are often indicative of tumor proliferation and a poor clinical prognosis and outcome.⁽²⁻⁸⁾ IL-6 is a pleiotropic cytokine that is secreted by various cell types in the body including fibroblasts, macrophages, lymphocytes and activated keratinocytes.⁽¹²⁾ It has been identified as a key regulator of a cytokine network responsible for the progression of squamous cell skin carcinoma; IL-6 expression has been found to be upregulated when the tumor progressed from a benign to a highly malignant state.⁽¹³⁾ Our finding that IL-6 was higher in HNSCC cell lines when compared to NOK by 4, 5 or 6 orders of magnitude, is a strong indicator that IL-6 is significantly involved in head and neck carcinoma. Accordingly, IL-6 expression was greater in cell lines derived from metastatic HNSCC than in HNSCC established from nonmetastatic primary site tumors suggesting that IL-6 may be indicative of the metastatic potential of head and neck cancer. IL-6 was also significantly higher in SCCs derived from tumors diagnosed with high/late staging when compared with SCCs established from early-stage tumors, suggesting that IL-6 increases in concentration when the tumor stage advances.

IL-6 is a key mediator of a complex cytokine network heavily involved in signaling pathways. It binds to IL-6R and then associates with gp130 receptors. The complex becomes active and it triggers three main signaling pathways: the JAK/STAT, the MAP kinase, and the PI3-kinase/Akt pathway.^(14, 15) Since IL-6R binds IL-6, it is, to some extent, complementary to IL-6 and is present in relatively high concentrations when IL-6 is up-regulated. Our study shows that IL-6R expression is elevated in the supernatants from head and neck cancer cell lines, whereas NOK registered very low values of IL-6R. IL-6 and its receptor were high in concentration in OSCC, which is in agreement with a previous study that focused exclusively on IL-6 and IL-6R expression in oral cancers.⁽¹⁶⁾ IL-6R expression in late stage tumors was higher than in early stage carcinomas. We still do not fully understand the effects of IL-6 and IL-6R on cancer. Certain studies have led to speculation that IL-6R might have binding partners that are different than IL-6, such as a novel synthetic peptide, S7, which is known to inhibit VEGF.^(17, 18)

Interestingly, our data also shows that overall, IL-6R has higher expression levels in non-oral carcinomas than the oral ones. In addition, in the oral-derived cancer cell lines we also noticed an increase in its secretion dependent on staging, suggesting that IL-6

overproduction that mirror tumor progression is mostly in its free unbound form, which may be indicative for the poorer clinical outcome of oral cavity cancers as opposed to other head and neck carcinomas. However, opposing trends are seen with staging for the IL-6/IL-6R ratio for non-oral versus oral HNSCCs; for instance, the stage IV non-oral HNSCC cells 11A and 11B show much lower expression of IL-6R than stage III HNSCCs. As a consequence, the IL-6/IL-6R ratio is 3 orders of magnitude higher in Stage IV non-oral HNSCC compared to NOK. When the cytokine is in a vast excess to its receptor, it may not have a significant autocrine effect on proliferation, but it may potentiate the local inflammatory response that facilitates invasion and metastasis. Although oral cancer is known to be more aggressive than non-oral cancers, there may be other factors that explain the differences between the 2 categories such as VEGF-independent tumor angiogenesis mediated via either the intracellular IL-6 or platelet-derived growth factor, or the chemokines that contain the glutamine-leucine-arginine (Glu-Leu-Arg: the ELR motif) with IL-8 being one of the representatives.⁽¹⁹⁻²¹⁾ Members with this ERL motif are potent inducers of angiogenesis (for review see⁽²⁰⁾).

It is notable that among oral cancer cell lines, IL-6R was lowest in a cell line derived from a recurrent tumor (UMSCC-14B) but the IL-6/IL-6R ratio was very high for this cell line. With the exception of values for stage IV cell lines, IL-6 values and IL-6/IL-6R ratios tend to increase during the progression of the tumor from primary to recurrence/metastasis (as is indicated by the rising slope of the line connecting the value of primary versus recurrent tumor/metastasis illustrated in Figures 2A, C, D and F). The IL-6/IL-6R ratios are therefore strategic determinants of tumorigenesis because they are always present at high levels in HNSCC cells when compared to normal cells, regardless of the primary site or stage, and their levels may also be a determinant of lymph node metastasis.

In various types of cancer, IL-6 is either a direct or an indirect stimulant of the release of inflammatory and angiogenic factors, such as IL-8, granulocyte-macrophage colony-stimulating factor, VEGF, and MCP-1. In cervical cancer, IL-6 can induce angiogenesis indirectly through the release of VEGF.⁽²²⁾ The combination of IL-6 and IL-6R was shown to increase VEGF production in fibroblast-like synovial cells from patients with rheumatoid arthritis, whereas IL-6 by itself did not.⁽²³⁾ In our study VEGF follows the trends of IL-6 expression supporting the idea of VEGF dependence on IL-6 (illustrated in Figure 1). VEGF has been shown to promote lymphogenesis, angiogenesis, and vascular permeability in cancer.^(24, 25) Furthermore, our data indicate that VEGF values arise as the differentiating factor between early and late stage tumors in both oral and non-oral cancer (summarized in Table 1).

To our knowledge, there are no other studies evaluating the cytokines levels of expression at multiple time points. The current study analyzes five different time points over 72-hour period with consistent results, confirming the stability of the increase noted in the cytokines expression levels and the reliability of multiplex ELISA as a highly sensitive method of detection. Our data indicate that IL-6, IL-6R, and VEGF were significantly higher in supernatants from HNSCC cell lines derived from patients with lymph node metastasis than in those with negative lymph node metastasis at all the analyzed time-points, confirming the

plausibility of using cytokine profiling as possible diagnostic tool of HNSCC metastatic potential.

TGF- β acts as an anti-proliferative agent in normal cells in the body. In cancer, some of the components of the TGF- β signaling pathway may become mutated, resulting in a loss of TGF- β function in the cancer cells. Consequently, TGF- β starts acting on nearby stromal, immune and endothelial cells. The effect may be harmful to cancer patients, as it suppresses the immune system and induces angiogenesis.⁽²⁶⁻²⁷⁾ Our data indicate that TGF- β expression was 3 times greater in head and neck cancer cell line supernatants than in NOK, which is in agreement with previous studies done on TGF- β in different types of cancer (for review see⁽²⁸⁾). Given the significant amounts of the protein secreted by normal cells, small-fold differences may have significant long-term effects in tumor growth. Increased stage and aggressiveness give rise to higher TGF- β levels with a maximum threshold reached in the highest expressing cell line, the stage IV non-oral recurrent UMSSC 11B, in which TGF- β activity was about 10 times that of NOK.

TNF is not expressed in NOK or most HNSCC cell lines except in the 4 late stage cell lines, with the highest values correlating to the most advanced stage. In a diagnostic implementation of our results TNF expression would be a late-stage determinant.

Although these results are exciting, they are based on cell lines and further clinical or preclinical studies are warranted to fully verify these hypotheses. Previous studies supported by the University of Michigan Head and Neck SPORE have reported independently VEGF and IL-6 detected in pretreatment serum as valuable biomarkers for predicting recurrence and overall survival in patients with HNSCC, thus indicative of tumor proliferation and a poor clinical prognosis and outcome (6,8). Further development of cytokine profiling will require a larger statistical sample of sera from oral, head and neck squamous cell carcinoma patients, assigning threshold values and designing a test for clinical application. Our results suggest that the serum cytokine expression profile in head and neck cancer should be explored as a potential clinical test that is simple to perform and inexpensive to complete. The cytokine release profiles of HNSCC cell lines with high levels of IL-6 and IL-6R together with VEGF expression characterize their relative metastatic potential. This work also suggests that TGF- β and TNF testing might be useful for the categorization of late stage non-oral origin cancers. Thus, a combination test of these various soluble factors may provide a stronger predictive value for the HNSCC tumor progression and clinical outcome.

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Abbreviations

ATCC	American Type Culture Collection
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HNSCC	Head and neck squamous carcinomas
IFN-γ	Interferon gamma
IL	Interleukin
KGM	Keratinocytes Growth Factor
NOK	Normal oral keratinocytes
OC	Oral cancer
PMSF	Phenylmethylsulfonyl fluoride
TGF-β	Tumor growth factor-beta
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
UM-SCC	University of Michigan Squamous Cell Carcinoma

Figure 1.

Mean cytokine levels in head and neck squamous cell carcinomas (HNSCCs). Cytokine concentrations subjected to statistical analysis as described in the Methods section. Although the results of our analysis were consistent for all the time points analyzed, the figure highlights the conclusions by analysis of cytokine values corresponding to the 48-hour time point for each of the cell lines shown. Values are expressed in pg/mL. Basis for comparison included: (A) cytokine values for HNSCC cell lines as compared to normal oral keratinocytes (NOKs); (B) staging (late = stages III and IV vs early = stages I and II); (C) oral vs non-oral origin of tumors; and (D) whether lymph node metastasis was present (N+) or not (N0) at the time of cell line derivation. Among the cytokines tested, interleukin (IL)-6, IL-6R, vascular endothelial growth factor (VEGF), and transforming growth factor-

beta (TGF- β) show significant differences in HNSCC versus NOK. The increase in VEGF expression was the most notable for late-stage and metastatic HNSCC.

Figure 2.

Interleukin (IL)-6, IL-6R, and their ratio values in head and neck squamous cell carcinoma (HNSCC) supernatants (normalized to normal oral keratinocytes [NOKs]). IL-6 and IL-6R activities reported for the 48-hour time point of each analyzed cell line were averaged and the mean and SD were divided by corresponding NOK values. Values are expressed in pg/mL. The graphs were generated using Microsoft Excel. IL-6 values used for graphs shown in (A) and (D) were divided by IL-6R values (shown in graphs B and E) to calculate the IL-6/IL-6R ratio (C, F). Lines are used in the graphs to connect values for cell lines

originating from the same patient at progressive stages of the cancer, for example, University of Michigan-squamous cell carcinoma (UM-SCC10A) and 10B. These illustrate a trend for increase or decrease with cancer progression (discussed in Results and Discussion sections).

Figure 3.

Transforming growth factor-beta (TGF- β) and vascular endothelial growth factor (VEGF) values in head and neck squamous cell carcinoma (HNSCC) supernatants (normalized to normal oral keratinocytes [NOKs]). TGF- β (A, C) and VEGF (B, D) activities reported for the 48-hour time point of each cell line illustrated were averaged and the mean and SD were divided by corresponding NOK values and graphed using Microsoft Excel. Values are expressed in pg/mL.

Figure 4.

Tumor necrosis factor (TNF) values in head and neck squamous cell carcinoma (HNSCC) supernatants (not normalized to normal oral keratinocytes [NOKs]). TNF activities reported for the 48-hour time point of each cell line were averaged and the mean and SD were graphed using Microsoft Excel.

Table 1

Cell lines and their characteristics.

Cell line	Grade/differentiation	Stage	Specimen site	Type of lesion	Primary site	Previous Treatment
UMSCC 10A	Moderate to well	III	True vocal cord	Primary	True vocal cord	None
UMSCC 10B	Moderate to well	III	Lymph node	Lymph node metastasis	Larynx	Surgery
UMSCC 11A		IV	Epiglottitis	Primary	Epiglottitis	None
UMSCC 11B		IV	Supraglottic	Persistent	Supraglottic	Chemotherapy
UMSCC 12	Moderately well	III	Larynx	Recurrence	Larynx	Surgery
UMSCC 22A	Moderate	III	Hypopharynx	Primary	Hypopharynx	None
UMSCC 22B	Moderate	III	Neck metastasis	Metastasis	Hypopharynx	None
UMSCC 14A	Poorly to moderately well differentiated	I	Floor of mouth	Recurrence	Floor of the mouth	Surgery
UMSCC 14B	Poorly differentiated	I	Floor of mouth	Recurrence	Floor of the mouth	Surgery, surgery, radiation therapy
UMSCC 47	Moderately well	III	Lateral tongue	Primary	Lateral tongue	None
CAL27	Poorly differentiated	IV	Tongue	Primary	Tongue	Radiotherapy, partial glossectomy, neck dissection, chemotherapy
SCC4	Moderate	III	Tongue	Primary	Middle of tongue	Radiotherapy, chemotherapy-little response
6	Moderate to poorly	II	Tongue	Primary	Back of tongue	None
103	Well	IV	Tongue	Primary	Right lateral tongue	None
NOK	-	-	-	-	-	-

Abbreviation: UMS-SCC, University of Michigan-squamous cell carcinoma.

Note: Table indicates the cell lines used in this study and their complete information: the degree of differentiation as shown by cytology (Grade/Differentiation), the stage of the cancer at the time of cell line derivation (Stage), the head and neck site of the biopsy (Specimen Site), whether the biopsy was of primary, recurrent or metastatic lesion (Type of lesion), what the primary site of occurrence had been (Primary Site) and the treatment received by the patient prior to the biopsy (Previous Treatment).

Table 2

Summary of statistical comparisons.

Basis for comparison	IL-6	IL-6R	VEGF	TGF- β
<i>Oral vs Non Oral</i>	No significant dif. (p=0.224)	ORAL<NON-ORAL (p=0.043)	ORAL<NON-ORAL (p=0.000)	ORAL<NON ORAL (p=0.011)
<i>Stage Early (I-II) vs Late (III-IV)</i>	No significant dif. (p=0.286)	No sig. dif. (p=0.113)	EARLY < LATE (p=0.002)	No significant dif.(p=0.825)
<i>Lymph Node “NO” vs “N+”</i>	N0 < N+ (p=0.000*)	N0 < N+ (p=0.040)	N0 < N+ (p=0.000)	No significant dif. (p=0.336)
<i>Oral vs NOK</i>	ORAL > NOK (p=0.000)	ORAL > NOK (p=0.000)	ORAL > NOK (p=0.000)	No significant dif. (p=0.244)
<i>Non-oral vs NOK</i>	NON ORAL > NOK (p=0.000)	NON ORAL > NOK (p=0.000)	NON ORAL > NOK (p=0.000)	NON ORAL > NOK (p=0.000)
<i>Stage Early (I-II) vs NOK</i>	EARLY (I-II) > NOK (p=0.000)	EARLY > NOK (p=0.000*)	EARLY > NOK (p=0.000)	No significant dif.(p=0.129)
<i>Stage Late (III-IV) vs NOK</i>	LATE > NOK (p=0.000)	LATE > NOK (p=0.000)	LATE (III-IV) > NOK (p=0.000)	LATE > NOK (p=0.004)

Abbreviations: IL, interleukin; VEGF, vascular endothelial growth factor; TGF- β , transforming growth factor-beta; dif., difference; NOK, normal oral keratinocyte.

Note: Cytokine concentrations subjected to statistical analysis are as described in the Methods section. Data are presented as an average for all the time points tested. Statistical values are listed for each comparison with *p* values < .05 considered significant. Basis for comparison included oral versus non-oral origin tumors, staging (late = stages III and IV vs early = stages I and II) and whether lymph node metastasis was present at the time of cell line derivation (N+) or not (N0).