Original Article

Matrix metalloproteinase (MMP) and immunosuppressive biomarker profiles of seven head and neck squamous cell carcinoma (HNSCC) cell lines

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Background: Biomarkers like programmed death ligand-1 (PDL1) have become a focal point for immunotherapeutic checkpoint inhibition in head and neck squamous cell carcinoma (HNSCC). However, it's only part of the total immunosuppressive biomarker profile of HNSCC cells. Matrix metalloproteinases (MMPs) are enzymes that break down the basement membrane allowing cancer cells to metastasize and play an important role in the tumor microenvironment. MMPs can also activate certain cytokines, growth factors, and chemokines post-translationally. The objective of this study was to determine MMP and biomarker profiles of seven different HNSCC cell lines.

Methods: Authenticated cell lines were grown in minimal media at 1×10^6 viable cells/mL and incubated at 37 ℃. After 24 hrs supernatants were collected, and adhering cells were lysed. Multiplex immunoassays were used to determine MMP1, MMP7, MMP9, IL-6, VEGFA, IL-1α, TNF-α, GM-CSF, IL-1RA, and IL-8 concentrations in supernatants. ELISAs were used to determine PDL1, CD47, FASL, and IDO concentrations in cell lysates. A one-way ANOVA was fit to examine log-transformed concentrations of biomarkers between seven HNSCC cell lines, and pairwise group comparisons were conducted using post-hoc Tukey's honest significance test ($\alpha=0.05$).

Results: Significant differences ($P<0.05$) in MMP and biomarker concentrations were found between the seven HNSCC cell lines. For example, MMP9 was highest in SCC25 and UM-SCC99, MMP7 was highest in SCC25 and UM-SCC19, and MMP1 was highest in SCC25.

Conclusions: These results suggest different patients' HNSCC cells can express distinct profiles of select biomarkers and MMPs, which could be due to metastatic stage of the cancer, primary tumor site, type of tissue the tumor originated from, or genomic differences between patients. MMP and biomarker expression profiles should be considered when choosing cell lines for future studies. The results support the reason for personalized medicine and the need to further investigate how it can be used to treat HNSCC.

Keywords: Biomarkers; cell culture techniques; head and neck squamous cell carcinoma (HNSCC); immunosuppression; matrix metalloproteinases (MMPs); tumor microenvironment

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common form of cancer by incidence worldwide (1). In HNSCC, matrix metalloproteinases (MMPs) degrade extracellular matrix and the basement membrane, which is associated with tumor invasion and metastasis (2). MMPs have also been implicated in tumor progression due to their ability to activate growth factors and enhance angiogenesis (3). Despite this known association, the expression of these MMPs in relation to other biomarkers has not been fully investigated in HNSCC (4).

In HNSCC and other cancers, tumor cells create an immunosuppressive environment through various immunosuppressive mediators and strategies (5-7). Cancer cells use a variety of mechanisms to evade host defenses and metastasize. In this study, we have focused on the tumor microenvironment by examining differences in the biomarkers expressed by cancer cells to induce immunosuppression and the MMPs expressed by cancer cells that aid in metastasis. Specifically, we examined the production of immunosuppressive biomarkers: interleukin-6 (IL-6), vascular endothelial growth factor A (VEGFA), interleukin (IL)-1 alpha (IL-1α), tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1 receptor antagonist (IL-1RA), and IL-8; and MMPs: MMP1, MMP7, and MMP9. We also looked at the cell surface marker production of programmed death ligand-1 (PD-L1), Fas ligand (FASL), indoleamine 2,3 dioxygenase (IDO), and cluster of differentiation (CD47). Not only have these biomarkers been suggested as helping tumor cells in creating an immunosuppressive environment, they also contribute to other biological functions like migration, angiogenesis, and cell growth regulation (5).

MMPs are the major enzymes implicated in degradation of the extracellular matrix and the basement membrane (2). MMPs are from a large family of calcium-dependent, zinc-containing endopeptidases. There are 24 MMPs in humans, which are categorized into six groups based on their substrate preferences and domain structure: collagenases (MMP1, 8, 13), gelatinases (MMP2, 9), stromelysins (MMP3, 10), matrilysins (MMP7, 26), membrane-type metalloproteinases (MT-MMPs), and others (2,8). MMPs play important roles in wound healing, tissue repair, angiogenesis, bone remodeling, morphogenesis, tooth eruption, cell communication, and remodeling after injury, but are also implicated in inflammatory diseases such as rheumatoid arthritis, atherosclerosis, and periodontal disease (2).

MMPs also play critical roles in immune responses by either modifying proteins post-translationally to promote rapid delivery to other cells or by inactivating these proteins to initiate or terminate the immune process (9). MMPs can release cytokines, growth factors, and chemokines from their pro-forms (10). These functions allow MMPs to direct systemic inflammation and regulate cytokine biosynthesis through activation of signal transduction pathways. In cancer, MMPs play a role in tumor progression by breaking down the basement membrane barrier leading to metastasis (11). For example, MMP9 is expressed in carcinoma and inflammatory cells in oral squamous cell carcinoma (OSCC, a subtype of HNSCC) tumors (12,13). MMP9 degrades type IV collagen, a main component of the basement membrane, hence its association with cancer metastasis (2,10).

MMP activity is tightly regulated transcriptionally and post-transcriptionally (8). They are also regulated through compartmentalization of MMP release, enzyme activation by removal of the pro-domain, and inhibition by tissue inhibitors of MMPs (TIMPs) or by non-specific proteinase inhibitors. This tight regulation is necessary due to the wide array of substrates. When this regulation is not controlled, it leads to diseases such as cancer, which is why MMPs could serve as potential therapeutic targets (8,14).

The purpose of this study was to elucidate differences in the expression of proteins expressed by HNSCC cancer cell lines, which are representative of differences that could be seen between patient tumor cells, and to better understand the role of biomarkers and MMPs expressed by cancer cells in the tumor microenvironment. Studying these cell responses may lead to potential biomarkers to target for future clinical applications.

Methods

Cell lines

HNSCC cell lines SCC4 (ATCC, Manassas, VA, USA), SCC15 (ATCC), SCC19 (University of Michigan), SCC25 (ATCC), SCC84 (University of Michigan), SCC92 (University of Michigan), and SCC99 (University of Michigan) were used in this study. SCC4, SCC15, SCC25, SCC84, and SCC92 are from the oral cavity, while SCC19 and SCC99 are from the oropharynx (*Table 1*) (15). TNM classifications of malignant tumors (TNM, where T describes the size of the primary tumor, N describes the nearby lymph nodes, and M describes the metastasis) are listed for the selected cell lines in

Table 1 HNSCC cell line information gathered from the literature. The cell lines' anatomical sites from which they originated are listed. The TNM classifications of malignant tumors (TNM, where T describes the size of the primary tumor, N describes the nearby lymph nodes, and M describes the metastasis) are listed for the selected cell lines

Cell line	Gender	Anatomical site	TNM stage
SCC4	M	Oral cavity: tongue	T3N0M0
SCC15	M	Oral cavity: tongue	T4N1M0
SCC25	M	Oral cavity: tongue	T2N1
UM-SCC19	M	Oropharynx: base of tongue	T2N1M0
UM-SCC84	M	Oral cavity	T2N0M0
UM-SCC92	F	Oral cavity: lateral tongue	T2N0M0
UM-SCC99	M	Oropharynx	T3N0M0

HNSCC, head and neck squamous cell carcinoma.

Table 1. SCC19, SCC84, SCC92, and SCC99 from the University of Michigan have been previously genotyped (16,17). These cell lines were each grown and maintained in a humidified atmosphere of 5% CO_2 at 37 ℃ in T75 flasks. SCC4 cells were grown in complete Dulbecco's Modified Eagle's Medium: F-12 (DMEM:F-12) containing 2 mM L-glutamine, 1% nonessential amino acids (ATCC), 400ng/mL hydrocortisone (Sigma-Aldrich Corp., St. Louis, MO, USA), 100 units/mL penicillin (Life Technologies, Madison, WI, USA), 100 units/mL streptomycin (Life Technologies), and 10% fetal bovine serum (ATCC) (18). SCC15, SCC25, and SCC84 cells were grown in complete Lymphocyte Growth Media-3 (LGM-3) (Lonza, Walkersville, MD, USA), 100 units/mL penicillin (Life Technologies), 100 units/mL streptomycin (Life Technologies), and 10% fetal bovine serum (ATCC) (18). SCC19, SCC92, and SCC99 were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine, 1% nonessential amino acids (ATCC), 100 units/mL penicillin (Life Technologies), 100 units/mL streptomycin (Life Technologies), and 10% fetal bovine serum (ATCC). These media were chosen because they ensured growth and survival of the HNSCC cells.

The seven cell lines were authenticated as described in 2012 in ANSI Standard (ASN-0002) Authentication of Human Cell Lines: Standardization of STR Profiling by the ATCC Standards Development Organization (*Table S1*).

Cell culture supernatants

Once the cells reached approximately 60–80% confluence in T75 flasks, we harvested the adherent HNSCC cells with 0.25% trypsin containing 0.53 mM EDTA (Cat. No. 30-2101, ATCC, Manassas, VA, USA). We counted the cells and tested them for viability using propidium iodide. We centrifuged the cells (400 RCF, Eppendorf 5810R, Westbury, NY, USA) at 4 ℃ for 10 minutes and resuspended the cells in high-glucose RPMI-1640 with L-glutamine and HEPES (ATCC, Manassas, VA) to 1×10^6 viable cells/mL. By using this minimal media to characterize the biomarker production for all seven cell lines we can eliminate changes caused by the presence of additives like serum. We added 1.0 mL of this suspension to 3 wells on a 12-well tissue culture plate (Corning Inc., Corning, NY, USA). The plate was incubated in a humidified atmosphere of 5% CO_2 at 37 ℃. After 24 hours, the cells were examined via tissue culture scope to confirm they had adhered. We removed the tissue culture medium and centrifuged it (3000 RCF, Eppendorf 5415D, Westbury, NY, USA) at room temperature for 5 minutes to remove any residual cells or cell fragments. The supernatant was stored at –80 ℃ until it was time to determine the concentration of the biomarkers.

Cell lysates

After the supernatant was removed from the wells, we added 1.0 mL of cell lysis buffer (Cell Signaling Technologies, Danvers, MA, USA) containing phenylmethanesulfonyl fluoride (1.0 mM PMSF, Cell Signaling Technologies) to the adhered cell layer in each well. The lysed cell suspension (i.e., cell lysate) was removed and stored at –80 ℃.

Biomarker determination

To determine the concentrations of MMP1, MMP7, MMP9, IL-6, VEGFA, IL-1α, TNF-α, GM-CSF, IL-1RA, and IL-8 in cell culture supernatants, we used multiplex immunoassays (R&D Systems, Minneapolis, MN, USA) read on a Luminex^{100} (Luminex, Madison, WI, USA), which are routinely used in our laboratory (19,20). These immunoassay kits use antibody-coated magnetic beads to bind the desired analytes in a solution and uses a standard

curve of known concentrations to determine the unknown concentrations of the samples as previously described (21). We used enzyme-linked immunosorbent assay (ELISA, Cusabio Biotech Co., Ltd., Wilmington, DE, USA) to determine the concentrations of CD47, IDO1, FASL, and PDL1 in cell lysates.

Human papillomavirus (HPV) and p16^{Ink4a} determination

Presence of HPV antigen in the cell lines was assessed using a double-sandwich ELISA (MyBioSource, Inc., San Diego, CA, USA). Both cell lysates and supernatants were tested in the ELISA. Immunohistochemistry (IHC) was used to check p16^{Ink4a} status among the seven cell lines. After the 24-hour incubation in a replicate experiment as the one described above, the supernatant was removed and the remaining adhering cells were fixed in 10% neutral buffered formalin. The cells were then placed in agar and fixed in a paraffin block. Sections of this block were deparaffinized for IHC staining with an antibody for p16^{Ink4a}. Squamous cell carcinoma of the uterus was used as a positive control.

Statistical analysis

Three replications were done for each group and a log-10 transformation was applied to all of the biomarker concentrations to attenuate for positive skew and to make the normality assumption more defensible (19). We first analyzed the log values of the biomarker concentrations using a one-way multivariate analysis of variance (MANOVA) to detect the overall effect for the different cell lines. As the MANOVA reached significance, a univariate one-way ANOVA was performed to explore the impact of the differing cell lines on the concentration within each biomarker, followed by the pairwise group comparisons using the post-hoc Tukey's honest significant differences test. A 0.05 level was used determine statistically significance.

Results

In both the cell supernatants and cell lysates, statistically significant differences were seen among the seven cell lines in all 14 biomarkers (*Tables 2*,*S2*). The MANOVA revealed a significant multivariate effect for the different cell lines, Wilk's Lambda =0.0001 and P<0.0001. The one-way ANOVAs revealed significant differences between the seven cell lines in each of the 14 biomarkers (P<0.05 in each instance).

SCC25 was the highest producer of MMP1, MMP7, and MMP9 (*Figure 1*, P<0.05). MMP1 production was significantly different in all seven cell lines except SCC19 and SCC92. MMP7 production was significantly different in all seven cell lines except SCC15 and SCC84. MMP9 production was significantly different between several cell lines but not all, with SCC25 being the highest and SCC4 and SCC19 being the lowest. Other than SCC25 being the highest producer of all three MMPs, the only apparent trend is that these cell lines are able to produce more than one type of MMP and at significantly different levels than each other.

GM-CSF and TNF-α, both inflammatory biomarkers, were produced by SCC92 at significantly higher levels than all of the other cell lines, while SCC4 and SCC99 produced the lowest amounts of GM-CSF and TNF-α (*Figure 2*). SCC15 produced significantly higher amounts of IL-1α and IL-8 than the other six cell lines. IL-1α was produced in moderate amounts by SCC4, SCC25, SCC84, and SCC92 and at significantly lower amounts by SCC19 and SCC99. IL-8 was produced at moderately high amounts in SCC19, SCC84, and SCC92, and SCC4 produced the lowest amount of IL-8. IL-1RA production was highest in SCC4 and SCC15 and lowest in SCC99 and SCC92. IL-6 production was highest in SCC19 and lowest in SCC99 and SCC4. VEGFA production was highest in SCC25 and SCC19 and lowest in SCC4, SCC15, and SCC84. MIP1α, MIP1β, and IL-12 p40 concentrations were also determined, but they were produced at very low levels (*Figure S1*).

When looking at the cell surface markers detected in the cell lysates, we see that PDL1 production was highest in SCC25 and lowest in SCC19 and SCC84 (*Figure 3*, P<0.05). This trend was also seen with FASL production, although not significant. SCC15 produced significantly more IDO and CD47 than any of the other cell lines. It is interesting to note that SCC25 was highest in MMP1, MMP7, and MMP9 compared to the other six cell lines (P<0.05) and highest in PDL1 and VEGFA production and SCC15 was highest in IDO, CD47, IL-1α, and IL-8 compared to the other six cell lines (P<0.05).

Human papillomavirus is an emerging risk factor for HNSCC (22). The HPV ELISA showed that these cell lines are HPV negative. Similar studies have also confirmed that four of these cell lines (e.g., SCC4, SCC15, SCC25, and SCC19) were HPV negative (23). The IHC staining revealed that all seven cell lines were negative for p16^{Ink4a} expression, a tumor suppressor protein often used as a surrogate marker for HPV infection (24).

Table 2 Comparisons of observed concentrations between seven cell lines within each biomarker

Cell lines	CD47	FASL	GM-CSF	IDO	IL-1α	IL-1RA	IL-6	IL-8	MMP1	MMP7	MMP9	PD-L1	TNF-α	VEGFA
SCC4, mean (SD)	1.72 (0.04): B, C	2.80 (0.12): A	1.14 (0.03): C	4.47 (0.13): B, C	3.31 (0.02): B	4.00 (0.00): A	0.37 (0.18): E	2.39 (0.05): E	2.36 (0.05): C	2.90 (0.04): D	2.98 (0.03): E	2.38 (0.10): A, B	0.28 (0.15): C	0.85 (0.73): D
SCC15, mean (SD)	2.38 (0.01): A	2.72 (0.03): A, B	1.94 (0.04): B	5.32 (0.16): A	4.00 (0.00): A	3.82 (0.00): B	1.55 (0.07): C	4.00 (0.00): A	1.82 (0.12): E	3.73 (0.01): C	3.28 (0.02): C, D	2.35 (0.11): A, B	0.85 (0.07): B	1.33 (0.16): C, D
SCC19, mean (SD)	1.54 (0.05): C	2.50 (0.19): A, B	2.08 (0.02): B	4.28 (0.06): B, C	2.49 (0.05): C	2.56 (0.02): C	3.61 (0.06): A	3.85 (0.01): B	1.58 (0.04): F	3.91 (0.02): B	2.93 (0.02): E	1.97 (0.07): C	0.71 (0.07): B, C	2.81 (0.13): A, B
SCC25, mean (SD)	1.82 (0.12): B	2.80 (0.19): A	2.09 (0.04): B	4.52 (0.07): B	3.02 (0.03): B	2.39 (0.06): D	1.97 (0.03): C	3.12 (0.04): D	4.13 (0.01): A	4.42 (0.03): A	4.13 (0.01): A	2.57 (0.14): A	0.94 (0.01): B	3.45 (0.05): A
SCC84, mean (SD)	1.52 (0.17): C	2.44 (0.16): B	1.92 (0.07): B	4.26 (0.10): B, C	3.02 (0.08): B	2.64 (0.01): C	2.49 (0.09): B	3.85 (0.02): B	2.15 (0.05): D	3.69 (0.03): C	3.35 (0.03): B	1.97 (0.17): C	1.10 (0.09): B	0.42 (0.73): D
SCC92, mean (SD)	1.67 (0.11): B, C	2.54 (0.03): A, B	2.76 (0.48): A	4.24 (0.07): C	3.47 (0.47): B	1.99 (0.02): E	2.79 (0.48): B	3.88 (0.02): B	1.46 (0.11): F	2.74 (0.03): E	3.25 (0.03): D	2.21 (0.12): B, C	2.67 (0.44): A	2.27 (0.08): B, C
SCC99, mean (SD)	1.57 (0.09): B, C	2.65 (0.04): A, B	0.13 (0.12): D	4.37 (0.03): B, C	2.21 (0.09): C	1.62 (0.05): F	0.97 (0.09): D	3.46 (0.02): C	3.67 (0.01): B	2.37 (0.05): F	3.31 (0.01): B, C	2.31 (0.10): A, B	0.23 (0.04): C	2.11 (0.13): B, C

Three replications were done for each group and a log-10 transformation was applied. Data were first analyzed using one-way multivariate analysis of variance (MANOVA) to detect the overall effect for the different cell lines (i.e., to determine whether there was a difference across cell lines with respect to the observed concentration values). The results revealed a significant multivariate effect for the different cell lines, Wilk's Lambda =0.0001 and P<0.0001. As the multivariate test is significant, the univariate one-way ANOVA was carried out. All 14 univariate one-way procedures indicated a significant effect for all seven cell lines (P<0.05 in each case). For each univariate one-way ANOVA procedure (i.e., within each biomarker), column means with the same letter are not significantly different using the post-hoc Tukey's HSD test (P>0.05). Different letters indicate significantly different means (P<0.05).

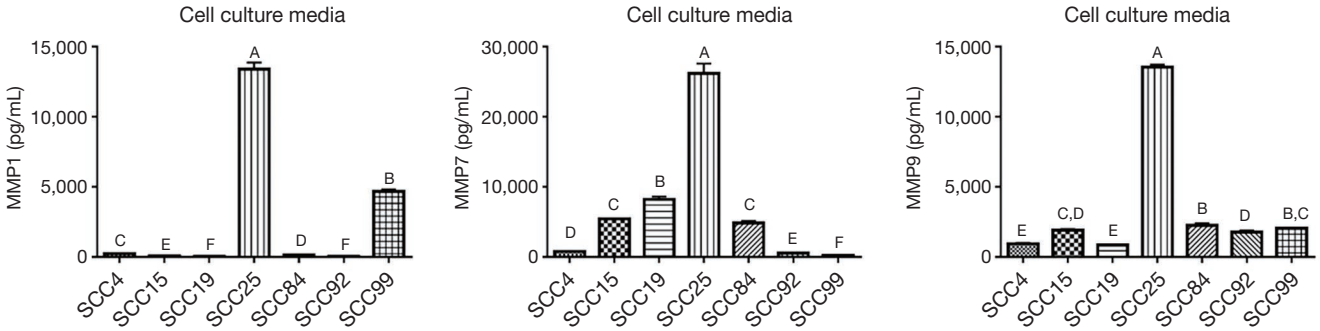

Figure 1 Matrix metalloproteinase (MMP) production by seven HNSCC cell lines. After 24 hours of incubation at 1×10^6 viable cells/mL at 37 ℃, the cell media was removed. We used multiplex immunoassays (R&D Systems, Minneapolis, MN, USA) read on a Luminex100 (Luminex, Madison, WI, USA) to determine the concentrations of MMP1, MMP7, and MMP9 in cell supernatants. A one-way ANOVA procedure was performed on the log10 values and pairwise group comparisons were made using the post-hoc Tukey's HSD test ($P<0.05$). Bars with the same letter are not significantly different. HNSCC, head and neck squamous cell carcinoma.

Discussion

As shown here, and in other studies, tumor cells can express several MMPs and at varying amounts (3). Besides contributing to the metastasis of cancer cells, MMPs can contribute to angiogenesis as well; specifically, MMP7 and MMP9 have been shown to have distinct roles in vascularization events taking place in the same tumor. MMP9 can also increase VEGF bioavailability, thereby increasing the amount of VEGF in the tumor microenvironment. MMPs also regulate inflammation in cancer (3). Several MMPs, including MMP1 and MMP9, can cleave pro-TNF-α to activate this proinflammatory cytokine. As we've seen here, HNSCC cells can release varying amount of TNF-α. Tumor cells produce TNF-α to promote cell survival. MMP9 can also interact with IL-8 to increase the recruitment of neutrophils to the tumor microenvironment (25).

MMPs also present as desirable drug targets because of the major role they play in disease progression. Early clinical trials for MMP inhibitors have been unsuccessful, but more studies are being conducted using novel approaches to target MMPs in cancer (14). MMPs have also been investigated as prognostic markers for HNSCC. For example, transcription of MMP9 has been proposed as a prognostic marker for treatment response to radiotherapy and chemo-radiotherapy (26). MMP9 has also been proposed as a possible diagnostic marker for early detection of HNSCC (27).

Cytokines, chemokines, and MMPs play a major role in both the inflammatory and tumor microenvironments. These proteins can act on each other and in synchronization. Secretion of transforming growth factor beta (TGF-β), IL-6, IL-10, and TNF-α from OSCC cells has recently been reported to create a favorable environment for tumor growth (28,29). TNF-α has been found to be secreted by stage IV, metastatic, and reoccurrence-derived HNSCC cell lines, suggesting it may serve as an indicator of late stage cancer (29). IL-6 is involved in inflammatory processes indicative of tumor proliferation, but it also has immunosuppressive effects on dendritic cells by preventing dendritic cell maturation (29,30). High expression of IL-6 in OSCC cells has been suggested as a predictive factor of poor response to chemoradiotherapy (31). The anti-inflammatory cytokine IL-1RA, which competes with IL-1β by binding to the IL-1 receptor, is also found in elevated levels in the saliva of patients with OSCC (32). IL-1β is secreted by tumor cells and is a substrate of MMP9 (10). TGF-β and TNF-α are also substrates of MMP9. Programmed death ligand 1 (PDL1) is expressed on tumor cells and its expression can be induced by IFN-γ (33). PDL1 of tumor cells binds to the immune-inhibitory receptor programmed death-1 (PD1) on activated B-cells or T-cells, which allows the tumor cells to evade destruction (33). Vascular endothelial growth factor (VEGF), known to stimulate angiogenesis, is a marker for tumor invasion and metastasis as it too can be detected in HNSCC (29). VEGF may also promote immune tolerance (34). Higher levels of IL-6 and VEGF are produced in late-stage HNSCC cell lines compared to early-stage and metastatic cell lines compared to nonmetastatic cell lines (29).

It was important for us to determine HPV status of our cell lines as HPV is considered a positive risk factor for oropharyngeal squamous cell carcinoma, a subset of HNSCC, and is a current focus of many HNSCC studies (35). While there has been a decrease in the number of tobacco

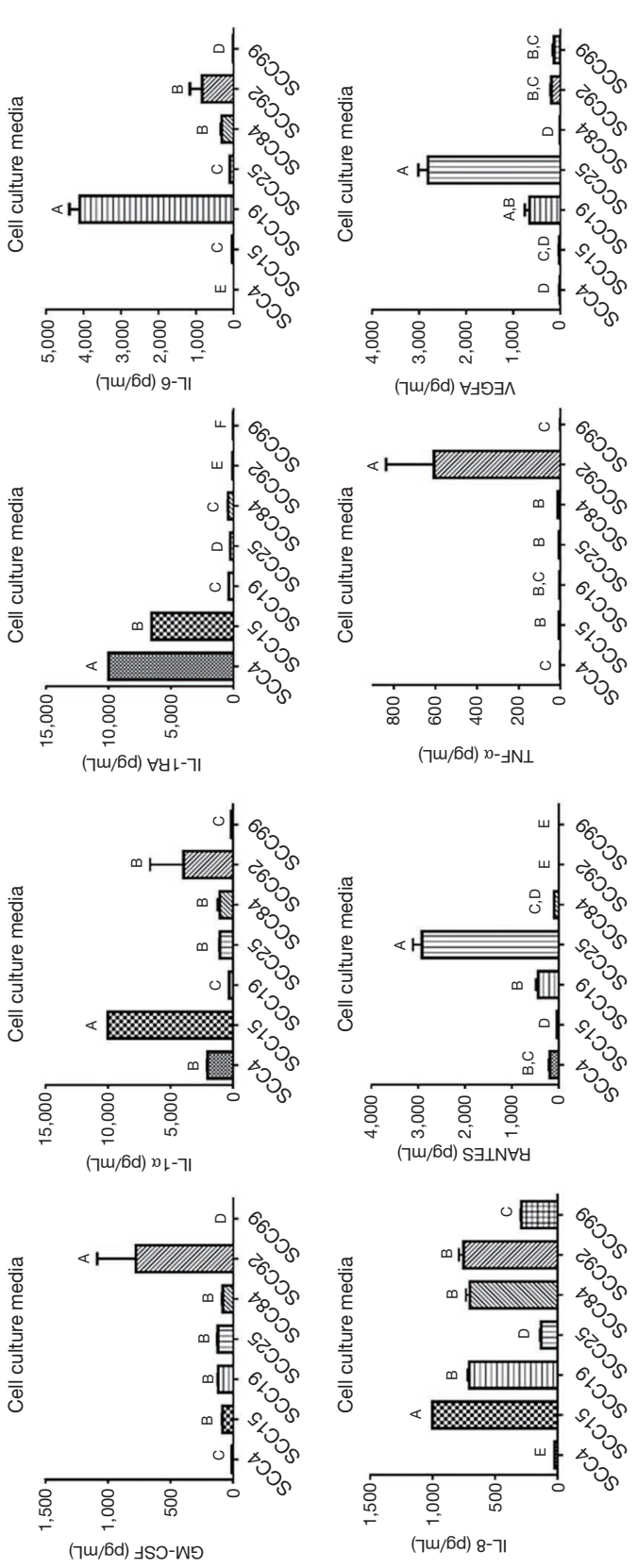

Figure 2 Biomarker production by seven HNSCC cell lines. After 24 hours of incubation at 1×10^6 viable cells/mL at 37 °C, the cell media was removed. We used multiplex immunoassays (R&D Systems, Minneapolis, MN, USA) read on a Luminex100 (Luminex, Madison, WI, USA) to determine the concentrations of GM-CSF, IL-1α, IL1RA, IL6, IL8, RANTES, TNFα, and VEGFA in cell supernatants. A one-way ANOVA procedure was performed on the log10 values and pairwise group comparisons were made using the post-hoc Tukey's HSD test ($P<0.05$). Bars with the same letter are not significantly different. HNSCC, head and neck squamous cell carcinoma.

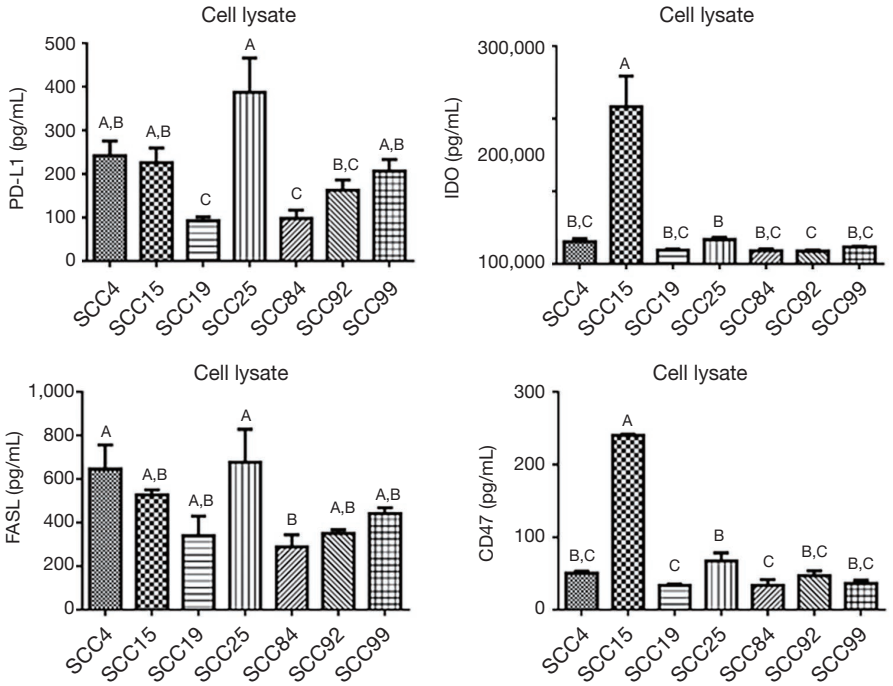

Figure 3 Cell surface biomarker production by seven HNSCC cell lines. After 24 hours of incubation at 1×10^6 viable cells/mL at 37 ℃, the cell media was removed, and the remaining adhering cells were treated with cell lysis buffer. We used enzyme-linked immunosorbent assay (ELISA, Cusabio Biotech Co., Ltd., Wilmington, DE, USA) to determine the concentrations of CD47, IDO, FASL, and PDL1 in cell lysates. A one-way ANOVA procedure was performed on the log10 values and pairwise group comparisons were made using the post-hoc Tukey's HSD test ($P<0.05$). Bars with the same letter are not significantly different. HNSCC, head and neck squamous cell carcinoma.

and alcohol related HNSCC over the last few decades, there has been an increase in the number of HPV-related HNSCC (36,37). HPV+ and HPV– HNSCC differ in terms of their mutational profiles (38). Typically, HPV+ HNSCC has a better survival rate than HPV– HNSCC (36,37). Patients with HPV+ tumors have been shown to respond to chemotherapy and chemoradiation treatments at higher rates than patients with HPV– tumors, and those with HPV+ tumors have shown lower risks of progression and death than those with HPV– tumors (39).

The negative $p16^{Ink4a}$ status was not surprising because it is a tumor suppressor protein (40). In a normal cell, $p16^{Ink4a}$ inhibits cyclin dependent kinase (CDK) activity. Without $p16^{Ink4a}$ inhibition, CDK4 and CDK6 are able to phosphorylate the retinoblastoma tumor suppressor (Rb), and this leads to the cell cycle shifting into S phase. Without $p16^{Ink4a}$ there is inappropriate cell division and cell proliferation, which is why it is not surprising to find our immortalized cancer cell lines lacking $p16^{Ink4a}$. However, $p16^{Ink4a}$ overexpression is sometimes used as a surrogate marker for high-risk-HPV-associated HNSCC (24,41,42). This is because the E7 HPV oncogene protein will induce $p16^{Ink4a}$ expression. The seven cell lines were identified as HPV negative, so there was no overexpression of $p16^{Ink4a}$ seen, as expected.

One limitation of this study is that the passage number of the cell lines is unknown. Cell lines can acquire more mutations overtime which can change their protein expression. However, a number of other labs use these cell lines too, and knowing the expression levels of MMPs and immunosuppressive biomarkers in minimal media conditions can aid in the selection of cell lines to use in future studies.

MMP and biomarker expression profiles should be carefully considered when choosing HNSCC cell lines for future studies as they can vary greatly. Because these cell lines are from different hosts, we have shown how different patients' HNSCC cells can express varying amounts of certain biomarkers and MMPs. These differences could be due to the metastatic stage of the cancer, the primary tumor site, the type of tissue the tumor originated from, or genomic differences between patients. These results support the reason for personalized medicine and the need for further investigation into how it can be used to treat HNSCC.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.05.09). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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Supplementary

Table S1 HNSCC cells were authenticated using short tandem repeat (STR) analysis as described in 2012 in ANSI Standard (ASN-0002) Authentication of Human Cell Lines: Standardization of STR Profiling by the ATCC Standards Development Organization (SDO) and in Capes-Davis *et al.* ("Match criteria for human cell line authentication: Where do we draw the line?" Int J Cancer 2013;132:2510-9)

Locus	SCC4	SCC15	SCC19	SCC25	SCC84	SCC92	SCC99
D3S1358	18	–	14	17	17	15	18
THO1	9.3	9, 9.3	6, 7	8	6	9, 10	6
D21S11	32.2	–	28, 30	30	29, 30	29, 31	27
D18S51	15	–	16	16	15, 18	14, 15	18
Penta_E	14	–	7, 11	14, 15	11, 17	7, 12	13
D5S818	13	12	11	12	12, 13	11	10, 13
D13S317	11, 13	9, 14	11	13	11	12, 14	8, 11
D7S820	9, 11	10, 11	9	12	8	11, 12	9, 12
D16S539	12	12, 15	9, 12, 13	11, 12	12	10, 11	12, 13
CSF1PO	11	10, 13	11	10	11, 12	10, 13	10, 12
Penta_D	12	–	9, 12	13	8, 13	9,12	9
AMEL	X, Y	X, Y	X, Y	X	X	X	X
vWA	15, 17	15, 17	16	17, 19	14, 16	17, 20	15, 18
D8S1179	14	–	10, 12	13	12, 16	10, 13	16
TPOX	8	8	8, 11	8, 12	8, 9	8, 12	8, 11
FGA	21	–	24	20, 24	22	19, 21	18
D19S433	12	–	14	13	15	14	12
D2S1338	16	–	19	17, 19	19,22	20, 24	20, 22

For this 17 STR loci plus the gender determining locus, amelogenin, were amplified using the commercially available PowerPlex® 180 Kit from Promega. The cell line samples were processed using the ABI Prism® 3500xl Genetic Analyzer. Data were analyzed using GeneMapper® I0-X v1.2 software (Applied Biosystems). Appropriate positive and negative controls were run and confirmed for each sample submitted. Using these results, the HNSCC cell lines were unique and identical to their name sakes. HNSCC, head and neck squamous cell carcinoma.

Table S2 Descriptive statistics of observed concentrations of the 14 biomarkers from each cell line

Cell lines	Variables	N	Mean	SD	Minimum	Maximum	Median
SCC4	CD47	3	1.72	0.04	1.69	1.76	1.70
	FASL	3	2.80	0.12	2.71	2.94	2.75
	GM-CSF	3	1.14	0.03	1.12	1.17	1.13
	IDO	3	4.47	0.13	4.33	4.58	4.49
	IL-1α	3	3.31	0.02	3.28	3.32	3.32
	IL-1RA	3	4.00	0.00	4.00	4.00	4.00
	IL-6	3	0.37	0.18	0.17	0.52	0.41
	IL-8	3	2.39	0.05	2.34	2.43	2.40
	MMP1	3	2.36	0.05	2.31	2.40	2.36
	MMP7	3	2.90	0.04	2.87	2.94	2.88
	MMP9	3	2.98	0.03	2.95	3.00	2.98
	PD-L1	3	2.38	0.10	2.32	2.49	2.32
	TNF-α	3	0.28	0.15	0.11	0.40	0.34
	VEGFA	3	0.85	0.73	0.00	1.27	1.27
SCC15	CD47	3	2.38	0.01	2.37	2.39	2.38
	FASL	3	2.72	0.03	2.69	2.75	2.72
	GM-CSF	3	1.94	0.04	1.90	1.98	1.93
	IDO	3	5.32	0.16	5.15	5.46	5.35
	IL-1α	3	4.00	0.00	4.00	4.00	4.00
	IL-1RA	3	3.82	0.00	3.82	3.82	3.82
	IL-6	3	1.55	0.07	1.49	1.62	1.53
	IL-8	3	4.00	0.00	4.00	4.00	4.00
	MMP1	3	1.82	0.12	1.73	1.96	1.77
	MMP7	3	3.73	0.01	3.73	3.74	3.73
	MMP9	3	3.28	0.02	3.27	3.30	3.28
	PD-L1	3	2.35	0.11	2.24	2.46	2.34
	TNF-α	3	0.85	0.07	0.79	0.93	0.83
	VEGFA	3	1.33	0.16	1.24	1.52	1.24
SCC19	CD47	3	1.54	0.05	1.49	1.57	1.57
	FASL	3	2.50	0.19	2.39	2.72	2.40
	GM-CSF	3	2.08	0.02	2.07	2.11	2.07
	IDO	3	4.28	0.06	4.22	4.33	4.28
	IL-1α	3	2.49	0.05	2.45	2.54	2.48
	IL-1RA	3	2.56	0.02	2.54	2.58	2.56
	IL-6	3	3.61	0.06	3.55	3.66	3.62
	IL-8	3	3.85	0.01	3.84	3.86	3.85
	MMP1	3	1.58	0.04	1.56	1.63	1.56
	MMP7	3	3.91	0.02	3.89	3.93	3.92
	MMP9	3	2.93	0.02	2.92	2.95	2.92
	PD-L1	3	1.97	0.07	1.91	2.04	1.95
	TNF-α	3	0.71	0.07	0.63	0.76	0.74
	VEGFA	3	2.81	0.13	2.66	2.90	2.86
SCC25	CD47	3	1.82	0.12	1.72	1.95	1.79
	FASL	3	2.80	0.19	2.59	2.95	2.87
	GM-CSF	3	2.09	0.04	2.06	2.14	2.07
	IDO	3	4.52	0.07	4.46	4.59	4.52
	IL-1α	3	3.02	0.03	3.00	3.05	3.00
	IL-1RA	3	2.39	0.06	2.33	2.43	2.42
	IL-6	3	1.97	0.03	1.95	2.00	1.97
	IL-8	3	3.12	0.04	3.08	3.15	3.13
	MMP1	3	4.13	0.01	4.12	4.14	4.12
	MMP7	3	4.42	0.03	4.39	4.44	4.42
	MMP9	3	4.13	0.01	4.13	4.14	4.13
	PD-L1	3	2.57	0.14	2.45	2.73	2.54
	TNF-α	3	0.94	0.01	0.93	0.95	0.94
	VEGFA	3	3.45	0.05	3.40	3.50	3.44
SCC84	CD47	3	1.52	0.17	1.36	1.69	1.50
	FASL	3	2.44	0.16	2.26	2.54	2.53
	GM-CSF	3	1.92	0.07	1.85	1.99	1.91
	IDO	3	4.26	0.10	4.15	4.35	4.27
	IL-1α	3	3.02	0.08	2.92	3.07	3.06
	IL-1RA	3	2.64	0.01	2.63	2.65	2.64
	IL-6	3	2.49	0.09	2.39	2.55	2.52
	IL-8	3	3.85	0.02	3.83	3.87	3.85
	MMP1	3	2.15	0.05	2.11	2.20	2.13
	MMP7	3	3.69	0.03	3.66	3.71	3.69
	MMP9	3	3.35	0.03	3.32	3.37	3.36
	PD-L1	3	1.97	0.17	1.77	2.09	2.05
	TNF-α	3	1.10	0.09	1.00	1.18	1.13
	VEGFA	3	0.42	0.73	0.00	1.27	0.00
SCC92	CD47	3	1.67	0.11	1.59	1.79	1.63
	FASL	3	2.54	0.03	2.52	2.58	2.53
	GM-CSF	3	2.76	0.48	2.20	3.04	3.03
	IDO	3	4.24	0.07	4.16	4.30	4.27
	IL-1α	3	3.47	0.47	2.93	3.75	3.74
	IL-1RA	3	1.99	0.02	1.98	2.01	1.98
	IL-6	3	2.79	0.48	2.24	3.08	3.05
	IL-8	3	3.88	0.02	3.85	3.89	3.89
	MMP1	3	1.46	0.11	1.35	1.56	1.47
	MMP7	3	2.74	0.03	2.70	2.76	2.76
	MMP9	3	3.25	0.03	3.23	3.28	3.24
	PD-L1	3	2.21	0.12	2.07	2.29	2.26
	TNF-α	3	2.67	0.44	2.16	2.93	2.92
	VEGFA	3	2.27	0.08	2.19	2.35	2.28
SCC99	CD47	3	1.57	0.09	1.51	1.67	1.54
	FASL	3	2.65	0.04	2.60	2.67	2.67
	GM-CSF	3	0.13	0.12	0.02	0.25	0.11
	IDO	3	4.37	0.03	4.35	4.40	4.36
	IL-1α	3	2.21	0.09	2.12	2.29	2.22
	IL-1RA	3	1.62	0.05	1.56	1.65	1.64
	IL-6	3	0.97	0.09	0.88	1.05	0.97
	IL-8	3	3.46	0.02	3.45	3.48	3.46
	MMP1	3	3.67	0.01	3.66	3.68	3.68
	MMP7	3	2.37	0.05	2.33	2.42	2.35
	MMP9	3	3.31	0.01	3.31	3.32	3.31
	PD-L1	3	2.31	0.10	2.24	2.42	2.27
	TNF-α	3	0.23	0.04	0.21	0.28	0.21
	VEGFA	3	2.11	0.13	1.99	2.24	2.11

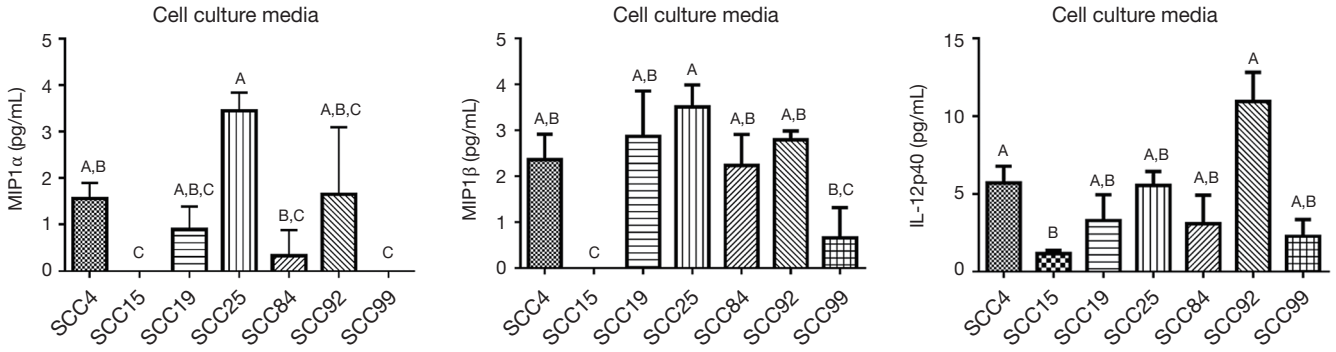

Figure S1 Additional biomarker production by seven HNSCC cell lines. After 24 hours of incubation at 1×10^6 viable cells/mL at 37 ℃, the cell media was removed. We used multiplex immunoassays (R&D Systems, Minneapolis, MN, USA) read on a Luminex100 (Luminex, Madison, WI, USA) to determine the concentrations of MIP1α, MIP1β, and IL12p40 in cell supernatants. A one-way ANOVA procedure was performed on the log10 values and pairwise group comparisons were made using the post-hoc Tukey's HSD test ($P<0.05$). Bars with the same letter are not significantly different. HNSCC, head and neck squamous cell carcinoma.