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Evaluation of Tyrosine Receptor Kinases in the Interactions of Head and Neck Squamous Cell Carcinoma Cells and Fibroblasts

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Summary

Despite treatment advancements, disease-free survival of head and neck squamous cell carcinoma (HNSCC) has not significantly improved. This may be a result of tumor-fibroblasts interactions providing protective pathways for oncogenic cells to resist therapy. Further understanding of these relationships in HNSCC may improve effectiveness of targeted therapies. In this article, we investigated the role of several receptor tyrosine kinases (RTK) in the interactions between HNSCC cells and supporting cells (fibroblasts). Evaluation of HNSCC tumor specimens, cell lines and fibroblasts found variable expression of multiple RTKs (fibroblasts growth factor receptor, platelet derived growth factor receptor and vascular endothelial growth factor receptor) and their ligands, supporting previous theories of paracrine and autocrine signaling within the microenvironment. In a dose-dependent fashion, RTK inhibition reduced proliferation of HNSCC cell lines and fibroblast in vitro. When HNSCC cells were cocultured with fibroblasts, RTK inhibition resulted in a smaller reduction in the proliferation relative to untreated conditions. In vivo, RTK inhibition resulted in significant tumor regression and growth inhibition ($p < 0.05$) and reduced the incidence of regional lymph node metastasis. Effective treatment of HNSCC, therefore, may require inhibition of multiple RTKs in order to adequately inhibit the microenvironment's various signaling pathways.

Keywords

aerodigestive squamous cell carcinoma; head and neck; TKI258; tyrosine receptor kinases

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous disease, from the cellular morphology to the array of expressed genes in individual tumors. The result is a complex set of relationships between the oncogenic cells and components of the surrounding environment, including the stroma and vasculature. Included in the oncogenic stroma are

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fibroblasts, which are functionally important for carcinogenesis by providing proliferative and anti-apoptotic regulatory factors. Transmembrane protein receptor tyrosine kinases (RTKs) are part of the tumor-stromal signal transduction pathways associated with oncogenic cell survival, proliferation and differentiation.^{1,2} Each of these receptors bind to specific ligands, resulting in dimerization of the receptor and subsequent intracellular signaling cascade. Several members of the RTK family are expressed by HNSCC cells, including fibroblast growth factor receptors (FGFR), platelet derived growth factor receptors (PDGFR) and vascular endothelial growth factor receptors (VEGFR).

FGFR-1, FGFR-2 and FGFR-3 are amplified and/or overexpressed in oral cavity squamous cell carcinomas.³⁻⁶ These receptors bind FGF-2 (FGF-basic), which has been found to be elevated in HNSCC specimens.⁶ Furthermore, FGF-2 and FGFR-1 expression in tumor associated fibroblast of HNSCC specimens correlate with higher grade, increased incidence of lymph node metastasis and poor prognosis.⁷

PDGFR has been found in 97% of HNSCC specimens and 100% of normal oral mucosa samples.⁸ In addition, PDGFR is found on endothelial cells, smooth muscle cells and pericytes.^{9,10} Binding of PDGFR by platelet derived growth factor (PDGF) leads to increased cell migration and proliferation.¹¹ Interestingly, the serum of patients with HNSCC malignancies have significantly higher levels of PDGF compared to controls. In addition, the inhibition of PDGFR in HNSCC cell lines lead to decreased PDGF and vascular endothelial growth factor (VEGF) levels.¹²

The pathologic growth and development of malignancies is dependent on angiogenesis for nutrition and oxygenation. HNSCC cell lines and fibroblasts secrete VEGF and FGF, both of which bind to the VEGFR found on endothelial cells.¹³⁻¹⁵ VEGF expression has been found in 87% of HNSCC specimens, with higher expression levels in neoplastic specimens compared to dysplastic or normal and its expression intensity predicted overall and disease free survival.^{16,17} In addition, there is an inverse correlation with VEGF expression and apoptosis in HNSCC.^{18,19}

Despite advancement in treatment options, HNSCC remains a challenge to treat. This may be the result of the complex set of interactions between the HNSCC cells and supporting cells, such as fibroblasts. Previous investigations have found fibroblasts to promote oncogenic cell survival and proliferation.^{20,21} Therefore, we hypothesize that inhibition of RTKs (PDGFR, FGFR, VEGFR) may prevent fibroblast mediated growth acceleration. Targeting the components of the oncogenic stroma in addition to cancer cells may deprive tumor cells of pro-malignant environment and consequently allow for improved treatment of HNSCC with better outcomes and decreased toxicity. To investigate our hypothesis, we chose dovitinib, a small molecule multi-targeted tyrosine kinase inhibitor with activity against FGFR, PDGFR, and VEGFR, as the therapeutic agent.^{2,22,23}

Materials and methods

Patient selection

Following Institutional Review Board approval, a retrospective review of patients with previous untreated HNSCC of the oral cavity (n=13) was performed at the University of Alabama at Birmingham. Histologic analyses of the tumor specimens from the primary lesion was preformed for each patient included. This histology of the primary tumor was confirmed by the pathology department.

Cell culture and reagents

SCC-1, SCC-6 and SCC-22A (University of Michigan), OSC-19 (University of Texas, MD Anderson), FADU, Cal27, HFL-1 and HS27 (American Type Culture Collection, Rockville, MD), and NDF (normal dermal fibroblast cultured from normal human skin) cells were maintained in DMEM and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. HFL-1, HS27 and NDF cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA) according to the manufactures instructions. The multitargeted tyrosine kinase inhibitor used was dovitinib (TKI258) by Novartis (Selleck Chemicals Co., Boston, MA). The anti-EGFR antibody used for imaging was panitumumab (Vectibix; Amgen, Thousand Oaks, California).

Cell proliferation, viability, cytotoxicity, and apoptosis assays

To determine cell proliferation in isolation, HNSCC cells (FADU, SCC-1, SCC-6, SCC-22A, OSC-19 and Cal27) and fibroblasts (HFL-1, NDF and HS27) were incubated alone. To determine cell proliferation in coculture, HNSCC cells (FADU, SCC-1, SCC-6, SCC-22A, OSC-19 and Cal27) were plated with CFSE labeled HFL-1, NDF or HS27 and incubated together. For both proliferation assays (isolation and coculture), HNSCC cells (3×10^4) and fibroblast (1.5×10^4) were seeded in 48 well tissue culture treated plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ), treated in triplicate with dovitinib at increasing concentrations (0, 0.01, 0.1, 1, 10, 100, 1000, and 5000 nM; day 0), and incubated for 48 h. On day 2 cells were trypsinized and counted with a flow cytometer (Accuri, C6, Ann Arbor, MI). The half maximal inhibitory concentration (IC₅₀) was calculated from dose-response curves consisting of 7 points (0, 0.01, 0.1, 1, 10, 100, 1000 nM), each in triplicate.

Cell viability, cytotoxicity and apoptosis were assessed. HNSCC cells and fibroblasts were grown to 80% confluence and then plated (5×10^4 /well) in triplicate in a 384 well plate (Costar, #3712) and treated with dovitinib at increasing concentrations (0, 10, 100, 1000, and 5000 nM) for 6, 24, and 48 hours. The ApoTox-Glo™ Triplex Assay (Promega, G6320) was then performed according to manufacture recommendations.

Immunohistochemical and immunofluorescence analysis

Immunohistochemical analysis was performed to determine PDGFR-β and FGFR-3 expression. Samples were rehydrated in xylene, 95% ethanol, and 70% ethanol. Antigen retrieval was accomplished in 1 mM EDTA, pH 9.0, for 5 minutes at 100°C. Samples were then allowed to cool at room temperature and blocked with 5% BSA in TBST for 5 minutes at room temperature. Primary antibodies PDGFR-β (Santa Cruz, 9974) or FGFR-3 (Santa Cruz, 13121), was applied at the concentrations recommended and allowed to incubate for 1 hour. Secondary antibodies (horseradish peroxidase) was applied for one hour in a humidified chamber at room temperature. DAB substrate was then applied to slides and allowed to incubate at room temperature until appropriate color developed. Samples were then counterstained with Harris Hematoxylin diluted 1:1 with tap water for 45 seconds. Finally, samples were dehydrated and mounted with permount and allowed to dry overnight.

Immunofluorescence analysis was performed to determine membrane expression levels of FGFR-2 and VEGFR-1/2. Samples were rehydrated in xylene (3×), 95% ethanol, and 70% ethanol. Antigen retrieval was accomplished in 1mM EDTA, pH 9.0, for 10 minutes at 100°C. Samples were then allowed to cool at room temperature in EDTA solution and then blocked with 5% BSA in TBST for 10 minutes at room temperature. The sections were incubated for one hour at room temperature in a humidified chamber with antibody to both 1:100 FGFR-2 (Abcam, 10648) and VEGFR-1/2 (Abcam, 36844). Slides were then washed

3× in 0.05 M Tris-Buffer, pH 7.6 for 10 min. and then incubated in the dark for 1h using a mixture of fluorophore-linked secondary anti-bodies 1:100 (AlexaFluor 546-conjugated goat anti-mouse IgG1 and AlexaFluor 488-conjugated goat anti-rabbit IgG). The slides were then washed 33× in .05 M Tris-Buffer, pH 7.6 for 10 min each and mounted using Gel Mount Aqueous Mounting Medium (Sigma, G0918). Fluorescence microscopy was performed for each field using Olympus IX70 fluorescence microscope with Olympus DP72 camera. Images were then processed using Olympus D2-Basic Imaging Software.

Western blot

Cells were grown to 70%-80% confluence, washed twice with cold PBS, and lysed in lysis buffer [50mM Tris-HCl (pH7.5), 150mM NaCl, 1%(v/v) NP40, 0.5 % (w/v) sodium deoxycholate, 1mM EDTA, 0.1% SDS], and a protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN) was added. The cleared lysates were collected by centrifugation at 12000 × g for 20 mins at 40°C. The protein concentrations were measured by BCA protein assay (Thermo Scientific, Rockford, IL). Lysates with 10µg of total protein were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with a primary antibody (see immunohistochemistry and immunofluorescence above; FGFR-1 [Abcam, ab832]; PDGF-B [Santa Cruz, 7878]; FGF-2 [Santa Cruz, 271847]). After washing and incubating with horseradish peroxidase conjugated secondary antibodies, the membranes were washed again and detected by the Amersham ECL Western blotting detection system (GE healthcare, Buckinghamshire, UK). The membranes were reprobred with horseradish peroxidase-conjugated mouse monoclonal antihuman β-actin to ensure equal protein loading.

Animal models

Athymic female nude mice aged 6 to 8 weeks (Charles River Laboratories and National Cancer Institute–Frederick) were obtained and housed in accordance with our institution's Institutional Animal Care and Use Committee (IACUC) guidelines. To assess the efficacy of dovitinib in vivo, an orthotopic tongue tumor model was established by injecting OSC-19 or SCC-1 cells (2×10^5) suspended in 30 L serum-free DMEM into the proximal tongue. Treatment groups were divided into two cohorts, control and dovitinib treated ($n = 5$ per group). Tumors were measured every 48–72 hrs using calipers to approximate surface area, and treatment was initiated after xenografts reached an average size of ~10 mm² or at the time of implantation (post-surgical model; OSC-19). Those mice being treated with dovitinib (20 mg/kg/d) received oral gavage doses daily for 12–14 days.²

Imaging of primary lesions and lymph node metastasis was achieved as previously described.^{25–29} Briefly, an anti-EGFR antibody was conjugated to IRDye800CW (LI-COR Biosciences, Lincoln, Nebraska) in 1.00 M potassium phosphate buffer (pH 9.0) for 2 hrs. The unconjugated dye was removed by desalting spin columns. The mice were systemically injected with 50 µg of the conjugate and imaged 72 hrs later using the Pearl Impulse (LI-COR Biosciences, Lincoln, Nebraska).

Statistical analyses

Data analysis of in vitro cell growth, IC₅₀ and in vivo xenografts growth was done using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered significant in unpaired t-test analysis used to determine differences between means of treated versus control groups. All results are expressed as the mean ± standard error (SE).

Results

Expression of RTKs in HNSCC

In order to determine the relative importance of FGFR, PDGFR and VEGFR expression levels in HNSCC we evaluated human tumor specimens (n=13) by immunohistochemical and immunofluorescence analysis (Fig. 1). The majority of HNSCC tumors specimens analyzed expressed PDGFR- β (94%), VEGFR-1/2 (71%), and FGFR-2 (65%). Although there was no evidence of FGFR-3 expression in the HNSCC regions, there was positive FGFR-3 staining in the surrounding stroma. There was no evidence of FGFR-1 staining in either the stroma or by the HNSCC cells (data not shown).

Inhibition of RTKs reduces HNSCC growth in an orthotopic nude mouse model

The antitumor efficacy of broad spectrum RTK inhibition was evaluated in HNSCC xenografts dosed with dovitinib (20 mg/kg/day) orally for 12–14 days. Treatments were initiated when orthotopic tongue tumors average 10 mm² (Fig. 2). Significant antitumor activity was seen in both OSC-19 and SCC-1 models. Growth stabilization was seen by day 2 in the SCC-1 xenografts (Fig. 2A) and by day 8 of treatment in the OSC-19 xenografts (Fig. 2B). In addition, growth reduction was seen in both SCC-1 ($p < 0.0001$; day 12) and OSC-19 ($p < 0.0001$; day 15) xenografts. During the final week of treatment, the tumors in the treatment cohort had areas of necrosis evident on gross examination which were not present in the tumors of untreated control mice.

Inhibition of RTKs reduces HNSCC growth and lymph node metastasis in a post-surgical orthotopic nude mouse model

A post-surgical model was implemented in order to evaluate the effect of broad spectrum RTKs inhibition on HNSCC xenograft tumor growth and lymph node metastasis (Fig. 3A). Treatment was initiated at the time of tumor cell (OSC-19) inoculation to mimic a post-surgical scenario in which margins are negative, however the patient will go on to recur at a later date. Many feel this is likely the result of small islands of tumor cells unknowingly being missed at the time of initial resection. The mice were dosed with dovitinib (20 mg/kg) orally for 14 days. Treatments were initiated at time of orthotopic implantation. Growth stabilization was seen by day 8 of treatment (Fig. 3B). On day 17, tumors were harvested and histological analysis was performed (Fig. 3C). Following Ki67 staining, tumors from control xenografts were found to have a higher percentage of proliferating cells (62%) compared to those treated with dovitinib (14%; $p=0.005$). The incidence of lymph nodes metastasis was greater in the control cohort (n=15) compared to those treated with dovitinib (n=5). Further analysis revealed positive bilateral lymph nodes in all the control xenografts (n=5) versus only one from the treated cohort (Fig. 3D).

Expression of RTKs in HNSCC cell lines and fibroblasts

In order to assess expression levels of RTKs and their ligands in HNSCC cell lines and fibroblasts, western blot analysis were performed (Fig. 4). Inconsistent receptor expression was found among oncogenic cell lines and among various stromal cell lines. Although histologic analysis demonstrated PDGFR- β expression was present in HNSCC tumor specimens from humans and xenografts, it was absent on western analysis of lysates from isolated HNSCC cell lines. Conversely, fibroblasts demonstrated high levels of PDGFR- β expression on western blot analysis. Interestingly, the ligand PDGF was expressed by HNSCC cell lines and fibroblasts. The OSC-19, FADU and HS27 cells demonstrated the greatest FGFR-1 expression, followed by low expression in the SCC-1, SCC-6 and SCC-22A cells. The greatest FGFR-2 expression was demonstrated by the SCC-6 and SCC-22A cell lines, followed by low expression in the FADU cells. The greatest expression

of FGFR-3 was demonstrated by the Cal27, FADU and SCC-22A cell lines, followed by moderate expression by SCC-1 cells, and low expression by SCC-6 cells. Additionally, there was very faint expression of FGFR-3 by the OSC-19 and HS27 lines. The ligand FGF-2 was only expressed by the OSC-19, FADU and HS27 cells. Although histologic analysis demonstrated VEGFR-1/2 expression was present in HNSCC tumor specimens from humans, it was also absent on western analysis of lysates from isolated HNSCC cell lines and fibroblasts (data not shown).

Inhibition of RTKs decreased HNSCC and fibroblast cell proliferation in vitro

Inhibition of RTKs by dovitinib reduced proliferation in all HNSCC and fibroblast cell lines in a dose-dependent fashion (Fig. 5). The IC_{50} values ranged from 1.4 to 4.0 nM. Cell proliferation was significantly decreased at 10 nM concentrations of dovitinib for all HNSCC cell lines and fibroblasts ($p < 0.0001$). With the exception of HS27, HNSCC cell lines and fibroblasts proliferation was reduced by 70–80% at 100 nM. The fibroblasts were the most significantly affected by treatment with dovitinib, particularly HS27 ($IC_{50} = 1.4$ nM) and NDF ($IC_{50} = 2.1$ nM). This may be attributed to their very high expression of RTKs (PDGFR- β and FGFR).

Inhibition of RTKs increased cytotoxicity, decreased viability and induction of apoptosis in HNSCC cells

Treatment with dovitinib (10 nM) for 6 hrs increased cytotoxicity and decreased cell viability of OSC-19, Cal-27 and SCC-1 cells while the FADU cell line required 12 hrs of treatment to achieve similar findings. A higher dose (100 nM) and longer treatment duration (12 hrs) was required for induction of apoptosis in FADU, OSC-19, Cal-27 and SCC-1 cell lines. Following 24 hrs of treatment with 10 nM there was increased cytotoxicity and decreased viability and with 100 nM there was induction of apoptosis of fibroblasts (data not shown).

Role of RTKs in interactions between HNSCC cells and fibroblast in vitro

It has been previously established that surrounding stromal fibroblast provide a growth advantage to tumor cells.²⁰ This phenomenon was confirmed in our HNSCC cell lines using flow cytometry to assess cell counts. When comparing the proliferation rate of HNSCC cells cultured in isolation to proliferation rates of HNSCC cells cocultured with fibroblasts, there was a significant increase seen (Fig. 6). When cocultured with HFL-1, proliferation of FADU cells doubled ($p = 0.0003$), OSC-19 cells increased 1.5 fold ($p < 0.0001$), Cal27 cells increased by 6% ($p = 0.01$) and SCC-1 cells increased by 20% ($p = 0.006$) (Fig. 6A). When cocultured with NDF, proliferation of FADU increased by 80% ($p < 0.0001$), OSC-19 increased by 40% ($p < 0.0001$), Cal27 more than doubled ($p < 0.0001$) and SCC-1 almost doubled ($p < 0.0001$) (Fig. 6B). When cocultured with HS27, proliferation of FADU tripled ($p < 0.0001$), OSC-19 increased by 60% ($p < 0.0001$), Cal27 more than doubled ($p < 0.0001$) and SCC-1 doubled ($p < 0.0001$) (Fig. 6C).

The population of HNSCC cells grown in isolation was significantly reduced by broad spectrum inhibition of RTKs with dovitinib (100 nM) for 48 h (Fig. 6). Interestingly, in the coculture setting, HNSCC cell populations were reduced relative to untreated cocultures, but the cell counts were similar to untreated isolated cultures (Fig. 6). Following treatment, proliferation of FADU and OSC-19 cocultured with HFL-1 exceeded proliferation of isolated cultures by 60% ($p < 0.0001$). Similarly, following treatment, proliferation of Cal-27 and SCC-1 cocultured with HFL-1 exceeded proliferation of isolated cultures by 40% ($p < 0.0001$) (Fig. 6A). When cocultured with NDF, the proliferation of FADU, OSC-19, Cal27 and SCC-1 remained higher than isolated cultures for both treated and untreated conditions ($p < 0.0001$; Fig. 6B). Although proliferation of treated FADU, OSC-19 and Cal27

cells was greater when cocultured HS27 relative to isolated cultures ($p < 0.001$), there was still a significant reduction in proliferation compared untreated cocultures ($p < 0.001$; Fig. 6C). This greater sensitivity of HNSCC cell lines to dovitinib when cocultured with HS27 relative to the other fibroblast lines (Fig. 6D) is likely the result of HS27 demonstrating the greatest expression of RTKs (Fig. 4) and sensitivity to treatment (Fig. 5).

In vivo expression levels of RTKs and phosphorylated-RTKs

In order to assess the effect dovitinib treatment had on expression levels of RTKs and phosphorylated-RTKs, tumors harvested from the post-surgical model underwent immunofluorescence analysis. There was a trend towards decreased levels of PDGFR- β ($p = 0.10$), FGFR-1 ($p = 0.20$), FGFR-2 ($p = 0.30$), and FGFR-3 ($p = 0.44$) and a significant decrease in VEGFR-1/2 ($p = 0.04$) in the treated xenografts relative to controls. The phosphorylated RTKs expression levels were similar for both cohorts (data not shown).

Discussion

The survival of malignant neoplasms is dependent on a complex set of interactions and signaling pathways which allow for increased cell proliferation, anti-apoptotic phenotypes, cell migration and invasion, and increased angiogenesis. Supporting cells, such as fibroblasts, may facilitate oncogenic cell survival by promoting proliferation and migration via intercellular signaling with oncogenic cells. These communication pathways may be facilitated by growth factor receptors and their ligands. In this investigation, we provide a pre-clinical evaluation RTKs in HNSCC cells and fibroblasts and the effects of RTK inhibition in these cell populations. Our study, as well as previous publications, found variable expression of various RTKs in HNSCC cells and fibroblasts.^{3–8,16–19} Consequently, we choose a treatment agent which targeted multiple RTKs (dovitinib).

Dovitinib is a target-driven small molecule inhibitor of growth factor tyrosine kinases (FGFR-1/2/3, PDGFR- β and VEGFR-1/2).² In this study we show that dovitinib is pharmacologically active against HNSCC cells in vivo. Xenografts treated with 20 mg/kg/d had a significant improvement in disease stabilization and tumor regression compared to untreated controls. As seen in previous studies, the antitumor activity of dovitinib translated into improved survival and disease stabilization.^{2,22,30} In addition, treatment with dovitinib reduced regional lymph node metastasis. The efficacy of dovitinib in treating HNSCC is in part conferred by the reduction of growth factor receptor activation in the supporting stroma. This is likely the result of FGFR-1/2/3 and PDGFR- β inhibition disrupting HNSCC cell and fibroblast interactions and VEGFR-1/2 and PDGFR- β inhibition disrupting neovascularization.^{2,12,22,31,32}

Similarly to other investigations, we found immunohistochemical and immunofluorescence analysis demonstrated expression of these receptors in the majority of HNSCC specimens.^{4,8,12,15,16,31,32} Of interest is the variation in receptor expression patterns demonstrated on histology of tumor samples relative to expression pattern demonstrated by western blot analysis of isolated cell lines. We hypothesize this is due to the heterogeneity of HNSCC tumors as well as the varying components of the tumor microenvironment. In contrast, immortalized cell lines represent a homogenous cell population, and as such, are unable to be a comprehensive representation of the actual pathogenesis occurring.

On western blot analysis it was found that fibroblasts highly express PDGFR- β while HNSCC cells express the corresponding ligand, PDGF-B. These findings provide support for a symbiotic relationship between stromal and oncogenic cells.¹² Interestingly, fibroblast themselves also produced PDGF-B which may provide evidence for an autocrine loop.

Similarly, FGFR-1 and FGF-2 expression was found in the OSC-19, FADU and HS27 cells. This may also be an indication of an autocrine loop in these cell lines.

Further investigation found dovitinib significantly inhibited HNSCC cells and fibroblasts proliferation in vitro. The anti-proliferative effects are likely due to a dose-dependent inhibition of growth factor receptor kinases.² The result is increased cytotoxicity, decreased viability and induction of apoptosis causing cell death and a reduction in cell proliferation. The fibroblasts, particularly HS27, were the most significantly affected by the treatment. This is presumably due to their high expression of several of the RTKs being targeted by dovitinib (PDGFR- β and FGFR-1) and their ligands (PDGF-B and FGF-2).

In order to create an environment more representative of what occurs in HNSCC tumors when the oncogenic cells interact with the surrounding stroma, HNSCC cells were cocultured with fibroblasts. When compared to isolated cultures, the proliferation of both HNSCC cells and fibroblasts increased significantly in coculture. These findings provide support for the synergistic relationship between fibroblast and HNSCC.⁴ In addition, proliferation in dovitinib treated cocultures surpassed the proliferation found in dovitinib treated isolated cultures. This suggests the paracrine signaling between fibroblast and HNSCC cells allows for resistance to targeted therapies and more resilient cells. As a result, treatment with a broad spectrum RTK inhibitor, such as dovitinib, suppresses fibroblasts stimulatory effects transmitted by paracrine loops.

Histologic analysis of orthotopic xenografts revealed elevated VEGFR-1/2 expression in the control cohort ($p=0.04$) and a trend towards higher expression levels of FGFR-1/2/3 and PDGFR- β compared to the dovitinib treated cohort. The lack of statistical significance is likely due to the low power ($n=5$) of the study. Despite inhibition of targeted RTKs, the expression levels of phosphorylated receptors were similar between the two cohorts. We may extrapolate that dovitinib is leading to a down-regulation or decreased expression of the RTKs without impacting their phosphorylation.

Despite improvement in locoregional control, disease-free survival of HNSCC has not significantly improved, highlighting a need for novel and innovative therapies. Evaluation of HNSCC tumor specimens, cell lines and supporting fibroblasts found variable expression of multiple RTKs. Therefore, treatment may require inhibition of multiple RTKs in order to adequately inhibit the autocrine and paracrine signaling of the microenvironment. Inhibition of RTKs by dovitinib resulted in decreased survival and cell proliferation of HNSCC cells. This study provides scientific rationale for combination tyrosine kinase inhibitors as a potential chemotherapeutic agent in HNSCC.

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Figure 1. Expression of FGFR, PDGFR- β , and VEGFR in human HNSCCs specimens. Tumor samples from 13 patients with HNSCC were stained by (A) immunohistochemistry or (B) immunofluorescence.

Figure 3.

Orthotopic OSC-19 xenografts were treated immediately following implantation with dovitinib (20 mg/kg/day). Tumor growth was followed for 17 days and imaged following systemic injection of labeled anti-EGFR antibody on day 17 (A). There was a significant stabilization of growth by day 8, which continued for the remainder of the study (B). Following treatment, tumors were harvested and histologically analyzed for proliferation by Ki67 staining (C). Lymph nodes were also harvested at the conclusion of the study and analyzed histologically for lymph node metastasis by H&E staining (D). Marker or columns, mean for triplicate; bars, SE. Statistical significance by unpaired t-test, * $p < 0.05$.

Figure 4. Western blot analysis of receptor tyrosine kinases and their ligands in HNSCC cell lines and fibroblasts.

Figure 5. Dose-response curves for HNSCC cells and fibroblasts treated in vitro with dovitinib (0–1000nM). Boxes, mean for triplicate; bars, SE.

Figure 6. Cell proliferation of (A-C) HNSCC cells grown alone and cocultured with fibroblasts +/- dovitinib (100 nM). (D) Fibroblasts provide protection from proliferation inhibition by dovitinib. Columns, mean for triplicate; bars, SE.