Original Article

Cytogenetic and Genome Research

Cytogenet Genome Res 2010;128:192–198
DOI: 10.1159/000308303

Accepted: October 30, 2009
by A. Geurts van Kessel
Published online: June 2, 2010

Overexpression of *KLF13* and *FGFR3* in Oral Cancer Cells

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Key Words
FGFR3 · KLF13 · miRNA · Oral cancer

Abstract
KLF13 and FGFR3 have important cellular functions and each is believed to play a role in cancer. KLF13 is a transcription factor required for the expression of several oncogenes. FGFR3 is a fibroblast growth factor receptor that initiates a signaling cascade leading to the activation of numerous cellular pathways. Here we show that KLF13 and FGFR3 are overexpressed in oral cancer cells. We also show that artificially reducing cellular levels of KLF13 and FGFR3 decreases cell proliferation and increases sensitivity to ionizing radiation. These data suggest that KLF13 and FGFR3 contribute to malignancy in oral cancer cells and may be useful biomarkers for early detection and possible targets for therapy.

Oral squamous cell carcinoma (OSCC) is the 8th most common cancer worldwide, and includes tumors within the oral cavity [Jemal et al., 2009]. Causes of OSCC include tobacco and alcohol use as well as human papillomavirus infection. Although diagnosis and treatment of OSCC have improved, the survival rate has not increased substantially in 40 years. Like other cancers, OSCC is characterized by genomic instability and altered gene expression. Recently, we examined how the altered expression of the microRNAs (miRNA) miR-125b and miR-100 contributes to malignancy in OSCC [Henson et al., 2009]. We found that expression levels of miR-125b and miR-100 are substantially lower in OSCC cell lines and tumors than in normal human oral keratinocyte (NHOK) controls. We also found that increasing cellular levels of miR-125b and miR-100 affected the expression of both predicted target genes and non-target genes. This included *KLF13* and *FGFR3*, both of which had reduced expression levels in response to increased levels of miR-125b and miR-100, respectively. Public databases predict that *KLF13* and *FGFR3* are targets of miR-125b and miR-100, respectively. Thus, the reduced expression of *KLF13* and *FGFR3* was presumably due to them being targets of miR-125b and miR-100; however, this has yet to be validated experimentally.

The altered expression of *KLF13* and *FGFR3* is intriguing since they are involved in a number of important biological processes. KLF13 (Krüppel-like factor 13) is a zinc finger transcription factor known to play a role in proliferation, differentiation, cell cycle progression, and apoptosis [Chen et al., 2001; Kaczynski et al., 2003; Nemer and

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Horb, 2007]. KLF13 is also involved in T and B lymphocyte development [Outram et al., 2008], and in proliferation and differentiation of the heart [Lavallee et al., 2006; Nemer and Horb, 2007]. FGFR3 (fibroblast growth factor receptor 3) is a member of the fibroblast growth factor receptor family of tyrosine kinases. It is a transmembrane protein that binds fibroblast growth factors, initiating a signaling cascade that leads to the activation of a number of cellular pathways, including the PI3K, MAPK, p38, STAT1, STAT3, and STAT5 pathways [Eswarakumar et al., 2005; Toll and Real, 2008; Matsushita et al., 2009].

In addition, *KLF13* and *FGFR3* have been implicated in a number of malignancies. For instance, altered expression of Krüppel-like transcription factors is believed to contribute to tumorigenesis [Black et al., 2001; Dang et al., 2001; Miller et al., 2001; Martin et al., 2003]. KLF13 can play the role of an activator, as it does for *CCL5* (RANTES) [Song et al., 1999], or that of a repressor, as it does for *CYP1A1* [Kaczynski et al., 2003]. This is of particular importance, since CCL5 is a chemotactic chemokine in inflammatory cells and is overexpressed in lung cancer, breast cancer, prostate cancer, melanoma, T-cell leukemia, ovarian cancer, and oral cancer [Negus et al., 1997; Robinson et al., 2003; Mori et al., 2004; Vaday et al., 2006; Karnoub et al., 2007; Borczuk et al., 2008; Chuang et al., 2009], and CYP1A1 plays a role in head and neck and lung cancers [Agundez, 2004; Li et al., 2004; Hiyama et al., 2008]. KLF13 is also required for the expression of cyclin D1, which is a known oncogene in OSCC [Nemer and Horb, 2007]. A mutated form of *FGFR3* is overexpressed in some bladder cancers, urothelial cancer, multiple myeloma, and cervical cancer [Dailey et al., 2005; Knowles, 2007, 2008; Tomlinson et al., 2007b], whereas the wild-type *FGFR3* is overexpressed in some bladder cancers [Tomlinson et al., 2007b] and multiple myeloma [Chesi et al., 2001]. Reducing FGFR3 levels has been shown to significantly decrease cell proliferation and clonogenicity in bladder cancer [Tomlinson et al., 2007a; Martinez-Torrecuadrada et al., 2008]. Karoui et al. [2001] showed that oral cancer cells do not contain mutations in *FGFR3*; however, expression levels were not examined.

Although KLF13 and FGFR3 are believed to be involved in other cancers, it is not known whether they contribute to malignancy in oral cancer cells. Our objective was to examine the expression of KLF13 and FGFR3 in OSCC cell lines and tumors, and determine what, if any, role they play in cell proliferation and radiosensitivity in oral cancer cells. We hypothesize that KLF13 and FGFR3 are overexpressed in oral cancer cells and that they contribute to malignancy.

Materials and Methods

Cell Culture

Fourteen OSCC cell lines were chosen from our collection [White et al., 2007; Martin et al., 2008] and cultured in Minimal Essential Medium (MEM) (Invitrogen, Carlsbad, Calif., USA) supplemented with gentamicin, L-glutamine, nonessential amino acids, and 10% fetal bovine serum. As a control, we used 2 independent NHOK samples, which were derived from fresh uvulopalatopharyngoplasty (UP3) specimens from consenting individuals undergoing corrective surgery for sleep apnea. NHOK were cultured in keratinocyte serum-free medium (Ker-sfm) (Invitrogen) supplemented with hEGF in 0.1% BSA, Penicillin/Streptomycin, L-Glutamine, and bovine pituitary extract. OSCC primary tumor samples and NHOK controls were obtained from the University of Pittsburgh Head & Neck SPORE Tissue Bank under our consolidated IRB approval. Table 1 lists the OSCC cell lines, tumors, and NHOK samples used in this study, along with demographic information.

Transfections

For all transfections in this study, we used the OSCC cell line UPCI:SCC029. To establish optimal transfection efficiencies, UPCI:SCC029 cells were transfected with Alexa Fluor 488-labeled AllStars Negative Control siRNA (Qiagen, Valencia, Calif., USA) under a variety of experimental conditions followed by fluorescence microscopy. In 6-well plates, we seeded 1×10^6 cells, and in 96-well plates, we seeded 2.5×10^4 cells on the day before transfection in MEM without antibiotics. Transfections were carried out in Opti-MEM® I (Invitrogen) with Lipofectamine™ 2000 (Invitrogen) at a dilution of 1:100. Transfections were carried out for 24 h, after which time the cells were either harvested or the medium was replaced with antibiotic-free MEM. To decrease cellular levels of KLF13 and FGFR3, we transfected UPCI:SCC029 cells with *KLF13* and *FGFR3* siRNAs (Ambion, Austin, Tex., USA) at a final concentration of 200 μm. As a control, we transfected cells with the AllStars Negative Control siRNA (Qiagen) at a final concentration of 200 μm.

Cell Proliferation Assays

To assess the ability of KLF13 and FGFR3 to alter proliferation, we carried out cytotoxicity assays (MTT) using the MTT Cell Growth Assay Kit (Millipore, Billerica, Mass., USA). We transfected UPCI:SCC029 cells with the *KLF13* siRNA, *FGFR3* siRNA, or the negative control siRNA and examined them at 0 h, 24 h, 48 h, and 72 h post-transfection in 3 biological replicates. To eliminate any effect that Lipofectamine 2000 might have on proliferation, all experimental groups were normalized to the mock-transfection control at all time points. Our plates were read using a Spectramax M2/M2e Microplate Reader (Molecular Devices, Sunnyvale, Calif., USA).

Western Blotting

OSCC cells were collected and washed with cold PBS and lysed (on ice) with a solution containing 50 mM Tris, 1% Triton X-100, 10 μg/ml leupeptin, 150 mM NaCl, 1 mM DTT, 0.1% sodium dodecyl sulfate, 10 μg/ml pepstatin, and 1 nM PMSF. Protein concentrations were established using the Bio-Rad Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, Calif., USA) with the SmartSpec 3000 (Bio-Rad Laboratories). Normal-

Table 1. Characteristics of the OSCC cell lines, OSCC tumors, and NHOK samples used in this study

	Sex	Race	Age	Smoking	Alcohol	Family history	Site	Stage
Cell line								
UPCI:SCC029	M	Cau	85	Yes	Yes	No	Buc	T4N2
UPCI:SCC032	M	Cau	60	Yes	Yes	Yes	RMT	T2N2B
UPCI:SCC040	M	Cau	51	No	Yes	Yes	Tong	T2N2
UPCI:SCC066	F	Cau	75	Yes	Yes	Yes	Alv	T1N0
UPCI:SCC070	F	Cau	34	Yes	Yes	Yes	RMT	T3N1
UPCI:SCC078	M	Cau	60	No	Yes	Yes	FOM	T2N0
UPCI:SCC084	M	Cau	53	Yes	Yes	No	RMT	T2N2B
UPCI:SCC103	F	Cau	27	Yes	No	No	Tong	T1N0
UPCI:SCC104	M	Cau	58	No	No	No	FOM	T4NX
UPCI:SCC116	M	Cau	58	Yes	Yes	Yes	Alv	T2N0
UPCI:SCC122	M	Cau	63	Yes	Yes	No	Tong	T1N1
UPCI:SCC131	M	Cau	73	No	No	No	FOM	T2N2
UPCI:SCC142	M	Cau	58	No	No	No	FOM	T4NX
UPCI:SCC182	M	Cau	71	Yes	Yes	No	RMT	T2N1
Tumor								
04-1555	M	Cau	65	Yes	Yes	Yes	Tong	T2N1
05-1797	M	Cau	42	Yes	Yes	Yes	Buc	T2N0
05-1872	M	Cau	79	Yes	No	No	Buc	T4N0
05-1902	M	Cau	68	Yes	No	Yes	Tong	T3N2B
05-1980	M	Cau	41	No	Yes	Yes	Tong	T3N2
NHOK								
06-2665	M	N/A	35	No	No	N/A	UP3	nl
07-2729	F	Cau	19	No	No	N/A	UP3	nl

Included are demographic data as well as smoking history, alcohol use, family history of cancer, tumor stage, and tumor location.

RMT = Retromolar trigone; Tong = tongue; FOM = floor of mouth; Alv = alveolar ridge; Buc = buccal mucosa; N/A = data not available; nl = normal histopathology; Cau = Caucasian; UP3 = uvula, palate, and/or pharynx.

ized cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Immobilon-P membranes (Millipore), and incubated in primary antibody for 2–4 h at room temperature. We used KLF13 (RFLAT-1, C-13) and FGFR3 (C-15) antibodies from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) at a 1:1,000 dilution. To verify equal protein loading, membranes were sectioned and probed for β-tubulin (H-235) (Santa Cruz Biotechnology) at a dilution of 1:1,000. Proteins were visualized using the Western Lightning™ Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences, Boston, Mass., USA). Densitometric analyses were done using Un-Scan-It Gel™ software (Silk Scientific, Orem, Utah, USA).

Clonogenic Survival Assays

To determine if reducing the levels of KLF13 and FGFR3 would increase the sensitivity of OSCC cells to ionizing radiation, we carried out clonogenic survival assays [Parikh et al., 2007]. Cells were seeded into 60-mm dishes and transfected with *KLF13* siRNA, *FGFR3* siRNA, or the negative control siRNA. At 48 h post-transfection, 1,000 cells were seeded into 60-mm dishes and allowed to attach overnight. The following day, the cells were irradiated with gamma radiation, using the Gammacell 1000 Elite irradiator (MDS Nordion, Ottawa, Ont., Canada) at 0, 1, 2.5, 5, and 10 Gy. The medium was changed 7 days after irradiation. At 12 days post-irradiation, dishes were stained with Giemsa stain (Sigma, St. Louis, Mo., USA) and colonies were counted. We defined a colony as a cluster of 50 or more cells that presumably originated from a single cell.

QRT-PCR

To determine the expression levels of *KLF13* and *FGFR3* in tumors, we carried out QRT-PCR. Reverse transcription reactions were performed as previously described [Huang et al., 2002] with 2 total RNA inputs (400 ng and 100 ng) for each sample using MMLV Reverse Transcriptase (Epicentre, Madison, Wisc., USA) and random hexamers. For Quantitative PCR (QPCR), we used TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, Calif., USA) for *KLF13*, *FGFR3*, and the 18s RNA control, using TaqMan 2× Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). All QPCR samples were

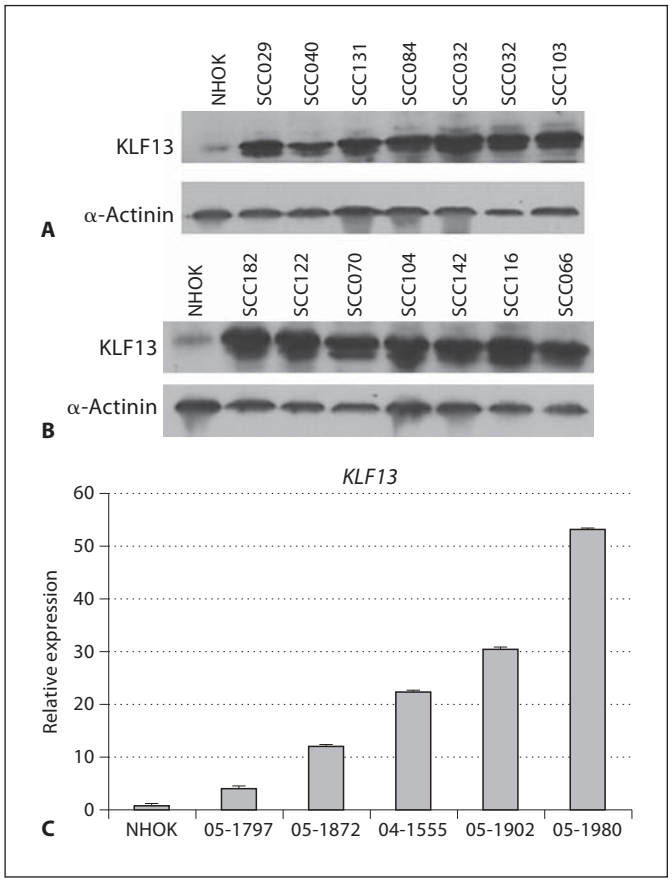

Fig. 1. Overexpression of KLF13 in UPCI:SCC oral cancer cell lines, tumors and NHOK controls. **A**, **B** Comparison of KLF13 protein levels in OSCC cell lines to NHOK controls. **C** Comparison of *KLF13* transcript levels between OSCC primary tumors and NHOK controls, with standard error of the mean shown.

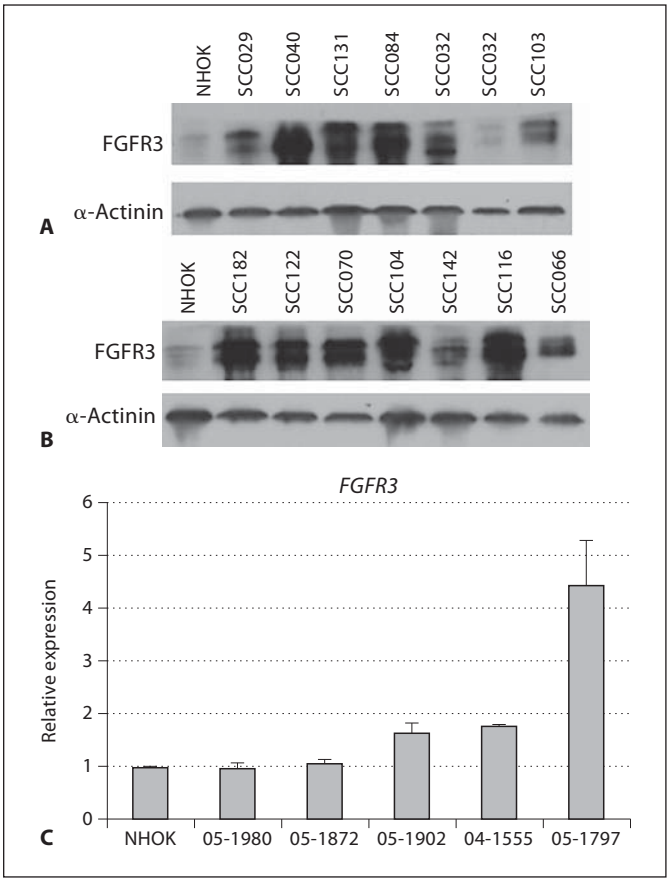

Fig. 2. Overexpression of FGFR3 in UPCI:SCC oral cancer cell lines, tumors and NHOK controls. **A**, **B** Comparison of FGFR3 protein levels in OSCC cell lines to NHOK controls. **C** Comparison of *FGFR3* transcript levels between OSCC primary tumors and NHOK controls, with standard error of the mean shown.

done in triplicate and each QPCR plate included no reverse transcriptase and no template controls. The data were analyzed using the comparative CT method.

Statistical Analysis

Data are presented as either the mean ± standard error or the mean ± standard deviation, as noted. Assessment of statistical significance in all of our experiments was done using 2-sided t tests with p values <0.05 being considered statistically significant.

Results

We found that KLF13 protein levels were substantially higher in all OSCC cell lines than in NHOK controls (fig. 1A, B). We also found that *KLF13* transcript levels were considerably higher in all OSCC tumors than in NHOK controls (fig. 1C). Our data also indicate the levels of FGFR3 protein were considerably higher in all but one of our cell lines than in the controls (fig. 2A, B), and that *FGFR3* mRNA levels were higher in 3 of the 5 tumors than in the controls (fig. 2C). Given the elevated levels of KLF13 and FGFR3 in our OSCC cell lines and tumors, we sought to determine whether they play a role in cell proliferation and radiosensitivity in oral cancer cells.

To determine if the overexpression of KLF13 and FGFR3 contributes to malignancy, we reduced cellular levels of KLF13 and FGFR3 (fig. 3) and assessed changes in cell proliferation and sensitivity to ionizing radiation. We found that reducing levels of KLF13 led to a ~20% decrease in proliferation, and reducing levels of FGFR3 resulted in a ~35% decrease in proliferation (fig. 4). Therefore, our data suggest that KLF13 and FGFR3 con-

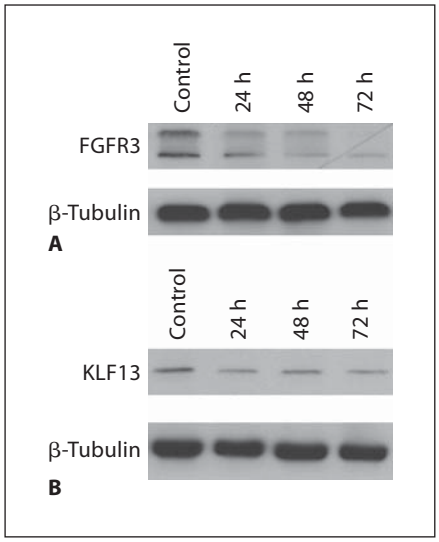

Fig. 3. Knockdown of FGFR3 (**A**) and KLF13 (**B**) levels in UPCI:SCC029 cells transfected with *FGFR3* siRNA and *KLF13* siRNA, respectively. Cells were collected at 24 h, 48 h, and 72 h post-transfection and compared to cells transfected with the negative control.

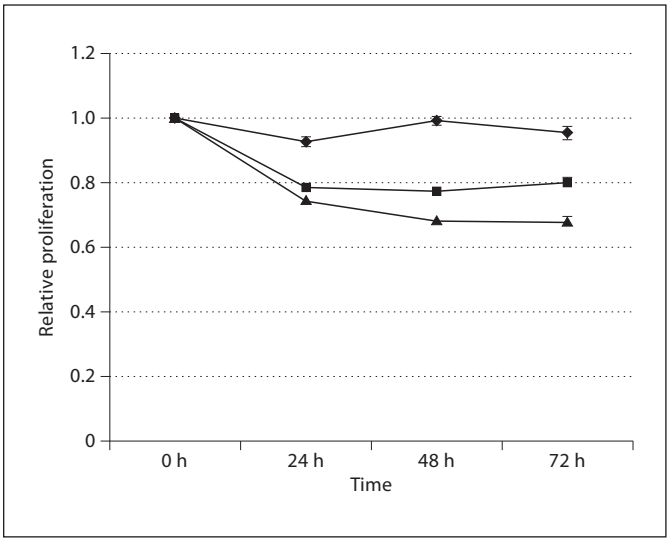

Fig. 4. Results of the MTT assays. Cells transfected with *KLF13* siRNA are represented by squares, cells transfected with *FGFR3* siRNA are represented by triangles, and cells transfected with the negative control are represented by diamonds, with standard error of the mean indicated. All experimental groups were normalized to the mock-transfection control at all time points.

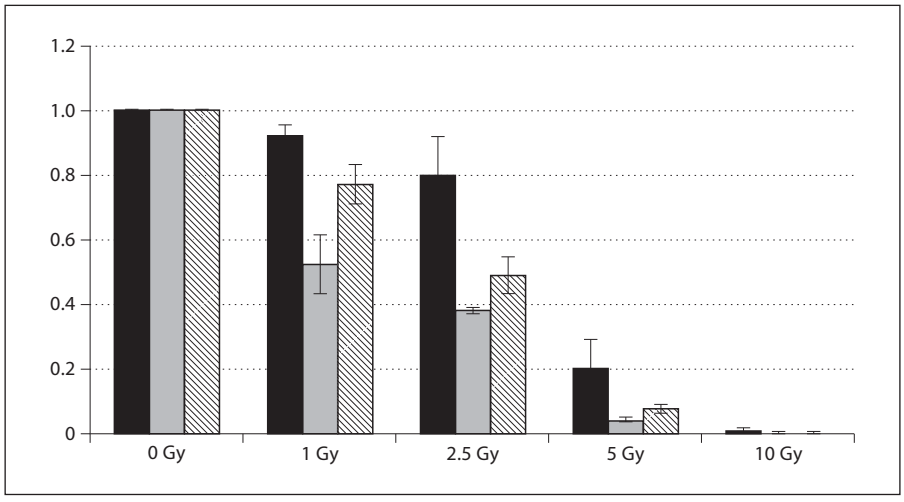

Fig. 5. Results of clonogenic survival assays showing the relative survival of UPCI:SCC029 cells transfected with the negative control (black bars), *FGFR3* siRNA (gray bars), or *KLF13* siRNA (hatched bars), and treated with ionizing radiation, with standard deviation shown.

tribute to increased cell proliferation in oral cancer cells. Reducing levels of KLF13 in conjunction with ionizing radiation led to a 25% reduction in colony formation with 1 Gy, a 50% reduction in colony formation with 2.5 Gy, and a 93% reduction in colony formation with 5 Gy, all of which were substantially lower than the negative control (fig. 5). Reducing levels of FGFR3 in conjunction with ionizing radiation led to a 50% reduction in colony formation with 1 Gy, a 60% reduction in colony formation with 2.5 Gy, and a 96% reduction in colony formation with 5 Gy, all of which were considerably lower than the negative control (fig. 5). Thus, the overexpression of KLF13 and FGFR3 in OSCC appears to contribute to resistance to ionizing radiation.

Discussion

Both KLF13 and FGFR3 have important biological functions and are altered in a number of malignancies. Here we show that KLF13 and FGFR3 are overexpressed in most of the OSCC cell lines and tumors we examined. This suggests that KLF13 and FGFR3 could be used as biomarkers for early detection of this debilitating disease. Others have suggested that FGFR3 be used as a biomarker for early detection of bladder cancer [Knowles, 2007]. Our data indicate that artificially reducing KLF13 and FGFR3 levels negatively impacted cell proliferation and resistance to ionizing radiation, indicating that the overexpression of KLF13 and FGFR3 contributes to malignancy in OSCC. This could be due in part to KLF13 being required for the expression of the oncogenes *CCL5*, *CYP1A1*, and *CCND1*, and that FGFR3 is known to stimulate the PI3K, MAPK, p38, STAT1, STAT3, and STAT5 pathways [Eswarakumar et al., 2005; Toll and Real, 2008; Matsushita et al., 2009], which have known roles in cancer.

KLF13 and FGFR3 probably do not hold any great promise as individual therapeutic targets in OSCC; however, in combination with other therapeutic agents they may be beneficial. For instance, reducing cellular levels of KLF13 and FGFR3, in conjunction with ionizing radiation, led to a substantial decrease in cellular survival. Others have suggested that FGFR3 be used as a therapeutic target to treat bladder cancer [Tomlinson et al., 2007a; Martinez-Torrecuadrada et al., 2008]. This is of interest because FGFR3, among other proteins, has been implicated in contributing to resistance to ionizing radiation in OSCC cells [Ishigami et al., 2007]. This point is supported by the data presented here. In summary, the overexpression of KLF13 and FGFR3 appears to contribute to malignancy in the oral cavity, and may be utilized as biomarkers for early detection and new targets for therapy.

Acknowledgements

This research was supported in part by NIH grant RO1DE14729 to S.M.G., NIH grant P50CA097190 to J.R. Grandis, and an NIH NRSA Kirschstein Fellowship Award to B.J.H., F32CA119725. We would like to thank Dr. Gerard Nau for the use of his laboratory equipment for these studies.

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