RESEARCH PAPER

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Decreased *SMAD4* expression is associated with induction of epithelial-to-mesenchymal transition and cetuximab resistance in head and neck squamous cell carcinoma

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Abbreviations: CDKN2A, cyclin-dependent kinase Inhibitor 2A; CTX, cetuximab; EGFR, epidermal growth factor receptor; EMT, epithelial-to-msenchymal transition; FDR, false delivery rate; HB-EGF, heparin-binding EGF-like growth factor; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; KD, knocked-down; mIR, microRNA; qRT-PCR, quantitative real-time polymerase chain reaction; RPPA, reverse phase protein arrays; RSEM, RNA-Seq by Expectation Maximization; shRNA, small hairpin RNA; SMAD4, mothers against decapentaplegic homolog 4; TCGA, The Cancer Genome Atlas.

Epidermal growth factor receptor (EGFR) is frequently overexpressed in head and neck squamous cell carcinoma (HNSCC) and cetuximab, a monoclonal antibody targeting this receptor, is widely used to treat these patients. In the following investigation, we examined the role of *SMAD4* down-regulation in mediating epithelial-to-mesenchymal transition (EMT) and cetuximab resistance in HNSCC. We determined that *SMAD4* downregulation was significantly associated with increased cell motility, increased expression of vimentin, and cetuximab resistance in HNSCC cell lines. In the HNSCC genomic dataset obtained from The Cancer Genome Atlas, *SMAD4* was altered in 20/279 (7%) of HNSCC via homozygous deletion, and nonsense, missense, and silent mutations. When *SMAD4* expression was compared with respect to human papillomavirus (HPV) status, HPV-positive tumors had higher expression compared to HPV-negative tumors. Furthermore, higher *SMAD4* expression also correlated with higher *CDKN2A* (p16) expression. Our data suggest that *SMAD4* down-regulation plays an important role in the induction of EMT and cetuximab resistance. Patients with higher *SMAD4* expression may benefit from cetuximab use in the clinic.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous disease associated with several anatomical sites including the oral cavity, pharynx, and larynx. Risk factors for HNSCC development include chronic tobacco exposure, heavy alcohol use, and human papillomavirus (HPV) infection.[1-4] Treatment intensification using multi-modality approaches can be effective for managing newly diagnosed HNSCC patients; however, recurrent and metastatic disease are often not curable and therapeutic options are limited.

Epidermal growth factor receptor (EGFR) is known to be an important prognostic marker and a therapeutic target in HNSCC. It has long been understood that EGFR overexpression as determined by immunohistochemistry is associated with poor survival in HNSCC patients.[5-8] Furthermore, recent comprehensive genomic analyses of HNSCCs have confirmed that *EGFR* is altered by increased copy number and mutations.[9-11] Based on these data, EGFR-targeted agents have been developed and cetuximab, a monoclonal antibody directed against EGFR, is currently FDA-approved for use in HNSCC patients.[12,13] Through binding the extracellular domain of EGFR, cetuximab competes with EGFR ligands and prevents downstream activation.[14] In addition to receptor inhibition, cetuximab may also promote apoptosis by inhibiting DNA damage repair and inducing antibody-dependent cellular cytotoxicity (ADCC).[15-17]

However, it has become apparent that a significant subset of HNSCC patients exhibit EGFR inhibitor resistance.[13] Several mechanisms of resistance have been investigated including activation of HER3, MET, and downstream AKT activation.[17,18] Previously, our laboratory has demonstrated multiple mechanisms of acquired cetuximab resistance in HNSCC cells including therapeutic target loss (downregulation of EGFR) and up-regulation of its cognate ligands, such as heparin-binding

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EGF-like growth factor (HB-EGF).[19] Furthermore, we reported that the deregulation of microRNA (miR) expression, such as decrease miR-212, may contribute to the observed HB-EGF upregulation.[19] We also observed that the cetuximab-resistant cells exhibit a mesenchymal phenotype *in vitro* compared to cetuximab-sensitive cells.

In the current study, we hypothesized that epithelial-to-mesenchymal transition (EMT) is an important contributor for mediating acquired cetuximab resistance. We demonstrate that down-regulation of Smad4 is associated with an EMT phenotype and contributes to cetuximab resistance in HNSCC. In addition, we evaluated *SMAD4* genomic alterations in human HNSCC tumors which revealed higher *SMAD4* expression in HPV-positive tumors suggesting that patients with HPV-positive tumors may benefit from cetuximab.

Results

Cetuximab-resistant cells exhibit a mesenchymal phenotype *in vitro* compared to cetuximab-sensitive cells

SCC1 and 1CC8 are an isogenic cell line pair, the latter developed as an acquired cetuximab resistance model. The sensitivity of SCC1 and 1CC8 to cetuximab treatment has previously been published.[18,19] To further investigate this phenomenon, we developed a second isogenic cell line panel of acquired cetuximab resistance using cetuximab-sensitive SCC25. Twelve cetuximab-resistant clones (CTX-R1-12) were generated by chronic exposure to cetuximab *in vitro* (**Fig. 1A**). After treatment selection, we observed the cell lines with acquired cetuximab resistance exhibited a mesenchymal morphology upon visual inspection and displayed increased migratory potential compared to the sensitive parent cell lines. These features are consistent with previous reports of EMT (**Fig. 1B**).[17,19]

To further evaluate the induction of EMT and subsequent cetuximab resistance, 218 probes representing 83 EMT-related genes (Gene Ontology set GO:0001837)[20] were analyzed for coordinated differential expression between SCC1/1CC8 using previously published global gene expression data (GSE21483, **File S1**). These genes associated with EMT were differentially expressed between SCC1 and 1CC8 with a statistical significance (p-value $= 9 \times 10^{-5}$). More specifically, 31 probes representing 21 genes were significantly associated with cetuximab resistance seen in 1CC8 (**Fig. 2**). Among these, *SMAD4* was chosen for further evaluation because *SMAD4* knockout mice develop spontaneous HNSCCs that histologically resemble the human disease.[21] Furthermore, with respect to our isogenic cell line pair, *SMAD4* was substantially down-regulated in 1CC8 compared to SCC1 (p-value of 8×10^{-9}). Lower 1CC8 *SMAD4* mRNA expression was also confirmed by qRT-PCR (**Fig. 1C**). To expand on this result, we evaluated the newly generated SCC25-derived cetuximab-resistant cell lines (CTX-R1-12) for *SMAD4* expression. While *SMAD4* expression was variable across the panel, qRT-PCR analysis determined that all 12 daughter cell lines expressed lower levels of *SMAD4* mRNA compared to the parental SCC25 cell line with an average decrease of 41% ± 3 (**Fig. 1C**).

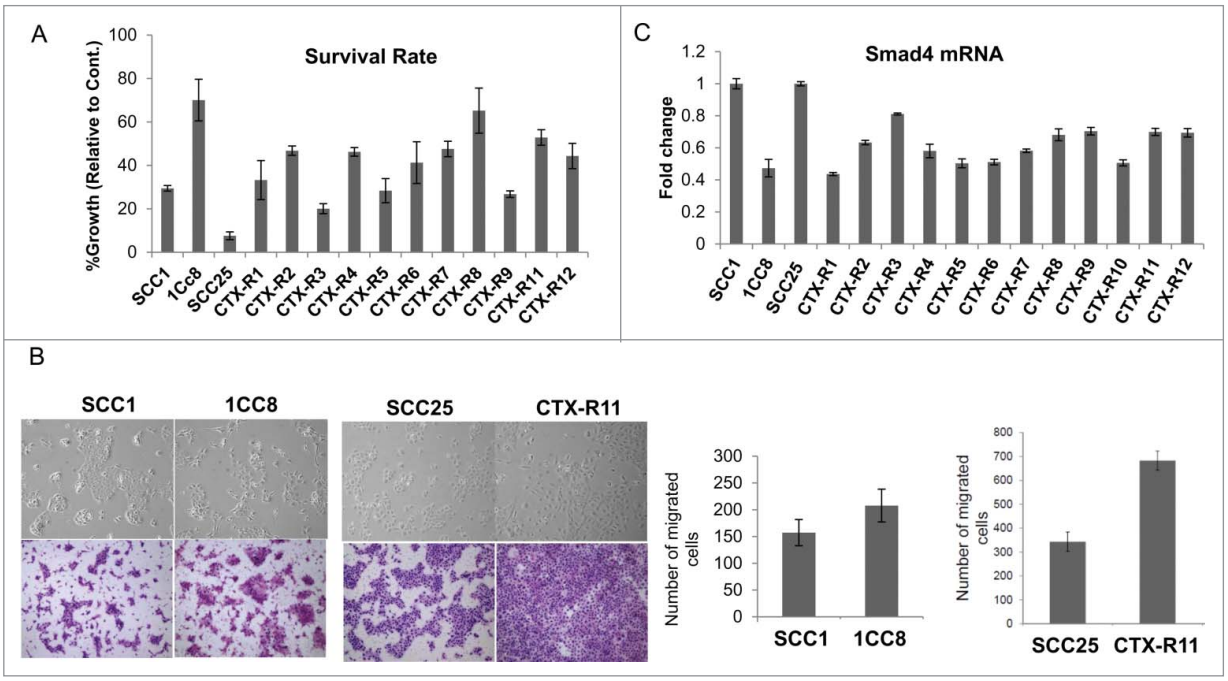
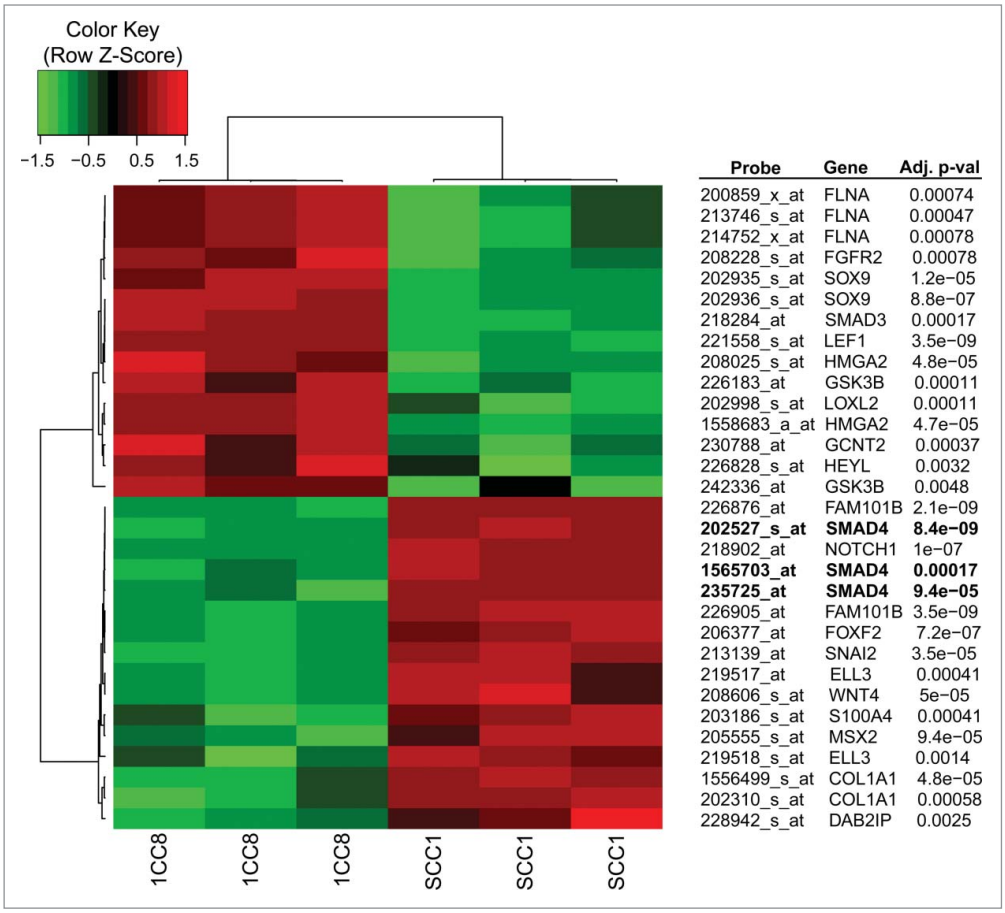


Figure 1. (**A**) Characterization of cetuximab (CTX)-resistant clones generated from SCC1 and SCC25 after chronic exposure to CTX *in vitro*. Cell survival was determined by Matrigel colony formation, (**B**) Increased cell mobility in CTX-resistant clones, 1CC8 and CTX-R11, compared to SCC1 and SCC25, respectively, determined by cell migration assay, and (**C**) *SMAD4* mRNA levels in CTX-resistant clones compared to the isogenic parent cell lines, SCC1/1CC8 and SCC25/CTX-Rs.

SMAD4 knock-down increases EMT phenotype and induces cetuximab resistance in HNSCC

To determine the functional consequences of *SMAD4* down-regulation in cetuximab-sensitive HNSCC cell lines, *SMAD4* was stably knocked-down (K_D) in SCC1 and SCC25 using shRNAs (**Fig. 3A–B**). In the *SMAD4* K_D groups, the number of migrating cells increased nearly 2-fold compared to scrambled shRNA controls ($p < 0.05$). These cells also demonstrated the EMT phenotype observed in the cetuximab-resistant cell lines (**Fig. 3C**). In SCC1, *SMAD4* K_D also caused a significant increase in vimentin mRNA, supporting our hypothesis that *SMAD4* down-regulation contributes to EMT in HNSCC (**Fig. 3D**). Additionally, down-regulation of *SMAD4* decreased cetuximab sensitivity of both SCC1 and SCC25, as determined by matrigel colony formation (**Fig. 3E–F**). These results suggest that decreased *SMAD4* expression is associated with EMT in HNSCC and the functional consequence of this transformation is increased motility and cetuximab resistance.



Probe	Gene	Adj. p-val
200859_x_at	FLNA	0.00074
213746_s_at	FLNA	0.00047
214752_x_at	FLNA	0.00078
208228_s_at	FGFR2	0.00078
202935_s_at	SOX9	1.2e−05
202936_s_at	SOX9	8.8e−07
218284_at	SMAD3	0.00017
221558_s_at	LEF1	3.5e−09
208025_s_at	HMGA2	4.8e−05
226183_at	GSK3B	0.00011
202998_s_at	LOXL2	0.00011
1558683_a_at	HMGA2	4.7e−05
230788_at	GCNT2	0.00037
226828_s_at	HEYL	0.0032
242336_at	GSK3B	0.0048
226876_at	FAM101B	2.1e−09
202527_s_at	**SMAD4**	**8.4e−09**
218902_at	NOTCH1	1e−07
1565703_at	**SMAD4**	**0.00017**
235725_at	**SMAD4**	**9.4e−05**
226905_at	FAM101B	3.5e−09
206377_at	FOXF2	7.2e−07
213139_at	SNAI2	3.5e−05
219517_at	ELL3	0.00041
208606_s_at	WNT4	5e−05
203186_s_at	S100A4	0.00041
205555_s_at	MSX2	9.4e−05
219518_s_at	ELL3	0.0014
1556499_s_at	COL1A1	4.8e−05
202310_s_at	COL1A1	0.00058
228942_s_at	DAB2IP	0.0025

Figure 2. Heatmap of expression values for probes that are differentially expressed between SCC1 and 1CC8 with a statistical significance and are annotated to the GO EMT pathway.

SMAD4 expression in human HNSCC

To further determine the relevance of *SMAD4* down-regulation in tumors obtained from HNSCC patients, we analyzed the genomic alteration of *SMAD4* in The Cancer Genome Atlas (TCGA) using DNA and RNA sequencing data obtained from 279 HNSCC samples.[9] There were 36 HPV-positive and 243 HPV-negative HNSCC with both DNA and RNA data within TCGA (**Table 1**). While HPV-positive samples did not demonstrate any homozygous deletions, 13 of 243 (5%) HPV-negative HNSCCs harbored homozygous deletions of *SMAD4*. One (3%) HPV-positive HNSCC had a missense mutation (P298S) but MutationAssessor found its probability of functional significance to be low.[22,23] Six of the HPV-negative HNSCCs (2%) had nonsense, missense, and silent mutations. MutationAssessor assigned the 3 missense mutations (P544L, R361H, and W99C) to have medium probability of functionally significance.[22,23] We found that samples with deletions and nonsense mutations of *SMAD4* had significantly reduced *SMAD4* mRNA expression ($p = 0.0002$, **Fig. 4A**). We also categorized the tumors with wild-type *SMAD4* into high and low expression cohorts relative to the expression levels in samples with homozygous deletion and nonsense mutations, and found 125 of the 279 samples (44.8%; **Table 1**) to have low *SMAD4* expression. Moreover, *SMAD4* mRNA expression was positively correlated with Smad4 protein expression (correlation coefficient 0.19, p-value 0.0057, **Fig. 4B**). In addition, we found *SMAD4* mRNA expression is significantly lower in HPV-negative HNSCC tumors relative to HPV-positive tumors (p-value of 1.5×10^{-12}, **Fig. 4C**). *SMAD4* mRNA expression was also significantly correlated with

Table 1. Genomic alterations of *SMAD4* in tumors obtained from HNSCC patients in TCGA. Samples are called SMAD4 low when their log transformed RSEM expression for SMAD4 from RNA-sequencing data is below the maximum value of SMAD4 expression for homozygous deletions or nonsense mutations

HPV	SMAD4	SMAD4.Low	SMAD4.High
HPV+	Homozygous Deletion	0	0
	Nonsense Mutation	0	0
	Missense / Silent Mutation	0	1 (p.P298S)
	WT	3	32
HPV-	Homozygous Deletion	13	0
	Nonsense Mutation	2 (p.S242*; p.Q461*)	0
	Missense / Silent Mutation	2 (p.W99C; p.R361H)	2 (p.P544L; p.K46K)
	WT	122	102

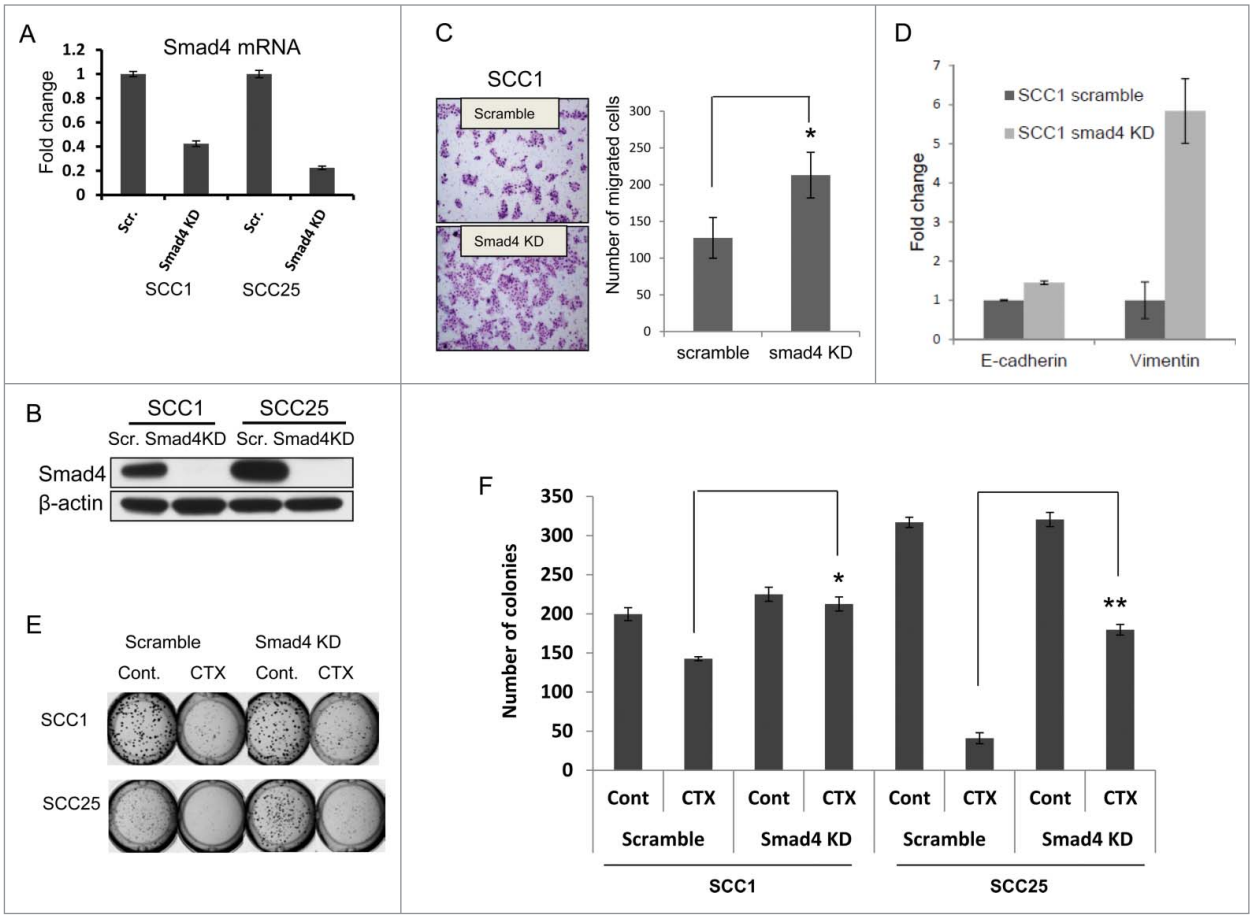


Figure 3. Smad4 knockdown (K_D) induces cetuximab (CTX) resistance in HNSCC cell lines. (**A**) Lower expression of *SMAD4* mRNA in CTX-sensitive cell lines, SCC1 and SCC25, after Smad4 shRNA K_D, (**B**) Lower expression of Smad4 protein in CTX-sensitive cell lines, SCC1 and SCC25, after Smad4 shRNA K_D, (**C**) SCC1- *SMAD4* K_D cells are more migratory, (**D**) SCC1- *SMAD4* K_D cells have increased expression of vimentin, and (**E–F**) *SMAD4* K_D cells are less sensitive to CTX treatment (100nM) than control (Cont) as measured by Matrigel colony formation assay. *statistically significant.

CDKN2A (p16) RNA expression which is commonly used as a surrogate marker of HPV status in HNSCC[24,25] (correlation coefficient 0.19, p-value 0.0018, **Fig. 4D**).

Discussion

Although the use of cetuximab has demonstrated positive clinical results in HNSCC treatment, *de novo* and acquired resistance to this targeted agent represent a significant challenges for improving patient outcome. Previous reports from our laboratory established ErbB-associated ligand upregulation and receptor crosstalk are correlated with acquired cetuximab resistance in HNSCC.[19] However, additional HNSCC-specific modifications associated with this resistance required further investigation.

EMT has been well studied in developmental biology where this process leads to a migratory mesenchyme that later establishes mesodermal and endodermal tissues.[26,27] In carcinogenesis, the migratory phenotype and increased plasticity associated with EMT provides many advantages for a developing malignancy to evolve and evade traditional therapies leading to distant metastasis. Thus, it is important to elucidate the molecular determinants of EMT in a disease-specific manner to improve therapeutic outcomes in HNSCC. Prior gene expression analysis of HNSCC from our laboratory demonstrated that a subset of this disease is associated with an upregulation of EMT-related genes, which then leads to poor clinical outcomes.[28] Supporting this hypothesis, changes in EMT downstream markers (i.e., E-cadherin, vimentin) are associated with the development of metastasis in HNSCC.[29] Additionally, putative HNSCC stem cells isolated from cell lines exhibit lower E-cadherin and higher vimentin levels, while also demonstrating increased invasiveness and EMT phenotypes.[30]

Smad4 is an established tumor suppressor in many cancer subtypes and its loss in HNSCC is associated with inactivating mutations or loss of heterozygosity at 18q21.[9,31-35] The significance of Smad4 in the development of HNSCC was recently strengthened by Bornstein, et al. as they demonstrated conditional Smad4 loss ($Smad4^{-/-}$) in the oral cavity of mice causes

spontaneous tumors. The histology ranged from moderately to poorly differentiated squamous cell carcinomas and closely mirrored that of analogous human disease.[21] The authors also showed that Smad4 loss resulted in genomic instability through the downregulation of DNA repair-related genes including Brca1, FancAm, FancD2 and Rad51, and subsequent abnormal centrosome amplification. In addition, Smad4 loss caused increased inflammation of the surrounding stroma characterized by numerous infiltrating leukocytes and increased inflammatory cytokines suggesting an upregulation of TGF-β1. Our current investigation adds to the existing data that *SMAD4* downregulation contributes to the induction of EMT and is a common feature of acquired cetuximab resistance in HNSCC cell lines supporting important therapeutic and prognostic consequences of *SMAD4* deregulation.

Lastly, we report that *SMAD4* expression is low in a significant number of HNSCC, especially in HPV-negative tumors, while the genomic alteration is relatively rare (7%). Also, tumors with lower *CDKN2A* (p16) expression are likely to have lower *SMAD4* expression. Considering that patients with HPV-negative HNSCC have worse survival compared to the HPV-positive tumors,[24,36] low *SMAD4* expression may contribute to poor outcome in HPV-negative patients. In addition, a retrospective analysis of a randomized phase III clinical trial comparing radiation to radiation plus cetuximab in locally advanced HNSCC patients demonstrated that p16 expression was strongly prognostic and HPV-positive patients benefited more from the addition of cetuximab to standard radiation therapy compared to the HPV-negative patients.[37] We are still waiting for the phase III clinical trial data comparing radiation plus cisplatin versus radiation plus cetuximab in HPV-positive patients to determine the efficacy of cetuximab in this patient setting; however, high expression of *SMAD4* in our data may suggest that HPV-positive patients may benefit from cetuximab.

To conclude, we have demonstrated that down-regulation of *SMAD4* is an important mediator of EMT and cetuximab resistance. Further investigation is warranted to translate the findings to therapeutic development of cetuximab in HNSCC patients.

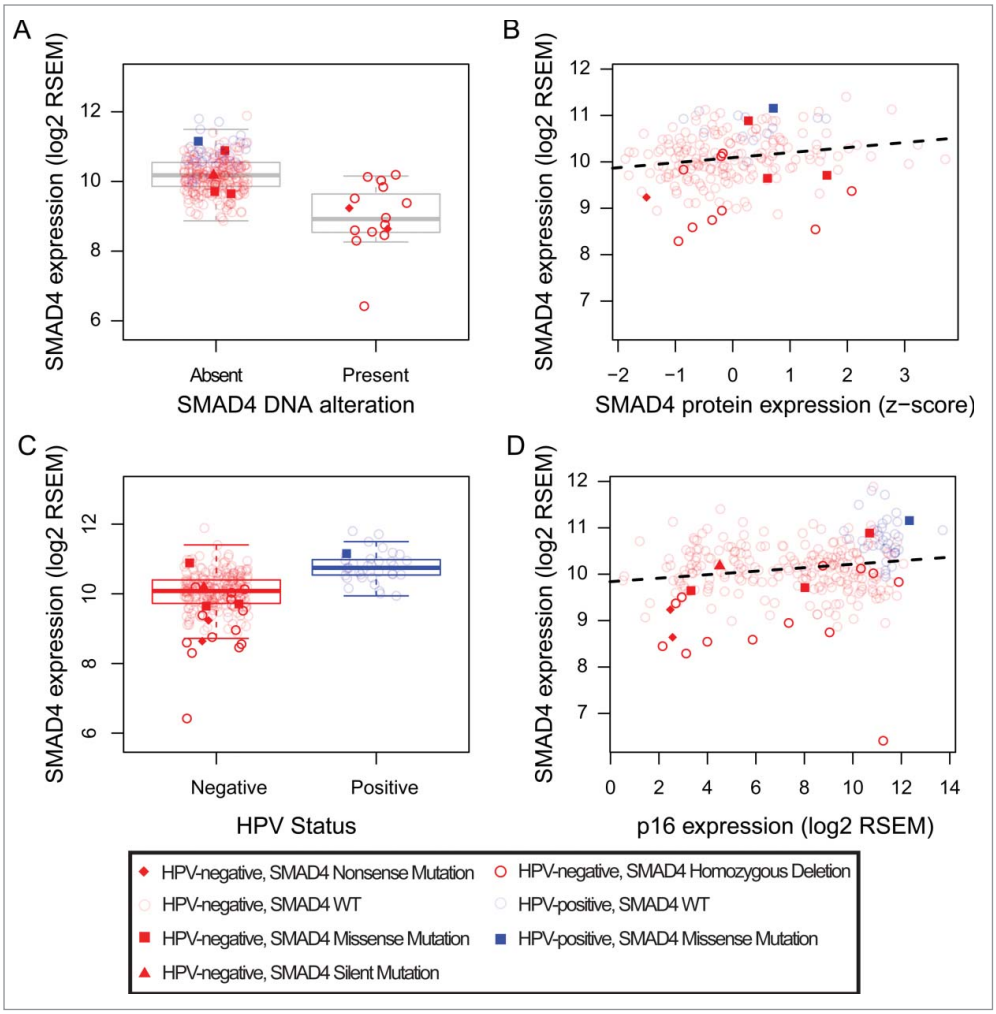


Figure 4. *SMAD4* expression by *SMAD4* alterations (homozygous deletions and nonsense mutations). Points are shaped and shaded as indicated in the figure and colored by HPV-status (red for HPV-negative, blue for HPV-positive). (**A**) HNSCCs with *SMAD4* alterations have lower *SMAD4* mRNA expression, (**B**) *SMAD4* mRNA expression is positively correlated with Smad4 protein expression, (**C**) *SMAD4* mRNA expression is higher in the HPV-positive HNSCCs compared to HPV-negative HNSCCs, and (**D**) *SMAD4* mRNA expression is positively correlated with *CDKN2A* (p16) mRNA expression.

Materials and Methods

Cell lines and materials

SCC1 and 1CC8 represent an isogenic cell line pair, in which 1CC8 was selected *in vitro* as a model for acquired cetuximab resistance.[18] SCC25 was purchased from American Type Culture Collection and additional cetuximab resistant clones of SCC25 were generated as previously published.[18] Each cell line was authenticated using short tandem repeat (STR) analysis kit GenePrint 10 (Promega, Madison, WI) through the Johns Hopkins University Genetic Resources Core Facility. Cetuximab (Bristol-

Myers Squibb, Princeton, NJ) was purchased from the Johns Hopkins Pharmacy.

Matrigel colony formation and cell migration assays

Matrigel colony formation assays were performed to assess the survival rate as previously described.[19] For the cell migration assay, cells were seeded in a Transwell chamber (8-μM pore) coated with Collagen I. Migration was initiated across the membrane with a 0–10% FBS chemoattractant gradient. After 36 hrs, remaining cells were removed with a cotton swab, while migratory cells were fixed, stained, and quantified from 6 fields of view at 200X magnification. Data are presented as the average number of cells per field of view.

Quantitative real-time PCR

Total RNA was extracted as previously described.[19] For real-time PCR, Taqman gene expression assays (Applied Biosystems, Foster City, CA) were performed per manufacturer's instructions. The assays utilized were SMAD4 (Hs00929647-m1) and β-actin (ACTB, Hs00357333-g1). Relative gene expression was normalized to β-actin by comparative C_t.

Stable knockdown of SMAD4

Cells were seeded in 6-well plates 24 hours prior to transduction. Per manufacturer's instructions, an appropriate amount of lentiviral transduction particles of *SMAD4* (Sigma, NY) were added into the media in the presence with hexadimethrine bromide. After puromycin selection, cell pools were collected and knockdown was confirmed with qRT-PCR and Western blot.

Statistical analysis of HNSCC cell line microarrays

Raw array data was obtained for SCC1/1CC8 from GSE21483 and normalized with fRMA version 1.16.0.[38] Differential gene expression associated with cetuximab resistance was assessed for SCC1/1CC8 using t-statistics moderated with empirical Bayes computed with the LIMMA Bioconductor package version 3.20.9. Gene set enrichment was inferred by applying a one-sided gene set test and implemented in the LIMMA package to the moderated t-statistics for SCC1/1CC8. The p-values reported for gene set enrichment of EMT were based upon the set of GO EMT probes from GO:0001837 identified in version 2.14.0 of the annotation for Affymetrix hgu133plus2.0.db package in Bioconductor. All reported probe-level p-values were adjusted for false discovery rate (FDR) using the Benjamini-Hotchberg correction. Probes with FDR adjusted p-value below 0.05 and absolute log fold-change greater than 0.5 were deemed significantly related to cetuximab resistance. This adjustment was performed only for the set of probes annotated to the EMT GO set, GO:0001837, hypothesized *a priori* to relate to cetuximab resistance. R code for all microarray preprocessing and analyses is provided in **File S1**.

Statistical analysis of TCGA data

Processed Level 3 mRNA expression, protein expression, copy number, and mutation data was obtained for the 279 TCGA HNSCC freeze-set samples used for the TCGA publication.[9] HPV status was assessed by integrating viral detection in sequencing data and p16 status, as described in the TCGA publication.[9] Samples were called to have a homozygous deletion in *SMAD4* when its GISTIC2.0[39] score estimated from the copy number data was −2. The MutationAssessor functional impact scores [23] for reported *SMAD4* mutations were obtained from cBioPortal.[40] Gene expression values were reported as log transformed RSEM values [41] using version 2 of data preprocessing for TCGA RNA-sequencing data. Protein expression values were reported as z-scores measured from reverse phase protein arrays (RPPA) and were obtained from cBioPortal.[40] The RPPA data were only available for 200 of the 279 samples (14 HPV-Positive and 186 HPV-Negative). *SMAD4* was called significantly differentially expressed when the p-value for t-statistics comparing log transformed RSEM values were below 0.05. Samples were deemed as having DNA alterations in SMAD4 when they had either homozygous deletions or nonsense mutations. The maximum log transformed RSEM value of SMAD4 expression for these altered samples (10.2) was used to establish a threshold for low SMAD4 expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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