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Inter- and intra-tumor heterogeneity of *SMAD4* loss in head and neck squamous cell carcinomas

Ariel L. Hernandez[1], **Ying Wang**[2], **Hilary L. Somerset**[1], **Stephen B. Keysar**[3], **Dara L. Aisner**[1], **Carrie Marshall**[1], **Daniel W. Bowles**[4], **Sana D. Karam**[5], **David Raben**[5], **Antonio Jimeno**[3], **Marileila Varella-Garcia**[2,3], and **Xiao-Jing Wang**[1,4]

[1]Department of Pathology, School of Medicine, Molecular Biology Program, Medical Scientist Training Program, University of Colorado, Aurora, Colorado

[2]University of Colorado Cancer Center, Molecular Pathology/Cytogenetics Shared Resource, University of Colorado, Aurora, Colorado

[3]Department of Medical Oncology, University of Colorado, Aurora, Colorado

[4]Veterans Affairs Medical Center, VA Eastern Colorado Health Care System, Aurora, Colorado

[5]Department of Radiation Oncology, University of Colorado, Aurora, Colorado

Abstract

Reports regarding the frequency of *SMAD4* loss in human head and neck squamous cell carcinoma (HNSCC) vary significantly. We have shown that *SMAD4* deletion contributes to HNSCC initiation and progression. Therefore, accurately detecting genetic *SMAD4* loss is critical to determine prognosis and therapeutic interventions in personalized medicine. We developed a *SMAD4* fluorescence in situ hybridization (FISH) assay to identify chromosomal *SMAD4* loss at the single cell level of primary HNSCC specimens and patient derived xenograft (PDX) tumors derived from HNSCCs. *SMAD4* heterozygous loss was detected in 35% of primary HNSCCs and 41.3% of PDX tumors. Additionally, 4.3% of PDX tumors had *SMAD4* homozygous loss. These frequencies of *SMAD4* loss were similar to those in The Cancer Genome Atlas (TCGA). However, we identified significant heterogeneities of *SMAD4* loss (partial or complete) among cells within each tumor. We also found that aneuploidy (monosomy and polysomy) contributed greatly to how to define chromosomal *SMAD4* deletion. Furthermore, in cultured PDX tumors, *SMAD4* mutant cells outcompeted *SMAD4* wildtype cells, resulting in establishing homogenous *SMAD4* mutant HNSCC cell lines with partial or complete genomic *SMAD4* loss, suggesting a survival advantage of *SMAD4* mutant cells. Taken together, our study reveals inter- and intra-tumor heterogeneities of *SMAD4* chromosomal loss in HNSCCs. Further, *SMAD4* FISH assay provides a platform for future clinical diagnosis of *SMAD4* chromosomal loss that potentially serves as a molecular marker for prognosis and therapeutic intervention in cancer patients.

Correspondence Xiao-Jing Wang MD, PhD, Director, Head and Neck Cancer Research Program, John S. Gates Endowed Chair of Cancer Stem Cell Biology, University of Colorado School of Medicine, 12800 East 19th Avenue, Mail stop 8104, Aurora, CO 80045. xj.wang@ucdenver.edu; Marileila Varella-Garcia, PhD, Professor, Department of Medicine, Division of Medical Oncology, University of Colorado School of Medicine, 12801 East 17th Avenue, Mail stop 8118, Aurora, CO 80045. Marileila.garcia@ucdenver.edu.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords

Aneuploidy; Chromosomal *SMAD4* deletion; genomic instability

1 | INTRODUCTION

Among all cancers worldwide, head and neck cancer is the sixth most common cancer type.[1,2] In the head and neck region, squamous cell carcinoma (HNSCC) represents over 90% of all head and neck cancers.[3] It is estimated that over 75% of HNSCCs are associated with chronic tobacco and alcohol use worldwide.[4] Human papillomavirus (HPV) associated HNSCC accounts for approximately 30-70% of all cases in developed countries.[5,6] SMAD4 protein is a tumor suppressor that regulates proliferation, apoptosis, and genomic stability.[7-9] *SMAD4* genetic loss was initially identified in 30-50% of pancreatic cancers[10-12] and colon cancers[13-15]; *SMAD4* loss does not play a role in initiation but promotes metastasis in pancreatic[11,16] and colon cancers.[17] In contrast, *SMAD4* loss is detected in early stage human HNSCC and initiates HNSCC development in mice.[8] We have shown that *SMAD4* null HNSCCs have extensive genomic instability, hyperproliferation, little apoptosis, and TGFβ- and SMAD3-dependent inflammation.[8] Given the significant functional impact of SMAD4 loss in HNSCC development, it is important to accurately determine the true frequency of SMAD4 loss in human HNSCC. Reported rates of SMAD4 loss in HNSCCs vary significantly. *SMAD4* point mutations occur in less than 5% of cases[18] and SMAD4 loss rates by IHC staining in HNSCC varies from 12% up to 67.8% SMAD4 protein loss[19-21]; however, antibody specificity remains unclear. In contrast, *SMAD4* loss of heterozygosity (LOH) is reported in ~30-50% of HNSCCs.[22-24] These variations make it difficult to define if SMAD4 loss affects clinical outcome. For example, reduced *SMAD4* expression in HNSCC is linked with poor clinical outcomes in one study,[20] but another study did not find such an association.[21] Recent TCGA data shows that, similar to what has been observed for other cancer types, large chromosome deletion of 18q is the most common cause of *SMAD4* loss in HNSCC.[25] Here, we present a technique alternative to sequencing for detecting chromosomal *SMAD4* loss at the single cell level in HNSCCs. Our study identified significant intra- and inter-tumor heterogeneity of *SMAD4* genetic loss that would be missed by genomewide sequencing of bulk tumors. Our study also provides an approach that can be developed into a Clinical Laboratory Improvement Amendments (CLIA)-certified assay to use for prognosis and personalized medicine.

2 | MATERIALS AND METHODS

2.1 | *SMAD4* FISH

A probe set was developed including genomic sequences from *SMAD4*, mapped at 18q21.2 (chr 18:48,478,290-48,634,496) labeled with SpectrumRed (SR2016-10-19) (Empire Genomics, Buffalo, NY), and centromere 18 (*CEN18*[26]) labeled with SpectrumGreen. Formalin-fixed, paraffin-embedded tissue sections were processed as previously reported.[27] Fluorescence microscopy with Z-stacking was used to survey the entire tumor section in three dimensions. *SMAD4* and *CEN18* FISH signals were counted in 50-100 nuclei from at least 4-5 tumor areas, each including 4-5 microscope fields under the 100× objective.

Quantification of these signals was averaged per specimen. In cell lines, quantification was performed in interphase and metaphase cells.

2.2 | *SMAD4* genomic status

The ratio of *SMAD4* signals to *CEN18* control signals was used to determine the tumor's *SMAD4* FISH status (*SMAD4/CEN18*) per the following criteria: gain: *SMAD4/CEN18* ratio ≥ 1.2; wildtype: *SMAD4/CEN18* ratio < 1.2 but ≥ 0.8; hemizygous: *SMAD4/CEN18* ratio < 0.8 > 0; homozygous loss: *SMAD4/CEN18* ratio = 0. These criteria were applied to primary HNSCCs ($n = 20$) and PDX tumors ($n = 46$). The prevalence of *SMAD4* FISH genotypes were compared to TCGA human HNSCC provisional sequencing data ($n = 496$). The TCGA HNSCC dataset was queried for wildtype, gained copy (GAIN), heterozygous loss (HET-LOSS), and homozygous deletion (HOMDEL) of *SMAD4* via cBioPortal. Copy numbers of *SMAD4* or *CEN18* were defined as follows: polysomy: ≥ 2.50; disomy: < 2.50 but ≥ 1.50; monosomy: < 1.50 but >0.50; nullisomy (complete loss): < 0.50. Copy number criteria were applied to cells and tumors. *SMAD4* and *CEN18* copy numbers were paired (*SMAD4.CEN18*) for polysomy analysis of cells and tumors.

2.3 | Specimens and cell lines

Primary HNSCC and PDX tumors were from consented patients at the University of Colorado Cancer Center and approval by the Colorado Multiple Institutional Review Board. Normal karyotype lymphoblastic cells (Coriell Institute for Medical Research, Camden, NJ) and HNSCC cell lines were grown in complete DMEM. HNSCC cell lines: CAL27 and FaDu were purchased from ATCC (Manassas, VA). PDX derived cell lines were generated at the University of Colorado as previously reported.[28] PDX derived cell lines were grown in RM_K Primary Cell Line Media comprised of 45% DMEM (ThermoFisher Scientific, Waltham, MA) #11965-092, 45% DMEM/F12 #11330-032 (ThermoFisher Scientific), 10% FBS, hEGF [10 ng/mL], transferrin [5 μg/mL], insulin [5 μg/mL], and hydrocortisone [400 ng/mL] sterile filtered (0.22 μm). Normal Oral Keratinocytes were grown in Defined Keratinocytes–SFM 10744019 (ThermoFisher Scientific) supplemented with EGF [2 ng/mL].

2.4 | qRT-PCR

At 90% confluence, cells were lifted with 0.4% TrypLE Select (ThermoFisher Scientific, #12563029). Trypsin was neutralized with RM_k media, and cells were spun down at 800 rpm for 5 min at 4°C. RNeasy Plus Mini Kit (Qiagen, Hilden, Germany, # 74136) was used to extract RNA from the cells. Total RNA was used to detect *SMAD4* expression with TaqMan probes (ThermoFisher Scientific) for *SMAD4* (FAM: Hs00232068_m1) and *GAPDH* (VIC: 4326317E). The Brilliant II QRT-PCR 1-step Master Mix kit (Agilent, Santa Clara, CA, #600809) was used for each 50 ng reaction of RNA on a 96 well plate (BioRad, Hercules, CA, #2239441). Ct values were used to determine relative fold change by $2^{-\Delta\Delta Ct}$.

2.5 | Statistical analysis

Statistical analyses were carried out using Graphpad software (PRISM 7). *SMAD4* and *CEN18* copy number average and standard deviation were determined by quantification of

50-100 nuclei per specimen. Inter-specimen heterogeneity was inferred from the standard deviation. Mean standard error was applied for all error bars. Comparison between subpopulations of *SMAD4* and *CEN18* for statistical significance was carried out by one-way ANOVA. Comparison between patient derived cell lines for relative SMAD4 mRNA were carried out by student *t*-test. *: $P < 0.05$; ****: $P < 0.0001$.

3 | RESULTS

3.1 | Frequency of genomic loss of *SMAD4* in human HNSCC

A lymphoblastic cell line with a normal karyotype was used for validation of the *SMAD4* probe set (Figure 1A). We also validated the probe set using two HNSCC cell lines with known *SMAD4* deletion: CAL27 that lacks one *SMAD4* allele and possesses an additional nonsense point mutation in the remaining *SMAD4* allele (LOH), and FaDu with homozygous *SMAD4* deletion.[29] The FISH assay identified one *SMAD4* allele per cell in CAL27 and no *SMAD4* alleles in FaDu cells (Figure 1A), indicating that the *SMAD4* probe set was suitable for detecting genomic *SMAD4* deletions of alleles but not point mutations.

We performed *SMAD4* FISH assay on primary HNSCC specimens ($n = 20$, Figure 1B, Supplementary Table S1) and PDX tumors ($n = 46$, Figure 1C, Supplementary Table S2) derived from HNSCC patients. *SMAD4* hemizygous deletion was found in 35.0% of primary tumors (Figure 1D) and in 41.3% of PDX tumors. Homozygous *SMAD4* loss was found in 4.3% PDX tumors. The frequency of *SMAD4* loss was similar to what was found by a TCGA query (Figure 1D) where either heterozygous (47.6%) or homozygous (4.0%) deletion of *SMAD4* occurred (ie, 51.6% in combination).[30,31] *SMAD4* was gained (>2 copies) in one primary HNSCC (5%), a frequency consistent with the *SMAD4* gain (6%) found by TCGA (Figure 1D). Together, these analyses show a prevalence of genomic *SMAD4* loss in HNSCCs.

We further examined copy numbers of *SMAD4* and *CEN18* by FISH on all primary HNSCCs (Supplementary Table S3) and PDX tumors (Supplementary Table S4). By considering both *SMAD4* and *CEN18* copy numbers per tumor to assess for *SMAD4* loss, we identified seven different *SMAD4/CEN18* patterns (Figure 1E). In total, *CEN18* polysomy occurred in 45% of primary HNSCCs and 37% of PDX tumors (Figure 1E). In contrast, *SMAD4* polysomy occurred at nearly half of the frequency, that is, 25% of primary HNSCCs and 10.9% of PDX tumors (Figure 1E), suggesting that some of these tumors have lost at least one copy of *SMAD4*. Indeed, cases with the ratio of *SMAD4/CEN18* < 0.8 occurred in 35% of primary HNSCCs and 45.7% of PDX tumors (Figure 1E). One PDX tumor lost one copy of both *SMAD4* and *CEN18* (monosomic loss, Figure 1E). The unbalanced increase in *CEN18* copy number (reflecting increased chromosomal copies) is potentially due to chromosomal instability caused by *SMAD4* loss.[8] Therefore, individual cells must be assessed for copy number of chromosome 18 to determine the actual copy loss of *SMAD4*.

3.2 | Inter- and intra-tumor heterogeneity of chromosome 18 and *SMAD4* in HNSCC

To further examine inter-cellular and inter-tumoral heterogeneity of *SMAD4* genotypes, we compared cellular *SMAD4/CEN18* ratios within and across tumors (Figure 2A). Among 10 different *SMAD4/CEN18* ratios in primary HNSCCs (Figure 2B) and 13 different *SMAD4/CEN18* ratios in PDX tumors (Figure 2C), we measured the subpopulation size (%) of each *SMAD4/CEN18* cell population within each tumor. It was difficult to determine a dominant subpopulation due to high heterogeneity within and across all primary HNSCCs and PDX tumors (Figures 2B and 2C). Therefore, we compared subpopulation size (%) of *SMAD4* or *CEN18* per primary HNSCC (Figure 3A) and PDX tumor (Figure 3B). In both primary HNSCCs (Figure 3C) and PDX tumors (Figure 3D), *SMAD4* loss subpopulations were significantly more abundant than *CEN18* loss subpopulations. In contrast, *SMAD4* polysomic subpopulations were smaller than *CEN18* polysomic subpopulations in primary HNSCC (Figure 3C) and PDX tumors (Figure 3D). There was no difference between *SMAD4* disomic and *CEN18* disomic subpopulations (Figures 3C and 3D). Overall, *SMAD4* loss correlated with *CEN18* polysomy (Figure 3) suggesting that genomic instability associated with *SMAD4* loss[8] leads to increased accumulation of centromere 18 in primary HNSCCs and PDX tumors.

3.3 | *SMAD4* loss is enriched in cancer cell lines derived from PDX with heterogeneous *SMAD4* loss

Five cell lines were established from PDX tumors (Figure 4). Three of the parental tumors (CUHN013, −036, and −065) had *SMAD4* deletions and two were *SMAD4* wild-type (CUHN-049 and −067) (Figure 4A). To determine if the *SMAD4* status of established cell lines faithfully replicates the *SMAD4* status of parental tumors, we performed *SMAD4* FISH on the derived cell lines. While CUHN-013, −036, and −065 cell lines were consistent with parental PDX tumors, CUHN-049 and −067 cell lines drifted from wild-type *SMAD4* in parental PDXs to *SMAD4* hemizygous deletions (Figure 4B), presumably due to enrichment from a small population of tumor cells with hemizygous *SMAD4* loss in CUHN-049 and −067 PDX tumors (Figure 4A). We assessed the mitotic spread of cells to determine ploidy status (Figure 4). All five cell lines had aneuploidy with an average chromosome count of 59.4 per cell line (Figure 4B). These cell lines had an average *SMAD4/CEN18* ratio of 0.44 with *SMAD4* loss relative to polyploidy *CEN18*. There was no variance (standard deviation = 0) of *SMAD4* in four out of five cell lines, suggesting a homogeneous cell population. One cell line (049C) had a standard deviation of 0.28 for *SMAD4* variants, suggesting that cells with heterozygous *SMAD4* loss largely outcompeted cells with wild-type *SMAD4* from the parental tumor. Loss of *SMAD4* expression, partially, or completely, in these lines was confirmed at the mRNA level compared to normal oral keratinocytes (Figure 4C). These data suggest that *SMAD4* loss by genomic deletion and reduced expression may enable these cells to permanently establish themselves in vitro.

4 | DISCUSSION

Loss of *SMAD4* is sufficient to initiate HNSCC in a mouse model[8]; however, reports regarding rates of *SMAD4* loss vary significantly.[19-21] In this report, we used a FISH assay to determine genomic *SMAD4* status in primary and PDX HNSCCs. We found that *SMAD4*

loss primarily occurred by heterozygous deletion in primary and PDX HNSCCs, similar to TCGA HNSCC sequencing data. Therefore, *SMAD4* FISH would provide a reliable but more rapid and cost-effective method for detecting *SMAD4* genomic loss at the single cell level compared with genomewide sequencing. Given the significant intratumor heterogeneities of *SMAD4* loss in HNSCCs identified from the current study, it would be difficult to determine if *SMAD4* heterozygous deletion correlates with HNSCC patient prognosis in either untreated patients or patients with the same treatment regimen(s). However, studies from mouse models have revealed that heterozygous *SMAD4* loss is sufficient to increase SCC susceptibility,[8,32] and that a small population of heterozygous *SMAD4* HNSCC cells were able to outcompete with wild-type *SMAD4* cells within the same tumor (Figure 4). Therefore, it is important to monitor HNSCCs with *SMAD4* heterozygous loss. Further, the remaining *SMAD4* allele in heterozygous *SMAD4* HNSCCs can be lost through LOH,[22-24] mRNA loss and protein loss.[8]

Although ploidy in tissue samples could not be assessed by either sequencing or FISH without mitotic spreads, we were able to assess *SMAD4* and *CEN18* copy numbers in our FISH assay. Copy number assessment revealed inter-tumor heterogeneity as noted by seven different *SMAD4/CEN18* copy number variants across all HNSCC specimens examined. We also found that tumors gained genetic copies of centromere 18 more frequently than tumors gained genetic copies of *SMAD4*, this may be explained by *SMAD4* loss-associated genomic instability and subsequent accumulation of centromeres as previously described.[8] Genomic instability can also increase tumor heterogeneity.[33]

Intra-tumor *SMAD4/CEN18* heterogeneity was also common with 6.27 ± 1.97 different copy number variants within the same tumor although nuclear truncation may account for some variation in tissue samples. As expected, no tumor was homogeneous across all 66 specimens. It was difficult to compare *SMAD4/CEN18* copy number variant populations due to high intra-tumor heterogeneity. By separating the *SMAD4* copy number variant populations from *CEN18* copy number variant populations per tumor, it is apparent that most tumors had lost one or more copies of *SMAD4*, but gained copies of *CEN18*. On average, the polysomy *CEN18* cell population was significantly larger than the polysomy *SMAD4* cell population. In contrast, the cell population with *SMAD4* loss was significantly larger than the cell population with *CEN18* loss, this further supported *SMAD4*-associated genomic instability resulting in accumulation of centromeres.

The use of PDX-derived cell lines allowed us to confirm that *CEN18* copy numbers are consistent with chromosomal numbers in chromosomal spreads, hence future molecular diagnosis could use *CEN18* as a surrogate marker for ploidy status to more accurately evaluate *SMAD4* copy number loss by FISH. Intriguingly, all five cell lines had partial or complete *SMAD4* loss suggesting a survival advantage for *SMAD4* mutant tumor cells. This may explain why *SMAD4* mutant HNSCCs are resistant to therapies primarily targeting tumor growth and survival.[34,35] Conversely, *SMAD4* mutant tumor cells are shown to be sensitive to certain DNA damaging agents.[3,36] Therefore, the *SMAD4* FISH analysis described here will provide a simple and cost-effective molecular diagnosis for solid tumors to readily determine genomic *SMAD4* deletion status and future clinical studies will

determine if partial or complete loss of genomic *SMAD4* serves as a biomarker for prognosis and therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CUHN	University of Colorado Head and Neck
FISH	fluorescence in situ hybridization
HNSCC(s)	head and neck squamous cell carcinoma(s)
HPV	human papillomavirus
LOH	loss of heterozygosity
PDX	patient derived xenograft
TCGA	The Cancer Genome Atlas

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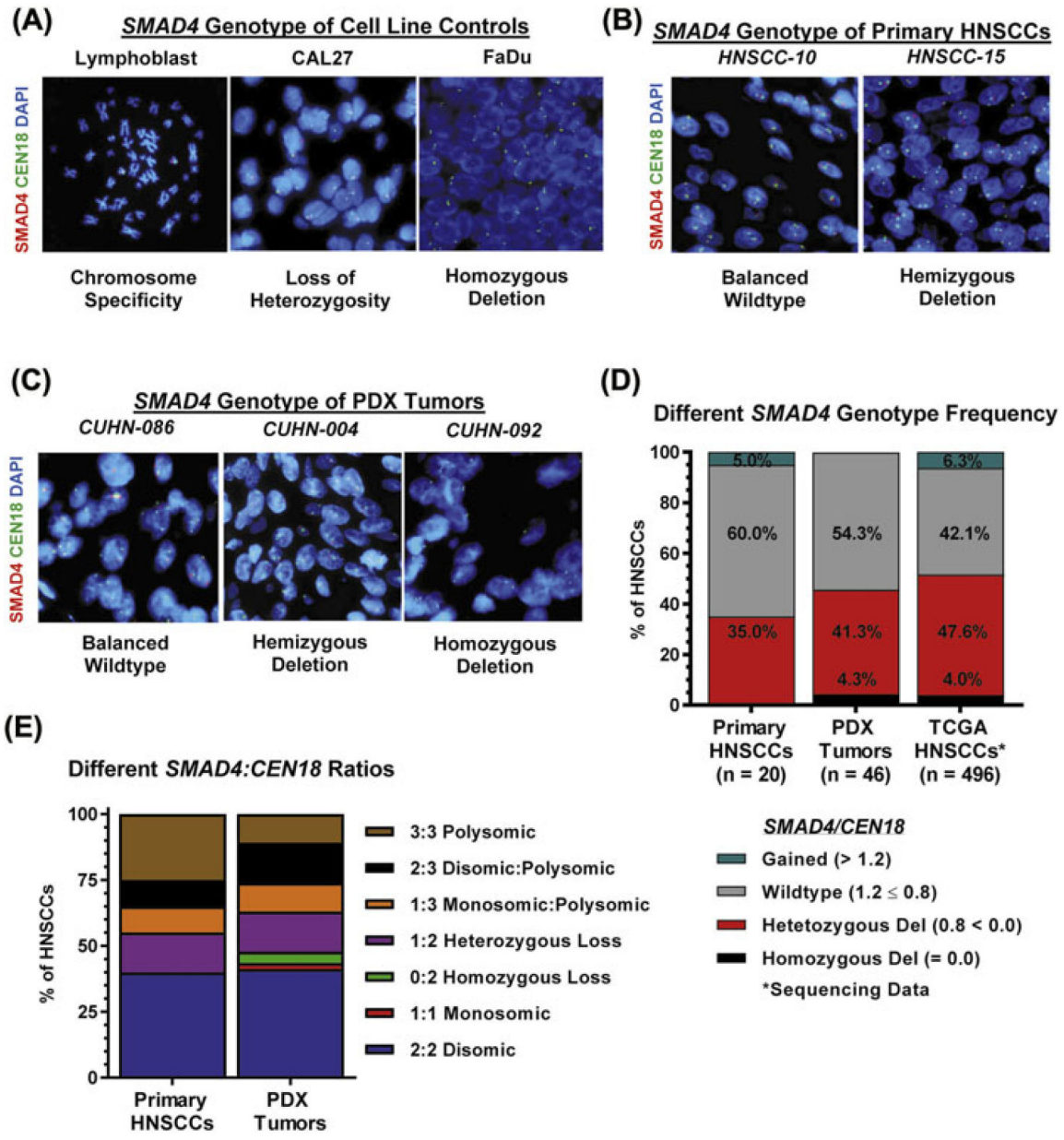

FIGURE 1.

Genomic *SMAD4* status in HNSCCs. *SMAD4* FISH of (A) lymphoblastoid cells with normal karyotype, CAL27 HNSCC with loss of heterozygosity by single point nonsense mutation to show one copy of genomic *SMAD4*, and FaDu HNSCCs with homozygous deletion of *SMAD4*. B) Representative images of primary HNSCCs designated *SMAD4* balanced-wildtype (HNSCC-10) and hemizygous deletion (HNSCC-15) by *SMAD4* FISH. C) Representative images of PDX tumors designated *SMAD4* balanced-wildtype (CUHN-086), hemizygous deletion (−004), and homozygous deletion (−092) by *SMAD4* FISH. D) *SMAD4* FISH: Prevalence of SMAD4 genotypes across primary HNSCC ($n = 20$) and PDX tumors ($n = 46$) compared to TCGA HNSCCs ($n = 496$) sequencing data. *SMAD4* loss includes hemizygous/heterozygous loss and homozygous loss. E) Frequency of different

SMAD4/CEN18 ratios across primary HNSCCs and PDX tumors. [Color figure can be viewed at wileyonlinelibrary.com]

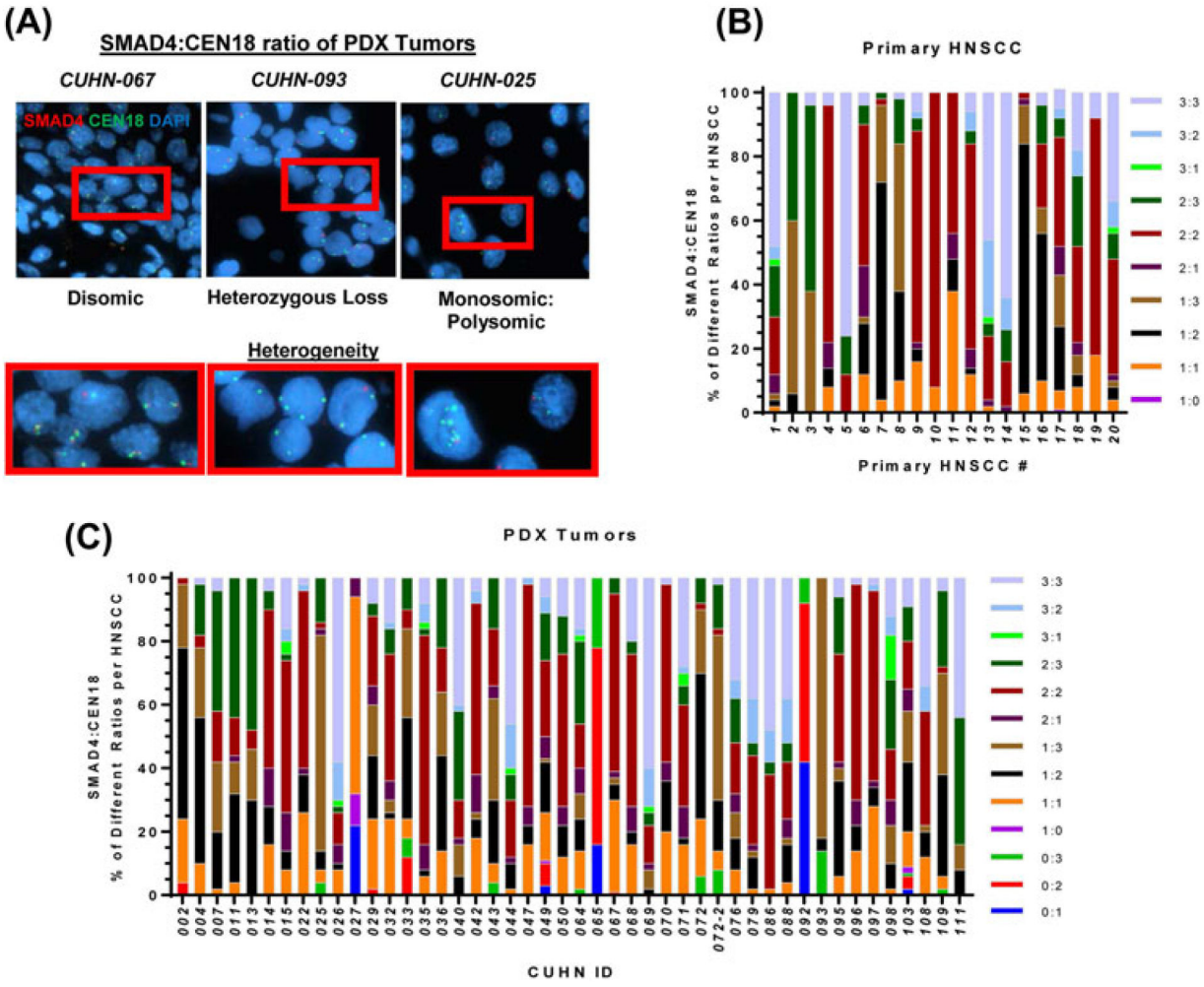

FIGURE 2.

Intra- and Inter-tumor heterogeneity of genomic *SMAD4* and centromere 18. A) Representative *SMAD4* FISH images of CUHN-067, −093, and −025 from left to right showing heterogeneity. Red boxes are magnified images. Subpopulations were defined by variations in copy numbers for *SMAD4* and *CEN18*, to identify 10 unique subpopulations across all 20 primary HNSCCs (B) and 13 unique subpopulations across all 46 PDX tumors (C). The *SMAD4:CEN18* subpopulations were organized by size (%) within each tumor specimen. [Color figure can be viewed at wileyonlinelibrary.com]

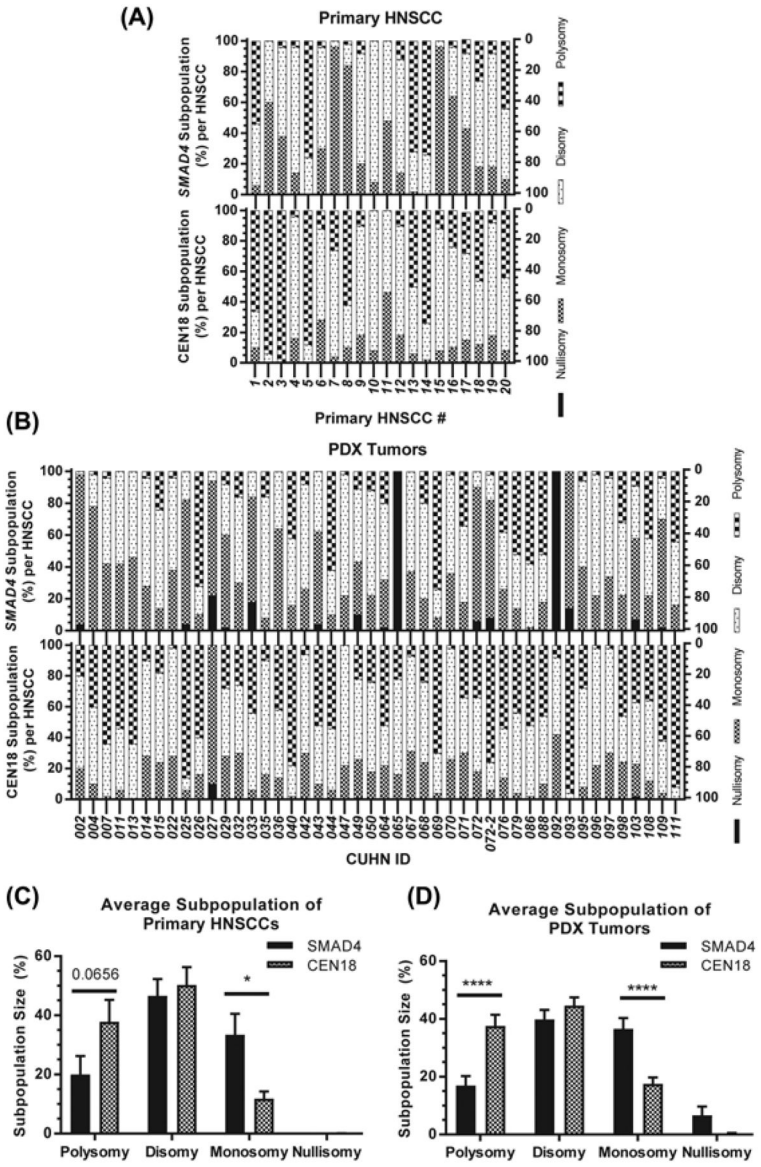

FIGURE 3.

Intra- and Inter-tumor heterogeneity *SMAD4* and *CEN18* copy numbers. *SMAD4* (top) and CEN18 (bottom) subpopulation size (%) per primary ($n = 20$) HNSCC (A) and PDX ($n = 46$) tumor (B). Comparison of *SMAD4* and *CEN18* subpopulation size (%) averaged across all 20 primary HNSCCs (B) and all 46 PDX tumors (D). Standard error was used for error bars and one-way ANOVA was conducted for statistical analysis and *P*-value was determined. * < 0.05, **** < 0.0001, absent = non-significant difference

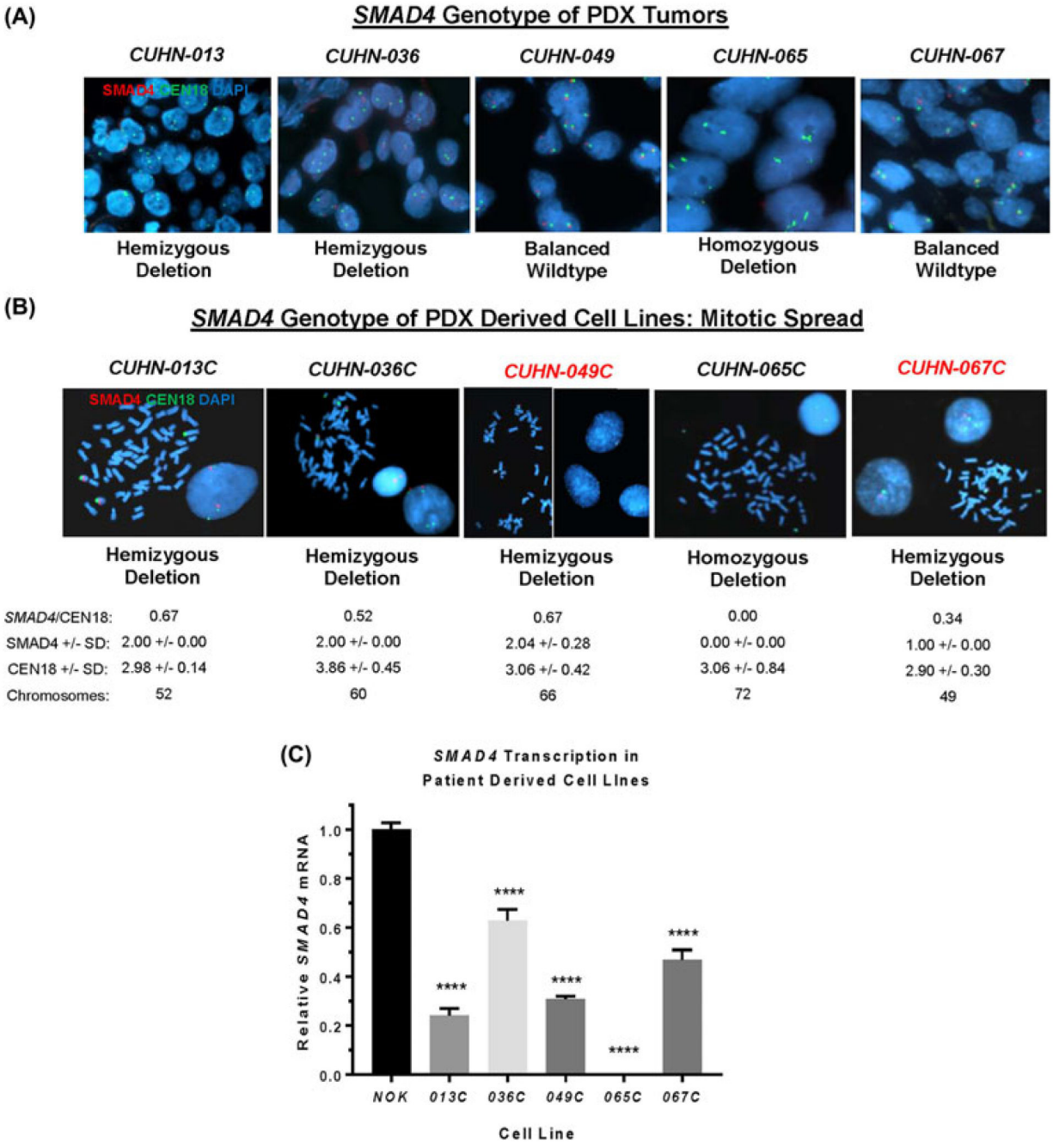

FIGURE 4.

Loss of genomic *SMAD4* is enriched in established HNSCC cell lines. A) Representative images of each parental PDX tumor and *SMAD4* genotype by FISH. B) Representative images of PDX derived cell lines in mitotic spread and *SMAD4* genotype by FISH. *SMAD4/CEN18* ratio, *SMAD4* copy number +/− standard deviation, *CEN18* copy number ± standard deviation, and chromosome count per cell line. C) qRT-PCR: *SMAD4* transcription relative to *GAPDH* per cell line compared to normal oral keratinocytes. Standard *t*-test: **** = *P*-value <0.0001. [Color figure can be viewed at wileyonlinelibrary.com]