Full Materials and Methods

RNA In Situ Hybridization

FGFR1, FGFR2, FGFR3 RNA in situ hybridization (ISH) expression was assessed in 107 patients from the UW Head and Neck SPORE oropharynx tissue microarray treated between 1989 and 2017 and in a panel of 22 HNSCC PDX samples using the RNAscope assay (Advanced Cell Diagnostics Inc., Hayward, CA). 5 µm formalin-fixed, paraffin-embedded tissue sections arranged in a tumor microarray were probed using the 2.5 HD-Brown kit (Advanced Cell Diagnostics) according to manufacturer instructions. Sections were counterstained with hematoxylin, dehydrated, and coverslipped with Cytoseal XYL (Thermo Fisher Scientific). The intensity of RNA ISH stains was scored by a board-certified head and neck pathologist with a 4-tier system (0 = absent, 1 & 2 = low expression and 3 & 4 = high expression). Scores were averaged for triplicates cores.

p16 immunohistochemistry

The expression of p16 (BD Pharmingen, #550834) was detected in PDX histologic sections by standard immunohistochemistry (IHC). Briefly, sections were deparaffinized, incubated in Bouins solution for 1 hour at 60ºC, washed in running tap water, and the nuclei stained with Weigert's iron hematoxylin for 15 minutes. Next, the slides were placed in Gomori's trichrome stain and incubated 20 minutes at room temperature. Finally, the tumor sections were rinsed with water, dehydrated, cleared, and cover slipped.

Cell lines and drug

Head and neck cancer cell lines (HPV-positive: UD-SCC-2, UM-SCC-47, UPCI-SCC-90, 93-VU-147T and HPV-negative: UM-SCC-6, SCC-9, UM-SCC22B, MDA-1483, CCL-30, TU-138) were obtained from the UW Head and Neck SPORE cell line repository and cultured at low passage in standard conditions. The identity of all cell lines was confirmed via short tandem repeat testing as listed in Supplemental Table 1. AZD4547, provided by AstraZeneca, was reconstituted in dimethyl sulfoxide (DMSO) and stored at −70 ºC for in vitro experiments.

Immunoblot analysis

Tissue and cells were harvested and washed with PBS before freezing at -80°C. Cells were lysed with RIPA buffer supplemented 1% (v/v) with phosphatase/protease inhibitor cocktail (Cell

Signaling Technologies, #5872) and sonicated. Equal amounts of protein were analyzed by SDS-PAGE, transferred to polyvinylidene diflouride membranes, and probed with specific primary antibodies overnight at 4°C. Targets were detected with NIR-conjugated anti-mouse and anti-rabbit secondary antibodies (LiCOR) and imaged on a LiCOR Odyssey FC. Specific antibodies and sources are listed in Supplementary Table 2.

Quantitative real time-polymerase chain reaction (qRT-PCR)

RNA was extracted from cultured cells using Allprep DNA/RNA Mini Kit (Qiagen, Valencia, CA) and measured by Nanodrop. First strand cDNA synthesis was performed using the SensiFAST kit (Bioline). Pre-designed TaqMan gene specific primers (Life Technologies, Carlsbad, CA) were used: FGFR1 (forward: 5'-ACACCTTACACATGAACTCCAC-3', reverse: 5'-AGCATCAACCACACATACCAG-3'), FGFR2 (forward: 5'-GCTTGTCTTTGTCAATTCCCA-3', reverse: 5'-GTCTCCGAGTATGAACTTCCAG), FGFR3 (forward: 5'-CCAGCAGCTTCTTGTCCATC-3', reverse: 5'-GTACTGTGCCACTTCAGTGT-3'). qPCR reactions were set up on an automated robot platform (Gilson, Inc Middleton, WI) and PIPETMAX qPCR assistant. The Gilson lab wizard was programmed to set up all qPCR reactions. Relative mRNA levels were quantified by RT-qPCR (Bio-Rad, S1000 Thermal cycler) and normalized to human tonsil epithelial cells (HTE). All reactions were performed in triplicate from RNA isolated from three independent biological experiments.

DNA-copy number by qPCR

DNA was isolated from cultured cells using DNeasy Mini Kit (Qiagen) and quantified by Nanodrop to 5 µg per sample. FGFR1 and FGFR2 copy number variation was determined by the TaqMan® Copy Number Assay (Life Technologies #Hs04933308_cn). RT-PCR was performed using the 7500 Fast Real-Time PCR System (Life Technologies). Samples were normalized to a positive control, mRNAseP, known to have two copies. All reactions were performed in quadruplicate from DNA isolated from three independent biological experiments.

Proliferation assay

Cells were plated in 96-well plates at densities ranging from 2,000 to 10,000 cells/well according to cell type growth rate. Twenty-four hours post-plating, cells were treated with indicated doses of AZD4547 control and incubated for 48-72 hours. Once control wells neared full confluence, Cell Counting Kit-8 (CCK8) reagent was added (Dojindo Molecular Technologies) and absorbance measured at 450 nm on a SpectraMax i3 plate reader (Molecular Devices). The absorbance of

treated wells was normalized to control wells and the half-maximal inhibitory concentration (IC50) values calculated.

Irradiation

Cells were irradiated with an Xstrahl X-ray System, Model RS225 (Xstrahl, UK) at a dose rate of 3.27 Gy/minute at 30 cm FSD, tube voltage of 195 kV, current of 10 mA and filtration with 3 mm Al. Animals were irradiated with an X-RAD320 (Precision X-Ray, North Branford, CT) with 1 Gy/minute delivered at 320 kV/12.5 mA at 50 cm FSD with a beam hardening filter with half-value layer of 4 mm Cu. The delivered dose rate was confirmed by ionization chamber. Mice were shielded with custom-built lead jigs to limit radiation exposure to the rear quarter of the body.

Clonogenic Survival Assay

Cells were seeded into 6-well plates at specific densities, incubated overnight, and treated with radiation after 1 hour of AZD4547 treatment and media refreshed 23 hours later. Once colonies averaged 50 or more cells (14-21 days) in the control wells, plates were fixed and stained with 1% (w/v) crystal violet in methanol, imaged, and colonies of 50 or more cells were counted. Survival curves were generated after normalizing for the amount of AZD4547-induced cell death. Clonogenic survival curves for each condition were fitted to a linear quadratic model ($Y = e-[A * X + B * X^2]$) according to a least squares fit, weighted to minimize the relative distances squared, and compared using the extra sum-of-squares F test. Radiation dose enhancement factors (DMF) were calculated at 10% survival levels by dividing the mean radiation dose for control conditions by the mean radiation dose after drug exposure. A value > 1.0 indicates enhancement of radiosensitivity.

Apoptotic cell death

Cells were seeded into 6-well plates at defined densities, incubated overnight, exposed to DMSO or 0.1 μM of AZD4547, and irradiated with 3 Gy one hour later. Treatment media was refreshed with drug-free medium the next day. 72 hours post-treatment, cells were trypsinized, washed twice with cold PBS, and stained with FITC Annexin V and propidium iodide according to manufacturer instructions (BD Biosciences #556547). Staining was detected with the Countess II FL Automated Cell Counter (Thermo Fisher Scientific) utilizing GFP and RFP EVOS Light Cubes. Cells stained with Annexin V were quantified and plotted.

Autophagy

Cells were plated, incubated overnight, and treated with DMSO or 0.1 μM AZD4547 1 hour before irradiation with 3 Gy. Drug-free medium was added the next day. Seventy-two hours post-treatment, cells were washed with PBS and stained using acridine orange (Sigma-Aldrich #318337) at a concentration of 1 μg/mL in PBS for 15 minutes at 37°C. After staining, cells were washed with PBS, trypsinized, and harvested to create a single cell suspension. Staining was detected with the Countess II FL Automated Cell Counter utilizing GFP and RFP EVOS Light Cubes. Acidic autophagic vesicles were stained bright orange/red, which contrasts with green stained nuclei and dim red stained cytoplasm. An RFP channel intensity threshold was established for each cell line using cells grown in serum (negative control) and serum-free conditions (positive control) in order to differentiate autophagic activity from cytoplasmic staining. Cells meeting or exceeding this intensity threshold were quantified and presented as a percentage of total detected cells in the GFP channel.

γH2AX immunofluorescence

Cells were plated in 8-well chamber slides at densities ranging from 30,000 to 40,000 cells/well depending on cell type size and growth rate. Twenty-four hours post-plating cells were treated with 0.1 μM AZD4547 or DMSO control, incubated for 1 hour, irradiated with 3 Gy and fixed with 70% methanol in PBS at indicated time points. Cells were permeabilized with 0.1% Triton-X 100 in TBST for 15 minutes, blocked with goat serum for 1 hour at 25°C, and incubated with anti-phospho-H2AX primary antibody overnight at 4°C (Supplementary Table 2). Cells were then probed with Alexa Fluor 555 conjugated secondary antibody (CST #4413) for 1 hour at 25°C in the dark, and cover-slipped with Fluoromount containing DAPI. The next day, they were imaged at 60x magnification using a Nikon A1RS inverted point scanning confocal microscope system. γH2AX foci were quantified using ImageJ. The number of cells were first counted using the DAPI-only image, and the foci were identified with the Analyze Particle function after optimizing the threshold for each image. Total foci per image was divided by the total cell count to calculate the average foci/cell. At least three images per treatment condition was analyzed.

Cell cycle

Cells were plated in 6-well plates at densities ranging from 200,000-300,000 cells/well depending on the cell type and growth rate. Twenty-four hours post-plating, cells were treated with 0.1 μM AZD4547 or DMSO control, incubated for 1 hour, irradiated with 3 Gy, and trypsinized and collected at indicated time points. Cells were centrifuged at 500 x g for 10 minutes, washed with PBS, pelleted again, and a single cell suspension was created in 0.5 ml PBS. 4.5 ml chilled

70% methanol was added to each tube and cells were stored in 4°C. Twenty-four hours later, cells were centrifuged at 500 x g for 10 minutes, and a single cells suspension was created in 1 ml solution of propidium iodide (Molecular probes # P3566), Triton X-100 (0.1%) (Sigma #T9284), and RNase A (Thermo Scientific #EN0531) in PBS. The samples were analyzed using the Attune NxT flow cytometer (Thermo Scientific), and the cell cycle distribution was calculated using ModFIT software (Verity Software House, Top-sham, ME).

Reverse-phase protein array

TU-138 and UM-SCC-6 cells were treated with DMSO or 0.1 μM for 24 hours. After treatment cells were lysed using lysis buffer (1%Triton X-100, 50 mmol/L HEPES pH 7.4, 150 nmol/L NaCl, 1.5 mmol/LMgCl2, 1 mmol/L EGTA, 100 mmol/L sodium pyrophosphate, 1 mmol/L Na3VO4, 10% glycerol, phosphatase and protease inhibitors) and centrifuged at 4ºC for 10 minutes at 12,700 x g. The lysate was mixed with 4ºC SDS sample buffer (40% glycerol, 8% SDS, 0.25 mol/L Tris-HCl pH 6.8), boiled for 5 minutes, and stored at -80ºC. Samples were sent to the Functional Proteomics Core Facility at MD Anderson for reverse-phase protein array (RPPA) analysis. In brief, cell lysate samples were serially diluted two-fold for 5 dilutions (undiluted, 1:2, 1:4, 1:8; 1:16) and arrayed on nitrocellulose-coated slides in an 11x11 format to produce sample spots. Sample spots were then probed with antibodies by a tyramide-based signal amplification approach and visualized by DAB colorimetric reaction to produce stained slides. Stained slides were scanned on a Huron 5. Relative protein levels for each sample were determined by interpolating each dilution curve produced from the densities of the 5-dilution sample spots using a "standard curve" (SuperCurve) for each slide (antibody). SuperCurve is constructed by a script in R, written by MD Anderson Bioinformatics. The relative protein level data points were normalized for protein loading and transformed to linear values, then transformed to log2, and then median-centered. The R package limma was used to determine the differentially expressed proteins.[13] The R package limma was used to determine the differentially expressed proteins.13 Gene Set Enrichment Analysis (GSEA)[14] was performed using the Molecule Signatures Database v6.2 (MSigDB) on the RPPA data by mapping the gene symbols to the RPPA probe identifiers and discarding those genes with no corresponding probe (custom perl scripts)[15].

Cell line xenograft and PDX growth delay studies

Six to eight-week-old male and female NOD-SCID gamma (NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}*/SzJ*) mice (Jackson Laboratories) were used for PDX revival and tissue amplification;

six to eight-week old female Hsd:athymic Nude-*Foxn1*^{nu} (Harlan Laboratories) mice were used for growth delay studies. Mice were kept in the Association for Assessment and Accreditation of Laboratory Animal Care-approved XXX Institute for Medical Research Animal Care Facility. Studies were carried out in accordance with an animal protocol approved by the University of XXX.

Cryopreserved UW-SCC-36 PDX tissue, previously established from an HPV-positive oropharynx human tumor[12], was reanimated and expanded in NSG mice, harvested and mixed 1:1 in Matrigel (BD Biosciences), and subcutaneously injected into bilateral flanks of female nude mice. TU-138 cells were grown *in vitro*, mixed 1:1 with Matrigel, and injected subcutaneously into bilateral flanks of nude mice at 1 x 10^6 cells/site. Tumor volumes were measured twice weekly with Vernier calipers and calculated according to the relationship $V = \left(\frac{\pi}{6}\right) x\ (large\ diameter)\ x\ (small\ diamter)^{\ 2}$. Once average tumor size reached ~200 mm^3, mice were randomized into treatment groups. Vehicle (1% (v/v) Tween80 in saline) or AZD4547 was administered once daily by oral gavage. Radiation was administered 1 hour after AZD4547. Eight to 10 mice (16-20 tumors) were used per treatment group.

Statistical analyses

Patient characteristics were analyzed using Chi-squared test for categorical variables and Mann–Whitney U test for comparison of medians. Univariate analyses for overall survival (OS) and disease free survival (DFS) were performed with the Cox regression model. Both OS and DFS were calculated from time of diagnosis. DFS was defined as any local, regional, distant recurrences or any death. *In vitro* experiments were repeated three times and statistical analysis was carried out using a Student's t-test. Data are presented as the mean ± standard error of the mean (SEM). Xenograft growth curves were fitted with an exponential growth equation and were compared using the extra sum of squares F-test. A probability level of a p value of <0.05 was considered significant. Statistical analyses were performed with GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA). and IBM SPSS Statistics for Windows, Version 25 (IBM Corp., Armonk, NY).

Supplementary Table 1. Short Tandem Repeat analysis of cell lines used in this study

Cell Line	Source	Passage sent	Date sent	# of allelic match	% allelic match
UD-SCC-2	ATCC	P8	10/5/2018	21/21	100
UM-SCC-47	ATCC	P4	5/12/2017	14/14	100
UPCI-SCC-90	ATCC	P3	7/6/2017	23/23	100
93-VU-147T	ATCC	P14	5/12/2017	25/25	100
UM-SCC-6	ATCC	P4	7/6/2017	24/25	98
SCC-9	ATCC	P4	7/21/2017	21/21	100
UM-SCC22B	ATCC	P1	7/6/2017	20/20	100
MDA-1483	ATCC	P14	5/12/2017	14/14	100
CCL30 (RPMI-2650)	ATCC	P6	10/5/2018	29/29	100
TU-138	ATCC	P16	6/16/2017	12/12	100
HTE	Klingelhutz at University of Iowa	P26	8/30/2017	See attached	

Supplementary Table 2. Antibodies used in this study

Antibody	Source	Company	Catalog no.	Dilutions
AKT	Mouse	Cell Signaling Technology	2920	1:2000
α-Tublin	Mouse	Calbiochem	CP06	1:5000
FGFR1	Rabbit	Cell Signaling Technology	9740	1:1000
Cleaved Caspase-3 (Asp175)	Rabbit	Cell Signaling Technology	9661	1:200
FGFR2	Rabbit	Cell Signaling Technology	11835	1:1000
FGFR3	Rabbit	Cell Signaling Technology	4574	1:1000
GAPDH	Mouse	Cell Signaling Technology	97166	1:10000
phospho-AKT (Ser473)	Rabbit	Cell Signaling Technology	4060	1:1000
phospho-Histone H2AX (Ser139) (20E3)	Rabbit	Cell Signaling Technology	9718	*In vitro* 1:1000 *In vivo*: 1:400
phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit	Cell Signaling Technology	4370	In vitro: 1:1000 *In vivo:* 1:200
phospho-p44/42 MAPK (Erk1/2)	Mouse	Cell Signaling Technology	4696	1:5000
phospho-S6 Ribosomal Protein (Ser235/236)	Rabbit	Cell Signaling Technology	4858	1:400

Supplemental Table 3. Patient Characteristics by FGFR1 mRNA expression

Patient Characteristic	No Expression	Low expression	High expression	*P* value
No. of patients (%)	63 (58.9%)	38 (35.5%)	6 (5.6%)	-
Age (y)				0.22
Median (range)	59 (44-80)	59 (37-83)	62 (55-70)	
Sex				0.70
Male	50 (79.4%)	31 (81.6%)	4 (66.7%)	
Female	13 (20.6%)	7 (18.4%)	2 (33.3%)	
Primary tumor Site				0.49
Base of Tongue	33 (52.4%)	21 (55.3%)	2 (33.3%)	
Tonsil	23 (36.5%)	16 (42.1%)	3 (50.0%)	
Oropharynx	7 (11.1%)	1 (2.6%)	1 (16.7%)	
Other				
p16				0.46
negative	21 (33.3%)	9 (23.7%)	1 (16.7%)	
positive	42 (66.7%)	29 (76.3%)	5 (83.3%)	
T classification				0.35
T1	30 (15.9%)	16 (42.1%)	1 (16.7%)	
T2	14 (22.2%)	15 (39.5%)	4 (66.7%)	
T3	8 (12.7%)	4 (10.5%)	1 (16.7%)	
T4	8 (12.7%)	3 (7.9%)	0 (0%)	
unknown	3 (4.8%)	0 (0%)	0 (0%)	
N classification				0.86
N0	12 (19.1%)	4 (10.5%)	1 (16.7%)	
N1	5 (7.9%)	3 (7.9%)	0 (0%)	
N2	38 (60.3%)	29 (76.3%)	5 (83.3%)	
N3	5 (7.9%)	2 (5.3%)	0 (0%)	
unknown	3 (4.8%)	0 (0%)	0 (0%)	
Tobacco use				0.97
Yes	56 (89.0%)	27 (71.0%)	5 (83.3%)	
No	14 (22.2%)	9 (23.7%)	1 (16.7%)	
Unknown	3 (4.8%)	2 (5.3%)	0 (0%)	

Supplemental Table 4. Patient Characteristics by FGFR2 RNA expression

Patient Characteristic	No Expression	Low expression	High expression	*P* value
No. of patients (%)	20 (18.7%)	61 (57%)	26 (24.3%)	-
Age (y)				0.58
Median (range)	63 (48-78)	59 (37-88)	59 (47-73)	
Sex				0.30
Male	14 (70%)	48 (78.7%)	23 (88.5%)	
Female	6 (30%)	13 (21.3%)	3 (11.5%)	
Primary tumor Site				0.97
Base of Tongue	11 (55.0%)	31(50.8%)	14 (53.8%)	
Tonsil	7 (35.0%)	25 (41.0%)	10 (38.5%)	
Oropharynx	2 (10.0%)	5 (8.2%)	2 (7.7%)	
Other				
p16				**0.010**
negative	9 (45.0%)	45 (73.8%)	22 (84.6%)	
positive	11 (55.0%)	16 (26.2%)	4 (15.4%)	
T classification				**0.012**
T1	3 (8.8%)	35 (57.4%)	9 (34.6%)	
T2	7 (35.0%)	13 (21.3%)	13 (50%)	
T3	4 (20.0%)	6 (9.8%)	3 (11.5%)	
T4	4 (20.0%)	6 (9.8%)	1 (3.8%)	
unknown	2 (10.0%)	1 (1.6%)	0 (0%)	
N classification				0.64
N0	4 (20%)	9 (14.8%)	4 (15.4%)	
N1	2 (10%)	5 (8.2%)	1 (3.9%)	
N2	10 (50%)	42 (68.9%)	20 (76.9%)	
N3	2 (10%)	4 (6.6%)	1 (3.9%)	
unknown	2 (10%)	1 (1.6%)	0 (0%)	
Tobacco use				0.23
Yes	17 (85%)	42 (68.9%)	19 (73.1%)	
No	1 (5%)	17 (27.9%)	6 (23.1%)	
Unknown	2 (10%)	2 (3.3%)	1 (3.9%)	

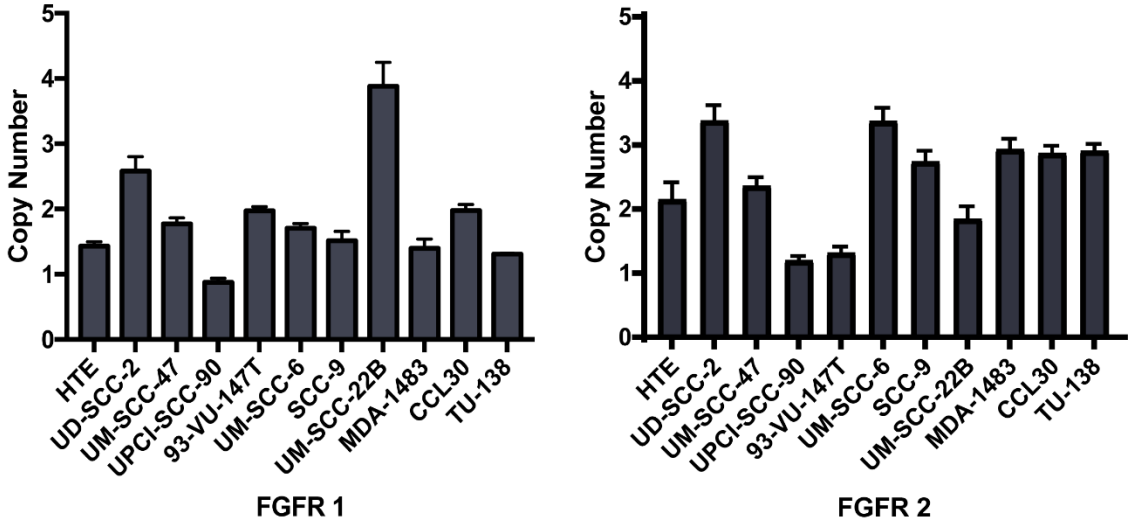
Supplemental Table 5. Patient Characteristics by FGFR3 RNA expression

Patient Characteristic	No Expression	Low expression	High expression	*P* value
No. of patients (%)	12 (11.2%)	48 (44.9%)	47 (43.9%)	-
Age (y)				0.40
Median (range)	64 (51-74)	59 (45-88)	58 (37-77)	
Sex				**0.017**
Male	6 (50%)	38 (79.2%)	41 (87.2%)	
Female	6 (50%)	10 (20.8%)	6 (12.8%)	
Primary tumor Site				0.15
Base of Tongue	4 (33.3%)	28 (8.3%)	24 (51.1%)	
Tonsil	5 (41.7%)	16 (33.3%)	21 (44.7%)	
Oropharynx	3 (25.0%)	4 (8.4%)	2 (4.3%)	
Other				
p16				**<0.001**
negative	4 (33.3%)	17 (35.4%)	6 (12.8%)	
positive	8 (66.7)	31 (64.6%)	41 (87.2%)	
T classification				**0.018**
T1	1 (8.3%)	21 (43.8%)	25 (53.2%)	
T2	4 (33.3%)	13 (27.1%)	16 (34.0%)	
T3	4 (33.3%)	7 (14.6%)	2 (4.3%)	
T4	3 (25.0%)	4 (8.3%)	4 (8.5%)	
unknown	0 (0%)	3 (6.3%)	0 (0%)	
N classification				0.06
N0	5 (41.7%)	8 (16.7%)	4 (8.5%)	
N1	1 (8.3%)	5 (10.4%)	2 (4.3%)	
N2	6 (50%)	28 (58.3%)	38 (80.9%)	
N3	0 (0%)	4 (8.3%)	3 (6.4%)	
unknown	0 (0%)	3 (6.3%)	0 (0%)	
Tobacco use				**0.002**
Yes	10 (83.3%)	40 (83.3%)	28 (59.6%)	
No	0 (0%)	6 (12.5%)	18(38.3%)	
Unknown	2 (16.7%)	2 (4.2%)	1 (2.1%)	

Supplemental Table 6. Univariate Analysis for overall survival and disease-free survival

	Overall Survival		Disease-Free Survival	
	HR (95% CI)	**P value**	**HR (95% CI)**	**P value**
FGFR1				
Negative	1.0	ref	1.0	ref
Low	1.18 (1.18-2.23)	0.62	1.17 (0.64-2.14)	0.60
High	1.42 (0.33-6.15)	0.64	1.04 (0.25-4.43)	0.96
FGFR2				
Negative	1.0		1.0	
Low	1.05 (0.54-2.03)	0.89	1.05 (0.55-2.00)	0.89
High	0.30 (0.083-1.10)	0.069	0.46 (0.16-1.33)	0.15
FGFR3				
Negative	1.0		1.0	
Low	0.96 (0.45-2.07)	0.92	1.01 (0.47-2.16)	0.99
High	0.58 (0.25-1.35	0.21	0.70 (0.31-1.59)	0.40
P16				
Negative	1.0		1.0	
positive	0.32 (0.18-0.55)	**<0.001**	0.29 (0.17-0.49)	**<0.001**

Supplemental Figure 1: FGFR1 and FGFR2 Copy Number



Supplemental Figure 2: Western blot downstream signaling analysis of AZD4547-treated HNSCC cell lines. UD-SCC-2 and MDA-1483 cells were treated for 10 minutes, 1 hour, 6 hours and 24 hours with 0.1 μM AZD4547.