Supplementary Methods and Data

Early tumor progression associated with enhanced EGFR signaling with bortezomib, cetuximab, and radiotherapy for head and neck cancer

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Methods

Serum Cytokine and Growth Factor Assays

Concentrations of serum cytokine and growth factors previously shown to be elevated in SCCHN patients and reduced in association with bortezomib, tumor response, and overall survival were determined as described previously.[18,26] Serum samples were prepared using the human extracellular protein buffer reagent kit and mixed bead kits containing IL-6, IL-8, and HGF (Invitrogen, Carslbad, CA), and analyzed using Luminex 100 instrument (Luminex, Austin, TX) according to the manufacturers' protocol. Serum cytokine concentrations for all time points from an individual patient were determined in the same assay to minimize inter-assay variability. Each of the standards and a blank were run in duplicate (patients #1, 2, 3, and 7) or triplicate (patients #4, 5, and 6), and patient serum samples were run in triplicate (patients #1, 2, 3, and 7) or quadruplicate (patients #4, 5, and 6). Lower limits of detection for each cytokine bead kit are as follows: IL-6, 3 pg/mL; IL-8, 3 pg/mL; HGF, 10 pg/mL. Serum levels of VEGF were

analyzed using an ELISA kit (Invitrogen, Cat. No. KHG0112), according to the manufacturer's protocol. The detection range of the kit is 5-1500 pg/mL. Each of the standards and a blank were run in duplicates and each patient serum sample was run in triplicate. For each patient, the log(10) of the concentration (+/-SD) is presented for comparison of the pre- and on-treatment timepoints, and potential relationship to treatment.

Immunoblotting of SCCHN tumor and cell line lysates

Tissue was immediately snap-frozen on dry ice and stored at -80 until use for Western immunoblot analysis. Cells were scraped into PBS containing a sodium orthovanadate and protease inhibitor mixture (Roche Diagnostic Co., Indianapolis, IN). Tumor or UMSCC1 cells were incubated for 15 min on ice in Laemmli buffer (63 mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.005% (w/v) bromphenol blue) containing 100 mM NaF, 1 mM Na_3Vo_4, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. After sonication, particulate material was removed by centrifugation at 13,000 rpm for 15 min at 4°C. The soluble protein fraction was heated to 95°C for 5 min, then applied to a 4-12% bis-tris precast gel (Invitrogen, Carlsbad, CA) and transferred onto a PVDF membrane. Membranes were incubated for 1h at room temperature in blocking buffer consisting of 3% BSA and 1% normal goat serum in Tris-buffered saline [137 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1% (v/v) Tween 20]. Primary antibodies including Phospho EGFR (2231 and 4407), phospho-ERK (4376), total ERK (9107), phospho AKT (9271), total AKT (9272), phospho STAT3 (9131), total STAT3 (9132), phospho P65 (3033), total P65 (3034), cleaved PARP (9541) and GAPDH (2118) were purchased from Cell Signaling Technology, Danvers, MA. EGFR antibody (SC-03) was acquired from Santa Cruz Biotechnology. Membranes were subsequently incubated overnight at 4°C with 1 μg/ml primary antibody in blocking buffer, washed, and incubated for 1h with horseradish peroxidase-

conjugated secondary antibody (Cell Signaling, Danvers, MA). After three additional washes in Tris-buffered saline, bound antibody was detected by enhanced chemiluminescence plus reagent (Amersham Biosciences, Piscataway, NJ).

Drug and radiation treatments of SCCHN in vitro

UMSCC1 cells, kindly provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI), were originally obtained in 2008. DNA sent for sequence genotyping in 2008 confirmed their unique origin from original stocks. Nine loci analyzed included D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, vWA, and amelogenin. Cells were treated individually or with double or triple combinations of Radiation (2Gy), Cetuximab (100 μg/ml; Imclone Systems Inc, New York, NY) and Bortezomib (5 nM; Millennium Pharmaceuticals, Cambridge, MA), for 24 hours. Irradiation was performed at room temperature at a dose rate of 3 Gy/min using a Pantak DXT300 orthovoltage unit (Experimental Irradiation Core, University of Michigan, Ann Arbor, MI). Dosimetry was carried out using an ionization chamber connected to an electrometer system that was directly traceable to a National Institute of Standards and Technology calibration. Following these treatments cells were harvested for clonogenic survival assays and immunoblotting for prosurvival signaling as below.

Clonogenic survival assays

Clonogenic assays were done using standard techniques in which cells were subcultured at clonal density immediately after drug treatments and single dose irradiation of 2Gy. The difference in the surviving fraction after combination of drug treatments and 2 Gy irradiation was compared, as the surviving fraction in cell lines has been shown to correlate with the radiocurability of the corresponding human tumors in vivo.[17, 27]

Supplemental Table 1 Treatment delivery and dose escalation

Patient number	Bortezomib doses	Cetuximab doses	Radiation fractions/days/total dose (Gy)	DLT
1	12	8	36 / 54 / 74	-
2	12	8	37 / 61 / 74	-
3	12	8	37 / 49 / 70	-
4	12 *	8	33 / 40 / 69.6	-
5	12	8	35 / 52 / 70	-
6	12	8	35 / 49 / 70	-
7	8**	6**	35 / 52 / 70	-**

*Bortezomib dose reduced 4 doses to 0.7 mg/m^2 weeks 7 and 8 for Grade 2 thrombocytopenia
**Off study week 6 due to recurrent cetuximab infusion reaction and regional progression of disease, not bortezomib-related DLT. Received one dose cisplatin and completed IMRT.