Mevalonate pathway activity as a determinant of radiation sensitivity in head and neck cancer

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Radioresistance is a major hurdle in the treatment of head and neck squamous cell carcinoma (HNSCC). Here, we report that concomitant treatment of HNSCCs with radiotherapy and mevalonate pathway inhibitors (statins) may overcome resistance. Proteomic profiling and comparison of radioresistant to radiosensitive HNSCCs revealed differential regulation of the mevalonate biosynthetic pathway. Consistent with this finding, inhibition of the mevalonate pathway by pitavastatin sensitized radioresistant SQ20B cells to ionizing radiation and reduced their clonogenic potential. Overall, this study reinforces the view that the mevalonate pathway is a promising therapeutic target in radioresistant HNSCCs.

1. Introduction
Radiotherapy remains one of the most widely used treatments for cancer as nearly half of patients receive it alone or in combination with other types of therapies (Delaney et al., 2005). For some tumor types, evidence indicates that dose escalation can improve the efficacy of radiotherapy (Andrews et al., 2004) (Bartelink et al., 2007) (Kuban et al., 2008), but the consequence is an increase in normal tissue toxicity, which has been documented as a major limitation of chemoradiation therapy (Eisbruch et al., 2002).

Head and neck squamous cell carcinomas include a broad category of neoplasms that primarily develop in the oral cavity, pharynx, and larynx and are the sixth leading cause of cancer worldwide with approximately

Abbreviations
a.u., arbitrary units; DSB, double-strand break; FDPS, farnesyl diphosphate synthase; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GGPS, geranylgeranyl diphosphate synthase; HMGCR, 3-hydroxy-methylglutaryl CoA reductase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HNSCC, head and neck squamous cell cancer; IPP, isopentenyl diphosphate; IR, ionizing radiation; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LFQ, label-free quantification; LRP1, pro-low-density lipoprotein receptor-related protein 1; M5P, mevalonate-5-phosphate; M5PP, mevalonate-5-diphosphate; MFI, mean fluorescence intensity; MVD, mevalonate decarboxylase; MVK, mevalonate kinase; NSDHL, NAD(P) dependent steroid dehydrogenase-like; PIT, pitavastatin; PMVK, phosphomevalonate kinase; SD, standard deviation; STR, short tandem repeats.
half a million new cases every year (Boyle and Levin, 2008) (Hunter et al., 2005). In the past decade, the survival rate for HNSCC has not significantly changed, with two-thirds of HNSCC patients presenting with locally advanced disease and five-year survival rates remaining below 50% (Carvalho et al., 2005). Radical radiotherapy in combination with platinum-based chemotherapy remains the standard treatment for HNSCC with locoregionally advanced carcinoma (J. Pignon et al., 2000). Common acute toxicities include dysphagia, mucositis, dermatitis, and dysgeusia (altered taste sensation), with xerostomia (reduced salivary output) the most common and most adversely affecting quality of life in patients (Bjordal et al., 2001). Other cytotoxic cancer agents including targeted drugs can be combined with radiotherapy (Bonner et al., 2006) (J.-P. Pignon et al., 2009), though toxicities may be dose-limiting and resistance is common and associated with a poor prognosis.

An attractive alternative strategy is to identify agents that are nontoxic and likely to be ineffective on their own but can serve as radiosensitizers (Dickreuter et al., 2016) (Khan et al., 2010) (Leiker et al., 2015) (Su et al., 2013), potentially allowing treatment of patients with safer radiation doses. An ideal outcome would be increasing the efficacy of radiation on the tumor while at the same time decreasing its adverse effects on normal tissue. One promising class of drugs is the statins, a family of chemically related competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), the first committed enzyme of the mevalonate pathway (Hristov et al., 2009) (Stancu and Sima, 2001) (Alagona, 2010). As the mevalonate pathway serves a key role in cholesterol biosynthesis, statins are commonly used to treat dyslipidemia and to prevent cardiovascular disease. Statins lead to an overall decrease in circulating low-density lipoprotein (LDL) cholesterol both by inhibiting cholesterol biosynthesis and increasing cholesterol uptake via compensatory upregulation of the LDL receptor. Their broad use and high overall safety have permitted large-scale studies to identify additional impacts on patients, revealing potential benefits in cancer prevention and treatment. Retrospective studies to identify factors affecting success of radiotherapy for prostate cancer have found that incidental use of statins, as for treatment of dyslipidemia, confers improved responses (Hutchinson and Marignol, 2017). Along with extensive preclinical studies, the clinical data suggest that statins may display cancer selectivity, increasing radiosensitization in tumor cells while protecting normal tissue from late radiation effects.

In earlier work, we identified several statin drugs as candidate radiosensitizers in a screen for ionizing radiation-induced foci (IRIF), a proxy for DNA double-strand break (DSB) repair (Labay et al., 2011). Following up on this screen, we tested effects of several statins on irradiated mammary and melanoma tumor cells, finding that lipophilic statins delayed DNA repair and promoted therapy-induced senescence in vitro and in vivo (Efimova et al., 2017). These results suggested examining the mevalonate pathway as a target to enhance radiation sensitivity in HNSCC, a malignancy where radiation resistance is a common clinical challenge. This stimulated a hypothesis-driven proteomics analysis comparing two radiosensitive HNSCC cell lines, JSQ3 and SQ20B (Weichselbaum et al., 1986, 1988), to a radiosensitive control, SCC61 (Weichselbaum et al., 1986), to explore contributions of the mevalonate pathway to the proteomic signature of radioresistance. Indeed, radioresistance was not only associated with dysregulated mevalonate pathway activity but also increased sensitivity to statins with respect to proliferation, viability, DNA damage response, and radiation sensitization. We found that cholesterol levels were increased in radioresistant cells, along with elevated LDL receptor abundance and uptake of extracellular LDL. Further, consistent with these in vitro results, in patients treated for HNSCC with radiotherapy, incidental use of statins was associated with improved local control of tumors. These studies suggest potential benefit to concomitantly prescribing lipophilic statin drugs along with radiation therapy in order to improve outcomes for head and neck cancers.

2. Materials and methods

2.1. Cell lines, cell culture, and agents

Head and neck squamous cell carcinoma cell lines SCC61 (radiosensitive) derived from a glossal tumor (Weichselbaum et al., 1986), JSQ3 (radioresistant) derived from a nasal vestibule tumor, and SQ20B (radioresistant) derived from a laryngeal tumor (Weichselbaum et al., 1988) were grown in DMEM/F-12 media (Life Technologies, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (Denville Scientific) and penicillin/streptomycin (100 units·mL⁻¹, 100 mg·mL⁻¹, Life Technologies). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ until approximately 70% confluence was reached. Authenticity was confirmed by short tandem repeats (STR) profiling (Center for Genomic Research, University of Illinois at Chicago). All the cell lines tested negative for mycoplasma contamination.

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Pitavastatin calcium (PIT) was obtained from Ato-
mole. Simvastatin, pravastatin, atorvastatin, lovastatin,
and rosuvastatin were from the NIH Clinical Collec-
tion (BioFocus, Mechelen, Belgium). FTI (FTI-276)
and GGTI (GGTI-2147) inhibitors were from CalBio-
chem.

Cellular irradiation was delivered using a 60Co
gamma irradiator (GammaCell, MDS Nordion) with a
dose rate ranging from 9.4 to 7.6 cGy s⁻¹, depending
on the date of the experiment.

2.2. LC-MS/MS proteomics

2.2.1. Sample preparation

SCC61, JSQ3, and SQ20B were seeded at 10⁶ cells in
100 mm Petri dishes and were grown under standard
conditions for 48 h. Proteins were isolated with M-
PER (Thermo Scientific, Fitchburg, WI, USA) in the
presence of protease and phosphatase inhibitors
(Thermo Scientific). Thirty microgram of the extract
was separated by SDS/PAGE electrophoresis on a
12% MOPS-buffered gel (Thermo Scientific) run for
10 min at 200V resulting in a ~2-cm ‘gel plug’. After
staining with Imperial Protein Stain (Thermo Scien-
tific), individual samples were excised by razor blade
and chopped into ~1-mm³ pieces. Each sample was
washed in distilled H₂O and destained using 100 mm
NH₄HCO₃ pH 7.5 and 10 mm iodoacetamide prepared fresh in 50 m M
NH₄HCO₃ pH 7.5 in 50% acetonitrile. A reduction
step was performed by addition of 100 µL 50 mm
NH₄HCO₃ pH 7.5 and 10 µL of 200 mm Tris (2-car-
boxyethyl) phosphine HCl at 37 °C for 30 min. The
proteins were alkylated by addition of 100 µL of
50 mm iodoacetamide prepared fresh in 50 mm
NH₄HCO₃ pH 7.5 buffer and allowed to react in the
dark at 20 °C for 30 min. The gel fragments were
washed in water, then acetonitrile, and vacuum-dried.
In-gel trypsin digestion was carried out overnight at
37 °C with a 1:50–1:100 enzyme–protein ratio of
sequencing grade-modified trypsin (Promega, Fitch-
burg, WI, USA) in 50 mm NH₄HCO₃ pH 7.5, and
20 mm CaCl₂. Peptides were extracted first with 5% for-
ic acid, then with 75% ACN: 5% formic acid, com-
bined, and vacuum-dried.

2.2.2. High-pressure liquid chromatography (HPLC)

All samples were re-suspended in Burdick & Jackson
HPLC-grade water containing 0.2% formic acid
(Fluka, Muskegon, Michigan, USA), 0.1% TFA
(Pierce, Rockford, IL, USA), and 0.002% Zwittergent
3–16 (Calbiochem, San Diego, CA, USA), a
sulfobetaine detergent that contributes the following
distinct peaks at the end of chromatograms: MH⁺ at
392, and in-source dimer [2 M + H⁺] at 783, and some
minor impurities of Zwittergent 3–12 seen as MH⁺ at
336. The peptide samples were loaded onto a 0.25 µL
C8 OptiPak trapping cartridge custom-packed with
Michrom Magic (Optimize Technologies, Oregon City,
OR, USA) C8, washed, then switched in-line with a
20 cm by 75 mm C₁₈ packed spray tip nanocolumn
packed with Michrom Magic C₁₈AQ, for a 2-step gra-
dient. Mobile phase A was water/acetonitrile/formic
acid (98/2/0.2), and mobile phase B was acetonitrile/
isopropanol/water/formic acid (80/10/10/0.2). Using a
flow rate of 350 nL·min⁻¹, a 90 min, 2-step LC gra-
dient was run from 5% B to 50% B in 60 min, followed
by 50–95% B over the next 10 min, 10 min at 95% B,
then back to the starting conditions and re-equili-
brated.

2.2.3. LC-MS/MS analysis

The samples were analyzed via electrospray tandem
mass spectrometry (LC-MS/MS) on a Thermo Q-
Exactive Orbitrap mass spectrometer, using a 70 000
RP survey scan in profile mode, m/z 360–2000 Da,
with lockmasses, followed by 20 MS/MS HCD frag-
mentation scans at 17,500 resolution on doubly and
triply charged precursors. Singly charged ions were
excluded, and ions selected for MS/MS were placed on
an exclusion list for 60 s.

Label-free quantification (LFQ) sample sets were ana-
lyzed as described previously (Truman et al., 2012). All
LC-MS/MS * .raw data files were analyzed with max-
quanz version 1.5.2.8, searching against the SPROT
Human database (Downloaded: 1/07/2016 with isoforms)
using the following criteria: LFQ was selected for quanti-
tation with a min of 1 high confidence peptide to assign
LFQ intensities. Trypsin was selected as the protease with
max miss cleavage set to 2. Carbamidomethyl(C) was
selected as a fixed modification. Variable modifications
were set to Oxidization (M), Formylation (n-term),
Acetyl (Protein n-term), Deamidation (NQ), GlyGly (K),
HNE (Budach et al., 2005), and Phosphorylation (STY).
Orbitrap mass spectrometer was selected using an MS
error of 20 ppm and a MS/MS error of 0.5 Da. 1%
FDR cutoff was selected for peptide, protein, and site
identifications.

Ratios were reported based on the LFQ intensities
of protein peak areas determined by MaxQuant and
reported in the proteinGroups.txt. Proteins were
removed from this results file if they were flagged by
MaxQuant as ‘Contaminants’, ‘Reverse’, or ‘Only
identified by site’. Three complete biological replicates
were performed. LFQ peak intensities were analyzed in each run to determine protein hits that fell into the category of single-condition-only hits and retained if they maintained this state across all runs. Proteins with two out of three observations were retained and quantitated based on LFQ intensity. Log2 transformation of ratios was performed, and a significance cutoff of 1.5-fold change was implemented ($\pm 0.58 \log_2$ ratio). Gene Ontology analysis was performed by submitting significant hits ($\geq \log_2 0.58$ and $\leq \log_2 -0.58$) and those only seen in either radiosensitive (SQ20B or JSQ3) or radioresistant (SCC61) lists to a statistical overrepresentation test using bioinformatics software (Panther 14.0) (The Gene Ontology Consortium, 2016) (Mi et al., 2013) (Muruganujan et al., 2016). Significant gene lists for (SQ20B/SCC61) and (JSQ3/SCC61) were compared to the Homo sapiens reference list using Fisher’s exact test for determining $P$ value and a Benjamini–Hochberg procedure to calculate false discovery rate (FDR). Results were sorted by fold-enrichment for GO_BP (biological pathway), GO_MF (molecular function), GO_CC (cell component), and Reactome Pathway annotations results.

2.3. Western blotting

About $1 \times 10^6$ cells were seeded in 10-cm-diameter culture dishes, incubated overnight, harvested, and pelleted by centrifugation at 1000 g. Total cell lysates were prepared using ice-cold RIPA buffer (Thermo Fisher) in the presence of protease inhibitors (Halt protein inhibitor cocktail, Thermo Fisher) including EDTA (Thermo Fisher). Protein concentration was determined by BCA assay (Thermo Fisher). About 20 µg of protein was loaded per well, separated using a 12% Bis-Tris gel (Life Technologies), and electroblotted onto PVDF membrane (Bio-Rad, Hercules, CA, USA). Equal loading was verified by actin detection (1:10 000 Sigma-Aldrich, St. Louis, Missouri, USA, A-5316). Immunoblotting was performed using a rabbit monoclonal primary antibody targeting HMGCR (1:1000, Abcam, Cambridge, UK, ab174830) followed by anti-rabbit peroxidase-conjugated secondary antibody (GE) followed by detection with chemiluminescent peroxidase substrate (ECL Femto, Thermo Fisher). Blot imaging was conducted using an iBright FL1000 Imaging System (Life Technologies).

For HMGCS1 and NSDHL 0.5 $\times 10^6$ cells were plated in 10-cm-diameter culture dishes, and after the indicated treatments, total cell lysates were prepared using M-PER cell lysis buffer (Thermo) in the presence of protease and phosphatase inhibitors (Thermo). About 20–30 µg of protein was loaded per well, separated using SDS/PAGE gels (Bio-Rad), and electroblotted onto a PVDF membrane (Millipore, Burlington, MA, USA). Equal loading was verified by actin detection (Sigma-Aldrich). Immunoblotting for targets was performed using primary rabbit, mouse, or goat anti-human antibodies and species-specific peroxidase-conjugated secondary antibodies (Millipore, Jackson Laboratories) followed by detection with ECL Femto chemiluminescent peroxidase substrate (Thermo). Antibodies: HMGCS1 (1:200, Santa Cruz, sc-32422), NSDHL (1:2000, Abcam, ab190353), Actin (1:10,000, Sigma-Aldrich, A-5316).

2.4. Cholesterol measurement

Total cholesterol content from $0.7 \times 10^6$ cells was measured per sample with a colorimetric assay kit (Cell Biolabs, Inc). Measurements were performed for three biological replicates per group. Cells were lysed in chloroform:isopropanol:NP-40 (7:11:0.1) for 30 seconds using a mini bead beater (BioSpec). Homogenized cells were centrifuged at 15,000 $\times$ g for 10 min. The resulting organic phase layer was air-dried at 50 °C, and the remaining organic solvent was removed by using a vacuum concentrator (Eppendorf) for 30 min. The lipid pellets were resuspended in the 1X assay diluent included in the kit. Following the kit protocol, relative cholesterol concentrations in the samples were determined by absorbance at 570 nm using a microplate reader (Synergy Neo, BioTek).

2.5. Colony formation assay

200 cells per well were plated in 6-well plates the day before treatment. Pitavastatin was added at the indicated doses 1 h prior to irradiation. Two weeks later, cells were fixed and stained with 0.1% crystal violet (Sigma-Aldrich) for 5 to 10 min. Wells were rinsed with water, dried, and imaged with a Nikon D70s camera. For quantitation plots, the number of cell colonies with greater than 50 cells was counted manually in each well.

2.6. Cell viability assays

For the ATP luminescence cell viability assay, 10,000 cells per well were plated in 96-well plates (Thermo) in a final volume of 100 µL. 24 h after plating, cells were treated with different pitavastatin concentrations and some plates were exposed to different radiation doses. 48 h after treatment, an ATP-based cell viability assay (Cell Titer-Glo Kit, Promega) was used to measure the relative amount of ATP by luminescence following manufacturer’s
instructions. Luminescence was measured with a microplate reader (Synergy Neo, BioTek). Three biological replicates were measured.

For the flow cytometry-based Calcein Violet viability assay, cells were plated overnight in 6-well culture plates at 0.2 x 10^6 cells per well in complete culture medium as described above. The following day, cells were treated with pitavastatin (10 μM) for 1 h followed by irradiation (0 or 10 Gy). Cells were then returned to a 37 °C, 5% CO_2 environment for 48 h prior to cell viability assay, conducted as follows. Cells were harvested by cell scraper into the medium contained in each culture well. The cell suspensions were transferred to sterile tubes, and cells were collected by centrifugation for 5 min at 1200 x g. Cell pellets were resuspended in Calcein Violet 450 AM viability stain (1 μM, eBioscience) in 1% BSA-DPBS for 20 min on ice in the dark. Flow cytometry analysis was then performed using a Fortessa flow cytometer (BD) equipped with a 405-nm excitation laser and a 450/50-nm emission detector. Fluorescence data for 10,000 cells were acquired per sample. Cellular viability data were analyzed using FlowJo software (FlowJo).

2.7. LDL receptor immunostaining and LDL uptake assays

HNSCC cells were plated at 0.2 x 10^6 cells per well in 6-well plates for 24 h in complete culture medium. For LDL receptor staining, cells were harvested by cell scraper to preserve cell surface receptors, counted, and resuspended in 0.5% BSA-DPBS at 0.5 x 10^6 cells per sample for staining. 0.5% BSA-DPBS served as the blocking agent for 10 min on ice. A fluorescently labeled antibody targeting LDLR (Abcam clone EP1553Y, Alexa Fluor 647 conjugate) was then added to cells at the titration recommended by manufacturer for 30 min on ice. Sytox Blue viability stain was added at 1 μM for 10 min on ice just prior to flow cytometry analysis. Two biological replicates were analyzed per cell line.

For LDL uptake assays, an LDL uptake assay kit was used according to manufacturer specifications (Cayman Chemical, Ann Arbor, MI, USA). Briefly, cells were plated at 0.1 x 10^6 cells per well in 6-well plates for 48 h in complete culture medium. At 48 h, serum-free medium was exchanged on all samples to provide a lipid-free growth environment, to which fluorescent LDL-DyLight 488 (Cayman Chemical) was added at manufacturer recommended dilution (1:200). Samples for microscopy were incubated for 6 h, washed 2X with kit assay buffer, and imaged using a fluorescent microscope with an appropriate fluorescence filter set (Zeiss). A set of biological replicates for flow cytometry were incubated for 6 h, harvested by cell scraper, pelleted by centrifugation for 5 min at 1,200 x g, and resuspended in kit assay buffer (Cayman Chemical). 7-AAD viability stain was added for 10 min on ice just prior to flow cytometry analysis.

Flow cytometry was conducted using a Fortessa cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with excitation lasers and emission detectors appropriate for the fluorophores used in the experiment. Data for 10,000 cells per sample were acquired using FACSDiva software. Postacquisition analysis was conducted using FlowJo software (FlowJo, LLC).

2.8. Neutral comet assay

To analyze DSB, cells were plated at 0.2 x 10^6 cells per well in 6-well plates the day before treatment. PIT was administered at the indicated doses 1 h prior to 10 Gy irradiation. After 20 h, cells were mixed with Comet LM agarose (Bio-Rad) and single-cell electrophoresis was performed on CometSlides (Trevigen). Slides were fixed, dried, stained with SYBR Green DNA stain (1/10 000x; Life Technologies), and imaged on an Axiovert 40 with a 20X Plan-NeoFluar objective and an AxioCam monochromatic fluorescence camera. Images were analyzed using a Comet assay macro for ImageJ software (http://www.med.unc.edu/microscopy/resources/imagejplugins-and-macros/comet-assay).

2.9. Senescence-associated β-galactosidase (SA-β-Gal) assay

0.5 x 10^6 cells were plated in a 100-mm dish the day before treatment. Pitavastatin (10 μM) was administered 1 h prior to 10 Gy irradiation. After 6 days, cells were washed with PBS, harvested by scraping, and resuspended in 1 ml of culture medium. Cells were incubated with Bafilomycin A1 (1 μM, Thermo) for 30 min at 37 °C with rotation to neutralize lysosomal pH. DDAO galactoside (10 μg·mL⁻¹) was added, and cells were incubated for 1 h at 37 °C with rotation. After centrifugation at 4 °C for 5 min at 1,200 x g, cells were resuspended in ice-cold 1% BSA-DPBS and centrifuged again. Supernatant was discarded, and cells were resuspended in serum-free media with 1 μM Calcein Violet 450 AM (eBioscience) for 15 min on ice. Stained cells were washed with a Fortessa flow cytometer and analyzed with FlowJo to measure DDAO to detect SA-β-Gal and calcein violet to identify viable cells.
2.10. Patient study population and analysis
We utilized a retrospective head and neck cancer database of 803 patients to identify patients with Stage III-IVB disease treated for curative intent at the University of Illinois Medical Center at Chicago between 1990 and 2012. Patients treated with medications other than a statin or having Stage I-II disease were excluded resulting in 518 patients for analysis. Analysis was performed under University of Illinois Medical Center IRB protocol 2011-1075 in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1999, as revised in 2000. Data were collected from all available physical and electronic medical records. Time to local control (LC) or distant control (RC) was determined from last date of radiotherapy. Patterns of failure were determined as the first failure with any component of local, regional, or distant recurrence, respectively.

2.11. Statistical analysis
For cell-based assays, statistical significance was determined using the unpaired t-test (cholesterol assays), unpaired multiple t-test (ATP viability assays), or Mann–Whitney U-test (Comet assays and flow cytometry). Most experiments were performed in biological duplicate or triplicate. Flow cytometry experiments comprised \( n > 3000 \) viable cells per sample. \( P \) value \( \leq 0.05 \) was considered statistically significant. Calculations were performed using Prism software (GraphPad).

For patient data analysis, discrete variables were compared with the Chi-square test and differences in the medians were assessed using the Wilcoxon rank sum test. We used JMP version 10 (SAS Institute) to perform statistical analysis using two-sided tests and defining significance as \( P < 0.05 \). For univariate and multivariate analyses, we used Cox proportional hazard models to compare differences in survival. Censoring is assumed to be noninformative. Variables with \( P \) value \( < 0.1 \) on univariate analysis were included on multivariate analysis. Survival curves were plotted using the Kaplan–Meier method, and significance was assessed using the Log Rank test.

2.12. Cholesterol uptake assay
For cholesterol uptake assays, an assay kit was used according to manufacturer’s specifications (Cayman Chemical). Briefly, cells were plated at \( 0.2 \times 10^6 \) cells per well in 6-well plates for 24 h in complete culture medium. At 24 h, serum-free medium was exchanged on all samples to provide a lipid-free growth environment. NBD cholesterol was then added to the serum-free media at the recommended dilution (20 \( \mu \)g.mL\(^{-1} \)). At this point, inhibitors were added, including PIT (2.5 \( \mu \)m) and U-18666A (1/1000X manufacturer stock solution, Cayman Chemical). Cells were incubated for 24 h, harvested by cell scraper, pelleted by centrifugation for 5 min at 1200 \( g \), and resuspended in kit assay buffer (Cayman Chemical) for flow cytometry analysis.

Flow cytometry was conducted using a Fortessa cytometer (Becton Dickinson) equipped with excitation lasers and emission detectors appropriate for the fluorophores used in the experiment. Data for 10,000 cells per sample were acquired using FACSDiva software. Postacquisition analysis was conducted using FlowJo software (FlowJo, LLC).

3. Results

3.1. Proteomic profiling of radioresistant HNSCCs
We set out to uncover molecular determinants of radiation resistance in HNSCCs by performing proteome profiling (Fig. 1A). Radiosensitive (SCC61) and radioresistant (JSQ3 and SQ20B) HNSCC human cancer cell lines were grown under standard culture conditions, and after protein isolation and digestion in biological triplicates, peptides were analyzed by LC-MS/MS mass spectrometry. Using MaxQuant software and accepting only protein identifications with a minimum FDR of 1%, a total of 4700 different proteins were identified, of which 4392 were present in the SCC61 samples, 4471 in JSQ3, and 4463 in SQ20B (Fig. 1B).

In order to analyze the proteomic signature of radioresistant cells, label-free quantitation (LFQ) was conducted using the mass spectrometry intensities for all the protein hits identified in a minimum of 2 of 3 biological replicates. Radioresistant LFQ values for each protein ID were compared to those of the radiosensitive cell line. A 1.5-fold (0.58 log2) change was considered statistically significant. We observed a total of 585 proteins significantly upregulated and 803 significantly downregulated in each of the radioresistant cell lines compared with the radiosensitive cell line (Fig. 1C). Individual proteins in these categories were then further evaluated (Fig. 1D). Proteins strongly upregulated in the radioresistant cell lines included RNPS1, SNCG, SEMA4D, HMGAI, HMGAI2, and several others. Downregulated proteins in radioresistant cell lines included HLA-B, SERPINB2, TGM2, IGFBP3, LAMA3, and others. The full proteomic data set was uploaded to the ProteomeXchange repository with identifier PXD007188. A supplementary
3.2. Proteomics analysis suggests a role for dysregulated mevalonate pathway activity in HNSCC radioresistance

To determine a proteomic ‘signature’ of radioresistant cells and evaluate potential links to mevalonate pathway activity, we first performed gene ontology (GO) analysis on the hit list of proteins that showed significant variability between radioresistant and radiosensitive cell lines. We then searched for GO terms of relevance to our hypothesis. Metabolism-related GO terms of significance included glucose metabolic process (GO:0006006, \( P = 2.06 \times 10^{-2} \) and \( 5.12 \times 10^{-3} \)), protein metabolic process (GO:0019538, \( P = 4.84 \times 10^{-17} \) and \( 3.13 \times 10^{-19} \)), and lipid metabolic process (GO:0006629, \( P = 1.73 \times 10^{-2} \) and \( 9.31 \times 10^{-5} \)) (Fig. S1). We also evaluated the significance of GO terms specifically related to mevalonate biosynthesis and metabolism (Fig. 2A), including mevalonate pathway (GO:00019287, \( P = 0.030 \) for SQ20B/SCC61 and 0.021 for JSQ3/SCC61), cholesterol biosynthetic process (GO:0006695, \( P = 0.075 \) and...
0.028), protein farnesylation (GO0018343, \( P = 0.043 \) and 0.033), and protein geranylgeranylation (GO0018344, \( P = 0.075 \), and 0.057).

Expression profiles of individual protein hits identified to be differentially regulated in the mevalonate pathway (Fig. 2A) suggest dysregulation of this pathway in radioresistant HNSCC. According to LFQ intensities, HMGCS1, the initiator enzyme of the pathway, was significantly downregulated in the JSQ3 radioresistant cells. We confirmed the downregulation of HMGCS1 in both radioresistant cell lines by western blotting (Fig. S2). Following the conversion of acetoacetyl-CoA to HMG-CoA by HMGCS1, the enzyme HMGCR converts HMG-CoA to mevalonate. HMGCR is a key rate-limiting enzyme in the mevalonate pathway. HMGCR was not detected by LC-MS/MS analysis, but western blotting revealed the enzyme to be upregulated in both radioresistant cell lines (Fig. 2B). Downstream from HMGCR are the enzymes MVK, PMVK, and MVD, which function to convert mevalonate to several intermediate metabolites (mevalonate-5-phosphate, M5P and mevalonate-5-diphosphate, M5PP) resulting in the formation of isopentenyl diphosphate (IPP). Of these enzymes, LC-MS/MS analysis indicated that MVK was downregulated, PMVK...
was variably regulated, and MVD was upregulated in radiosensitive cell lines. For enzymes required for conversion of IPP to geranyl pyrophosphate (GGPS), and then to farnesyl pyrophosphate (FDPS), differences across cell lines were insignificant. The enzyme NSDHL, which mediates the conversion of farnesyl pyrophosphate to cholesterol, was found to be downregulated in both radiosensitive cell lines. NSDHL downregulation was confirmed by western blotting (Fig. S2). Taken together, these results implicate dysregulation of mevalonate pathway protein expression in radiosensitive HNSCC.

An important metabolic outcome of mevalonate pathway activity is cholesterol biosynthesis. Suggesting a functional consequence of mevalonate pathway dysregulation on cell cholesterol content, analysis of cholesterol in untreated cell lysates (Fig. 2C) revealed significantly higher levels of cholesterol in the radiosensitive cell lines ($P < 0.05$ by unpaired $t$-test). These data raised the question whether cholesterol biosynthesis might offer a target to restore radiation sensitivity in radiosensitive HNSCC.

### 3.3. Lipophilic statins inhibit cell proliferation in radiosensitive HNSCC cell lines

To ascertain the role of the mevalonate pathway in survival of HNSCC cell lines, we tested a panel of FDA-approved statin drugs, which block mevalonate generation. Statins included lipophilic pitavastatin (PIT), simvastatin, lovastatin, and atorvastatin, as well as the hydrophilic statins pravastatin and rosuvastatin. Using a colony formation assay (Fig. 3A), we evaluated whether any statin might suppress cellular proliferation in HNSCC cell lines. Indeed, growth of cells was suppressed by PIT at doses as low as 2.5 $\mu$m, as well as the other lipophilic statins simvastatin, lovastatin, and atorvastatin at higher doses (10 $\mu$m). These effects were particularly dramatic for the radiosensitive cell lines. The hydrophilic statins pravastatin and rosuvastatin displayed only minimal effects. Given these results, PIT was selected as the most promising statin for further testing in the study.

Results of an ATP-based cell viability assay (Fig. 3B) further demonstrate that radiosensitive cell lines have increased sensitivity to PIT (0 to 10 $\mu$m), as measured after 48 hours of treatment. While PIT treatment reduced viability in all cell lines, its effects were more dramatic on radiosensitive cell lines. At 4 and 8 $\mu$m, PIT treatment significantly reduced viability in SQ20B cells compared with the same doses used on SCC61 cells ($P < 0.05$ by unpaired multiple $t$-test, $n = 3$ biological replicates per sample). At 10 $\mu$m, PIT reduced viability in both radiosensitive cell lines to approximately 50% of radiosensitive cells.

### 3.4. Cholesterol uptake via the LDL receptor is elevated in radiosensitive HNSCC cell lines

Cellular cholesterol uptake is commonly mediated by endocytosis of low-density lipoprotein (LDL) through the LDL receptor. Thus, we immunostained for cell surface LDL receptors (Fig. 4A) and examined uptake of fluorescently labeled LDL (Fig. 4B,C). Flow cytometry analysis revealed that levels of LDL receptors were upregulated (LDLR<sup>HI</sup>) on the surface of radiosensitive (JSQ3, 30.6%; SQ20B, 68.8%) vs. (SCC61, 23.9%) HNSCC cells. In turn, the radiosensitive cell lines demonstrated higher levels of LDL uptake (JSQ3, 98.4%; SQ20B, 99.0%; vs. SCC61, 48.3%). Differential uptake of fluorescently labeled LDL can also be seen in microscopy images taken with standardized exposure and equal cell density across cell lines (Fig. 4C). Statistical analysis of mean staining intensities for LDLR (Fig. 4D) and LDL-Dy488 uptake (Fig. 4E) confirmed significant upregulation of these features in the radiosensitive cell lines ($P < 0.05$ by Mann–Whitney $U$-test, $n > 3000$ viable cells per sample).

### 3.5. Inhibition of mevalonate pathway sensitizes radiosensitive HNSCC cell lines to radiation

To demonstrate the differences in radioresponse across cell lines, an ATP-based cell viability assay was performed. After a single 10 Gy dose of IR, the radiosensitive cell lines JSQ3 and SQ20B maintained viability according to cellular ATP assay for at least 50 h after irradiation (Fig. 5A), while the radiosensitive cell line SCC61 displayed a significant loss of viability over the same period ($P < 0.05$ by unpaired multiple $t$-test, $n = 3$ biological replicates per sample). The ATP assay further demonstrated the radiosensitizing effects of PIT at concentrations from 1 to 10 $\mu$m (Fig. 5B), particularly in radiosensitive cells. At 10 $\mu$m, PIT reduced ATP in irradiated cells by 15% (SCC61), 40% (SQ20B), or 50% (JSQ3). All dose conditions of PIT tested significantly reduced ATP levels in both radiosensitive cell lines as compared to the same dose in the radiosensitive cell line ($P < 0.05$ by unpaired multiple $t$-test, $n = 3$ biological replicates per sample).

In prior studies of PIT as a radiosensitizer (Efimova et al., 2017), we observed that when MCF7 mammary carcinoma cells were treated with PIT and then irradiated, they displayed a marked delay in double-strand break (DSB) repair. To examine whether PIT similarly affects DSB repair in HNSCC cells, we performed neutral comet assays after treatment with PIT and/or ionizing radiation (IR) (Fig. 5C,D). As expected, the combination of PIT + IR (68 ± 0.9% comet tail DNA) significantly increased the levels of persistent
DSBs over that of IR alone (60 ± 1.2%) in SQ20B cells (P < 0.05 by Mann–Whitney U-test). This effect was not seen in the sensitive cell line SCC61 (47 ± 1.5% for PIT + IR vs. 45 ± 1.8% for IR only). Further, even without radiation, PIT increased the basal level of DSBs in SQ20B cells. These effects were recapitulated in JSQ3 cells (Fig. S3A). These effects may underlie the loss of proliferation and viability induced by PIT in the radioresistant cell lines.

Along with increasing persistence of DNA damage, our prior work showed that combining PIT with radiation also promoted therapy-induced senescence in melanoma and breast cancer cells (Efimova et al., 2017). Here, senescence of HNSCC cells treated with PIT with or without IR was measured by a conventional flow cytometry SA-β-Gal assay at six days after treatment (Fig. 5E). While treating radioresistant SQ20B cells with PIT or IR alone slightly increased senescence (12.2% for PIT alone, 17.8% for IR alone, compared to < 2% in vehicle only treated SQ20B cells), combining statin with radiation induced senescence in 79.4% of cells. A similar pattern was observed in radioresistant JSQ3 cells (Fig. S3B). The senescence response in radiosensitive SCC61 cells was comparable for combination-treated cells above levels induced by IR treatment alone (75.9 vs 82.1%). Statistical analysis of mean senescence probe fluorescence (Fig. S3C) supports these conclusions (P < 0.05 for most conditions compared with controls, Mann–Whitney U-test, n > 3000 viable cells per sample).

To examine effects of treatment with various statins on radioresponse, SCC61, JSQ3, and SQ20B cell lines were treated with statins before irradiation (IR) and assayed for colony formation (Fig. S4). PIT alone decreased colony formation in radioresistant cells. The lipophilic statins simvastatin, lovastatin, and...
atorvastatin potentiated the effect of radiation on SQ20B cells. The hydrophilic statins tested failed to confer toxicity or radiosensitization in any cell line.

3.6. Clinically approved lipophilic statin drugs are associated with better local tumor control in HNSCC patients

Radiotherapy is widely used in the treatment of head and neck cancers, and our previous results suggest that its combination with statins may have beneficial effects. Consequently, we analyzed 518 HNSCC patients on Stage III–IV treated with radiotherapy of which 53 patients were also taking a statin drug. Table 1 describes patient characteristics. Patients receiving statin were significantly older, had higher comorbidity scores but less alcohol use, and fewer T3–T4 tumors and N2-3 nodal disease. Of the 53 patients incidentally taking a statin drug during their radiotherapy, 41 (77.4%) were taking a lipophilic statin and 3 (5.7%) were taking a hydrophilic statin. For 9 patients, the type of statin was not indicated. Table 2 shows univariate and multivariate analysis of survival. On univariate analysis, improved survival was associated with statin use (HR 0.47, 95% CI, 0.20–0.93; \( P = 0.03 \)). On multivariate analysis, statin use remained associated with improved survival (HR 0.29, 95% CI, 0.09–0.71; \( P = 0.004 \)).
For univariate and multivariate analyses, Cox proportional hazard models was used to compare differences in survival. Variables with $P$ value < 0.1 on univariate analysis were included on multivariate analysis. Significance was assessed using the Log Rank test. Use of statins, high - very high comorbidity, the size of the tumor and the use of post-operative radiotherapy have an impact on survival. The effect of statins on local and distant control by radiotherapy treatment of HNSCC tumors is shown in Fig. 6.
Table 1. Patient characteristics (n = 518).

<table>
<thead>
<tr>
<th>Statin (n = 53)</th>
<th>No statin (n = 465)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median follow-up (months)</td>
<td>18.4 (11.4–38.7)</td>
<td>18.7 (6.8–58.7)</td>
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<tr>
<td>Age ≤57</td>
<td>14 (26.4%)</td>
<td>258 (56.0%)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>39 (73.6%)</td>
<td>203 (44.0%)</td>
</tr>
<tr>
<td>Gender Male</td>
<td>41 (77.4%)</td>
<td>350 (75.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (22.6%)</td>
<td>115 (24.7%)</td>
</tr>
<tr>
<td>Comorbidity Medium</td>
<td>16 (30.2%)</td>
<td>366 (78.7%)</td>
</tr>
<tr>
<td>High-very high</td>
<td>37 (69.8%)</td>
<td>99 (21.2%)</td>
</tr>
<tr>
<td>Performance status &lt;70</td>
<td>5 (9.4%)</td>
<td>22 (4.7%)</td>
</tr>
<tr>
<td>≥70</td>
<td>40 (75.5%)</td>
<td>380 (81.7%)</td>
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<tr>
<td>Not stated</td>
<td>8 (15.1%)</td>
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<tr>
<td>Alcohol use ≥3 drinks/day</td>
<td>26 (63.4%)</td>
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<tr>
<td>&lt;3 drinks/day</td>
<td>15 (36.6%)</td>
<td>84 (23.3%)</td>
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<tr>
<td>Smoking</td>
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<tr>
<td>≥10 pack-year</td>
<td>39 (81.2%)</td>
<td>350 (79.2%)</td>
</tr>
<tr>
<td>&lt;10 pack-year</td>
<td>9 (18.8%)</td>
<td>92 (20.8%)</td>
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<td>T-stage T1–2</td>
<td>21 (39.6%)</td>
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<tr>
<td>T3–4</td>
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<td>N-stage N0–1</td>
<td>31 (58.5%)</td>
<td>199 (42.8%)</td>
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<tr>
<td>N2–3</td>
<td>22 (41.5%)</td>
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<tr>
<td>Primary site Oral cavity</td>
<td>15 (28.3%)</td>
<td>116 (25.0%)</td>
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<tr>
<td>Oropharynx</td>
<td>18 (34.0%)</td>
<td>119 (25.6%)</td>
</tr>
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<td>Larynx</td>
<td>12 (22.6%)</td>
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<td>Hypopharynx</td>
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<td>Other</td>
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<td>Induction chemotherapy</td>
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<td>9 (17.0%)</td>
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<td>44 (83.0%)</td>
<td>304 (65.4%)</td>
</tr>
<tr>
<td>Concurrent chemotherapy</td>
<td></td>
<td></td>
</tr>
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<td>47 (88.7%)</td>
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</tr>
<tr>
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<td>6 (11.3%)</td>
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</tr>
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<td>Post-operative radiotherapy</td>
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<td></td>
</tr>
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<td>18 (34.0%)</td>
<td>180 (38.7%)</td>
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<tr>
<td>No</td>
<td>35 (66.0%)</td>
<td>285 (61.3%)</td>
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<td>Type of statin Lipophilic</td>
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<tr>
<td>Hydrophilic</td>
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<td>N.D.</td>
</tr>
<tr>
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<td>9 (17.0%)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Included in propensity matching: age, gender, year of diagnosis, facility, path stage grouping, path T-stage, path N-stage, LN > 2, LVI, LND and site. For patient data analysis (table 1) discrete variables were compared with the Chi-square test and differences in the medians were assessed using the Wilcoxon Rank Sum test, significance was defined as P < 0.05. In patients under statin treatment, variables such as age, comorbidity, T and N-stage and success in induction and concurrent chemotherapy, were significantly changed compared to those who where not in treatment.

## 4. Discussion

Although many head and neck squamous cell cancers (HNSCC) initially regress upon treatment with conventional or targeted therapies and display a complete or partial response, a large fraction recur locally or at distant sites with metastatic disease. For recurrent HNSCC, the prognosis remains poor as these tumors typically display acquired therapy resistance. This pattern likely reflects the heterogeneity of epithelial tumors and treatment driving selection for cancer cells able to survive and proliferate even in the presence of therapy. When local recurrence occurs after radiotherapy, this can be particularly problematic, given the limited tolerance of normal tissue for reirradiation. Were it possible to prevent the selection of resistant clones and thus suppress the emergence of resistant disease, recurrence might be rarer and easier to treat. In the case of locoregional recurrence, sensitizers targeting resistance might permit reirradiation at tolerable doses.

This work identifies the mevalonate pathway as a promising target to overcome radiation resistance in HNSCC. Quantitative proteomic profiling to compare two radioresistant HNSCC cell lines, SQ20B and JSQ3, to the radiosensitive cell line SCC61 revealed altered expression of several mevalonate pathway enzymes. Further analysis revealed increased expression of HMG-CoA reductase (HMGCR), the rate-limiting enzyme of mevalonate synthesis, in the radioresistant HNSCC cells. In turn, the radioresistant cells also displayed increased cholesterol levels. Treating SQ20B and JSQ3 with PIT only partly suppressed cholesterol levels, suggesting active uptake of cholesterol along with increased biosynthesis. Import of free cholesterol was similar between the three cell lines, but SQ20B and JSQ3 displayed higher expression of LDL receptors and greater LDL uptake compared with SCC61. Despite the moderate impact on cellular cholesterol, PIT and other lipophilic statins decreased cell proliferation and viability in the radioresistant HNSCC cells. Further, when combined with IR, PIT restored radiation sensitivity to SQ20B and JSQ3 cells, increasing cell death, DNA damage persistence, and senescence. These latter effects

### References

may be partly independent of cholesterol levels, potentially mediated by other metabolic pathways down-stream of HMGCR and mevalonate biosynthesis such as protein prenylation as previously observed (Efimova et al., 2017). Even so, the active uptake of cholesterol by the radioresistant cell lines SQ20B and JSQ3 reinforces the impression that high cellular cholesterol may be critical for their survival and proliferation.

Prior studies have shown that the mevalonate pathway in cancer cells can impact cell division, tumor growth, and metastatic potential (Clendening et al., 2010) (Thurnher et al., 2012) and modulate immune response and mTOR signaling (Bekkering et al., 2018). Recently, novel links between the mevalonate pathway and apoptosis, cell cycle arrest, and DNA damage response have been observed (Horlbeck et al., 2018; Martin Sanchez et al., 2015; Stine et al., 2016). Our prior work demonstrated strong radiosensitization by statin drugs in breast cancer and melanoma cell lines both in vitro and in vivo (Efimova et al., 2017). While connections have been noted between the mevalonate pathway and tumor radioresistance in prostate and pancreatic cancer (Chen et al., 2018; Souchek et al., 2014), this study is the first to extend these links to HNSCC.

Given the strong influence of advanced age and smoking history on head and neck cancer, it is not surprising that a large fraction of patients also display intercurrent cardiovascular disease and/or hypercholesterolemia, leading to high rates of incidental statin use. In our analysis, among HNSCC patients treated

<table>
<thead>
<tr>
<th>Table 2. Univariate and multivariate analysis of survival (n = 518).</th>
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<tr>
<td><strong>Univariate analysis for survival</strong></td>
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<tr>
<td><strong>Hazard ratio</strong></td>
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<td>Statin use</td>
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<td>Yes</td>
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<td>Age</td>
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<td>Alcohol use</td>
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<td>&lt;3 drinks/day</td>
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<td>N2–3</td>
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<td>Induction chemotherapy</td>
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<tr>
<td>No</td>
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<tr>
<td>Yes</td>
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<tr>
<td>Concurrent chemotherapy</td>
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<td>No</td>
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<td>Yes</td>
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<tr>
<td>Post-operative radiotherapy</td>
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<tr>
<td>No</td>
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<td>Yes</td>
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</table>

For univariate and multivariate analyses (Table 2), Cox proportional hazard models was used to compare differences in survival. Variables with P value < 0.1 on univariate analysis were included on multivariate analysis. Significance was assessed using the Log Rank test. Use of statins, high - very high comorbidity, the size of the tumor and the use of post-operative radiotherapy have an impact on survival.
radiotherapy, taking statins appeared to improve locoregional control, suggesting radiosensitization in vivo. Additional studies would be useful to reduce potentially confounding variables such as HNSCC subtype, HPV status, and patient age. Expanding the analysis to include more patients taking statins would allow analysis of the potential impact of specific types of statin, if any. However, our data mining approach utilizing currently available patient data revealed promising findings regarding local control of tumors. We hope these findings will help to justify future clinical studies involving co-treatment with lipophilic statins for HNSCC patients undergoing radiotherapy.

5. Conclusions
In radioresistant HNSCC cell lines, we observed dysregulation of proteins in the mevalonate pathway, as well as elevated cholesterol content, increased LDL receptor abundance, and high levels of LDL uptake. Radioresistant cells displayed loss of cell viability, decreased clonogenic potential, an increase in DNA damage, and induction of cell senescence when treated with the mevalonate pathway inhibitor pitavastatin along with radiation. As a clinical correlate, head and neck cancer patients taking statins displayed improved local tumor control after radiation therapy, suggesting value for administering lipophilic statins to HNSCC patients as a radiosensitization strategy.

Acknowledgements
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Conflict of interest
S.J.K. is a founder of OncoSenescence. The other authors have no conflicts to declare.

Data Accessibility
Research data pertaining to this article is located at figshare.com: https://doi.org/10.6084/m9.figshare.8145971.

Author contributions
NR, EVE, AWT, and SJK involved in the conception and design. NR, DW, AF, EVE, and SJK involved in the development of methodology. NR, DW, AF, EVE, AR, OKA, JB, and MTS involved in the acquisition of data. NR, DW, AF, EVE, AR, OKA, MTS, and SJK analyzed and interpreted the data. NR, AF, DW, EVE, AR, OKA, JB, AWT, MTS, and SJK wrote, reviewed, and/or revised the manuscript.

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brain radiation therapy with or without stereotactic radiosurgery boost for patients with one to three brain metastases; phase III results of the RTOG 9508 randomised trial. *Lancet* **363**(9422), 1665–1672.


Supporting information

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

Fig. S1. Metabolism-related Gene Ontology (GO) analysis of radioresistant HNSCC proteomes. Proteins were separated into functional categories and represented as the log2 ratio for JSQ3/SCC61 (left) and SQ20B/SCC61 (right). Significantly differentiated metabolism-related GO categories found in both radioresistant cell lines include glucose metabolic process, protein metabolic process, and lipid metabolic process. P values for each GO category were determined using the Panther GO database and are shown in the figure.

Fig. S2. Downregulation of critical mevalonate pathway enzymes HMGCS1 and NSDHL. Cells were harvested in active growth phase, lysed, and subjected to Western blotting for targets as shown. Actin is shown with 2.5, 5 or 10 μM PIT, simvastatin, lovastatin, atorvastatin, pravastatin or rosuvastatin for 1 h prior to 10 Gy irradiation. After 4 d of culture, crystal violet staining was conducted and plates were imaged.

Fig. S3. Combination treatment of pitavastatin and irradiation induces persistent DNA damage and accelerated senescence in JSQ3 radioresistant cells. (A) Plots representing mean percent of DNA in comet tail ± SEM for 100 cells per treatment condition. Significance is indicated by *, P < 0.05 (Mann–Whitney U-test). Results indicate that PIT alone enhances persistent DSBs in radioresistant JSQ3 cells. PIT + IR had a similar effect. (B) Flow cytometric senescence assay data of cells treated with 10 μM PIT (PIT), 10 Gy (IR), or PIT + IR. Vehicle-treated cells (VEH) were stained as controls. PIT + IR significantly induced senescence in the radioresistant cells above IR or PIT only treatments. (C) Statistical significance was determined using Mann–Whitney U-test. Mean fluorescent intensity (MFI) of the senescence probe is shown. Error bars, SEM. *, P < 0.05; ns, not significant. n > 3000 viable cells per sample.

Fig. S4. Colony formation assay of SCC61, JSQ3, and SQ20B treated with statin ± IR. Cells were treated with 2.5, 5 or 10 μM PIT, simvastatin, lovastatin, atorvastatin, pravastatin or rosuvastatin for 1 h prior to 10 Gy irradiation. After 4 d of culture, crystal violet staining was conducted and plates were imaged.