Clinical Cancer Research

The CD98 Heavy Chain Is a Marker and Regulator of Head and Neck Squamous Cell Carcinoma Radiosensitivity

David Digomann[1], Ina Kurth[1,2], Anna Tyutyunnykova[1], Oleg Chen[1,3], Steffen Löck[1,4,5], Ielizaveta Gorodetska[1], Claudia Peitzsch[1,6], Ira-Ida Skvortsova[7,8], Giulia Negro[7,8], Bertram Aschenbrenner[7,8], Graeme Eisenhofer[9,10], Susan Richter[9,10], Stephan Heiden[1], Joseph Porrmann[4,6,11], Barbara Klink[4,6,11,12], Christian Schwager[2,13,14], Adam A. Dowle[15], Linda Hein[1], Leoni A. Kunz-Schughart[1,6], Amir Abdollahi[2,13,14], Fabian Lohaus[1,4,5,6], Mechthild Krause[1,4,5,6,16], Michael Baumann[1,2,5], Annett Linge[1,4,5,6], and Anna Dubrovska[1,4,16]

Abstract

Purpose: The heavy chain of the CD98 protein (CD98hc) is encoded by the *SLC3A2* gene. Together with the light subunit LAT1, CD98hc constitutes a heterodimeric transmembrane amino acid transporter. High *SLC3A2* mRNA expression levels are associated with poor prognosis in patients with head and neck squamous cell carcinoma (HNSCC) treated with radiochemotherapy. Little is known regarding the CD98hc protein–mediated molecular mechanisms of tumor radioresistance.

Experimental Design: CD98hc protein expression levels were correlated with corresponding tumor control dose 50 (TCD_{50}) in HNSCC xenograft models. Expression levels of CD98hc and LAT1 in HNSCC cells were modulated by siRNA or CRISPR/Cas9 gene editing. HNSCC cell phenotypes were characterized by transcription profiling, plasma membrane proteomics, metabolic analysis, and signaling pathway activation. Expression levels of CD98hc and LAT1 proteins were examined by IHC analysis of tumor tissues from patients with locally advanced HNSCC treated with primary radiochemotherapy (RCTx). Primary endpoint was locoregional tumor control (LRC).

Results: High expression levels of CD98hc resulted in an increase in mTOR pathway activation, amino acid metabolism, and DNA repair as well as downregulation of oxidative stress and autophagy. High expression levels of CD98hc and LAT1 proteins were significantly correlated and associated with an increase in radioresistance in HNSCC *in vitro* and *in vivo* models. High expression of both proteins identified a poor prognosis subgroup in patients with locally advanced HNSCC after RCTx.

Conclusions: We found that CD98hc-associated signaling mechanisms play a central role in the regulation of HNSCC radioresistance and may be a promising target for tumor radiosensitization.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with about 550,000 new cases diagnosed annually (1). Despite advances in treatment management, patients with locally advanced disease often experience locoregional and distant recurrence and have a 5-year survival rate of only about 50% (1–3). Primary radiochemotherapy (RCTx) or postoperative radiochemotherapy (PORT-C) are

[1]OncoRay–National Center for Radiation Research in Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Helmholtz-Zentrum Dresden-Rossendorf, Dresden, Germany. [2]German Cancer Research Center (DKFZ), Heidelberg, Germany. [3]Department of Cell Signaling, Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv, Ukraine. [4]German Cancer Consortium (DKTK), partner site Dresden and German Cancer Research Center (DKFZ), Heidelberg, Germany. [5]Department of Radiotherapy and Radiation Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany. [6]National Center for Tumor Diseases (NCT), Partner Site Dresden: German Cancer Research Center (DKFZ), Heidelberg; Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, and Helmholtz-Zentrum Dresden-Rossendorf (HZDR), Dresden, Germany. [7]EXTRO-Lab, Department of Therapeutic Radiology and Oncology, Medical University of Innsbruck, Innsbruck, Austria. [8]Tyrolean Cancer Research Institute, Innsbruck, Austria. [9]Department of Medicine III, Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany. [10]Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany. [11]Institute for Clinical Genetics, Faculty of Medicine Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany. [12]Laboratoire National de Santé, National Center of Genetics, Dudelange, Luxembourg, Germany. [13]Department of Radiation Oncology, Heidelberg Ion-Beam Therapy Centre (HIT), University of Heidelberg Medical School, Heidelberg, Germany. [14]Heidelberg Institute of Radiation Oncology (HIRO), National Center for Radiation Research in Oncology (NCRO), Heidelberg, Germany. [15]Bioscience Technology Facility, Department of Biology, University of York, York, United Kingdom. [16]Helmholtz-Zentrum Dresden-Rossendorf, Institute of Radiooncology-OncoRay, Dresden, Germany.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

M. Baumann, A. Linge, and A. Dubrovska are the co-senior authors of this article.

Corresponding Author: Anna Dubrovska, OncoRay, TU Dresden, Fetscherstr.74, Dresden 01307, Germany. Phone: 4935-1458-7150; Fax: 4903-5145-87311; E-mail: Anna.Dubrovska@OncoRay.de

doi: 10.1158/1078-0432.CCR-18-2951

Translational Relevance

Radiotherapy is one of the key modalities in the management of HNSCC. Nevertheless, only few biological parameters have been identified so far as potent prognostic biomarkers for radiotherapy outcome and as potential targets for personalized treatment approaches of radiotherapy combined with chemotherapy. The heavy chain of the CD98 protein (CD98hc) is encoded by the *SLC3A2* gene. Together with the light subunit LAT1, CD98hc constitutes a heterodimeric transmembrane amino acid transporter. High expression levels of CD98hc and LAT1 proteins were significantly correlated and associated with an increase in radioresistance in HNSCC *in vitro* and *in vivo* models. High expression of both proteins identified poor prognosis subgroup in patients with locally advanced HNSCC after primary radiochemotherapy. We found that CD98hc-associated signaling mechanisms play a central role in the regulation of HNSCC radioresistance and may be a promising target for tumor radiosensitization.

standard treatments for patients with locally advanced HNSCC (4–8). Because of the biological heterogeneity of HNSCC, the patients' response to treatment is highly diverse and reliable stratification of patients with HNSCC for prediction of outcomes is of utmost importance (9).

In addition to clinical stage, other clinical, pathologic, and biological indicators that may have a prognostic value in HNSCC have been reported, such as expression of hypoxia-associated gene signatures, tumor metabolic volume measured by 2-[18F]fluoro-2-deoxy-D-glucose (FDG) uptake, cancer stem cell (CSC) marker expression, $CD8^+$ tumor-infiltrating lymphocytes, and human papillomavirus (HPV) infection (10–16). A number of clinical studies demonstrated that infection with HPV is a strong prognostic factor in HNSCC after PORT or primary RCTx (10–12). To further stratify patients with HPV-negative HNSCC for the risk of recurrence, additional markers are warranted.

Retrospective biomarker analyses in patients with locally advanced HNSCC treated with curatively intended cisplatin-based PORT (PORT-C) or primary RCTx revealed that the putative CSC marker *SLC3A2* is a prognostic biomarker of locoregional tumor control (LRC) in patients with HPV-negative tumors, with significantly higher LRC rates in tumors with low *SLC3A2* mRNA expression (11, 17).

SLC3A2 encodes for the heavy chain of a cell surface, transmembrane protein that is also known as CD98 (CD98hc). Together with the large neutral amino acid transporters LAT1 (*SLC7A5*), CD98hc constitutes a heterodimeric transmembrane amino acid transporter LAT1/CD98hc that preferentially transports large neutral amino acids including isoleucine, leucine, methionine, valine, histidine, cysteine, tryptophan, and tyrosine (18, 19). A recent study also showed an interaction of CD98hc with other proteins: LAT2 (*SLC7A8*), which is an amino acid transporter specific for isoleucine and leucine and a mediator of glutamine efflux (20); and xCT (*SLC7A11*), which is a cystine/glutamate exchange transporter essential for synthesis of the antioxidant glutathione (GSH; ref. 21). Owing to its function, a high expression of CD98hc is associated with the development and aggressiveness of a number of cancers including HNSCC (19, 22, 23). Although the role of CD98hc as a potential regulator of CSC maintenance in HNSCC has been reported previously (22), its contribution to the molecular mechanisms governing tumor response to radiotherapy is not yet understood.

The main objective of this study is the biological validation of *SLC3A2* as a potential biomarker of HNSCC radioresistance and identification of potential molecular mechanisms driving survival of *SLC3A2*-overexpressing cells during radiotherapy.

Materials and Methods

Additional methods not described here are included in the Supplementary Data.

Patients, treatment, and tissue samples

In this study, a total of 197 patients from two different patient cohorts were included. All patients were diagnosed with locally advanced HNSCC arising in the oral cavity, oropharynx, hypopharynx, or larynx and received curative-intended, state-of-the-art primary radiotherapy (10/197), or cisplatinum-based radiochemotherapy (187/197) between 1999 and 2015, covering the tumor region and regional neck nodes as well as a boost to the tumor region and involved regional lymph nodes. Inclusion criteria have been described in detail before (11). For staging, the 7th edition of the UICC classification was used. The first patient cohort included 134 patients selected according to available tumor material from an original population of 158 patients of the retrospective, multicenter study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG), all of whom received primary radiochemistry as described previously (11). From the remaining 24 patients, insufficient tumor material was available. Thus, this patient cohort was further supplemented by a second, monocentric cohort including 63 patients, who were treated with primary radio(chemo)therapy between 1999 and 2015 at the Dresden University Hospital (Dresden, Germany). The latter cohort was not part of the DKTK cohort but fulfilled the same inclusion criteria. Patient characteristics are shown in Supplementary Table S1. All patients were followed-up for a minimum of 24 months.

Written informed consent was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki. Ethical approval for retrospective analyses of clinical and biological data was granted by the local ethics committee (Dresden) as well as by the ethics committees of all DKTK partner sites for the respective patients of the multicenter study.

Cell lines and cell culture conditions

In this study, we used established human squamous cell carcinoma (hSCC) cell lines including Cal33 derived from squamous cell carcinomas of the tongue (Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ GmbH), FaDu derived from pharyngeal squamous cell carcinoma (ATCC), UTSCC5 originated from squamous cell carcinomas of the tongue (established at the University Turku, Finland) and SAS derived from squamous cell carcinomas of the tongue (Health Science Research Resources Bank, Osaka, Japan). All cell lines including CD98hc wild-type (WT) and monoallelic knockout (maKO) clones of Cal33 RR cells were maintained in DMEM (Sigma-Aldrich, GE) containing 10% FBS (PAA Laboratories, GE) and supplemented with 1 mmol/L L-glutamine (Sigma-Aldrich, GE), 1% HEPES (1 mol/L, PAA Laboratories, GE), 1% sodium pyruvate

(100 mmol/L, Sigma-Aldrich, GE), 1% MEM nonessential amino acids (100×, Sigma-Aldrich, GE). Radioresistant (RR) cell lines of Cal33 and FaDu cells were established as described previously (24). All cell lines were cultured in a humidified 37°C incubator supplemented with 5% CO_2. The cell lines (i.e., FaDu, FaDu RR, SAS, UTSCC5, Cal33, Cal33 RR, Cal33 RR WT1, Cal33 RR WT2, Cal33 RR maKO1, Cal33 RR maKO2) were genotyped using microsatellite polymorphism analyses by Eurofins Medigenomix Forensik GmbH and tested for *Mycoplasma* directly prior to experimentation.

Statistical analysis

The results of colony formation assays, γH2AX foci assay, Seahorse metabolism analysis, metabolic mass spectrometry analysis, flow cytometry, and Western blotting were analyzed by paired *t* tests. The differences between cell survival curves were analyzed using the statistical package for the social sciences (SPSS) v23 software as described by Franken and colleagues (25) by fitting the data into the linear-quadratic formula $S(D)/S(0) = \exp(\alpha D + \beta D^2)$ using stratified linear regression. Multiple comparison analysis for the data depicted on Figs. 3A, 4A, and 5B was performed using one-way ANOVA analysis by GraphPad Prism software. A $P < 0.05$ was regarded as statistically significant. Correlation was evaluated by SUMO software using the Pearson correlation coefficient. For the presented patient cohort, the primary endpoint was LRC, which was calculated from the first day of radiotherapy to the occurrence of a local or regional recurrence or censoring. Corresponding survival curves were estimated by the Kaplan–Meier method and compared by log-rank tests. In addition, the impact of CD98 and LAT1 on LRC was evaluated using univariable and multivariable Cox regression. In multivariable regression, CD98 or LAT1 were included together with nodal stage (0,1 vs. 2,3), p16 status and the natural logarithm (ln) of tumor volume, as performed in ref. 11. Patients were classified according to the fraction of CD98- or LAT1-positive tumor cells using the cutoff ≥10%. This cutoff was adapted from the literature (26) to give the highest power for a significant patient stratification regarding LRC as described in ref. 17.

Results

CD98hc protein expression identifies a poor prognosis subgroup in patients with HNSCC after primary radiochemotherapy

Previous retrospective multicenter studies demonstrated that *SLC3A2* mRNA levels are significantly associated with LRC in patients with locally advanced HNSCC, who were treated with PORT-C or primary RCTx (11, 17). As the correlation between mRNA and protein levels has been reported to be notoriously low (27), we attempted to assess the levels of the *SLC3A2*-encoding protein CD98hc by IHC analysis of tumor tissues from patients with locally advanced HNSCC treated with primary RCTx ($n = 197$, DKTK cohort and monocentric Dresden cohort; Fig. 1). First, the percentage of CD98hc-positive tumor cells within the specimens was evaluated. Patients with at least 10% of CD98hc-positive tumor cells showed significantly lower LRC in univariable and multivariable analyses ($P = 0.002$ and $P = 0.005$, Fig. 1A and B; Supplementary Table S2) compared with those with less positive or negative tumors. Tumors with more intensive staining for CD98hc protein showed significantly lower LRC rates

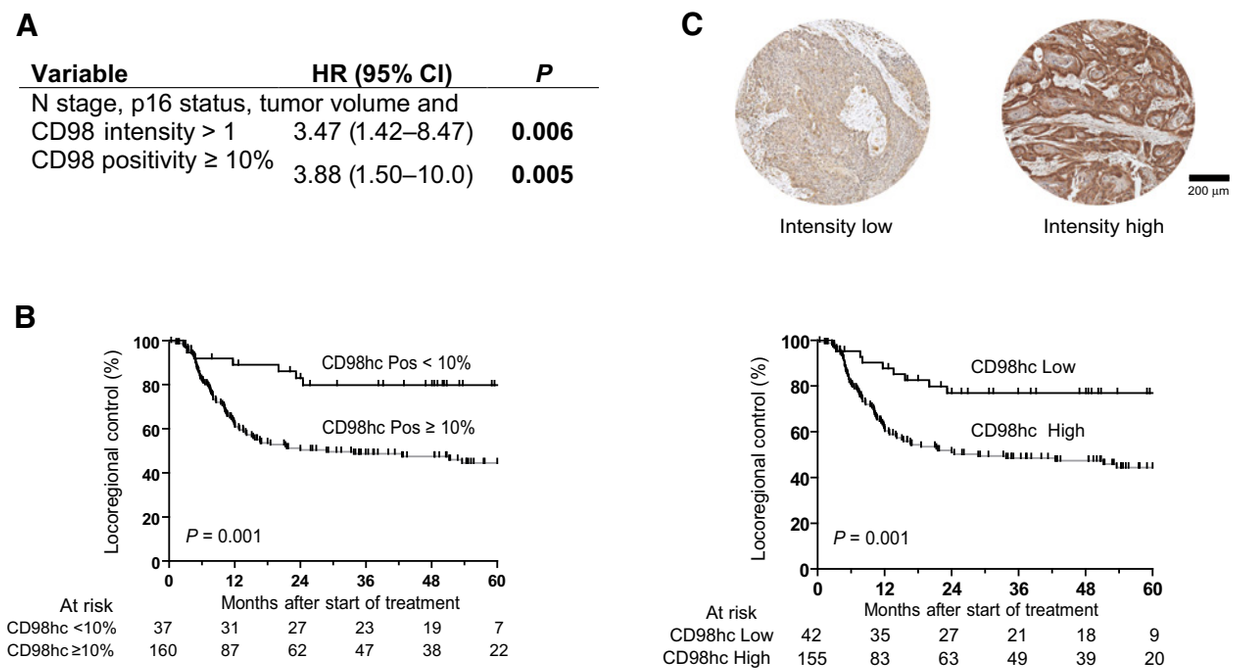


Figure 1.

Low CD98hc expression identifies a good prognosis subgroup in locally advanced HNSCC treated with primary R(C)Tx. **A,** Multivariable Cox regression of LRC. In each model, one CD98hc parameter was combined with N stage (0.1 vs. 2.3), p16 status, and the natural logarithm (ln) of tumor volume. Only the result for the CD98hc parameter is reported. 95% CI, 95% confidence interval. Kaplan–Meier estimates of LRC for patients with locally advanced HNSCC treated with primary R(C)Tx regarding percentage of CD98hc-positive tumor cells in primary tumor specimens (IHC analyses; **B**), and regarding CD98hc protein expression level (**C**).

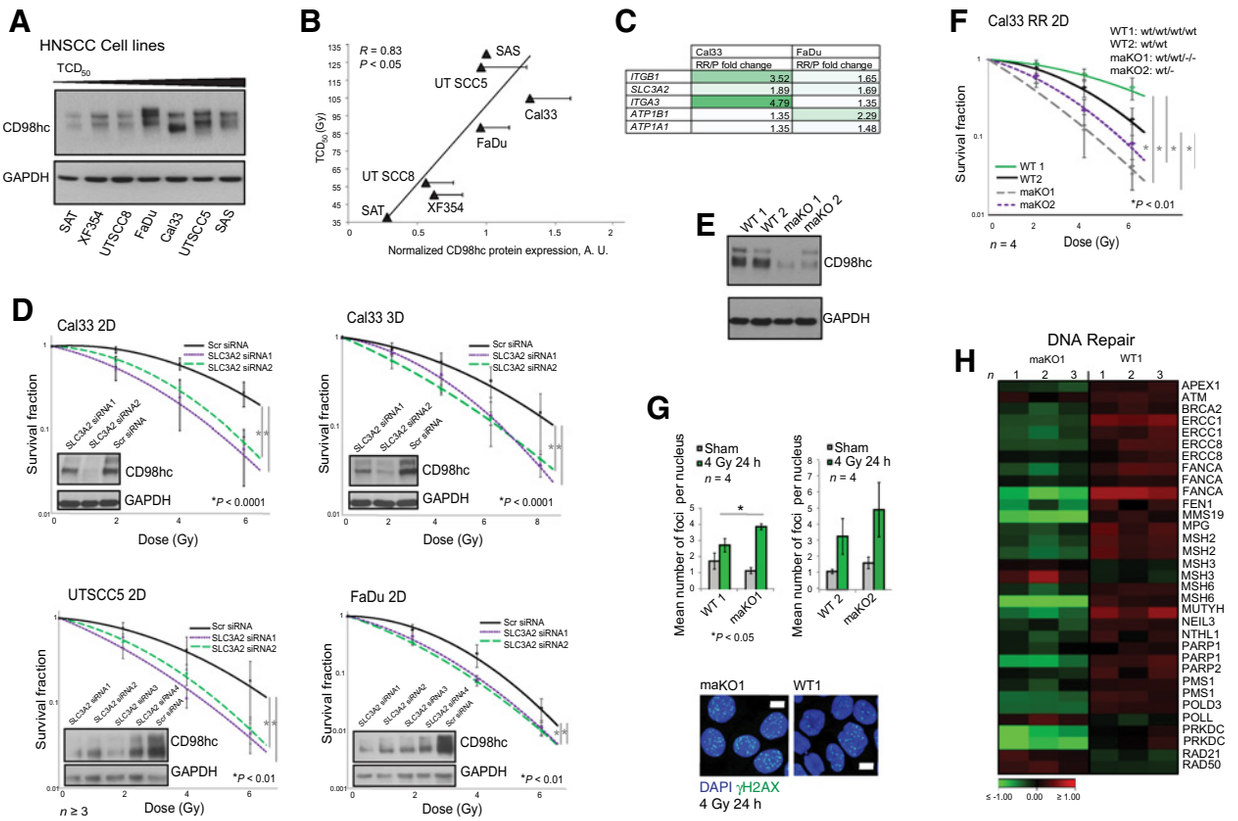


Figure 2.

Expression levels of CD98hc correlate with radiotherapy response in experimental HNSCC models. **A,** Western blot analysis of CD98hc expression in HNSCC cell lines. **B,** Correlation of TCD_{50} of xenograft tumors with expression levels of CD98hc protein in corresponding HNSCC cell lines ($R = 0.83$; $P < 0.05$; A.U., arbitrary units); error bars, SEM. **C,** CD98hc was identified by LC/MS-MS as one of the top scoring proteins upregulated in Cal33 and FaDu radioresistant (RR) sublines as compared with their parental counterparts. **D,** siRNA-mediated knockdown of *SLC3A2* expression in HNSCC cells results in cell radiosensitization. Relative cell radiosensitivity was analyzed by radiobiological clonogenic assay. Cells transfected with unspecific scrambled siRNA were used as control. Reduction of *SLC3A2* expression was validated by Western blot analysis. $n \geq 3$; error bars, SD. **E,** Validation of gene silencing by Western blot analysis. **F,** Analysis of relative cell radiosensitivity of two validated monoallelic *SLC3A2* knockout (maKO) and two wild-type (WT) clones by radiobiological clonogenic assay; error bars, SD. **G,** Analysis of residual γH2AX foci number 24 hours after irradiation or after sham irradiation; error bars, SEM. Scale bars, 10 μm. **H,** Heatmap of DNA repair genes significantly up- or downregulated in WT1 as compared with KO1 cells.

compared with those with less intensive staining in univariable analyses ($P = 0.002$, Supplementary Table S2; Fig. 1C) and in multivariable analyses ($P = 0.006$, Fig. 1A). Taken together, these data suggest that both overall expression of CD98hc in tumor bulk and the size of cell population, which is highly positive for CD98hc expression, are prognostic biomarkers of LRC in patients with locally advanced HNSCC treated with primary RCTx.

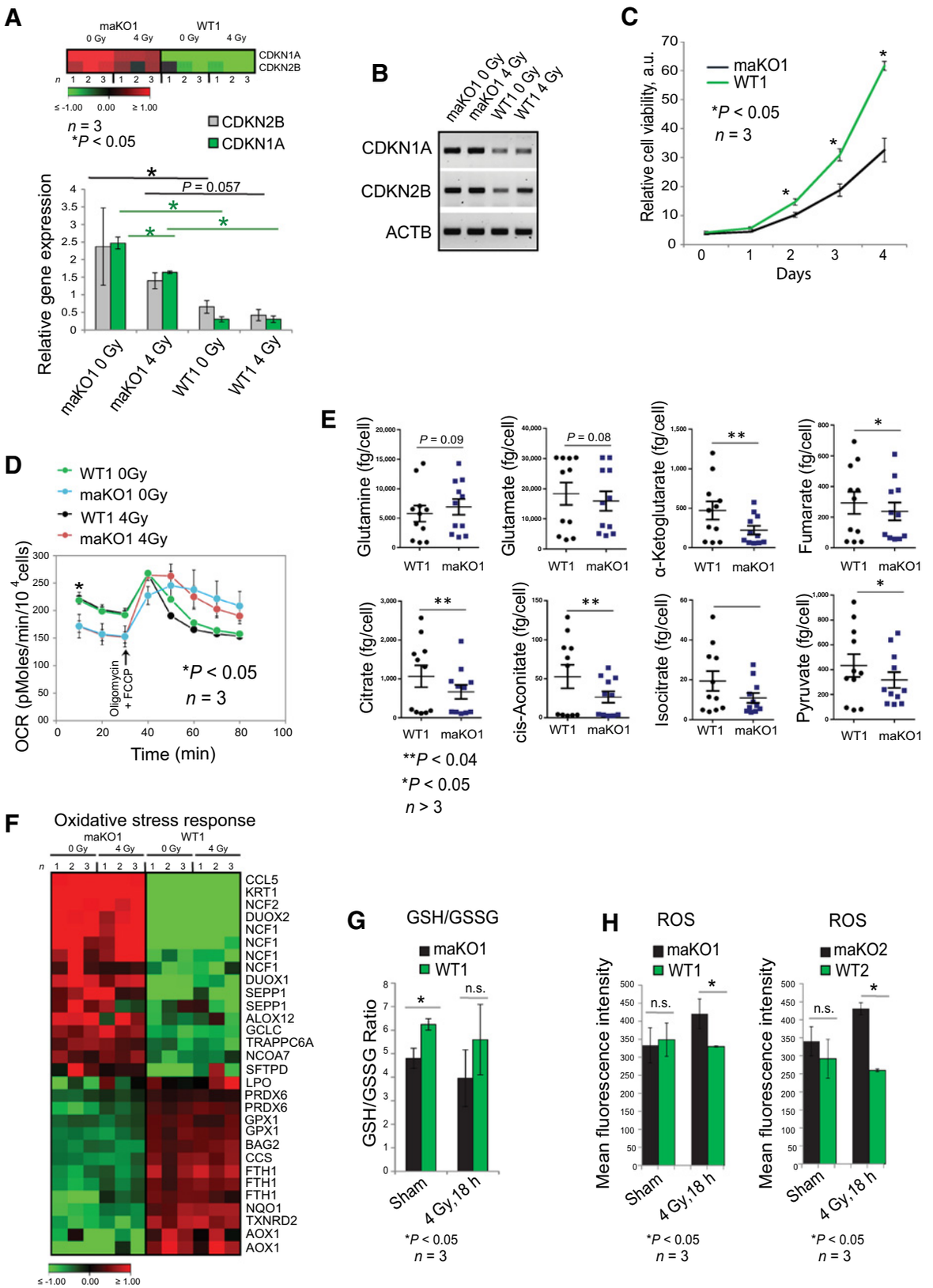
Expression level of CD98hc correlates with radiotherapy response in experimental models

Next, we analyzed whether radiation response of the established HNSCC cell lines xenografted into mice correlates with CD98hc expression. We assessed the protein expression levels of CD98hc in seven different HNSCC cell lines (SAS, UTSCC5, Cal33, FaDu, UTSCC8, XF354, and SAT) by Western blot analysis (Fig. 2A) and found a positive correlation between previously determined tumor control dose 50 (TCD_{50}) values after fractionated irradiation of HNSCC xenograft tumors (28, 29) and CD98hc protein levels in corresponding cell lines ($R = 0.83$; $P < 0.05$; Fig. 2B).

Plasma membrane proteins from the parental Cal33 and FaDu cell lines and their previously described radioresistant (RR) derivative sublines (24, 30) were enriched by differential centrifugation and analyzed by LC/MS-MS as described in refs. 31 and 32 (Supplementary Tables S3 and S4). CD98hc was identified as one of the top scoring proteins upregulated in the RR sublines as shown in Fig. 2C. Western blot analysis revealed that expression of CD98hc protein is dynamically regulated after X-ray irradiation, which can indicate its role in the tumor response to radiotherapy (Supplementary Fig. S1A).

Downregulation of CD98hc expression results in HNSCC cell radiosensitization

To validate the role of CD98hc in regulation of cell radiosensitivity, siRNA-mediated knockdown of *SLC3A2* expression in several HNSCC cell lines including Cal33, Cal33 RR, FaDu, UTSCC5, and SAS was used. The results of 2D and 3D clonogenic assays demonstrated that knockdown of CD98hc led to a significant increase of radiosensitivity in all analyzed cell lines (Fig. 2D; Supplementary Fig. S1B and S1C). Taking into account that the



effect of siRNA-mediated gene silencing is transient, CRISPR/Cas9 gene editing technology was employed to achieve a stable knockout of CD98hc expression in the Cal33 RR cell line (Supplementary Fig. S2A). Analyses of the cell clones demonstrated that homozygous knockout was not achievable. This may be explained by a nearly-tetraploid genotype of the Cal33 RR cell line and, potentially, also by the special importance of CD98hc expression for survival and proliferation. Two wild-type clones (WT1, WT2) and two clones with monoallelic knockout of *SLC3A2* expression (maKO1, maKO2) were further used. Metaphase chromosome analysis of each of these selected clones showed that both WT2 and maKO2 have a hyperdiploid karyotype, and the WT1 and maKO1 are near-tetraploid (Supplementary Fig. S2B). Therefore, WT and maKO cells were compared according to their chromosome numbers (WT1 with maKO1, and WT2 with maKO2, respectively).

Radiobiological clonogenic survival assays showed that both maKO1 and maKO2 clones are more radiosensitive than the corresponding WT1 and WT2 clones (Fig. 2E and F; Supplementary Fig. S2C). Residual DNA double-strand breaks (DSB) were analyzed by γH2A.X foci count 24 hours after cell irradiation with 4 Gy of X-rays (Fig. 2G). *SLC3A2* maKO cell lines showed less efficient DNA DSB repair as compared with their wild-type counterparts. Gene expression analyses showed that the *SLC3A2* loss-of-function phenotype is associated with downregulation of key DNA repair genes (Fig. 2H).

SLC3A2 maKO cells showed inhibition of amino acid metabolism and induction of oxidative stress

Gene expression analyses showed that the *SLC3A2* maKO1 phenotype is associated with inhibition of cell proliferation and deregulation of amino acid metabolism (Fig. 3A–C; Supplementary Fig. S3A). Cell energy metabolic analyses using seahorse technology revealed that maKO cells have a low basal oxygen consumption rate (OCR) and a more quiescent phenotype, which is in line with a reduced proliferation rate of maKO1 compared with WT1 cells (Fig. 3C and D; Supplementary Fig. S3B).

The intracellular levels of Krebs cycle metabolites were measured in *SLC3A2* maKO1 and WT1 cells by LC/MS-MS (33). Most Krebs cycle metabolites such as α-ketoglutarate, fumarate, citrate, cis-aconitate, isocitrate, and pyruvate were found to be significantly downregulated in *SLC3A2* maKO1 cells, whereas the level of glutamine was increased. These data demonstrate that maKO1 cells have a decreased CD98hc/LAT1-mediated uptake of amino acids, which serve as a source for Krebs cycle intermediates and are transported in exchange with internal glutamine (Fig. 3E; Supplementary Fig. S3C). Inhibition of amino acid transport resulted in the activation of p53 signaling mechanisms and in the increase of expression of the common stress-responsive transcriptional factor *ATF3* (Supplementary Fig. S4A–S4D). Of importance, *ATF3* expression was found to be downregulated in HNSCC-radioresistant cell lines (Supplementary Fig. S4E).

A recent study demonstrated that CD98hc-associated amino acid transport is crucial to control reactive oxygen species (ROS; Supplementary Fig. S3C; ref. 34). Gene expression analysis of maKO1 and WT1 cells showed that *SLC3A2* loss-of-function results in deregulation of a number of genes involved in oxidative stress response (Fig. 3F). To determine whether CD98hc silencing disrupts redox balance in HNSCC cells, the levels of ROS, as well as reduced and oxidized glutathione (GSH and GSSG, respectively) were measured in SLC3A2 maKO and WT cell lines. Reduced CD98hc expression was associated with decreased basal GSH/GSSG ratio and significant upregulation of ROS after irradiation (Fig. 3G and H).

The role of LAT1-dependent amino acid transport for regulation of cell radioresistance is mediated by CD98hc

CD98hc (*SLC3A2)* and LAT1 (*SLC7A5*) constitute a heterodimeric transmembrane complex that mediates amino acid transport (18, 34, 35). Previous studies showed that CD98hc plays a crucial role for transport activity by regulating LAT1 trafficking to the plasma membrane (36–38). Indeed, analyses of the LAT1 and CD98hc protein expression in *SLC3A2* maKO and WT cell lines revealed a more pronounced cytoplasmic localization of LAT1 in maKO cells compared with WT clones (Fig. 4A). Analyses of tumor tissues from patients with locally advanced HNSCC treated with primary R(C)Tx ($n = 63$; Dresden monocentric cohort) showed a significant correlation of membrane localization of CD98hc and LAT1 proteins (Fig. 4B). In addition to the membrane colocalization, protein and mRNA levels of CD98hc also significantly correlate with LAT1 expression in *SLC3A2* maKO and WT clones and in HNSCC cell lines ($R = 0.78$; $P < 0.05$; Fig. 4C and D; Supplementary Fig. S5A and S5B). Similar to CD98hc, TCD_{50} values of the xenograft models were found to significantly correlate with expression levels of LAT1 in the corresponding HNSCC cell lines ($R = 0.73$; $P < 0,05$; Fig. 4E).

Recent studies showed that the tumorigenic potential of CD98hc KO cells can be attributed to residual LAT1 transport activity (34). To validate the role of LAT1-mediated amino acid transport in regulation of radiation response in *SLC3A2*-deficient cells, *SLC3A2* maKO1 and WT1 cell lines were transfected with *SLC3A2* siRNA or *SLC7A5* siRNA alone or in combination, and analyzed by clonogenic survival assays after different doses of X-rays (Supplementary Fig. S6A and S6B). Knockdown of both proteins resulted in a significant increase in cell radiosensitivity. Strikingly, the effect of *SLC7A5* knockdown on cell radiosensitivity depends on the levels of CD98hc protein and is more pronounced in WT cells. Knockdown of expression of both *SLC3A2* and *SLC7A5* results in inhibition of the mTOR signaling pathway in WT cells (Fig. 4F and G). In contrast, *SLC3A2* maKO cells showed low basal level of PI3K/mTOR pathway activation, which was not inhibited but rather increased upon *SLC3A2* or *SLC7A5* knockdown (Fig. 4F

Figure 3.

SLC3A2 maKO cells have deregulated expression of genes involved in DNA repair, proliferation, and oxidative stress response. **A,** Gene expression analysis of maKO1 and WT1 cells showed that *SLC3A2* loss-of-function results in upregulation of the cell-cycle inhibitors *CDKN1A* and *CDKN2B*; error bars, SD. **B,** PCR analysis validating the expression levels of *CDKN1A* and *CDKN2B*. **C,** Reduced proliferation rate in *SLC3A2* maKO1 cells compared with WT1 cells; error bars, SEM. **D,** Agilent Seahorse XF showed that reduced *SLC3A2* expression is associated with low basal oxygen consumption rate (OCR); error bars, SEM. **E,** The intracellular levels of Krebs cycle metabolites and amino acids glutamine and glutamate measured in maKO1 and WT1 by LC/MS-MS. **F,** Gene expression analysis showed that *SLC3A2* loss of function results in deregulation of the genes involved in oxidative stress response. **G,** *SLC3A2* maKO cells have a low intracellular GSH/GSSG ratio; error bars, SD. **H,** *SLC3A2* maKO cells have a high intracellular level of ROS after irradiation with 4 Gy of X-rays; error bars, SD.

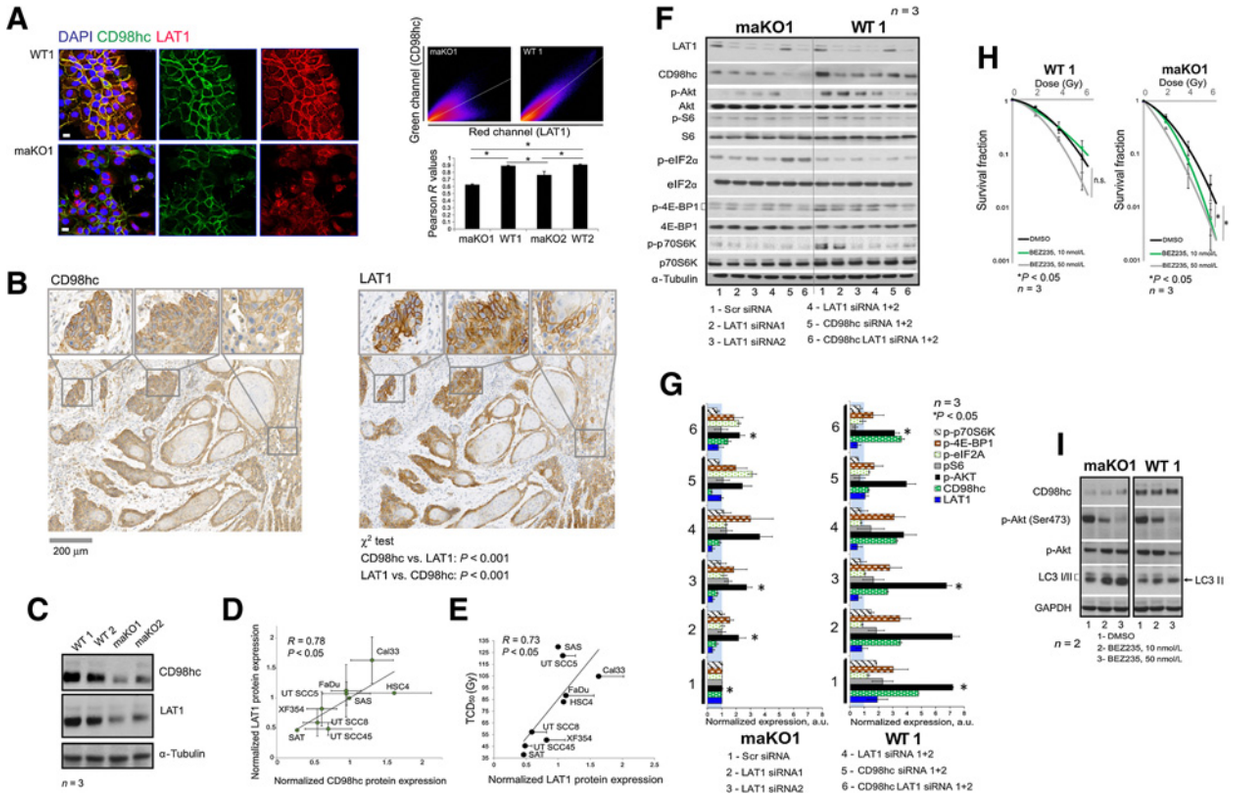


Figure 4.

LAT1 and CD98hc colocalize in HNSCC cell lines and tumor tissues and regulate cell radioresistance by activation of mTOR signaling pathway. **A,** Fluorescence microscopy analysis revealed more cytoplasmic localization of LAT1 in maKO cells as compared with WT clones; error bars, SEM. Scale bars, 25 µm. **B,** Correlation of CD98hc and LAT1 membrane expression levels in HNSCC tumor tissues from patients with locally advanced HNSCC treated with primary RCTx ($n = 63$). **C,** Expression of CD98hc and LAT1 proteins are coregulated in WT and maKO cell lines. **D,** Expression levels of CD98hc and LAT1 proteins correlate in nine HNSCC cell lines; error bars, SEM. **E,** Expression levels of LAT1 in HNSCC cell lines significantly correlate with TCD_{50} values of the corresponding xenograft models; error bars, SEM. **F,** Knockdown of expression of *SLC3A2* and *SLC7A5* genes results in the inhibition of mTOR signaling pathway. **G,** Quantification of the Western blot data; error bars, SEM. **H,** Targeting of the PI3K/Akt/mTOR pathway with BEZ235 results in significant increase in radiosensitivity of maKO1 and WT1 cells. Cells were pretreated with BEZ235 for 72 hours; error bars, SD. **I,** Representative Western blot analysis of maKO1 and WT1 cells treated with BEZ235 inhibitor for 24 hours.

and G). To test whether low basal activation levels of the prosurvival PI3K/mTOR signaling pathways may lead to a higher sensitivity of *SLC3A2* maKO1 cells to inhibition, the effect of the dual PI3K/mTOR inhibitor BEZ235 was investigated. It was found to be more potent for the inhibition of viability of *SLC3A2* maKO1 cells as compared with their WT1 counterparts (Supplementary Fig. S6C). Consistent with these results, targeting of the PI3K/mTOR pathways with BEZ235 yielded a significant increase in cell radiosensitivity only in maKO1 cells (Fig. 4H; Supplementary Fig. S6D). Of importance, treatment of maKO1 cells with BEZ235 results in activation of autophagy, a prosurvival mechanism which was also described for other types of cancer after inhibition of PI3K/AKT/mTOR pathway (Fig. 4I; refs. 39–42). Taken together, these data suggest that expression of CD98hc in HNSCC is crucial for the LAT1 membrane localization, activation of the prosurvival mTOR/PI3K signaling pathway and regulation of cell radiosensitivity. Similar to knockout of CD98hc, inhibition of mTOR/PI3K signaling results in increase of cell radiosensitivity and induces autophagy as a mechanism of cellular stress response.

Activation of autophagy as a prosurvival mechanism in CD98hc-deficient cells

Gene expression analysis revealed that autophagy-associated genes are activated in *SLC3A2* maKO1 cells (Supplementary Fig. S7A). A high basal level of autophagy in maKO cells was confirmed by Western blotting and by cytometry-based analysis of Autophagy Green as an autophagosome marker (Fig. 5A and B; ref. 43). In contrast to WT1 cells, which showed radiation-induced autophagy, maKO1 cells exhibit a high basal level of autophagy, which did not further increase after irradiation (Fig. 5B).

Knockdown of the key autophagy-regulating protein ATG5 has been previously shown to suppress autophagy in HNSCC cells (44). Furthermore, ATG5 was found to be significantly overexpressed in HNSCC-radioresistant cells as compared with their parental counterparts (Supplementary Fig. S7B). Analysis of the TCGA gene expression dataset for 517 patients with HNSCC revealed that low expression of *ATG5* significantly correlates with better overall survival (Supplementary Fig. S7C). Inhibition of autophagy by knockdown of *ATG5* and by Bafilomycin A1 (45) in WT1 and maKO1 cells resulted in significant radiosensitization in both cell lines (Fig. 5C–F; Supplementary Fig. S7D and S7E).

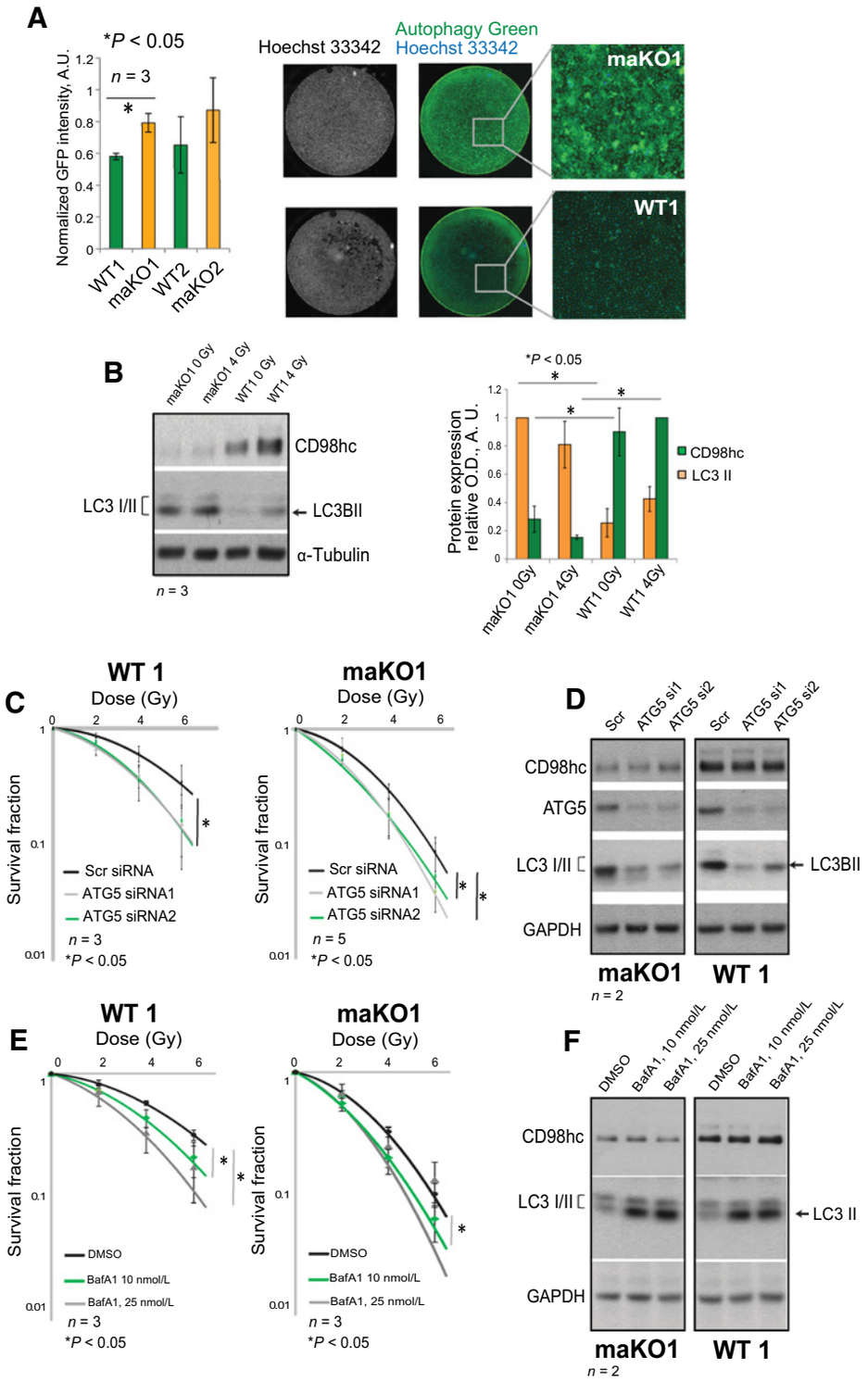


Figure 5.

Activation of autophagy as a prosurvival mechanism. **A,** Celigo cytometry-based analysis of autophagy in maKO and WT cells; error bars, SEM. **B,** Autophagy level in WT1 cells significantly increases in response to irradiation; error bars, SEM. **C,** Radiobiological clonogenic analysis of maKO1 and WT1 cell lines after transfection with ATG5 siRNA; error bars, SD. **D,** Representative Western blot analysis of maKO1 and WT1 cells after transfection with ATG5 siRNA. **E,** Inhibition of autophagy with Bafilomycin A1 (BafA1) results in significant increase in cell radiosensitivity in maKO1 and WT1 cells. Cells were treated for 3 hours and irradiated 2 hours after start of treatment; error bars, SD. **F,** Representative Western blot analysis of maKO1 and WT1 cells treated with Bafilomycin A1 (BafA1) for 3 hours.

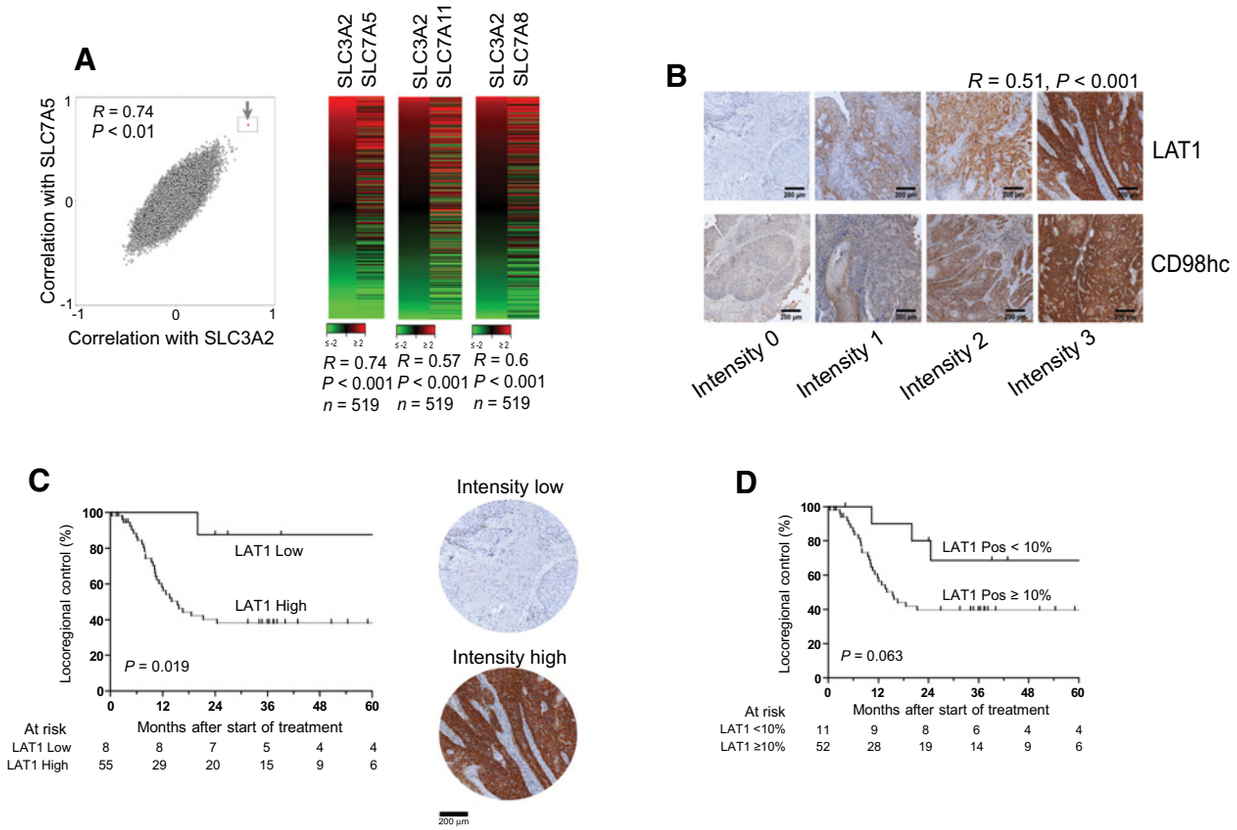


Figure 6.

Expression of light subunit LAT1 correlates with CD98hc expression and identifies a poor prognosis subgroup in patients with HNSCC after primary R(C)Tx. **A,** Analysis of the TCGA dataset for patients with HNSCC shows a significant correlation of CD98hc gene (*SLC3A2*) expression with expression of other CD98hc-associated amino acid transporters, such as LAT1 (*SLC7A5*), LAT2 (*SLC7A8*), and xCT (*SLC7A11*). **B,** Expression levels of CD98hc and LAT1 proteins are highly correlated in patients receiving primary R(C)Tx. Kaplan–Meier estimates of LRC for patients receiving primary R(C)Tx regarding LAT1 protein expression (**C**), and percentage of LAT1-positive tumor cells in primary tumor specimens (IHC analyses; **D**).

Autophagy inhibitors Bafilomycin A1 and Chloroquine, which prevent autolysosome maturation more potently reduce the viability of *SLC3A2* maKO1 cells compared with their WT counterparts (Supplementary Fig. S7F). Consistent with these results, treatment with Bafilomycin A1 resulted in activation of apoptosis with more pronounced effect in maKO1 than in WT cells (Supplementary Fig. S7G). Taken together, these data suggest that activation of autophagy in CD98hc-deficient cells acts as a pro-survival mechanism, and its inhibition could be an effective approach for tumor cell radiosensitization.

Correlation of LAT1 expression with LRC after primary RCTx in patients with HNSCC

Expression levels of LAT1 and CD98hc were significantly correlated in the HNSCC cell lines studied here (Fig. 4C and D). Consistent with these data, analysis of the TCGA HNSCC dataset ($n = 519$) revealed the highest correlation between the expression values of *SLC3A2* and *SLC7A5* compared with the correlation of these genes' expression to the rest of the tumor transcriptome (Fig. 6A). This analysis also revealed a significant correlation of the *SLC3A2* gene and other CD98hc-associated amino acid transporters such as LAT2 (*SLC7A8*) and xCT (*SLC7A11*; Fig. 6A). IHC analyses of tumor tissues from patients with locally advanced HNSCC treated with primary R(C)Tx confirmed that expression levels of CD98hc and LAT1 proteins are highly correlated ($P < 0.001$; monocentric Dresden cohort; Fig. 6B; Supplementary Table S5). In univariate analyses, LAT1 overexpression was also found to be significantly associated with poor LRC (all patients: $P = 0.047$; patients with HPV-negative HNSCC only: no events, Cox model did not converge; Supplementary Table S2; Fig. 6C). A statistical trend was also revealed for an association of the percentage of LAT1-positive tumor cells with LRC. Patients with at least 10% of LAT1-positive tumor cells were found to have a poor prognosis compared with those with low or negative LAT1 staining (all patients: $P = 0.077$; Supplementary Table S2; Fig. 6D). However, in multivariate analyses corrected for N stage, tumor volume and p16 status, no significant impact on LRC was revealed for LAT, which may, in part, be explained by the small patient cohort (Supplementary Table S6).

Discussion

Our previous studies demonstrated that mRNA levels of *SLC3A2* gene are significantly associated with LRC in patients with locally advanced HNSCC who received PORT-C or primary RCTx (11). In this study, we found that the protein levels of

CD98hc correlate with the TCD_{50} of HNSCC xenograft tumors, which is a functional endpoint of CSC inactivation *in vivo* (46). Consistent with these data, IHC analysis of pretherapeutic tumor specimens from patients with locally advanced HNSCC, who underwent primary R(C)Tx revealed a correlation between LRC and CD98hc protein levels. Furthermore, high expression levels of CD98hc protein were found to be associated with a poor prognosis after primary RCTx compared with those patients with low CD98hc-expressing tumors.

A recent study by Rietbergen and colleagues has been shown that CD98 overexpression in p16/HPV16 DNA-positive oropharyngeal carcinoma is significantly associated with decreased overall survival and shorter progression-free survival (26). We could not show this effect, which is likely due to the low number of p16-positive/HPV16 DNA-positive oropharyngeal tumors in the cohorts analyzed in our study. Our cohorts are mainly composed of patients with HPV-negative HNSCC that is characterized by a less favorable outcome after radio(chemo)therapy. Additional biomarkers and treatment modifications for patients with HPV-negative HNSCC are urgently needed to improve outcome of RCTx in these patients (13, 16, 17).

The curative potential of radiotherapy depends on its ability to induce irreparable DNA damage in tumor cells by direct ionization of DNA or by production of ROS (47, 48). For the defense against ROS-induced oxidative stress, cancer cells produce a high level of an antioxidant GSH (49). CD98-related amino acid transporter complexes such as CD98hc/xCT are essential for the transport of cystine and thus for the synthesis of GSH and control of ROS level (50). Our study showed that downregulated CD98hc expression is associated with a significant decrease in the level of reduced GSH and upregulation of ROS levels after irradiation suggesting that CD98hc is important for tumor cell protection against oxidative stress.

Restriction of amino acid availability in CD98hc-deficient cells, which is associated with inhibition of mTOR signaling and decreased levels of Krebs cycle intermediates as a source of energy and biosynthesis, also contributes to the activation of autophagy. When autophagy is activated upon amino acid restriction, cells start to utilize its own components, which provide the building blocks and energy to survive (51). Our data suggest that restriction of amino acid availability in CD98hc-deficient cells is associated with inhibition of mTOR/PI3K signaling and significant increase in basal levels of autophagy, which can be increased further by mTOR/PI3K inhibition. In agreement with these findings, recent studies revealed that targeting of mTOR in HNSCC results in feedback autophagy activation, which serves as a prosurvival mechanism (40–42). Conversely, autophagy inhibition can sensitize HNSCC cells to anticancer therapies targeting mTOR/PI3K signaling (40, 41). Consistently, we found that expression levels of one of the key autophagy regulators, the *ATG5* gene correlates with overall survival of patients with HNSCC, and inhibition of autophagy by *ATG5* knockdown or by Bafilomycin A1 resulted in tumor cell radiosensitization and induction of apoptosis. Interestingly, global gene expression analyses revealed that cells with monoallelic knockout of *SLC3A2* show significantly decreased expression levels of key DNA repair genes and upregulation of the wild-type p53-dependent signaling. This might suggest additional mechanisms for the CD98-dependent regulation of tumor radiosensitivity that warrant further studies. For further discussion on the possible role of CD98hc for the p53-dependent signaling, see Supplementary Data. Of note, we did not reveal a correlation between overexpression of CD98hc and 11q13 chromosomal locus amplification, which is common in HNSCC and is associated with a deregulation of DNA damage response in HNSCC (Supplementary Fig. S8A and S8B; ref. 52).

We found a significant coregulation and membrane colocalization of CD98hc and LAT1 in HNSCC cell lines and tumor tissues. This is in line with previous observations showing that expression of CD98hc and LAT1 are coregulated, and that CD98hc is required for the membrane localization of LAT1 (53). Inhibition of LAT1 expression resulted in a significant downregulation of cell radioresistance only in wild-type but not in *SLC3A2* maKO cells, suggesting that the role of LAT1 in regulating tumor radiosensitivity may be mediated by the CD98hc protein. IHC analysis of LAT1 protein expression in tumor tissues from patients with locally advanced HNSCC treated with primary RCTx showed poor LRC rates in patients with high LAT1 expression levels. Analysis of the TCGA dataset for patients with HNSCC revealed a significant correlation of the *SLC3A2* gene and other CD98hc-associated amino acid transporters such as LAT2 and xCT. The potential role of these genes as biomarkers for radiotherapy warrants further investigation.

Previously published data demonstrated the role of CD98 as a marker of the HNSCC CSC population (22). Consistent with this finding, our data suggest that not only the overall expression of CD98hc in the tumors, but also the size of CD98hc-positive cell populations are prognostic biomarkers of LRC in patients with locally advanced HNSCC treated with primary R(C)Tx. Overall, our findings indicate that CD98hc is not only a potential biomarker of the CSC number, but also a promising target for tumor radiosensitization. Current clinical trials are testing CD98-targeted treatment in a variety of malignancies (Supplementary Table S7). Integration of such approaches into radiotherapy might open up a promising avenue for clinical translational studies.

Future research is needed to validate our findings by using additional *SLC3A2* knockout cell cultures, by combination of CD98hc-targeted therapy with radiotherapy in patient-derived HNSCC xenograft mouse models and by prospective validation of CD98hc and LAT1 expression levels as prognosticators of LRC after radio(chemo)therapy.

Disclosure of Potential Conflicts of Interest

M. Baumann attended an advisory board meeting of Merck KGaA (Darmstadt), for which the University of Dresden received a travel grant; received funding for his research projects and for educational grants to the University of Dresden by Teutopharma GmbH, IBA, Bayer AG, Merck KGaA, and Medipan GmbH; as former chair of OncoRay (Dresden) and present CEO and Scientific Chair of the German Cancer Research Center (DKFZ, Heidelberg), signed/s contracts for his institute(s) and for the staff for research funding and collaborations with a multitude of companies worldwide; and for the German Cancer Research Center (DKFZ, Heidelberg), is on the supervisory boards of HI-STEM gGmbH (Heidelberg). None of these funding sources were involved in the study design or materials used, nor in the collection, analysis and interpretation of data nor in the writing of the paper.

M. Krause received funding for her research projects by Merck KGaA and Medipan GmbH, and as chair of OncoRay - National Center for Radiation Research in Oncology she signs contracts for her institutes and for the staff for research funding and collaborations with a multitude of companies worldwide. None of these funding sources were involved in the present study. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D. Digomann, I. Kurth, L.A. Kunz-Schughart, M. Baumann, A. Linge, A. Dubrovska

Development of methodology: D. Digomann, I. Kurth, A. Tyutyunnykova, C. Peitzsch, A. Linge, A. Dubrovska
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Digomann, I. Kurth, A. Tyutyunnykova, O. Chen, I. Gorodetska, C. Peitzsch, I.-I. Skvortsova, B. Aschenbrenner, G. Eisenhofer, S. Richter, S. Heiden, J. Porrmann, A.A. Dowle, L. Hein, F. Lohaus, A. Linge, A. Dubrovska
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Digomann, I. Kurth, A. Tyutyunnykova, S. Löck, C. Peitzsch, I.-I. Skvortsova, G. Negro, B. Aschenbrenner, S. Richter, B. Klink, A.A. Dowle, A. Abdollahi, A. Linge, A. Dubrovska
Writing, review, and/or revision of the manuscript: D. Digomann, A. Tyutyunnykova, O. Chen, S. Löck, I.-I. Skvortsova, G. Eisenhofer, S. Richter, B. Klink, A. Abdollahi, F. Lohaus, M. Krause, M. Baumann, A. Linge, A. Dubrovska
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Digomann, I.-I. Skvortsova, G. Negro, C. Schwager, L.A. Kunz-Schughart, F. Lohaus, M. Krause, M. Baumann, A. Linge, A. Dubrovska
Study supervision: I. Kurth, M. Krause, M. Baumann, A. Linge, A. Dubrovska

Acknowledgments

The authors thank Vasyl Lukiyanchuk for analysis of gene expression data, TCGA datasets and Celigo imaging, co-localization analysis, PCR analysis of DNA repair genes as well as for design and cloning of sgRNAs, screening for clones with SLC3A2 deletion, and their genotyping. They thank Liane Stolz-Kieslich for her technical assistance with IHC staining. They thank Lydia Koi (DKTK) for providing the HNSCC cell samples and Aliona Bogdanova (MPI-CBG, Dresden) for providing the Cas9-HF1 plasmid construct. They also thank the DKTK-ROG for providing access to the tumor material and to the clinical data of the subset of patients from previously published work (11). Work in the Dubrovska laboratory was partially supported by grants from the Deutsche Forschungsgemeinschaft (DFG; 273676790, 401326337, and 416001651), from Wilhelm Sander-Stiftung (2017.106.1), the DLR Project Management Agency (01DK17047), and the German Federal Ministry of Education and Science (BMBF; 03Z1NN11). D. Digomann was supported by the Preiss Daimler foundation Medical Equipment and Research and by the *Else Kröner*-Promotionskolleg. The York Centre of Excellence in Mass Spectrometry was created thanks to a major capital investment through Science City York, supported by Yorkshire Forward with funds from the Northern Way Initiative, and subsequent support from EPSRC (EP/K039660/1; EP/M028127/1). Work in Baumann and Krause laboratories was supported by German Federal Ministry of Education and Science (BMBF, Oncoray), the German Cancer Consortium DKTK, and by the Deutsche Forschungsgemeinschaft (DFG) grants BA 1433/5-2 and BA 1433/6.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 7, 2018; revised December 12, 2018; accepted January 17, 2019; published first January 22, 2019.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359–86.
2. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer 2011;11:9–22.
3. Brockstein B, Haraf DJ, Rademaker AW, Kies MS, Stenson KM, Rosen F, et al. Patterns of failure, prognostic factors and survival in locoregionally advanced head and neck cancer treated with concomitant chemoradiotherapy: a 9-year, 337-patient, multi-institutional experience. Ann Oncol 2004; 15:1179–86.
4. Begg AC. Predicting recurrence after radiotherapy in head and neck cancer. Semin Radiat Oncol 2012;22:108–18.
5. Department of Veterans Affairs Laryngeal Cancer Study Group, Wolf GT, Fisher SG, Hong WK, Hillman R, Spaulding M, et al. Induction chemotherapy plus radiation compared with surgery plus radiation in patients with advanced laryngeal cancer. N Engl J Med 1991;324:1685–90.
6. Forastiere AA, Goepfert H, Maor M, Pajak TF, Weber R, Morrison W, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med 2003;349:2091–8.
7. Bernier J, Domenge C, Ozsahin M, Matuszewska K, Lefebvre JL, Greiner RH, et al. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. N Engl J Med 2004;350: 1945–52.
8. Corvo R. Evidence-based radiation oncology in head and neck squamous cell carcinoma. Radiother Oncol 2007;85:156–70.
9. Razzouk S. Translational genomics and head and neck cancer: toward precision medicine. Clin Genet 2014;86:412–21.
10. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92:709–20.
11. Linge A, Lohaus F, Löck S, Nowak A, Gudziol V, Valentini C, et al. HPV status, cancer stem cell marker expression, hypoxia gene signatures and tumour volume identify good prognosis subgroups in patients with HNSCC after primary radiochemotherapy: A multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). Radiother Oncol 2016;121:364–73.
12. Kimple RJ, Harari PM. The prognostic value of HPV in head and neck cancer patients undergoing postoperative chemoradiotherapy. Ann Transl Med 2015;3(Suppl 1):S14.
13. Lohaus F, Linge A, Baumann M. HPV and beyond-looking out for biomarkers for distinguishing the good prognosis from the bad prognosis group in locally advanced and clinically high risk HNSCC. Ann Transl Med 2015;3:255.
14. Brandwein-Gensler M, Smith RV. Prognostic indicators in head and neck oncology including the new 7th edition of the AJCC staging system. Head Neck Pathol 2010;4:53–61.
15. Worsham MJ. Identifying the risk factors for late-stage head and neck cancer. Expert Rev Anticancer Ther 2011;11:1321–5.
16. Lohaus F, Linge A, Tinhofer I, Budach V, Gkika E, Stuschke M, et al. HPV16 DNA status is a strong prognosticator of loco-regional control after postoperative radiochemotherapy of locally advanced oropharyngeal carcinoma: results from a multicentre explorative study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). Radiother Oncol 2014;113:317–23.
17. Linge A, Löck S, Gudziol V, Nowak A, Lohaus F, von Neubeck C, et al. Low cancer stem cell marker expression and low hypoxia identify good prognosis subgroups in HPV(-) HNSCC after postoperative radiochemotherapy: a multicenter study of the DKTK-ROG. Clin Cancer Res 2016;22: 2639–49.
18. Napolitano L, Scalise M, Galluccio M, Pochini L, Albanese LM, Indiveri C. LAT1 is the transport competent unit of the LAT1/CD98 heterodimeric amino acid transporter. Int J Biochem Cell Biol 2015;67:25–33.
19. Verrey F, Jack DL, Paulsen IT, Saier MH Jr, Pfeiffer R. New glycoprotein-associated amino acid transporters. J Membr Biol 1999;172:181–92.
20. Rosell A, Meury M, Alvarez-Marimon E, Costa M, Perez-Cano L, Zorzano A, et al. Structural bases for the interaction and stabilization of the human amino acid transporter LAT2 with its ancillary protein 4F2hc. Proc Natl Acad Sci U S A 2014;111:2966–71.
21. Lewerenz J, Hewett SJ, Huang Y, Lambros M, Gout PW, Kalivas PW, et al. The cystine/glutamate antiporter system x(c)(-) in health and disease: from molecular mechanisms to novel therapeutic opportunities. Antioxid Redox Signal 2013;18:522–55.
22. Martens-de Kemp SR, Brink A, Stigter-van Walsum M, Damen JM, Rustenburg F, Wu T, et al. CD98 marks a subpopulation of head and neck squamous cell carcinoma cells with stem cell properties. Stem Cell Res 2013;10:477–88.
23. Ip H, Sethi T. CD98 signals controlling tumorigenesis. Int J Biochem Cell Biol 2016;81(Pt A):148–50.

24. Kurth I, Hein L, Mabert K, Peitzsch C, Koi L, Cojoc M, et al. Cancer stem cell related markers of radioresistance in head and neck squamous cell carcinoma. Oncotarget 2015;6:34494–509.
25. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells *in vitro*. Nat Protoc 2006;1:2315–9.
26. Rietbergen MM, Martens-de Kemp SR, Bloemena E, Witte BI, Brink A, Baatenburg de Jong RJ, et al. Cancer stem cell enrichment marker CD98: a prognostic factor for survival in patients with human papillomavirus-positive oropharyngeal cancer. Eur J Cancer 2014;50:765–73.
27. Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 2003;4:117.
28. Koch U, Hohne K, von Neubeck C, Thames HD, Yaromina A, Dahm-Daphi J, et al. Residual gammaH2AX foci predict local tumour control after radiotherapy. Radiother Oncol 2013;108:434–9.
29. Koi L, Löck S, Linge A, Thurow C, Hering S, Baumann M, et al. EGFR-amplification plus gene expression profiling predicts response to combined radiotherapy with EGFR-inhibition: a preclinical trial in 10 HNSCC-tumour-xenograft models. Radiother Oncol 2017;124:496–503.
30. Cojoc M, Peitzsch C, Kurth I, Trautmann F, Kunz-Schughart LA, Telegeev GD, et al. Aldehyde dehydrogenase is regulated by beta-Catenin/TCF and promotes radioresistance in prostate cancer progenitor cells. Cancer Res 2015;75:1482–94.
31. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 2005;4:1265–72.
32. Dowle AA, Wilson J, Thomas JR. Comparing the diagnostic classification accuracy of iTRAQ, peak-area, spectral-counting, and emPAI methods for relative quantification in expression proteomics. J Proteome Res 2016;15:3550–62.
33. Richter S, Gieldon L, Pang Y, Peitzsch M, Huynh T, Leton R, et al. Metabolome-guided genomics to identify pathogenic variants in isocitrate dehydrogenase, fumarate hydratase, and succinate dehydrogenase genes in pheochromocytoma and paraganglioma. Genet Med 2018 Jul 27. [Epub ahead of print].
34. Cormerais Y, Giuliano S, LeFloch R, Front B, Durivault J, Tambutte E, et al. Genetic disruption of the multifunctional CD98/LAT1 complex demonstrates the key role of essential amino acid transport in the control of mTORC1 and tumor growth. Cancer Res 2016;76:4481–92.
35. Bhutia YD, Babu E, Ramachandran S, Ganapathy V. Amino Acid transporters in cancer and their relevance to "glutamine addiction": novel targets for the design of a new class of anticancer drugs. Cancer Res 2015;75:1782–8.
36. Nakamura E, Sato M, Yang H, Miyagawa F, Harasaki M, Tomita K, et al. 4F2 (CD98) heavy chain is associated covalently with an amino acid transporter and controls intracellular trafficking and membrane topology of 4F2 heterodimer. J Biol Chem 1999;274:3009–16.
37. Wagner CA, Lang F, Broer S. Function and structure of heterodimeric amino acid transporters. Am J Physiol Cell Physiol 2001;281:C1077–93.
38. Mastroberardino L, Spindler B, Pfeiffer R, Skelly PJ, Loffing J, Shoemaker CB, et al. Amino-acid transport by heterodimers of 4F2hc/CD98 and members of a permease family. Nature 1998;395:288–91.
39. Ji Y, Di W, Yang Q, Lu Z, Cai W, Wu J. Inhibition of autophagy increases proliferation inhibition and apoptosis induced by the PI3K/mTOR inhibitor NVP-BEZ235 in breast cancer cells. Clin Lab 2015;61:1043–51.
40. Zhou Y, Peng Y, Tang H, He X, Wang Z, Hu D, et al. Autophagy induction contributes to GDC-0349 resistance in head and neck squamous cell carcinoma (HNSCC) cells. Biochem Biophys Res Commun 2016;477:174–80.
41. Xie J, Li Q, Ding X, Gao Y. Targeting mTOR by CZ415 inhibits head and neck squamous cell carcinoma cells. Cell Physiol Biochem 2018;46:676–86.
42. D'Amato V, Rosa R, D'Amato C, Formisano L, Marciano R, Nappi L, et al. The dual PI3K/mTOR inhibitor PKI-587 enhances sensitivity to cetuximab in EGFR-resistant human head and neck cancer models. Br J Cancer 2014;110:2887–95.
43. Munafo DB, Colombo MI. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 2001;114(Pt 20):3619–29.
44. Sobhakumari A, Schickling BM, Love-Homan L, Raeburn A, Fletcher EV, Case AJ, et al. NOX4 mediates cytoprotective autophagy induced by the EGFR inhibitor erlotinib in head and neck cancer cells. Toxicol Appl Pharmacol 2013;272:736–45.
45. Mauvezin C, Neufeld TP. Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. Autophagy 2015;11:1437–8.
46. Krause M, Dubrovska A, Linge A, Baumann M. Cancer stem cells: Radioresistance, prediction of radiotherapy outcome and specific targets for combined treatments. Adv Drug Deliv Rev 2017;109:63–73.
47. Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene 2003;22:5734–54.
48. Azzam EI, Jay-Gerin JP, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett 2012;327:48–60.
49. Peitzsch C, Kurth I, Kunz-Schughart L, Baumann M, Dubrovska A. Discovery of the cancer stem cell related determinants of radioresistance. Radiother Oncol 2013;108:378–87.
50. de la Ballina LR, Cano-Crespo S, Gonzalez-Munoz E, Bial S, Estrach S, Cailleteau L, et al. Amino acid transport associated to cluster of differentiation 98 heavy chain (CD98hc) is at the cross-road of oxidative stress and amino acid availability. J Biol Chem 2016;291:9700–11.
51. White E. The role for autophagy in cancer. J Clin Invest 2015;125:42–6.
52. Parikh RA, White JS, Huang X, Schoppy DW, Baysal BE, Baskaran R, et al. Loss of distal 11q is associated with DNA repair deficiency and reduced sensitivity to ionizing radiation in head and neck squamous cell carcinoma. Genes Chromosomes Cancer 2007;46:761–75.
53. Wang Q, Holst J. L-type amino acid transport and cancer: targeting the mTORC1 pathway to inhibit neoplasia. Am J Cancer Res 2015;5:1281–94.