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

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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 (TCD50) 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

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CD98 as a Marker and Regulator of HNSCC Radiosensitivity

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 RTx (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 RTx 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 (GS; 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 cisplatin-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 Consortia 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 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). Radioreistant (RR) cell lines of Ca33 and FaDu cells were established as described previously (24). All cell lines were cultured in a humidified 37°C incubator supplemented with 5% CO2. The cell lines (i.e., FaDu, FaDu RR, SAS, UTSCC5, Ca33, Ca33 RR, Ca33 RR WT1, Ca33 RR WT2, Ca33 RR maKO1, Ca33 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.

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](image)

**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).
CD98 as a Marker and Regulator of HNSCC Radioresistance

Expression level of CD98hc correlates with radiotherapy response in experimental HNSCC models. A, Western blot analysis of CD98hc expression in HNSCC cell lines. B, Correlation of TCD50 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 ≥ 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 K01 cells.

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 (TCD50) 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...
A. Relative gene expression

B. Relative cell viability, a.u.

C. Oxidative stress response

D. OCR (pMoles/min/10 cells)

E. Glutamine and glutamate (fg/cell)

F. Oxidative stress response

G. GSH/GSSG Ratio

H. ROS Mean fluorescence intensity

CDKN1A ≤ -1.00 0.00 ≥ 1.00

C

00 50 100 150 200 250 300 OCR (pMoles/min/10 cells)

WT1 4Gy maKO1 4Gy

WT1 0Gy maKO1 0Gy

0 20 40 60 80 100 n.s.

Time (min)

α GTP

Isocitrate (fg/cell)

** P < 0.04

* P < 0.05

n > 3

maKO1 WT1

0 Gy 4 Gy 0 Gy 4 Gy

CDKN1A

CDKN2B

ACTB

01 2 3

0 10 20 30 40 50 60 70

maKO1 WT1

0 Gy 4 Gy 0 Gy 4 Gy

n = 3

* P < 0.05

P < 0.05

n = 3

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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 radiosensitivity 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 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(CT)x (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, TCD98 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).
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 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. S7A). 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.

**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 TCD50 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.

**Activating 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.
Analysis of the TCGA dataset for patients with HNSCC shows a significant percentage of LAT1-positive tumor cells in primary tumor specimens (IHC analyses; Digomann et al.).

Consistent with these data, analysis of the TCGA HNSCC dataset related in the HNSCC cell lines studied here (Fig. 4C and 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 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).

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 TCD50 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)T(x) 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/SCL are essential for the transport of cysteine 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/P13K signaling and significant increase in basal levels of autophagy, which can be increased further by mTOR/P13K 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/P13K 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 knockout 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 in vitro, 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 targeting CD98-directed 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 prognostic markers 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/sponsored 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; as former chair of OncoRay (Dresden) and present CEO and Scientific Chair of the German Cancer Research Center (DKFZ, 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.

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References


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