Epidermal growth factor receptor

Acquired resistance to cetuximab is associated with the overexpression of Ras family members and the loss of radiosensitization in head and neck cancer cells

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Abstract

Purpose: Cetuximab in combination with radiation therapy is used to treat patients with head and neck squamous cell carcinoma (HNSCC). In the present study, the mechanism of acquired resistance to cetuximab in HNSCC cells was investigated in vitro.

Material and methods: The HNSCC cell lines UT5 and SAS and UT5 cells with acquired resistance to cetuximab (UT5R9) were used. The radiotoxicity potentials of cetuximab and inhibitors of PI3K, MAPK and farnesylation were tested using a clonogenic survival assay. Western blotting was used to evaluate protein expression. The levels of EGFR ligands were detected by ELISA.

Results: Cetuximab inhibited proliferation and induced radiosensitization in UT5 cells but not in SAS cells. In comparison with UT5 cells, cetuximab-resistant SAS cells markedly overexpressed the K-Ras, H-Ras and N-Ras proteins, as detected by Western blotting. Resistance in UT5R9 cells was associated with the overexpression of the K-Ras, H-Ras and N-Ras proteins as well as an increase in the autocrine production of the EGFR ligands amphiregulin and transforming growth factor α (TGFα). UT5R9 cells were significantly more radioresistant than UT5 cells. Radioresistant UT5R9 cells were not radiosensitized by cetuximab, but knocking down H-RAS and N-RAS with siRNA and targeting Ras farnesylation using the farnesyltransferase inhibitor lonafarnib induced radiosensitization in these cells. Targeting PI3K and MEK revealed that the activation of the PI3K/Akt pathway but not the MAPK/ERK pathway is associated with radioresistance in UT5R9 cells.

Conclusion: Targeting Ras and PI3K activity improves the outcome of irradiation in cetuximab-resistant HNSCC cell lines in vitro.

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The mutation of RAS genes, especially K-RAS, has been demonstrated to be associated with a lack of response to cetuximab in colorectal cancers [27]. Therefore, the use of cetuximab as a monotherapy has been restricted to tumor patients with wild-type K-RAS [27,28]. Among HNSCC patients, the frequency of K-RAS mutations is very low [29,30]; therefore, there is no clinical recommendation for determining the K-RAS mutation status before using cetuximab [31]. In the current study, we investigated the potential role of Ras signaling in the acquired resistance to cetuximab of head and neck tumor cells in vitro. The results obtained indicate that acquired resistance to cetuximab in HNSCC cells is associated with the overexpression and enhanced signaling of Ras family members.

Materials and methods

Reagents

The antibodies against phospho-Akt (Ser-473), Akt, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, EGFR, actin, K-Ras and GAPDH; the control-siRNA and K-RAS-siRNA; the PI3K inhibitor LY294002; the MEK inhibitor PD98059; and the ELISA kits for TGFα and AREG have been previously described [32–36]. The EGFR tyrosine kinase inhibitor gefitinib was provided by Selleck Chemicals (Houston, TX, USA). Erlotinib was provided from Hoffman-La Roche Ltd. (Basel, Switzerland), and cetuximab was provided by Merck (Darmstadt, Germany). The anti-H-Ras antibody and the Ras-activity assay reagent were purchased from Millipore. The farnesyltransferase inhibitor (lonafarnib) was gratefully provided by Dr. Meier, Dept. of Dermatology, University of Tuebingen. H-RAS- and N-RAS-siRNAs were purchased from Thermo Fisher Scientific (Bonn, Germany).

Cell lines

The human HNSCC cells (UT5 and SAS cell lines) were cultured in DMEM routinely supplemented with 10% fetal calf serum and 1% penicillin–streptomycin. Cells were incubated in a humidified atmosphere of 93% air/7% CO₂ at 37°C. To establish acquired cetuximab resistance, UT5 cells were continuously treated with increasing concentrations of cetuximab. Treatment started with 5 nM of the antibody and was doubled after every cell culture passage up to 100 nM. Resistance to cetuximab was achieved after 10 passages (approximately 2 months), and the resistant cell line (UT5R2) was then incubated continuously with 100 nM cetuximab over a period of 9 months (UT5R9).

Proliferation kinetics and clonogenic assay

Cells (2 × 10⁴ cells/well) were treated with 20 nM of cetuximab or left untreated and incubated for the indicated time intervals. On days 1, 2, 4 and 6 after treatment, cells were harvested and counted. The post-irradiation survival of cells was analyzed using a colony formation assay. Pre-plated cells in 6-well plates were

Fig. 1. Acquired resistance to cetuximab is associated with accelerated proliferation and radioresistance. (A) Cultures were treated with 20 nM cetuximab or left untreated. At the indicated times after treatment, cells were counted, and the data were graphed. The data points shown represent the mean ± SD of two independent experiments (n = 6) (DT: doubling time). (B) UT5, UT5R9 and SAS cells were pretreated with cetuximab (20 nM) for 1 h and irradiated with single dose of 2 or 4 Gy. Colonies formed within 9 (UT5R and SAS cells) to 13 (UT5 cells) days were counted, and the survival fractions were calculated as previously described [55]. The data points shown represent the mean surviving fraction ±SD of two independent experiments (n = 12) (**p < 0.01, Student’s t-test).
treated with the indicated EGFR-antagonists for one hour or left untreated. Subsequently, the cells were irradiated and then incubated to assess colony formation as described previously [35].

Western blotting, Ras activation assay, preparation of conditioned medium and enzyme-linked immunosorbent assay

The detailed protocols for the Western blotting, Ras activation assay, preparation of the conditioned medium and enzyme-linked immunosorbent assay have been described previously [33,34,35].

Sequencing of K-RAS

Total RNA was isolated from cell pellets with the RNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed with the Reverse-iT 1st strand synthesis kit (ABgene, Surrey, UK) using anchored oligo-dT primers. Thereafter, sequencing was performed as described previously [36].

SIRNA transfection

SiRNA transfection was performed as described previously [37,38]. At 48 h after transfection, the cells were plated in 6-well plates, and 24 h later, the cultures were treated with 20 nM cetuximab or left untreated and then irradiated. The cells were then incubated to assess colony formation for 8 (UT5R9) and 12 (UT5) days. The colonies were then fixed, stained and counted.

Results

Acquired resistance to cetuximab is associated with increased proliferation and post-irradiation survival as well as the loss of cetuximab-induced radiosensitization

The effects of cetuximab on the proliferation and clonogenic activity of the HNSCC cell lines UT5 and UT5R9 (UT5 cells with acquired resistance to cetuximab after treatment with cetuximab for 9 months) and primary cetuximab-resistant SAS cells were investigated. The acquired resistance of UT5R9 cells to cetuximab was associated with increased proliferation activity, with a doubling time of 26 h compared with 45 h for the UT5 control cells (Fig. 1A). Cetuximab significantly inhibited the proliferation of UT5 cells but did not affect the proliferation of either UT5R9 or SAS cells (Fig. 1A). The analysis of post-irradiation survival indicated that UT5R9 cells were more radioresistant than UT5 or SAS cells, as shown by the D37, which is the dose of radiation required to reduce survival to 37% (UT5-D37 = 2.15 Gy, UT5R9-D37 = 3 Gy, SAS-D37 = 2.42). Cetuximab (20 nM) increased the radiosensitivity of UT5 cells but not SAS cells. Interestingly, and as shown for UT5R9 cells, acquired resistance to cetuximab was associated with the loss of radiosensitization (Fig. 1B).

Cetuximab resistance in HNSCC cells is associated with the overexpression of Ras family members and the autocrine production of EGFR ligands

Acquired resistance to cetuximab has been reported to be associated with the increased accumulation of EGFR in the nucleus in non-small cell lung cancer (NSCLC) cell lines [21]. As alternative mechanisms, the overexpression of erbB2 in NSCLC, HNSCC and colorectal cancer cells [25]; erbB3 in HNSCC cells [39]; and IGF1R in colorectal cancer cells [40] has also been described. In the present study, we showed that none of these mechanisms appear to be involved in the acquired resistance of HNSCC to cetuximab (Fig. S1). The lack of response to cetuximab is assumed to be linked to the mutational activation of the K-RAS gene [41]. Because cetuximab-resistant SAS cells overexpress wild-type K-RAS, the pattern of expression of different Ras isoforms, i.e., K-Ras, H-Ras and N-Ras, was analyzed in UT5 and UT5R9 cells. Although no K-RAS mutation was detected in UT5R9 cells, these cells exhibited overexpression and increased activity of K-Ras, H-Ras and N-Ras (Fig. 2A and B).

Previous results from our laboratory indicate that the continuous activation of K-Ras due to a point mutation in codon 12 or 13 mediates radioresistance in tumor cells via the autocrine activation of EGFR [34,36]. In line with these previous reports, in the present study we showed that the overexpression and activation of Ras family members is accompanied by the autocrine...
production of the EGFR ligands AREG and TGFα (Fig. 2C). The autocrine release of EGFR ligands was confirmed by the inhibition of P-Akt and P-ERK1/2 using the EGFR-TK inhibitors erlotinib and gefitinib in UT5 cells when treated with UT5R9-conditioned media (UT5R9-CM) (Fig. 2D).

**Targeting Ras abrogates the radioresistance of UT5R9 cells**

Because RAS mutations are associated with radioresistance [32,36,42], we asked whether the overexpression of wild-type RAS family members is involved in the radioresistance of UT5R9 cells. To answer this question, the farnesyltransferase inhibitor (FTI) lonafarnib and siRNAs against different RAS family members were used. As shown in Fig. 3A, lonafarnib (100 nM) strongly radiosensitized UT5R9 and SAS cells, but only a slight effect was observed in UT5 cells. The differential levels of radiosensitization are reflected by the different values of the dose-modifying factor (DMF) for UT5R9 (DMF 2.25), UT5 (DMF 1.2) and SAS (DMF 1.7) cells.

The radiosensitizing effect of lonafarnib was confirmed using an siRNA-based approach. The knockdown of H-Ras and N-Ras (Fig. 3B) led to strong significant radiosensitization of UT5R9 cells but not UT5 cells, whereas K-RAS-siRNA induced only slight radiosensitization.

It is known that the autocrine activation of EGFR leads to the activation of the PI3K/Akt and MAPK/ERK pathways [34]. In this study, we investigated the role of these pathways in the radioresistance of UT5R9 cells. To this end, the radiosensitizing effects of the PI3K inhibitor LY294002 and the MEK inhibitor PD98059 were analyzed. As shown in Fig. S2A, the inhibition of PI3K by LY294002 induced radiosensitization in both UT5R9 and SAS cells. The LY294002-dependent $D_{0.7}$ value was 2 Gy for UT5 cells, 1.65 Gy for UT5R9 cells and 1.5 Gy for SAS cells. These data indicate that the activation of the PI3K pathway most likely plays a prominent role in the acquisition of resistance to cetuximab. Interestingly, in neither of cell lines tested did targeting the MAPK/ERK pathway using the MEK inhibitor PD98059 result in radiosensitization (Fig. S2B).

**Discussion**

Acquired resistance to cetuximab has been reported to be associated with resistance to ionizing radiation [17] and to the EGFR-TK inhibitor gefitinib [22]. K-RAS mutations that result in the constitutive activation of K-Ras are one of the most important predictive markers for the lack of a tumor cell response to cetuximab, as shown for colorectal cancer [41,43,44]. In the current study, we showed that cetuximab radiosensitizes UT5 cells but not SAS cells. A similar result has been reported previously for SAS cells in vitro [45]. In contrast to these in vitro data, Gurtner et al. [8] have shown that cetuximab in combination with fractionated radiotherapy improves local tumor control in SAS xenografts in vivo but not in UT5 xenografts in vivo. The varying effects of cetuximab on radiation sensitivity in vitro and on local tumor control in vivo have also been reported for FaDu HNSCC cells. Gurtner et al. [8] have shown that cetuximab in combination with radiotherapy does not improve local tumor control for FaDu xenografts in vivo, whereas significant radiosensitization of FaDu cells in vitro by cetuximab has been reported by another laboratory [46]. Based on these studies, it appears that for the cell lines tested, the radiosensitizing effect of
cetuximab in vitro is not correlated with local tumor control in vivo. Because in our current study and in studies conducted in other laboratories [45,46] the radiosensitizing effect of cetuximab was tested in combination with single-dose radiation treatment, one potential reason for the varying effects might be differences in the experimental procedure, i.e., fractionated radiotherapy in vitro vs. single-dose radiation treatment in vitro. Another factor that might result in a differential effect of cetuximab is the difference in the behavior of the cells in vitro and in vivo. Likewise, the differential effects of cetuximab on the tumor microenvironment might potentially trigger a differential response with respect to local tumor control.

In the present study, it was demonstrated that acquired resistance to cetuximab in HNSCC cells is associated with the increased activation of Ras family member proteins. At least for K-Ras, it was demonstrated that the increased activity is due to the overexpression of the wild-type protein. Our in vitro results are in line with and are supported by data from in vitro and in vivo studies reported by Misale et al. for colorectal cancer [47]. These authors showed that cetuximab resistance is associated with increased Ras activity due to the presence of a K-RAS mutation or the overexpression of the wild-type RAS protein.

Our previous report and reports from other laboratories indicate that mutations in RAS genes, i.e., K-RAS and H-RAS, lead to radiosensitivity in tumor cells [32,36,48]. Furthermore, the underlying mechanism of this radioresistance could be demonstrated; this mechanism is the stimulation of the autocrine production of the EGFR ligands AREG and TGF-αx, resulting in the activation of PI3K/Akt-mediated survival [32,34,36,49]. Likewise, as shown in the present study, the overexpression and increased activity of the wild-type K-Ras protein stimulate the autocrine production of these EGFR ligands. Furthermore, as expected, acquired resistance to cetuximab in association with the overexpression of the wild-type K-Ras protein was accompanied by increased resistance to radiation treatment. Data from the FTI and siRNA approaches support the role and function of Ras proteins in the radioresistance of K-Ras-overexpressing cells with acquired cetuximab resistance.

Although the influence of Ras activity on resistance to cetuximab is widely accepted, we were not able to resensitize UT5R9 cells to cetuximab after siRNA knockdown of K-Ras, H-Ras and N-Ras. This result might be due to additional mechanisms that affect the cellular response to cetuximab. In this context, Bonner et al. [50] reported that the knockdown of STAT-3 improves the antiproliferative effects of cetuximab and reduces radiation survival in HNSCC cells [50]. Moreover, src activity, which has previously been shown to be involved in acquired resistance to cetuximab by mediating the translocation of EGFR into the nucleus [21,51], may improve cetuximab sensitivity and induce radiosensitization in HNSCC cells [51,52].

Variable clonal repopulation dynamics due to tumor heterogeneity may also alter the cellular response to cetuximab. Recent reports have shown that tumor cells display inherent functional variability. Such variability contributes to differential therapeutic responses [53,54]. Kreso et al. [54] reported that in addition to genetic diversity, tumor cells exhibit functional heterogeneity, even in uniform genetic lineages. According to this report, functional cells do not continuously contribute to tumor growth; some are held in reserve, whereas others are able to switch between periods of dormancy and activity, especially during periods of stress [54]. Taking this behavior into account, the distinct behavior of sub-clones may affect the responses of HNSCC to cetuximab. Based on this hypothesis, proliferating cetuximab-sensitive clones are preferentially killed, whereas the relatively cetuximab-resistant cells become dominant during or after treatment.

In summary, acquired resistance to cetuximab in HNSCC cells was demonstrated to be accompanied by the activation of Ras family member proteins. Based on the data presented, the inhibition of Ras and PI3K signaling might be effective approaches to overcome cetuximab-resistance and radioresistance in HNSCC tumor cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2013.06.023.

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Mechanism of cetuximab resistance in HNSCC cells


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