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EGFR-amplification plus gene expression profiling predicts response to combined radiotherapy with EGFR-inhibition: A preclinical trial in 10 HNSCC-tumour-xenograft models

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ABSTRACT

Background and purpose: Improvement of the results of radiotherapy by EGFR inhibitors is modest, suggesting significant intertumoural heterogeneity of response. To identify potential biomarkers, a preclinical trial was performed on ten different human squamous cell carcinoma xenografts of the head and neck (HNSCC) studying *in vivo* and *ex vivo* the effect of fractionated irradiation and EGFR inhibition. Local tumour control and tumour growth delay were correlated with potential biomarkers, e.g. *EGFR* gene amplification and radioresponse-associated gene expression profiles.

Material and methods: Local tumour control 120 days after end of irradiation was determined for fractionated radiotherapy alone (30 f, 6 weeks) or after simultaneous EGFR-inhibition with cetuximab. The *EGFR* gene amplification status was determined using FISH. Gene expression analyses were performed using an in-house gene panel.

Results: Six out of 10 investigated tumour models showed a significant increase in local tumour control for the combined treatment of cetuximab and fractionated radiotherapy compared to irradiation alone. For 3 of the 6 responding tumour models, an amplification of the *EGFR* gene could be demonstrated. Gene expression profiling of untreated tumours revealed significant differences between amplified and non-amplified tumours as well as between responder and non-responder tumours to combined radiotherapy and cetuximab.

Conclusion: The *EGFR* amplification status, in combination with gene expression profiling, may serve as a predictive biomarker for personalized interventional strategies regarding combined treatment of cetuximab and fractionated radiotherapy and should, as a next step, be clinically validated.

The epidermal growth factor receptor (EGFR) is overexpressed in many tumours such as in head and neck squamous cell carcinoma (HNSCC) and therefore represents a target in cancer therapy [1]. The overexpression of EGFR is associated with aggressive tumour growth and leads to a poor prognosis of patients with HNSCC treated with radiotherapy [2]. Patients with locally advanced HNSCC are routinely treated by concurrent, cisplatin-based radiochemotherapy [3]. Alternatively, combined irradiation and inhibition of the EGFR with a chimeric monoclonal antibody (cetuximab) has also been shown to be superior over radiotherapy alone [4,5]. The magnitude of the effect of cetuximab on local control after radiotherapy is moderate, and not superior to the effect of platinum-based simultaneous radiochemotherapy [4]. Furthermore combination of radiochemotherapy with simultaneous application of cetuximab could not further improve the outcome compared to radiochemotherapy alone in unselected patients with HNSCC [6] as well as in an early trial on other cancer entities including lung cancer or anal cancer [7,8]. Taken together these observations of only modest efficacy of combining radiotherapy with cetuximab suggest a shallow population-dose response curve which often hints at significant intertumoural heterogeneity of response [9]. Thus to allow for a better selection of patients who are likely to benefit from the treatment with cetuximab, biological

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tumour characteristics predicting the response to cetuximab-related treatment need to be identified [10]. Such biomarkers would provide a basis to include cetuximab into the portfolio of personalized radiation oncology trials and strategies [11]. Another open research question is the systematic comparison of the curative effects of combined irradiation with anti-EGFR antibodies versus tyrosine kinase inhibitors (e.g. erlotinib), which block signal transduction by inhibiting the intrinsic kinase activity of the EGFR on the intracellular domain [12,13].

In the present preclinical trial, the efficacy of combining EGFR-inhibition by cetuximab or erlotinib with fractionated irradiation was analysed in a total of 10 HNSCC xenografts regarding their impact on local tumour control as well as tumour growth delay. The results obtained on five of these HNSCC have already previously been reported [14]). We address the question whether *EGFR* amplification and gene expression profiling predict the response of the combination of cetuximab and radiotherapy was evaluated.

Materials and methods

Animals and tumour models

All experiments were performed using 7–14 week old male and female NMRI (nu/nu) mice obtained from the pathogen-free animal breeding facility (Experimental Centre, Faculty of Medicine, Technische Universität Dresden) and were approved according to the institutional guidelines, the German animal welfare regulations and followed the ARRIVE guidelines.

To further suppress the residual immune system, the mice received whole-body irradiation (WBI) with 4 Gy (200 kV X-rays, 0.5 mm Cu-filter, ~1 Gy/min) 2–5 days before tumour transplantation. Source tumours of the five established human head and neck squamous cell carcinoma xenografts were cut into small pieces and transplanted subcutaneously into the right hind leg of anesthetized mice (120 mg/kg body weight ketamine and 16 mg/kg xylazine intraperitoneal). Tumour characteristics and origin as well as the exclusion of immunogenic effects of the tumour cell line UT-SCC-15, UT-SCC-8, UT-SCC-45 and XF 354 have previously been described in detail [15,16]. SAT (HSRRB Osaka Cell No. JCRB 1027) is a non-metastatic undifferentiated cell line derived from the oral cavity with a median volume doubling time of 72 h in cell culture. To identify residual immune response reaction of the nude mice against SAT, tumours were irradiated under clamp conditions with anaesthesia using single doses between 20 and 62 Gy (Suppl. Fig. 1) [16]. This tumour model evokes also no residual immune response (TCD_{50}: single dose with WBI 34.7 Gy, 95% confidence interval (CI) [21 Gy–42 Gy], single dose without WBI 33.0 Gy [24 Gy–39 Gy] p-value 0.83). DNA-microsatellite analyses, histological examination and volume doubling time confirmed the identity of the transplanted xenograft line. All tumour models were negative for the type-III mutated variant of the EGFR (EGFRvIII; exon 8) and the EGFR–TK site (exon 19–21) [14,17].

Treatment and analysis in vivo

Tumour sizes were measured twice per week using a calliper. When the tumour volume reached ~180 mm^3, animals were randomly allocated to different groups in the respective experiments. The tumour volume was calculated for each time point as $V = Pi/6 \cdot a \cdot b^2$, where a is the longest and b the perpendicular shorter tumour diameter. For evaluation of the growth delay (GD), animals were treated daily with the tyrosine kinase inhibitor erlotinib (Tarceva®) or with the monoclonal antibody cetuximab (Erbitux®) for one (day 0) or four (day 0, 2, 5, 7) times. Erlotinib was given by oral gavaging (50 mg/kg b.w.) up to the final size of the tumour (reaching 15 mm one diameter) and cetuximab (1 mg/mouse) intraperitoneally. For evaluation of local tumour control, applications of the drugs were combined with a fractionated irradiation performed with 30 fractions in 6 weeks (30 f/6 w). Erlotinib was applied daily during radiotherapy (4 h before each fraction) and cetuximab weekly 6 h before irradiation. All fractionated irradiations were given using 200 kV X-rays (0.5 mm Cu-filter, ~1 Gy/min) with a total dose between 18 Gy and 120 Gy. The procedure of irradiation under normal blood flow conditions without anaesthesia was described previously [14,18]. After radiotherapy, tumour diameters were measured twice a week until day 90 and once per week thereafter. Recurrences were scored when the volume increased for at least three consecutive measurements after passing a nadir. Animals were observed until the mean diameter of the untreated or unirradiated tumours exceeded 15 mm, until day 120 after end of fractionated irradiation, or until death. Animals that appeared to suffer were sacrificed before reaching these endpoints.

DNA fluorescence in situ hybridization (FISH) analysis

FISH analyses have been performed as previously described [14]. Briefly, sections of 2-μm formalin-fixed paraffin-embedded (FFPE) material were used for analysing the FISH *EGFR*/CEP-7 ratio. Adequate areas with tumour cells were identified using consecutive sections, stained with Haematoxylin/Eosin (H&E). After hybridization of the defined area, fluorescence signals were evaluated by fluorescence microscope (AxioCam, Carl Zeiss, Jena) and images were prepared with a laser scanning microscope (Axiovert 200 M, LSM 510 Meta, Carl Zeiss). Specimens were considered amplified for *EGFR* with a gene to CEP ratio ≥ 2; and non-amplified with a gene to CEP ratio < 2.

Gene expression analysis

For gene expression analysis, 10-μm frozen cross-sections of untreated tumours of the 5 different tumour models from this study as well as from the previous study (including UT-SCC-5, SAS, FaDu UT-SCC-14 and CAL-33) [14] were used. Per tumour model, 6 individual tumours were used for RNA analyses. Total RNA was extracted according to the manufacturer's instructions (Quiagen, RNeasy Mini Kit), and 80 ng total RNA was used per sample. Gene expression analyses were performed using nanoString technologies using an in-house radiobiological gene panel as described previously [19]. The gene panel has been composed in a hypothesis-driven approach and included 209 genes which have previously been reported in the literature to be associated with mechanisms of radioresistance such as proliferation, invasion and metastasis, epithelial-mesenchymal transition (EMT), tumour hypoxia, cancer stem cells and DNA repair. For analysis, raw counts were logarithmized and normalized to the mean of the internal level of reference genes *ACTR3, B2M, GNB2L1, NDFIP1, POLR2A, RPL11, RPL37A.*

Statistics

Median tumour volumes and their 95% confidence intervals (CI) were calculated for each treatment arm as a function of time after the start of treatment. Growth delay (GD) was evaluated from tumour growth curves of the individual animals as the time needed after the start of treatment to reach five times the starting volume (GT_{V5}). Comparisons of the medians between the treatment groups were done by Mann–Whitney-U tests using GraphPad Prism (Prism 5 for Windows version 5.03). Statistical analysis and comparison of local tumour control data was performed as described previously [20] using the commercial software package STATA/SE 8.0 (STATA Corporation, College Station, TX, USA). For correlation