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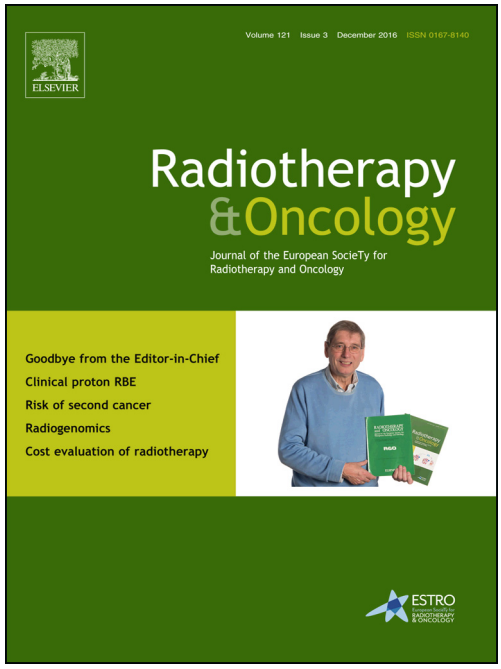
Originally published:

August 2017

Radiotherapy and Oncology 124(2017), 496-503

DOI: https://doi.org/10.1016/j.radonc.2017.07.009



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PII: S0167-8140(17)32468-4
DOI: http://dx.doi.org/10.1016/j.radonc.2017.07.009
Reference: RADION 7222

To appear in: *Radiotherapy and Oncology*

Received Date: 15 May 2017
Revised Date: 3 July 2017
Accepted Date: 6 July 2017

Please cite this article as: Koi, L., Löck, S., Linge, A., Thurow, C., Hering, S., Baumann, M., Krause, M., Gurtner, K., EGFR-amplification plus gene expression profiling predicts response to combined radiotherapy with EGFR-inhibition: a preclinical trial in 10 HNSCC-tumour-xenograft models, *Radiotherapy and Oncology* (2017), doi: http://dx.doi.org/10.1016/j.radonc.2017.07.009

Fast track paper Green journal

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

Lydia Koi [1,2,3], Steffen Löck [1,2], Annett Linge [1,2,4,5,6], Cedric Thurow [1], Sandra Hering [7],Michael Baumann [1,2,3,5,6], Mechthild Krause [1,2,3,4,5,6], Kristin Gurtner [1,2,5,6]

Affiliation:
[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]Department of Radiotherapy and Radiation Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany
[3]Helmholtz-Zentrum Dresden - Rossendorf, Institute of Radiooncology – OncoRay, Dresden, Germany
[4]German Cancer Consortium (DKTK), partner site Dresden
[5]German Cancer Research Center (DKFZ), Heidelberg, Germany
[6]National Center for Tumor Diseases (NCT), partner site Dresden, Germany
[7]Institute for Legal Medicine, Faculty of Medicine, University Hospital Carl Gustav Carus, Technische Universität Dresden, Germany

Corresponding author at:
Department of Radiotherapy and Radiation Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Fetscherstraße 74, 01307 Dresden, Germany.
E-mail address: Kristin.Gurtner@uniklinikum-dresden.de

Running title: Biomarkers for EGFR-directed radiotherapy
Keywords: Radiotherapy; Head and neck cancer; Biomarker; Cetuximab; Gene expression

Abstract:

Background and Purpose: Improvement of the results of radiotherapy by EGFR inhibitors is modest, suggesting significant intertumoral 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 (30f, 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.

Introduction:

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 intertumoral heterogeneity of response [9]. Thus to allow for a better selection of patients who are likely to benefit from the treatment with cetuximab, biological 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 to 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 to 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 hours 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 caliper. 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 (30f/6w). 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 has 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 hybridisation 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 of

the enhancement ratio (ER) of the TCD_{50} values with the FISH *EGFR*/CEP-7 ratios, a spearman correlation coefficient was calculated. Differences in gene expressions between response groups were evaluated by Mann-Whitney-U tests. For all analyses, two-sided tests were performed and p-values <0.05 were considered as statistically significant. Twenty-seven of 209 genes were found to be differentially expressed between tumour models responding or non-responding to the combined treatment with cetuximab and radiotherapy, using a minimum mean fold change of ±1.4 (0.485 on log2-scale). Of these 27 genes, 18 were significantly different expressed between both groups after Bonferroni-Holm correction for multiple testing. To visualize gene expression in a heatmap and compare variability between the tumour models, expressions were z-normalised to mean 0 and variance 1.

Results:

In total, 10 different tumour models (5 of them already published [14]) were investigated for tumour growth delay after erlotinib or cetuximab application (Table 1). Both EGFR-inhibitors led to a significantly prolonged growth time compared to untreated tumours. Erlotinib given up to final size of the tumour leads to a significant growth delay in 7 of 9 treated tumour models. In 5 of the models, growth delay could not even be quantified because most the tumours did not recur during treatment time. After a single application of cetuximab, in 7 of 10 treated tumour models a significantly longer growth time to reach the 5-fold of the starting volume (GT_{V5}, 3 of them also did not recur) compared to untreated tumours was observed. Impact of cetuximab on growth delay, with an overall higher magnitude of the effect, was confirmed in the four injections schedule. In contrast to tumour growth delay, erlotinib in combination with irradiation had no impact on local tumour control (TCD_{50} value) compared to irradiation alone (Figure 1, Table 2). It should be noted that the local control experiments with erlotinib were terminated after no improvement was observed in eight HNSCC models, five of them reported previously [14]. The impact of cetuximab on local control after radiotherapy was investigated in all 10 tumour models, five of these have been previously reported [14]. In the cohort reported here, increased local tumour control was observed for

UT-SCC-15, UT-SCC-8 and SAT. For UT-SCC-8 and SAT, TCD_{50} values were estimated to be smaller than the lowest dose level of 24 Gy because all of the tumours were cured with combined treatment even at the lowest dose (Figure 1, Table 2). In total Cetuximab increased local tumour control in 6 out of the 10 HNSCC models investigated. The increase in local tumour control was represented by the enhancement ratios (ER) of the TCD_{50} after combined radiotherapy plus cetuximab versus radiotherapy alone, calculated as the ratio of TCD_{50} control and TCD_{50} RT + cetuximab. Ratios greater than 1 indicate that the dose to achieve the same local control rate could be reduced by administration of cetuximab in comparison to irradiation alone (Table 2).

The *EGFR* amplification status, determined by FISH, differed between the tumour models. Among the 10 tumour models evaluated here and in our previous publication [14], UT-SCC-8, UT-SCC-14 and CAL-33 showed a higher copy number of *EGFR*. These three tumour models were responders to radiotherapy and cetuximab, whereas in non-amplified tumours 3/7 tumours were responders (Figures 2A and 2B). The comparison between the ER and the amplification-status (*EGFR*/CEP-7 ratio, Suppl. Fig. 2) resulted in a significant correlation with a p-value of 0.011 (Spearman r = 0.78, Figure 2C).

Gene expression analyses revealed a significant upregulation of *EGFR* in tumours responding to the combined treatment with cetuximab and radiotherapy, and in tumours with *EGFR* amplification (Figure 3A). To further explore potential radiobiological mechanisms of the response to radiotherapy and cetuximab, gene expression analyses have been performed using an in-house panel of genes which have previously been reported in the literature to be associated with radioresistance or radiosensitivity mediated by DNA-repair, proliferation, EMT, or hypoxia. Tumour models non-responding to the combined treatment with cetuximab and radiotherapy, showed a significant upregulation of DNA repair genes as well as hypoxia-associated genes (Table 3). In addition, genes contributing to cellular proliferation were also found to be significantly upregulated in non-responding versus responding tumour models. Interestingly, the differential gene expression was most distinct between non-responding tumour models and responding tumour models without *EGFR*

amplification (Figure 3B). Variability in gene expression differed between tumour models (lowest variability for SAT: mean standard deviation of z-normalised expressions: 0.30, highest for UT-SCC-5: 0.54).

Discussion

Our previous study with 5 different HNSCC xenografts showed heterogeneous effects of the combined treatment of EGFR-inhibition by the monoclonal antibody cetuximab and radiotherapy compared to radiotherapy alone on permanent local tumour control. This effect was confirmed for the additional 5 tumour models presented here showing a response in a total of 6 out of 10 tumour models and is in line with other preclinical studies evaluating local tumour control after single dose irradiation [21] or tumour volume and growth delay endpoints [18,22,23].

To the best of our knowledge, we present here the largest dataset on this combined treatment, enabling us to evaluate the highly important topic of predictive parameters for the improvement of local tumour control by cetuximab applied simultaneously to radiotherapy. It has already been shown by Gurtner et al. that the expression of the EGFR protein on the cell membrane does not correlate with the improvement of local tumour control after the combined treatment of radiotherapy and cetuximab [14]. These results were also observed in other studies on xenograft tumours with different EGFR protein expression levels [24,25] as well as in a phase III clinical trial [6]. Thus EGFR protein expression alone, although correlating with local tumour control after radiotherapy alone in preclinical and clinical studies [6,26,27], is not a predictive marker for biologically stratified treatment allocation to combined radiotherapy with cetuximab. However, the presence of the EGFR is an important factor since it is crucial for the binding of cetuximab and the resulting signalling cascade. Furthermore, it is known that the EGFR expression can change during fractionated radiotherapy [28–30], which can also influence the therapeutic impact. Thus, genetic *EGFR* expression may be the better candidate biomarker. In our study, 3 out of 10 investigated tumour models showed a gene amplification defined as *EGFR* gene to chromosome CEP-7

ratio greater two. All three tumour models with *EGFR* gene amplification were responders to combined irradiation and cetuximab, i.e. simultaneous cetuximab led to an improvement of local tumour control over radiotherapy alone. We could show a significant correlation of the enhancement ratio (TCD_{50} control/TCD_{50} RT + cetuximab) to the *EGFR* amplification determined as *EGFR* gene to chromosome CEP-7 ratio ($p = 0.011$, $r = 0.78$). It should be noted that the ERs of the tumour models UT-SCC-14, UT-SCC-8 and SAT are largely underestimated (to a lesser extent also CAL-33) as most tumours were cured with the combined treatment and the TCD_{50} could only be estimated to be lower than the lowest applied radiation dose. This aspect does not diminish the above-mentioned conclusion of a correlation of *EGFR* gene expression with response to the combined treatment, but we cannot conclude whether this correlation is linear or follows another kinetics.

Although *EGFR* gene amplification seems to have a substantial potential to predict improvement of local control when irradiation is combined with cetuximab, our preclinical trial shows that use of this parameter alone as a biomarker is not optimal because a significant proportion of responder tumours would be overseen. Nearly half of the tumour models without an amplification of the *EGFR* gene (UT-SCC-15, SAT and SAS) did benefit from the additional treatment with cetuximab. This observation is in line with other reports indicating that the use of single biomarkers such as the *EGFR* amplification status alone is not sufficient for patient stratification for personalized therapy [31]. Comparative gene expression analyses of untreated tumours revealed different radiobiological characteristics depicting their intertumoural heterogeneity. The gene expression patterns differed significantly between responders and non-responders, but also in between the responders with *EGFR*-amplification and the responders with non-*EGFR* amplification. In general, responders showed a significant downregulation of genes associated with DNA repair, cellular proliferation, hypoxia and EMT compared to non-responders. These mechanisms are known to determine radioresistance or -sensitivity (reviewed in [11]) [32]. Overall cetuximab seems to increase local control after radiotherapy particularly in those tumours which exhibit a more sensitive gene-expression profile. This notable observation deserves further mechanistic

investigations. A further interesting observation is that there was also a significant difference between *EGFR*-amplified responder tumours and *EGFR* non-amplified responder tumours regarding their gene expression profiles. Downregulation of genes assumed to be involved in radioresistance was most prominent in responder tumours without *EGFR* gene amplification. This leads to the hypothesis, that tumours with *EGFR* gene amplification are very likely to be responders to combined irradiation and cetuximab, as hypothesized earlier [17], and that the response of *EGFR* non-amplified tumours can be predicted by using the above mentioned gene panel.

Lastly, along with the anti-EGFR antibody cetuximab also the EGFR-TK inhibitor erlotinib was investigated regarding its effect on local tumour control and tumour growth delay for HNSCC xenografts. Similar to cetuximab alone, erlotinib alone led to a significantly prolonged growth time to reach the 5-fold of the starting volume compared to untreated tumours for 7 of our 10 tumour models. However, although previous in vitro data suggested radiosensitizing potentials of erlotinib in different cell lines [33], we could show no improvement of local tumour control in any of the 8 investigated xenografts after simultaneous radiotherapy and Erlotinib comparison to irradiation alone. Because of these findings, the extensive experiments for determination of the local tumour control on the tumour models UT-SCC-8 as well as UT-SCC-45 were not carried out for erlotinib in combination with radiotherapy and biomarker evaluation were not performed. The differential response in terms of local tumour control between anti-EGFR antibodies and EGFR-TK inhibitors have been shown in one tumour model already before [34–36]. These data underline the importance of using the endpoint local tumour control for studies on combined treatment with curative purposes as this endpoint reflects the inactivation of cancer stem cells [37–39].

Taken together, the results of our preclinical study on human HNSCC xenografts indicate that a biomarker panel combining *EGFR*-amplification with gene expression parameters can predict improvement of local tumour control by cetuximab applied simultaneously to

radiotherapy. These results bear considerable promise for establishing biomarkers useful for personalized radiation oncology approaches and, as a next step, need to be validated in clinical data. In a clinical study, genetic investigations could be performed on biological material (e.g. biopsies) prior to treatment. Depending on the result of the biomarker panel of *EGFR*-amplification with gene expression parameters, the treatment with cetuximab and RT could be performed. Patients with tumours which do not have these gene expression parameters would obtain standard therapy (e.g. radiochemotherapy). Another approach would be to stratify the patients for the gene expression status and then randomize radiochemotherapy versus radiotherapy plus cetuximab.

Acknowledgements:

We thank Ms. Katja Schumann, Ms. Elisabeth Jung, Ms. Anne Kluske and Ms. Daniela Friede for their excellent technical assistance. The authors thank Dr. Wolfgang Eicheler for verification of the tumour constancy. The study was supported by a Grant of the Deutsche Forschungsgemeinschaft (DFG PAK-190).

[1] Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183–232.
[2] Ang KK, Berkey BA, Tu X, Zhang H-Z, Katz R, Hammond EH, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. Cancer Res 2002;62:7350–6.
[3] Budach V, Stromberger C, Poettgen C, Baumann M, Budach W, Grabenbauer G, et al. Hyperfractionated accelerated radiation therapy (HART) of 70.6 Gy with concurrent 5-FU/Mitomycin C is superior to HART of 77.6 Gy alone in locally advanced head and neck cancer: long-term results of the ARO 95-06 randomized phase III trial. Int J Radiat Oncol Biol Phys 2015;91:916–24. doi:10.1016/j.ijrobp.2014.12.034.
[4] Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 2006;354:567–78. doi:10.1056/NEJMoa053422.
[5] Bonner JA, Harari PM, Giralt J, Cohen RB, Jones CU, Sur RK, et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. Lancet Oncol 2010;11:21–8. doi:10.1016/S1470-2045(09)70311-0.
[6] Ang KK, Zhang Q, Rosenthal DI, Nguyen-Tan PF, Sherman EJ, Weber RS, et al. Randomized phase III trial of concurrent accelerated radiation plus cisplatin with or without cetuximab for stage III to IV head and neck carcinoma: RTOG 0522. J Clin Oncol Off J Am Soc Clin Oncol 2014;32:2940–50. doi:10.1200/JCO.2013.53.5633.
[7] Walraven I, van den Heuvel M, van Diessen J, Schaake E, Uyterlinde W, Aerts J, et al. Long-term follow-up of patients with locally advanced non-small cell lung cancer receiving concurrent hypofractionated chemoradiotherapy with or without cetuximab. Radiother Oncol J Eur Soc Ther Radiol Oncol 2016;118:442–6. doi:10.1016/j.radonc.2016.02.011.
[8] Levy A, Azria D, Pignon J-P, Delarochefordiere A, Martel-Lafay I, Rio E, et al. Low response rate after cetuximab combined with conventional chemoradiotherapy in patients with locally advanced anal cancer: long-term results of the UNICANCER ACCORD 16 phase II trial. Radiother Oncol J Eur Soc Ther Radiol Oncol 2015;114:415–6. doi:10.1016/j.radonc.2015.02.008.
[9] Baumann M, Grégoire V. Molecular-targeted agents for enhancing tumour response. Basic Clin. Radiobiol. fourth edition, Hodder Arnold; 2009, p. 287–300.
[10] Bussink J, Kaanders JHAM, van der Kogel AJ. Microenvironmental transformations by VEGF- and EGF-receptor inhibition and potential implications for responsiveness to radiotherapy. Radiother Oncol J Eur Soc Ther Radiol Oncol 2007;82:10–7. doi:10.1016/j.radonc.2006.10.022.
[11] Baumann M, Krause M, Overgaard J, Debus J, Bentzen SM, Daartz J, et al. Radiation oncology in the era of precision medicine. Nat Rev Cancer 2016;16:234–49. doi:10.1038/nrc.2016.18.
[12] Bonner JA, Buchsbaum DJ, Russo SM, Fiveash JB, Trummell HQ, Curiel DT, et al. Anti-EGFR-mediated radiosensitization as a result of augmented EGFR expression. Int J Radiat Oncol Biol Phys 2004;59:2–10. doi:10.1016/j.ijrobp.2004.01.053.
[13] Baselga J. The EGFR as a target for anticancer therapy--focus on cetuximab. Eur J Cancer Oxf Engl 1990 2001;37 Suppl 4:S16–22.
[14] Gurtner K, Deuse Y, Bütof R, Schaal K, Eicheler W, Oertel R, et al. Diverse effects of combined radiotherapy and EGFR inhibition with antibodies or TK inhibitors on local tumour control and correlation with EGFR gene expression. Radiother Oncol J Eur Soc Ther Radiol Oncol 2011;99:323–30. doi:10.1016/j.radonc.2011.05.035.
[15] Yaromina A, Zips D, Thames HD, Eicheler W, Krause M, Rosner A, et al. Pimonidazole labelling and response to fractionated irradiation of five human squamous cell carcinoma (hSCC) lines in nude mice: the need for a multivariate approach in biomarker studies. Radiother Oncol J Eur Soc Ther Radiol Oncol 2006;81:122–9. doi:10.1016/j.radonc.2006.08.010.

[16] Yaromina A, Krause M, Thames H, Rosner A, Krause M, Hessel F, et al. Pre-treatment number of clonogenic cells and their radiosensitivity are major determinants of local tumour control after fractionated irradiation. Radiother Oncol J Eur Soc Ther Radiol Oncol 2007;83:304–10. doi:10.1016/j.radonc.2007.04.020.
[17] Kasten-Pisula U, Saker J, Eicheler W, Krause M, Yaromina A, Meyer-Staeckling S, et al. Cellular and tumor radiosensitivity is correlated to epidermal growth factor receptor protein expression level in tumors without EGFR amplification. Int J Radiat Oncol Biol Phys 2011;80:1181–8. doi:10.1016/j.ijrobp.2011.02.043.
[18] Stegeman H, Kaanders JH, van der Kogel AJ, Iida M, Wheeler DL, Span PN, et al. Predictive value of hypoxia, proliferation and tyrosine kinase receptors for EGFR-inhibition and radiotherapy sensitivity in head and neck cancer models. Radiother Oncol 2013;106:383–9. doi:10.1016/j.radonc.2013.02.001.
[19] 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 Off J Am Assoc Cancer Res 2016;22:2639–49. doi:10.1158/1078-0432.CCR-15-1990.
[20] Yaromina A, Kroeber T, Meinzer A, Boeke S, Thames H, Baumann M, et al. Exploratory study of the prognostic value of microenvironmental parameters during fractionated irradiation in human squamous cell carcinoma xenografts. Int J Radiat Oncol Biol Phys 2011;80:1205–13. doi:10.1016/j.ijrobp.2011.02.015.
[21] Nasu S, Ang KK, Fan Z, Milas L. C225 antiepidermal growth factor receptor antibody enhances tumor radiocurability. Int J Radiat Oncol Biol Phys 2001;51:474–7.
[22] Milas L, Mason K, Hunter N, Petersen S, Yamakawa M, Ang K, et al. In vivo enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. Clin Cancer Res Off J Am Assoc Cancer Res 2000;6:701–8.
[23] Harari PM, Huang SM. Head and neck cancer as a clinical model for molecular targeting of therapy: combining EGFR blockade with radiation. Int J Radiat Oncol Biol Phys 2001;49:427–33.
[24] Koi L, Bergmann R, Brüchner K, Pietzsch J, Pietzsch H-J, Krause M, et al. Radiolabeled anti-EGFR-antibody improves local tumor control after external beam radiotherapy and offers theragnostic potential. Radiother Oncol J Eur Soc Ther Radiol Oncol 2014;110:362–9. doi:10.1016/j.radonc.2013.12.001.
[25] Aerts HJWL, Dubois L, Perk L, Vermaelen P, van Dongen GAMS, Wouters BG, et al. Disparity between in vivo EGFR expression and 89Zr-labeled cetuximab uptake assessed with PET. J Nucl Med Off Publ Soc Nucl Med 2009;50:123–31. doi:10.2967/jnumed.108.054312.
[26] Milas L, Fang F-M, Mason KA, Valdecanas D, Hunter N, Koto M, et al. Importance of maintenance therapy in C225-induced enhancement of tumor control by fractionated radiation. Int J Radiat Oncol Biol Phys 2007;67:568–72. doi:10.1016/j.ijrobp.2006.09.044.
[27] Krause M, Baumann M. Clinical biomarkers of kinase activity: examples from EGFR inhibition trials. Cancer Metastasis Rev 2008;27:387–402. doi:10.1007/s10555-008-9141-z.
[28] Eicheler W, Krause M, Hessel F, Zips D, Baumann M. Kinetics of EGFR expression during fractionated irradiation varies between different human squamous cell carcinoma lines in nude mice. Radiother Oncol J Eur Soc Ther Radiol Oncol 2005;76:151–6. doi:10.1016/j.radonc.2005.06.033.
[29] Cerciello F, Riesterer O, Sherweif M, Odermatt B, Ciernik IF. Is EGFR a moving target during radiotherapy of carcinoma of the uterine cervix? Gynecol Oncol 2007;106:394–9. doi:10.1016/j.ygyno.2007.04.019.
[30] Petersen C, Eicheler W, Frömmel A, Krause M, Balschukat S, Zips D, et al. Proliferation and micromilieu during fractionated irradiation of human FaDu squamous cell carcinoma in nude mice. Int J Radiat Biol 2003;79:469–77.
[31] 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. doi:10.1016/j.addr.2016.02.002.

[32] de Jong MC, Ten Hoeve JJ, Grénman R, Wessels LF, Kerkhoven R, Te Riele H, et al. Pretreatment microRNA Expression Impacting on Epithelial-to-Mesenchymal Transition Predicts Intrinsic Radiosensitivity in Head and Neck Cancer Cell Lines and Patients. Clin Cancer Res Off J Am Assoc Cancer Res 2015;21:5630–8. doi:10.1158/1078-0432.CCR-15-0454.
[33] Kriegs M, Gurtner K, Can Y, Brammer I, Rieckmann T, Oertel R, et al. Radiosensitization of NSCLC cells by EGFR inhibition is the result of an enhanced p53-dependent G1 arrest. Radiother Oncol J Eur Soc Ther Radiol Oncol 2015;115:120–7. doi:10.1016/j.radonc.2015.02.018.
[34] Krause M, Schütze C, Petersen C, Pimentel N, Hessel F, Harstrick A, et al. Different classes of EGFR inhibitors may have different potential to improve local tumour control after fractionated irradiation: a study on C225 in FaDu hSCC. Radiother Oncol J Eur Soc Ther Radiol Oncol 2005;74:109–15. doi:10.1016/j.radonc.2004.10.011.
[35] Krause M, Gurtner K, Deuse Y, Baumann M. Heterogeneity of tumour response to combined radiotherapy and EGFR inhibitors: differences between antibodies and TK inhibitors. Int J Radiat Biol 2009;85:943–54. doi:10.3109/09553000903232835.
[36] Baumann M, Krause M, Zips D, Eicheler W, Dörfler A, Ahrens J, et al. Selective inhibition of the epidermal growth factor receptor tyrosine kinase by BIBX1382BS and the improvement of growth delay, but not local control, after fractionated irradiation in human FaDu squamous cell carcinoma in the nude mouse. Int J Radiat Biol 2003;79:547–59.
[37] Baumann M, Zips D, Krause M. [Experimental tumor therapy]. Strahlenther Onkol Organ Dtsch Rontgengesellschaft Al 2012;188 Suppl 3:291–4. doi:10.1007/s00066-012-0201-5.
[38] Baumann M, Krause M, Dikomey E, Dittmann K, Dörr W, Kasten-Pisula U, et al. EGFR-targeted anti-cancer drugs in radiotherapy: preclinical evaluation of mechanisms. Radiother Oncol J Eur Soc Ther Radiol Oncol 2007;83:238–48. doi:10.1016/j.radonc.2007.04.006.
[39] Baumann M, Krause M, Hill R. Exploring the role of cancer stem cells in radioresistance. Nat Rev Cancer 2008;8:545–54. doi:10.1038/nrc2419.

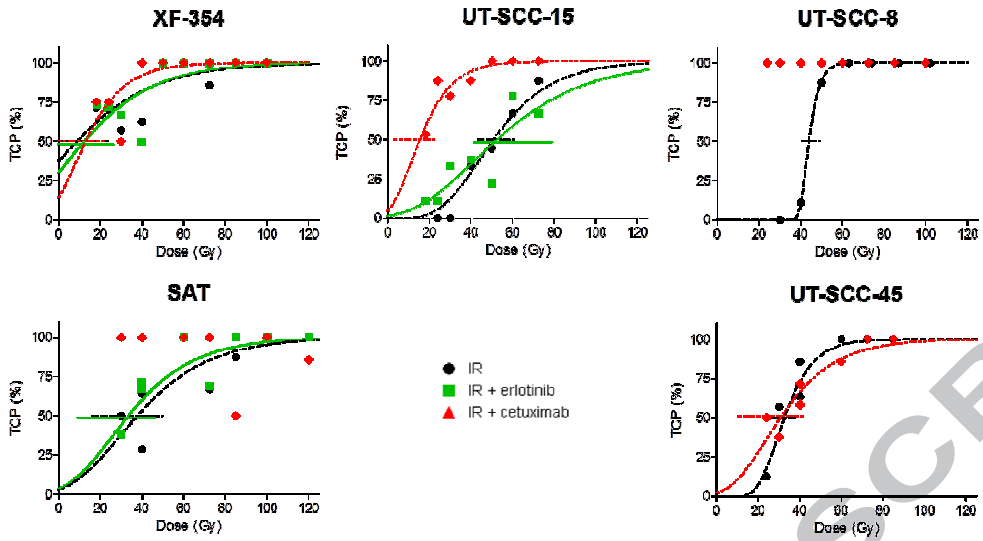
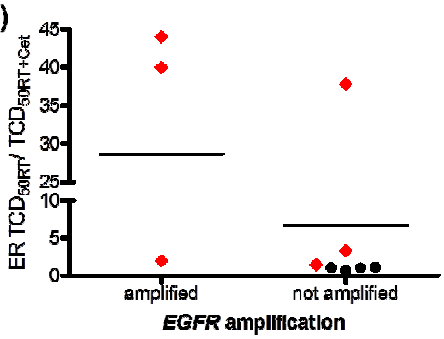


Figure 1: Local tumour control rates (symbols) and calculated local tumour control probabilities for XF-354, UT-SCC-15, UT-SCC-8, SAT and UT-SCC-45 tumours irradiated with 30f/6 weeks (solid line). Simultaneous to fractionated irradiation, animals received either daily erlotinib (50 mg/kg body weight; orally , dashed line) or weekly cetuximab (1 mg; intraperitoneal, dotted line). Error bars represent 95% confidence intervals (CI) of the tumour control dose 50% (TCD_{50}) value.

(A)



(B)

	ER	FISH
1	XF354: 0.67	XF354: 1.32
2	UT-SCC-15: 3.28	UT-SCC-15: 1.10
3	UT-SCC-8: 44.0	UT-SCC-8: 2.34
4	SAT: 37.8	SAT: 1.67
5	UT-SCC-45: 1.06	UT-SCC-45: 1.04
6	UT-SCC-5: 1.03	UT-SCC-5: 0.81
7	SAS: 1.45	SAS: 1.63
8	FaDu: 1.11	FaDu: 1.53
9	UT-SCC-14: 40.0	UT-SCC-14: 9.23
10	CAL-33: 1.97	CAL-33: 2.18

(C)

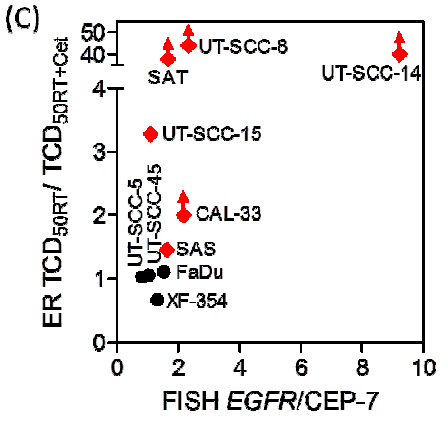


Figure 2: (A) Enhancement ratio (ER) of cetuximab in relation to *EGFR* amplification determined by FISH; (B) Summary of ER and FISH values of all investigated tumour models; in UT-SCC-8, UT-SCC-14 and SAT, the statistical calculations revealed a TCD_{50} < 24 Gy (Table 2). However, in these models more than 70% of the tumours did not recur in the growth delay assay after one injection of cetuximab alone. Thus, the TCD_{50} is assumed to be close to zero. Therefore we used a value of 1 Gy for the calculations of the ER; (C) Correlation of ER and the FISH *EGFR*-CEP-7 ratio. Red symbols indicate responders to cetuximab and radiotherapy. ER -and FISH values from UT-SCC-15, SAS, FaDu, UT-SCC-14 and CAL33 were taken from Gurtner et al. [14].

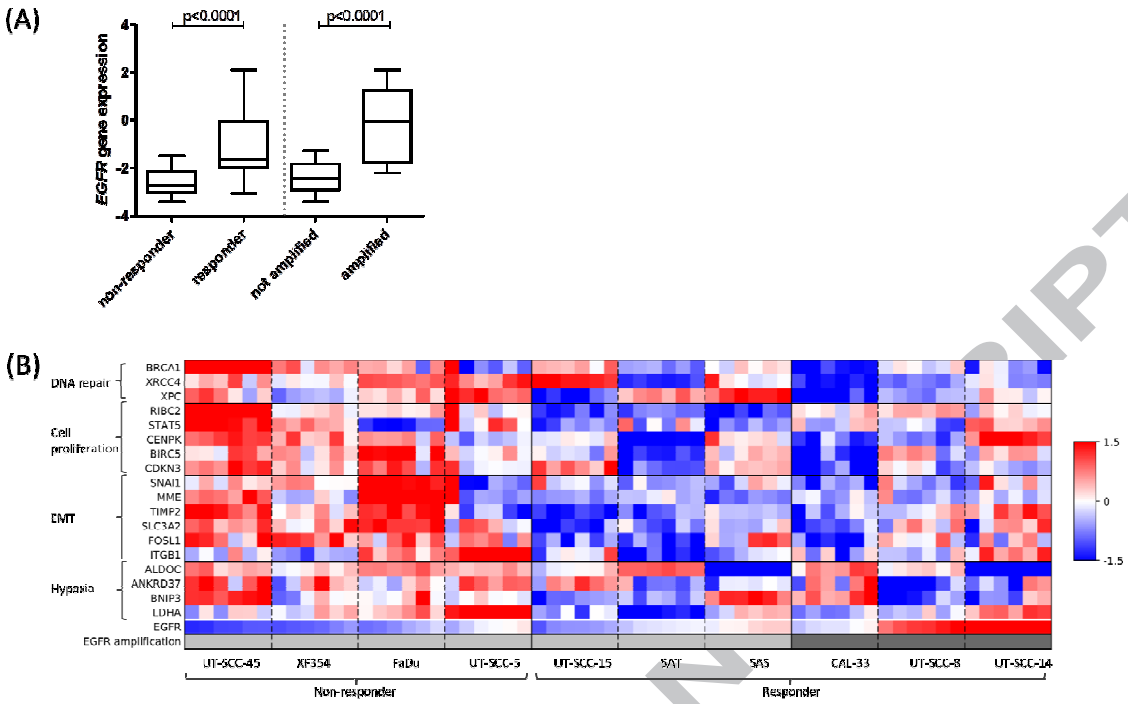


Figure 3: Gene expression determined by nanoString analyses. (A) *EGFR* gene expression of non-responders versus responders to cetuximab and fractionated radiotherapy, and tumours without *EGFR* gene amplification versus with amplification. (B) Heatmap of genes significantly differentially expressed between non-responders and responders to cetuximab and fractionated radiotherapy (minimum fold change of ±1.4, z-normalised, blue: low expression, red: high expression). *EGFR* gene amplification is additionally shown (light: no amplification, dark: amplification).

Table 1: Growth delay after EGFR-inhibition. Median time to reach fivefold the tumour volume at start of treatment (GT_{V5}) for 10 different HNSCC xenografts receiving either no treatment (control), erlotinib (50 mg/kg, body weight, orally) daily up to final size (f.s.), cetuximab (1 mg; intraperitoneal (i.p.) day 0) once or four times (1 mg; i.p., day 0, 2, 5, 7). GT_{V5} from the lower five models were already published in [14]. Bars represent medians and numbers show 95% confidence interval (CI).

*p-value in comparison to control group; **not evaluable. n.t. – not tested

Tumour	**GT_{V5} in days [95% CI] (p-value*)**			
	control	erlotinib up to f.s.	cetuximab d0	cetuximab d0,2,5,7
XF-354	42 [16-91]	122 [28-128] (0.10)	48 [30-129] (0.25)	93 [27-157] (0.080)
UT-SCC-15	18 [18-26]	**	130 [77-193] **(<0.001)**	**
UT-SCC-8	21 [18-28]	**	**	**
SAT	44 [38-60]	**	**	**
UT-SCC-45	67.5 [30-182]	n.t.	60 [41-105] (0.98)	90 [54-120] (0.64)
UT-SCC-5	17 [15-23]	21.5 [18-26] (0.14)	25 [20-27] (0.052)	22 [13-24] (0.13)
SAS	13 [10-14]	37.5 [26-63] **(<0.001)**	47.5 [34;55] **(<0.001)**	66 [49-75] **(<0.001)**
FaDu	13.5 [10-14]	21 [10-29] **(0.025)**	18 [14-23] **(0.005)**	22 [11-23] **(0.034)**
UT-SCC-14	30 [23-36]	**	64.5 [40-80] **(0.002)**	**
CAL-33	21 [16-52]	**	**	**

GT_{V5}:growth time to reach 5-fold of starting tumour volume
f.s.: final tumour size
d0,2,5,7: day 0,2,5,7
CI: confidence interval

Table 2: Calculated tumour control doses after fractionated radiotherapy alone or in combination with erlotinib or cetuximab and their 95% confidence interval (CI). The enhancement ratio (ER) was evaluated to assess the efficacy of the combination. Simultaneous to fractionated irradiation animals received either daily erlotinib (50 mg/kg body weight; orally) or weekly cetuximab (1 mg; intraperitonealy). The ER for cetuximab could not be evaluated for UT-SCC-8 and SAT because of the complete response (CR) with simultaneous radiotherapy. Thus, the TCD_{50} is assumed to be close to zero. TCD_{50} value from the lower 5 models were already published in [14]

Tumour	**TCD_{50}(Gy) [95%CI] (p-value*)**			**ER**
	30f/6 w	30f/6 w + erlotinib	30f/6 w + cetuximab	30f/6 w + erlotinib
XF-354	8.7 [0-25]	12.3 [0-26] (0.96)	13 [0-23] (0.25)	0.71
UT-SCC-15	50.9 [43-60]	52.6 [42-79] (0.84)	15.5 [3-23] **(<0.001)**	0.97
UT-SCC-8	44 [41-49]	n.t.	<24	n.t.
SAT	37.8 [38-60]	33.5 [9-46] (0.76)	<24	1.13
UT-SCC-45	32.6 [27-39]	n.t.	30.8 [11-41] (0.83)	n.t.
UT-SCC-5	121.7 [106-143]	107.7 [98-118] (0.12)	118.7 [103-139] (0.74)	1.13
SAS	118.8 [106-132]	128.5 [117-141] (0.23)	82.1 [70-94] **(<0.001)**	0.92
FaDu	64.8 [57-73]	62.5 [54;70] (0.65)	58.2 [47-66] (0.24)	1.04
UT-SCC-14	40 [33-48]	45.2 [38-53] (0.28)	<24	0.88
CAL-33	59 [24-79]	65.6 [31-92] (0.65)	<30	0.90

n.t.: not tested
TCD_{50}: tumour control dose 50%
ER: enhancement ratio
30f/6 w: 30 fractions in 6 weeks
CR: complete response

Table 3: Genes with a minimum of ±1.4-fold differential expression and a significant difference between non-responders and responders to cetuximab and fractionated radiotherapy. Given are the mean expressions for both groups and the corresponding standard deviations. The p-values originate from non-parametric Mann-Whitney-U tests. Shown are all genes, for which the single p-values <0.05 remained significant after Bonferroni-Holm correction for multiple testing

Gene	Non-responders cetuximab Mean expression (standard deviation)	Responders cetuximab Mean expression (standard deviation)	p-value
BRCA1	-4.44 (0.63)	-5.00 (0.43)	**<0.001**
XRCC4	-4.10 (0.34)	-4.76 (0.74)	**<0.001**
XPC	-5.41 (0.54)	-5.90 (0.82)	**0.024**
RIBC2	-6.86 (1.29)	-8.41 (1.05)	**<0.001**
STAT5	-6.01 (1.00)	-6.58 (0.57)	**0.013**
CENPK	-4.01 (0.48)	-4.57 (0.80)	**0.005**
BIRC5	-1.79 (0.38)	-2.31 (0.48)	**<0.001**
CDKN3	-2.11 (0.41)	-2.71 (0.65)	**<0.001**
SNAI1	-6.88 (1.03)	-7.61 (0.61)	**0.002**
MME	-7.62 (2.56)	-9.95 (0.76)	**<0.001**
TIMP2	-4.24 (1.76)	-5.94 (1.41)	**<0.001**
SLC3A2	-0.70 (0.27)	-1.20 (0.36)	**<0.001**
FOSL1	-2.04 (0.46)	-2.88 (0.66)	**<0.001**
ITGB1	0.16 (0.71)	-0.34 (0.55)	**0.015**
ALDOC	-2.85 (0.60)	-4.45 (2.39)	**0.027**
ANKRD37	-4.00 (0.76)	-4.86 (1.06)	**0.002**
BNIP3	-1.86 (0.78)	-2.46 (1.22)	**0.029**
LDHA	-4.64 (0.79)	-5.50 (0.90)	**0.001**
EGFR	-2.59 (0.57)	-1.07 (1.43)	**<0.001**