Synergistic In-Vitro Effects through Radiation and Blocking the Epidermal Growth Factor Receptor (EGFR) with the Monoclonal Antibody Cetuximab in Prostate Carcinoma Cell Line DU145

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Abstract

Background: The EGF-receptor is often overexpressed in advanced prostate carcinoma. In-vitro studies in prostate carcinoma cell-line DU145 demonstrated increased sensibilities to radiation with Cetuximab; in-vivo effects were not detected.

Methods: In vitro, we analyzed the effect of radiation and Cetuximab in cell-lines DU145 and A431 (reference), using a proliferation assay, colony-forming unit assay, and Annexin-V apoptosis assay. We analyzed changes in the protein expression of pEGFR and pERK1/2 post-radiation and Cetuximab. Additionally, we investigated the impact of Cetuximab long-term treatment on the development of secondary-resistance-mutations.

Results: DU145 cell counts were reduced by 44% after 4Gy (p=0.006) and by 55% after 4Gy and Cetuximab (p<0.001). The surviving fraction was 0.69 after 2Gy; 0.41, 4Gy; and 0.15, 6Gy (p<0.001). The additional Cetuximab-treatment did not significantly alter the impact on growth reduction or on the surviving fraction. After radiation and Cetuximab-treatment minor effects on the apoptotic cell-fraction in DU145 were detected. Using western blot, there were no pEGFR and pERK1/2 protein signals after Cetuximab-treatment. While no mutations of RAS, BRAF, PI3KCA and no amplifications of HER2 were detected, there were a TP53 mutation before and after long-term treatment with Cetuximab.

Conclusion: Radiation inhibits cell-proliferation and colony-growth and induces apoptosis in DU145. Despite blocking EGFR-MAP-Kinase pathway with Cetuximab, no significant radiation-sensitizing-effect was detected. Cetuximab-treatment did not cause typical resistance mutations in DU145. Further research must clarify whether
a combination of anti-EGFR therapeutics and immune-oncological approaches can increase the radiation-sensitizing-effect.

Background

Prostate carcinoma represents the most common cancer disease in men and affect 26% of all male cancer patients. In Germany every year prostate carcinoma is diagnosed in 60,000 men (mean age at onset is currently 71 years).

The commonly used therapeutic options for locally advanced prostate carcinomas is radical prostatectomy (Bill-Axelson et al. 2011) or percutaneous radiation therapy with 72–74 Gy (Kupelian et al. 2004), which are similarly effective with regard to overall survival. In addition to radiation therapy, radiated patients with high risk for recurrence and an advanced cT3 tumor should opt for hormone ablation therapy with a gonadotropin-releasing-hormon (GnRH) blocker for 2–3 years, because this treatment combination is associated with a significantly improved disease-specific survival (D’Amico et al. 2008, Widmark et al. 2009). Advanced metastatic hormone-sensitive prostate carcinoma is treated with a combination of androgen deprivation and either docetaxel or androgen receptor targeted therapy (Sweeney et al. 2015, James et al. 2016). Currently, newer therapies activating the immune system are tested in clinical studies. Among them are monoclonal antibodies directed against programmed death receptor 1 (PD-1) or its ligand (PD-L1) and the orally administered olaparib (De Felice et al. 2017). The latter inhibits Poly-Adenosine-Diphosphat-Ribose-Polymerase (PARP) in carcinomas with Breast Cancer 1/2 (BRCA 1/2) mutation.

As a primarily radiation-susceptible tumor, the advanced prostate carcinoma belongs to the group of late-responding tissues resp. tumors. The survival curve
contains a broad shoulder, fractionation effects and repair capacity are large. The effects of radiation therapy can be enhanced with simultaneous chemotherapy or targeted therapy. These effects can be additive or superadditive and are mostly explained with inhibition of tumor regrowth by chemotherapy in the intervals between radiation therapy. Interfering with DNA repair mechanisms is central in chemotherapy. Cisplatin and mitomycin C and substances like cetuximab, which target specific proteins and signal pathways, are typical radiosensitizers. Targeted proteins and pathways are involved in cell proliferation, neoangiogenesis, or immunotherapeutic sensitization.

The epidermal growth factor receptor (EGFR, ErbB1, HER1), the molecular target for cetuximab, is a transmembrane glycoprotein (170 kD, 1186 amino acids) and a member of the receptor tyrosine kinases. The EGFR contains a cystein-rich extracellular domain, a transmembrane domain, and an intracellular tyrosin kinase domain (Holbro et al. 2003). The EGFR gene contains 30 exons and is located on chromosome 7p11.2 (HUGO Gene Nomenclature Committee 2017). The EGFR triggers particularly in epithelial tissues, mitosis, apoptosis, migration, and differentiation (Wells 1999). EGFR is overexpressed in > 36% of the prostate carcinomas. EGFR expression levels increase as the tumor advances (Shah et al. 2006, Hernes et al. 2004) which is related to increased resistance to radiation therapy (Akimoto et al. 1999). Deletion of exons 2–7 affects the extracellular domain and results in the constitutively active EGFR variant III (EGFRvIII) present in prostate carcinomas (Olapade-Olaopa et al. 2000). Certain missense mutations of the tyrosine kinase domain lead to constitutive activation of the receptor and intracellular signaling pathways (Cai et al. 2008) independent of ligand binding. Cetuximab inhibits proliferation in DU145 prostate carcinoma cells (Prewett et al.}
1997, Dhupkar et al. 2010) and enhances effects of radiation on these cells
(Wagener et al. 2008, Liu et al. 2010), but no data is available from Phase III studies
on cetuximab-induced increased survival of patients with advanced prostate
carcinoma. In the work presented here, we aimed at developing basic
radiobiological in vitro tests, investigating the radiation-enhancing effect of
cetuximab in the DU145 prostate cell line (and A431 reference cell line), and
identifying cetuximab-specific resistance mutations. We discuss the mechanisms
potentially being responsible for the low clinical success rates and how these
mechanisms can be targeted.

Methods

Characterization and Quantification of Tumor Cells

DU145 – a human, adherent, androgen receptor-positive and androgen-independent
carcinoma cell line from a prostate carcinoma brain metastasis of a 69 year old
male patient (van Bokhoven et al. 2003, Alimirah et al. 2006) and A431 – a human,
hypertriploid, adherent, epithelial, EGFR-overexpressing epidermoid carcinoma cell
line from the epidermis of an 85-year-old female patient (Giard et al. 1973) were
obtained from Leibniz-Institute DMSZ – German Collection of Microorganisms and
Cell Cultures GmbH, Braunschweig, Germany.

The vitality of the tumor cells and the rate of cell growth (cells per ml) was
monitored regularly using the automatically Scepter™ cell count pipette. Cells were
always plated in 100 mm dishes and cultivated in 10 ml Medium RPMI 1640 +10%
FBS and used for experiments in their exponential growth phase.

Cetuximab and Radiation Treatment

For the quantification of colonies, cells were cultivated permanently in 100 nM
cetuximab (provided by Merck KGaA, Darmstadt)-containing cell culture medium. In experiments with combined radiation and cetuximab treatment, cells were cultivated in cetuximab-containing cell culture medium for four hours prior to radiation and permanently in cetuximab-containing cell culture medium after radiation. For resistance analyses, cells were cultivated in cetuximab-containing cell culture medium for up to one year with increasing cetuximab concentrations.

Standardized radiation doses were applied using the same settings at the radiation unit (Gulmay X-ray therapy unit D3225): 135 mm Polymethylmethacrylate (PMMA) camera plate, radiation tube K (20x20 cm), filter 9 as well as using a radiation stay time table (PMMA 5 mm, FHA=40cm, radiation stay time based on farmer chamber measurement 30013-0415 on 31.05.2012/27.06.2012). The desired dosage (in Gy) was achieved by defining radiation amount per time unit according to radiation stay time table.

_Proliferation and colony forming assay_

Cells of the tumor cell lines were harvested and transferred to a tube and diluted in cell culture medium for counting. Cells were seeded in 10 ml cell culture dishes (20,000 cells/dish) in duplicates per measurement 48 hours prior to the proliferation test. Cells had time to adhere during these first two days.

On Day 3, cetuximab was added four hours prior to radiation (4 Gy, once). Culture Medium, which was renewed one day after radiation, contained cetuximab for the whole proliferation period.

Cells of radiation alone, cetuximab and radiation±cetuximab group, were counted in three independent measurements for the following 8 days (exact 24-hour intervals) using the Scepter™ cell count pipette.
In preparation for the colony forming trials, an appropriate number of $5 \times 10^6$ – $1 \times 10^6$ cells were cultivated overnight and four hours before radiation, the cell culture medium was replaced with a medium containing the respective cetuximab concentration.

After radiation, the medium was removed, the cell layer was washed and dissociated from the dish through trypsination. At an initial density of 10,000 to 20,000 cells/ml the optimal plating concentrations for building cell colonies could be calculated and either 500 or 1,000 cells were seeded in two replicates per dose. The treatment of the tumor cells with cetuximab was maintained throughout the colony-building period by refreshing cetuximab containing medium. After twelve days, the colonies were stained and quantified. A control group not treated with radiation and cetuximab was cultivated throughout the entire experiment.

**Apoptosis Detection with Annexin V**

To determine the proportion of living cells in apoptosis after radiation±cetuximab with an Agilent 2100 Bioanalyzer based on phophatidylserine Annexin Cyanin 5 staining, the protocol after Preckel et al. 2002 and the Annexin V-Biotin Apoptosis Detection Kit was used.

The result was displayed in the form of histograms or Dot Plots after every measurement. Through manual gating between Calcein (blue) and Annexin (red), the proportion of the apoptotic cell fraction in living cells was determined.

To determine the proportion of apoptotic cells after radiation±cetuximab, a fluorescence-activated-cell-sorting (FACS) system BD Canto™ II was also used after staining with Annexin V-Allophycocyanin (APC) with Dead Cell Apoptosis Kit (Life Technologies) and SYTOX® Green as live/dead vitality staining.
Western Blot Experiments

Western Blot experiments served to verify the epidermal growth factor receptor (EGFR) on the protein level, its activated (phosphorylated) form, and its activated effector molecule pERK1/2. Prior to the Western Blot experiments, the exponential growing cells were treated with radiation±cetuximab, according to protocol and/or stimulated with epidermal growth factor (EGF, 10 min prior to cell lysis).

A Polyvinylidene Fluoride (PVDF) membrane was used for blotting. After that, primary antibodies anti-EGFR clone H9B4, anti-phospho-EGFR (pY1173), (Invitrogen\textsuperscript{TM}), phospho-p44 MAPK+p42 MAPK, pThr 202+pTyr 204 (pERK 1-44 kDa and pERK 2-42 kDa), (all Thermo Fisher Scientific) were added which was followed by the incubation with secondary antibodies, HRP-conjugated anti-mouse and anti-rabbit IgG whole antibodies (GE Healthcare), for a period of one hour and washing the PVDF membrane with PBS-TWEEN five times.

After removing the membrane and incubation in 1:1 Super Signal West\textsuperscript{®} Pico Stable Peroxide Solution and Super Signal West\textsuperscript{®} Pico Luminol/Enhancer Solution for one minute, the photograph was developed in Agfa CURIX 60 image processor.

Molecular Genetic Testing of Cetuximab Resistance

To verify secondary and cetuximab-induced resistance mutations, cells were incubated for up to nine months with a monthly increasing cetuximab concentration that progressed from 5, 10, 20, 50 and from month four with 100 µg/ml cetuximab. Two untreated control groups were run parallelly.

DNA preparation was done with QIAamp\textsuperscript{®} DNA Mini Kit 50 (Qiagen\textsuperscript{®}) according to the protocol. DNA concentration in the samples was determined through measuring optical density at 230 nm in ng/µl in the Nano Drop\textsuperscript{®} ND 1000 photometer. Thus, we
ensured that enough DNA was available for the following gene mutation analysis. After amplification and labelling, fifteen target genes from the DNA libraries of samples from treated and untreated DU145 and A431 cells were sequenced with a TruSight® Tumor 15 Panel and next generation sequencing (NGS, Illumina®) technologies. After this, the obtained sequences were aligned and mapped to a reference sequence matrix. Potential deviations from reference sequences were analyzed using Illumina Variant Studio Data Analysis Software.

**Statistical Evaluation**

Data from the proliferation and colony forming assay were analyzed with the Kruskal-Wallis-Test on independent samples. In order to compare cell lines with each other and to analyze the differences between the treatments and the cell lines in determining apoptosis and furthermore differences in EGFR gen amplification rate as well as tumor suppressor (TP)53 gene mutation frequencies, Mann-Whitney-U-Test was employed on independent samples. Multivariate analysis was performed to compare cell lines and proliferation between treatments over time.

**Results**

Without treatment, the daily increase of cells in both cell lines – DU145 and A431 – was significant over the observation period of nine days. From Day 7 we could determine a difference in cell count in DU145 between control and radiation at 4 Gy as well as control and radiation + cetuximab; there was no difference in cell count between control and cells treated with cetuximab alone. The differences between the different treatment groups were more pronounced in cell line A431 (Fig. 1). From Day 7, we measured a notable decrease in cell count after treatment with radiation and radiation + cetuximab. Additionally, cetuximab treatment alone
reduced the cell count in A431 significantly (p = 0.0012) on Day 9 in comparison to untreated control.

Relative reduction in cell count in DU145 on Day 7 in comparison to control was 44% after radiation with 4 Gy, p < 0.006, 55% after combined treatment with radiation and cetuximab, p = 0.001, and 24% after cetuximab treatment only. The reduction in cell count in A431 on Day 7 in comparison to control was 75% after radiation with 4 Gy, p = 0.02, 85% after combined treatment, p = 0.001, and 61% after cetuximab treatment. Figure 2 shows the relative cell count based on these results.

We determined the average plating efficiency (PE) in three independent experiments each in two replicates to be 0.53 in cell line DU145 and 0.33 in cell line A431. After radiation at 2 Gy the average survival fraction ([SF] = number of colonies formed after treatment divided by number of cells by PE) was 0.69 (DU145) and 0.54 (A431), at 4 Gy it was 0.41 and 0.25 respectively, and at 6 Gy it was 0.15 and 0.11. Comparing the control group with the radiated groups and the differently dosed groups with each other showed that the decline of the SF was significant in each case (p < 0.0001).

In the concentrations used, cetuximab had no (DU145) or only a low (A431) impact on the decline of SF after radiation with 4 Gy. Average SF was 0.42 (DU145) and 0.28 (A431) after radiation at 4 Gy; after radiation + cetuximab SF was 0.41 and 0.25 respectively. Cetuximab treatment alone resulted only in A431 in a decline of the average SF to 0.81. Figure 3 shows the logarithmic (log10) decline of SF in DU145 and A431 cells treated with radiation and treated with radiation and cetuximab.

After radiation treatment of the DU145 cells at 2 Gy, we measured that only a few
more cells went into apoptosis (10.8%) in comparison to untreated control (3–7%). After treatment with radiation (2 Gy) and cetuximab, we measured considerably more apoptotic cells (60.8%). Radiation at 4 Gy and 6 Gy led to a similarly high number of apoptotic cells; whereas additional treatment with cetuximab had no further impact. Radiation of A431 cells increased the apoptotic rate to 15.8% (2 Gy), 18.9% (4 Gy), and 20.8% (6 Gy) and after the combined treatment with radiation and cetuximab to 33.6% (2 Gy), 28.9% (4 Gy) and 36.9% (6 Gy); this was not significant in comparison to all radiation doses in treatment with radiation alone.

Following Fluorescence-Activated Cell Sorting (FACS) analyses, in part, brought differing observations and results. Radiated DU145 cells showed no dose-dependent increase of apoptotic cells in comparison to the untreated control group. Additional cetuximab treatment over all radiation doses had a minimal impact on the apoptotic rate in DU145 cells.

In cell line A431 there was a notable increase of the apoptotic fraction after radiation, in comparison to control. The average apoptotic rate during a combined treatment with radiation and cetuximab was 44.6% and thus showed in comparison with the rate during radiation treatment alone, 11.49%, an increasing trend (p = 0.057). Comparing the apoptotic rate in both cell lines measured by FACS with regard to all treatments (early apoptosis: p = 0.006, late apoptosis: p = 0.043), radiation alone (late apoptosis: p = 0.018), as well as combined treatment (early apoptosis: p = 0.009) showed differences with significantly higher apoptotic rate in A431 in each case.

The protein expression of the EGFR in DU145 is reduced after cetuximab treatment. This was also observed after a combined treatment of radiation and cetuximab and to a lesser degree, when cells were stimulated with EGF. The expression of the EGFR
on the protein level was pronounced in cell line A431 and was influenced by cetuximab only to a small degree. In DU145, the protein band of the pEGFR was completely suppressed after cetuximab treatment, independent from radiation. EGF (re)induced a weak signal. In A431, the pEGFR protein signal was considerably weakened after cetuximab treatment. After additional EGF treatment, a strong signal was visible.

The activated form pERK1/ERK2 is an important transmitter of information at the end of the intracellular signal cascade, which is, in DU145, completely suppressed after cetuximab treatment independent from radiation. In cell line A431 the suppression is not complete and EGF stimulates the phosphorylated ERK1/ERK2 protein as well.

The long-term treatment (L) of DU145 and A431 cells with cetuximab did not cause secondary mutations in the KRAS, NRAS- or BRAF-V600 genes. Likewise, no modifications were detected in Exon 9 and 20 of the PI3KCA nor typical amplifications in the HER2 receptor gene.

In the TP53 gene on chromosome 17 we detected different point mutations for both cell lines that were unrelated to the long-term cetuximab treatment. The TP53 gene in DU145 expressed the mutation c.820G > T with amino acid replacement p.Val274Phe with a frequency of 65%. In A431 it expressed the mutation c.818G > A with p.Arg273His and a frequency of 100%.

In contrast, only A431 cells showed a notable amplification of the EGFR gene. The sequencing rate for untreated A431 cells increased by the factor 4 to 77 in comparison to the standard value. After long-term cetuximab treatment the sequencing rate was reduced significantly to half the value of the untreated sample (Fig. 4). Parallelly we detected significantly lower TP53 mutation frequencies after
long-term cetuximab treatment compared to the untreated control group ($p = 0.015$).

Discussion

The effect of radiation and cetuximab treatment on cell line DU145 was determined with a classic proliferation assay. In comparison to the control (Relative Cell Count (RZW) = 1), the cell count measured on Day 7 after the begin of cultivation was significantly reduced by one radiation treatment at 4 Gy and by the combined treatment of radiation and cetuximab (RZW = 0.56, $p = 0.006$; resp. RZW = 0.45, $p < 0.001$). Treatment with cetuximab alone showed no significant reduction in cell count (RZW = 0.76); likewise, no difference in cell count occurred between radiation alone and in combination with cetuximab. In comparison to that, the cell count in cell line A431 was lowered significantly in all treatment branches (also cetuximab alone) from Day 8 after the begin of cultivation. In A431 the antiproliferative effect was, with RZW = 0.15, most effective after the combined treatment of radiation and cetuximab. These results are generally in accordance with the results presented by Dhupkar et al. (2010), who could determine a notably stronger suppression of cell proliferation through cetuximab in A431 than in DU145: Thus, DU145 cells appeared to be more radiation resistant and less susceptible to cetuximab in the proliferation assay.

The weak suppression of proliferation through cetuximab in DU145 cells can be attributed to different causes. Although the EGFR is expressed, recent data explains the incomplete suppression through cetuximab with the increased formation of heterodimers between EGFR and HER2, which are formed alongside EGFR homodimers especially after radiation (Kiyozuka et al. 2013). Furthermore, there is
evidence indicating upregulation of the EGFR-specific ligands amphiregulin and epiregulin in prostate carcinoma cells. Especially epiregulin is upregulated in hormone-resistant cells, which, however, can activate cell proliferation by stimulating not only the EGFR but all heterodimer complexes of the Human Epidermal Growth Factor Receptor (HER) family (Tørring et al. 2005). Additionally, it seems that the formation of HER2/HER3 heterodimers in combination with the upregulation of HER3's physiological ligand neuregulin-1 supports the alternative activation of the PI3K/Akt signaling pathway (Carrión-Salip et al. 2012). The expression of the epidermal growth factor (EGF) on circulating tumor cells and the formation of prostaspheres are factors for metastasis, while it is already known that the coexpression of Receptor Activator of NF-κB (RANK) and HER2 receptor plays a central role in the progression of bone metastases and, thus, fundamentally in the aggressiveness of the tumor (Day et al. 2017).

In 2010 Liu et al. found, after evaluating the colony formation assays, the relative biological effectiveness (RBE = ratio of the survival fraction between combined treatment [radiation + cetuximab] and radiation alone) to be 1.39 during the treatment of DU145 cells with 2 Gy radiation ± cetuximab. Similarly, Wagener et al. (2008) showed in their analyses that cetuximab effects DU145 cells to make them more radiation-susceptible and cytostatic after radiation. These results could not be verified to a sufficient extent in the colony formation assay carried out by us. While we could determine that radiation alone suppresses colony formation dependent on its dose in both cell lines, we could not detect any effect of cetuximab alone or in combination with radiation to increase its effect in DU145 cells (RBE at 4 Gy = 1.02). The suppression of colony formation in A431 cells, however, was numerically increased by an additional cetuximab treatment (RBE at 4 Gy = 1.12). These results
possibly indicate that a high variability and high biological dynamic is at work in regulating the proliferation of androgen-non-responsive DU145 cells through alternative signaling pathways. During the stimulation through radiation and the simultaneous blockage through cetuximab, the EGFR activation seems to be one possibility to quickly upregulate alternative pathways and activate resistance mechanisms. The combined application of substances that suppress the cyclin-dependent kinases (CDK)4/6 with an mechanistic target of rapamycin (mTOR)-antagonist during the cell cycle, speaks to this function of the regulation network. This experiment demonstrated that the androgen-responsive prostate carcinoma cell line LNCaP showed a more pronounced antiproliferative effect than androgen-non-responsive cell line DU145. Blockade of the PI3K-AKT-mTOR signaling pathway seems to amplify the effectiveness of the CDK4/6 suppression which is dependent on the activity of the androgen receptor. (Berrak et al. 2016).

Determining the apoptosis fraction of living DU145 cells using the Bioanalyzer Agilent 2100 and the Annexin V assay, showed in comparison to the untreated control group that considerably more cells went into apoptosis after radiation at 4 Gy and 6 Gy, without any increase through an additional cetuximab block. At 2 Gy the effect of the radiation was weak but could be increased to the level of higher radiation doses through an additional cetuximab treatment. This observation was only partly be confirmed during FACS analysis: The apoptosis fraction in DU145 cells was not significantly increased after radiation or combined treatment. Radiated A431 cells, however, showed in both tests pronounced apoptosis, which was further increased by additional cetuximab treatment. However, Brown and George (2003) highlighted that there is no sufficient evidence for the correlation between the extent of the apoptosis and the clinical response of solid tumors of epithelial origin.
Furthermore, there are more possibilities for tumor cells to be removed or arrested from the cell population. In this way, non-apoptotic ways such as necrosis, mitotic catastrophe, or senescence are often more important factors in determining the programmed cell death. While maintaining their metabolic functions, senescent cells are incapable of completing the cell cycle. This process comes with the effect of an increased apoptosis resistance (Campisi et al. 2007). Additionally, we would like to point out that the in vitro rate of apoptotic cells is recorded and analyzed only shortly after the exposure to radiation or cetuximab treatment, which means that evaluating the impact of apoptosis induction is limited because the effects of DNA reparation processes can not be accounted for in a sufficient manner.

The Western Blot showed in the DU145 cells after cetuximab treatment, independent from radiation, that the EGFR signals were downregulated and that the phosphorylation at Tyr-1173 site, the binding site of Shc adaptor protein/phospholipase C, was suppressed. Cetuximab also suppresses p44/p42 Erk1/Erk2 entirely in DU145 and partially in A431. This complete block of the signaling cascade stands in contrast to the weak suppression of cell proliferation and apoptosis induction in DU145, we observed. Apparently, DU145 cells are able to use alternative signaling pathways due to the EGFR/HER2 activity. Additionally, activating mutations in the PI3kinase, the loss of Phosphatase and Tensine Homologue deleted on chromosome 10 (PTEN) activity, or protein kinase B (PKB, Akt) overexpression can be present (Dhupkar et al. 2010). This has been shown primarily for the PTEN mutated (“PTEN-loss”) prostate carcinoma cell line PC-3 (McCubrey et al. 2007).

Data from Lehmann et al. (2007) describes the meaning of functional loss of one or both alleles of the TP53 gene due to missense mutations for DU145. After this loss,
these TP53 missense mutations in the prostate carcinoma split up early into one cell type that undergoes complete functional loss of tumor suppression and one “dominant negative” phenotype (Guedes et al. 2017). As a consequence of this alteration G2/M arrest in the cell cycle is absent, which, in turn, results in further accumulation of mutations, genetic instability, and reduction of repair capacity. Clinically, an increased radiation resistance and degeneration of tumor cells can be observed (Lehmann et al. 2007). With a TrueSight® mutation analysis using NGS technology, we found the c.820G > T mutation with amino acid replacement p.Val274Phe with a frequency of 65%, which leads to a functional loss of TP53 in the majority of cells. This mutation remained visible in the untreated cells as well as in those which underwent long-term cetuximab treatment. Data of Kumar et al. (2000) indicates that EGFR amplifications in benign prostatic hyperplasia cells and the prostate carcinoma do occur, but rarely. Expectedly, no gene amplification for the EGFR, the HER2, or the c-Met occurred, during our examination of DU145 cells. In cell line A431, however, EGFR gene amplifications play a superior role for the expression of the receptor in the cell membrane. We found in the untreated control group that the occurrence of amplifications of the EGFR gene was up to 77 times higher. After treating A431 cells for over nine months with cetuximab, the amplification level was reduced by half in comparison to the initial value. It is possible that during a long-term cetuximab treatment, cells with a high amount of gene copies and a high EGFR expression level go into apoptosis and, as a result, the number of amplicons is lower in the remaining cell population. Based on the findings of the past twenty years regarding the regulation of proliferation in prostate carcinoma cells and therapeutic intervention, one can, considering the progression to the hormone-independent, metastasizing state,
speak of a molecular-pathological shift. The decrease of radiosensibility during the progression from primary tumor to metastasizing carcinoma has to be taken into account for current therapeutic approaches as much as the presentation and activation of the EGFR and its corresponding signaling pathways (Bromfeld et al. 2003). The success of treating patients with advanced tumors of the prostate with targeted therapies in the future depends on a combined manipulation of several, interacting signaling pathways (EGFR, HER2, RAS-RAF-ERK, PI3k/Akt) and on observing resistance developments as the treatments progress. It follows that the improvement of tumor-specific survival by additional cetuximab treatment during radiation of locally progressed tumors in the head and neck region (Bonner et al. 2006) as well as the improvement of clinical overall survival of combination chemotherapy with cetuximab to treat metastasizing colorectal carcinomas of the RAS wild-type (Van Cutsem et al. 2009), can not easily be transferred to the therapy of advanced prostate carcinomas (Cathomas et al. 2012, Slovin et al. 2009, Fleming et al. 2012).

Recent findings indicate that germline mutations in the BRCA 1- and BRCA 2-genes can also occur in the prostate carcinoma, which affected by a more aggressive carcinoma type, more lymph nodes metastases, and a shorter disease-specific survival rate (Castro et al. 2013). When PARP is being suppressed by oral PARP-inhibitor olaparib it results in a clear response to treatment in pre-treated patients with metastatic prostate carcinoma (Mateo et al. 2015). Currently, substances are being tested which target the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) receptor, the PD-1 receptor, and the PD-L1 protein and which activate T-cell-mediated immune responses. Essential for these responses is an immunogenic tumor with many expressed or released neoantigens on its cell surface. Although
the principle of enhancing and activating immunogenic cells through radiation- or chemotherapy and cetuximab has been verified in preclinical studies (Pozzi et al. 2016), it is unclear if the advanced prostate carcinoma, a tumor with relatively low immunogenicity, can profit from this therapeutic approach (Schumacher 2015). Ongoing clinical studies of phases I to III (Powles et al. 2017) that use corresponding immune checkpoint inhibitors alone or in combination with other agents will show if this mechanism can lead to a successful tumor control in the advanced or metastasized, castrate-resistant prostate carcinoma (Modena et al. 2016).

Conclusions

Radiation inhibits cell-proliferation and colony-growth and induces apoptosis in DU145. Despite blocking EGFR-MAP-Kinase pathway an additive or synergistic effect of radiation and cetuximab on cell line DU145 could not be verified. Cetuximab long-term treatment did not cause typical resistance mutations in DU145. It remains to be seen, if the combined application of cetuximab and complementary substances can improve radiation results in prostate carcinoma and further research must clarify whether a combination with immune-oncological approaches can increase the radiation-sensitizing-effect.

Abbreviations

APC Allophycocyanin
BRCA1/2 Breast Cancer 1/2
CDK Cyclin-dependent kinases
CTLA-4 Cytotoxic T-lymphocyte-associated Protein 4
EGF Epidermal Growth Factor
EGFR Epidermal Growth Factor Receptor
ErbB Erythroblastic leukemia viral oncogene
ERK Extracellular-signal-regulated-kinases
FACS Fluorescence-Activated Cell Sorting
FBS Fetale Bovine Serum
GnRH Gonadotropin-Releasing-Hormon
Gy Gray
HER Human Epidermal Growth Factor Receptor
MAPK Mitogen-Activated Protein Kinase
mTOR Mechanistic Target of Rapamycin
NGS Next Generation Sequencing
PARP Poly-Adenosine-Diphosphat-Ribose-Polymerase
PBS Phosphate buffered saline
PD-1 Programmed Cell Death Protein-1
PD-L1 Programmed Death-Ligand 1
PE Plating Efficiency
PIK3CA (PI3K) Phosphatidylinositol-4,5-Bisphosphat-3-KinaseCatalytic Subunit Alpha
PMMA Polymethylmethacrylat
PTEN Phosphatase and Tensine Homologue deleted on chromosome 10
PVDF Polyvinylidene Fluoride
BRAF B-Raf proto-oncogene, serine/threonine kinases
RANK Receptor Activator of NF-κB
RAS Rat Sarcoma
RBE Relative Biological Effectiveness
RPRI 1640 Roswell Park Memorial Institute 1640 Medium

RZW Relative Cell Count

SF Surviving Fraction

Declarations

*Ethics approval*: not applicable.

*Consent for publication*: not applicable

*Availability of data and materials*: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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*Authors’ Contributions:*

RS Material preparation, data collection and data analysis, manuscript writing

HJO Conceptualization, methodology

KN Data analysis and interpretation

BJ Data analysis and interpretation

PH Data analysis and interpretation

MW Data analysis

MS Writing-review and editing

TB Resources

FC major contributor writing and editing manuscript

GG Conceptualization, resources

All authors read and approved the final manuscript.

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Figures

DU145 (left) and A431 (right) – Proliferation Assay-cell count-based growth curve
Figure 2

DU145-Proliferation Assay-Relative cell count day 7 after radiation 4 Gy ± Cetuximab (p<0.006, p<0.001), (RT=Radiation, Cet=Cetuximab)

Figure 3

Colony formation DU145 and A431-Logarithmic depiction of the impact of radiation
Mutation analysis DU145/A431 ± cetuximab (L)

![Graph showing mutation analysis-EGFR-gen amplification in chromosome 7 of cell lines DU145 and A431 with and without cetuximab treatment.](image)

**Figure 4**

Mutation analysis-EGFR-gen amplification in chromosome 7 of cell lines DU145 and A431 with and without cetuximab treatment.