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Radiation-Induced Activation of the Epidermal Growth Factor Receptor in Lung Cancer

Dissertation

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1. Hypothesis

About 80% of non small cell lung cancers (NSCLC) show an overexpression of the epidermal growth factor (EGF) receptor, which, due to its downstream pathways, can induce various pro-survival signals, such as increased proliferation and inhibition of apoptosis. Because of its signaling effects, the radiation-induced activation of the EGF receptor (EGFR) can reduce the anti-tumor effects of irradiation, and therefore the EGFR has been the target for different inhibitory treatment approaches with the aim of radiosensitization. Thus, the blockage of the EGFR has become an important strategy, not only in NSCLC but also in a variety of other malignancies.

There are no established molecular markers that can predict which NSCLC can be radiosensitized by EGFR inhibition. The presence of a KRAS mutation has been proposed to be a positive predictor of sensitivity to combined radiation and EGFR inhibition. In particular, delayed EGFR activation by KRAS dependent EGFR ligand expression has been suggested as an underlying mechanism. However, in several of these studies the importance of KRAS status has been deduced from the comparison of KRAS mutant lung cancer cells (A549) and KRAS wild-type head and neck squamous cell carcinoma cells (FaDu) (Toulany et al., 2005, 2006), which leaves open the possibility that other genetic changes between the two cell lines might have accounted for the radiosensitization of A549 but not FaDu cells.

Therefore, the objective of this thesis is to investigate the importance of mutant KRAS status for the radiosensitization of NSCLC cell lines by the EGFR tyrosine kinase inhibitor (TKI) erlotinib (Tarceva®). We will test the hypothesis that EGFR activation in irradiated NSCLC cell lines is associated with radiosensitization by erlotinib, and we will determine the role of a possible delayed EGFR activation for radioresistance of mutant KRAS cell lines.

2. Introduction

2.1. The Family of ErbB-Receptors

The group of the ErbB-Receptors consists of four family members, ErbB-1, which is also known as the epidermal growth factor receptor (EGFR), ErbB-2 (known as HER-2), ErbB-3 and ErbB-4 (Marshall et al., 2006). All the receptors of the ErbB family are transmembrane receptors, consisting of a cystein rich extracellular ligand binding domain, a hydrophobic intramembranous domain and a cytoplasmatic tyrosine kinase domain. These receptors are closely related to each other, with a considerable homology in their structure and the main differences to be found at their extracellular ligand binding domain. (Harari et al., 2007).

2.2. The Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) was one of the earliest growth factor receptors to be characterized and sequenced (Cohen at al., 1975; Ullrich et al., 1984).
The EGFR, as a member of the ErbB receptor family, is sharing its basic structural features with the rest of the family members. The EGFR is a transmembrane receptor with a molecular weight of 170kD and can be divided into three parts of different function: the extracellular domain (621 amino acids), the short transmembrane domain (23 amino acids) and the intracellular domain (542 amino acids).
While the extracellular domain is the site of ligand binding and the transmembrane domain anchors the receptor to the cellular membrane, the intracellular domain carries tyrosine kinase functions (Nyati et al., 2006). The kinase function enables the intracellular domain to act as a signal transducer, conducting the extracellular stimulus of a ligand binding to the extracellular domain downstream into the intracellular space. Besides the stimulation by extracellular ligand binding, irradiation is known to activate the EGFR by inducing autophosphorylation of the receptor (Contessa et al., 2002).
While the EGFR is of interest in treatment regimes of radiation oncology, the receptor is not only expressed in cells of malignant origin, but can be found on all cells of epithelial origin and thus the functions mediated by EGFR are also important in non malignant cells (Wells at al., 1999).

2.3. Ligands of EGFR

The cystein rich extracellular domain of the EGFR is the site of ligand binding, a stimulating signal that induces the activation of the receptor. The receptor activation is not solely based on one ligand, but instead various ligands can stimulate the receptor, with more than ten ligands known to bind to EGFR (Hynes et al., 2005; Yarden et al., 2001), such as amphiregulin, EGF and transforming growth factor-α (TGF-α**)** (Nyati et al., 2006).

The binding of an activating ligand to EGFR induces the dimerization of the receptor, with the consequence of activation of the tyrosine kinase domains and ultimately activation of a variety of downstream pathways. The dimerization can either involve two EGFR, then being called homodimerization, or the EGFR dimerizes with another receptor of the ErbB family, this process being referred to as heterodimerization (Bowers et al., 2001).

The heterodimerization is influenced by the bivalency of ErbB ligands, the varying binding affinities of ligands as well as the pH stability of the ligand-receptor complex (Beerli et al., 1996; French et al., 1995), making the dimerization a process depending on various parameters, not only being influenced by the microenvironment of the cell, but also by the various stimulating ligands. Therefore, there is considerable variability in the pairing of receptors during dimerization, and taking into account that the EGFR can be activated via various ligands, the signal inputs to the receptor system are of a great diversity (Olayioye et al., 2000). As a consequence of the diverse activation patterns of EGFR, there are a variety of downstream pathways that can be activated (Lemmon et al., 1994; Yarden et al., 2001), leading to a very complex pattern of cascade activations with partly crossing pathways, making the EGFR signal transduction a very complex and interconnected signaling network.

2.4. EGFR Mediated Pathways and Functions

Upon activation of EGFR, the phosphorylation of the tyrosine kinase domain of the receptor induces a variety of downstream pathways, with the major cascades visualized in Fig. 1. Besides these cascades of protein activations, EGFR itself has been shown to translocate to the nucleus, possibly acting as a transcription factor to mediate specific EGFR functions (Lin et al., 2001).

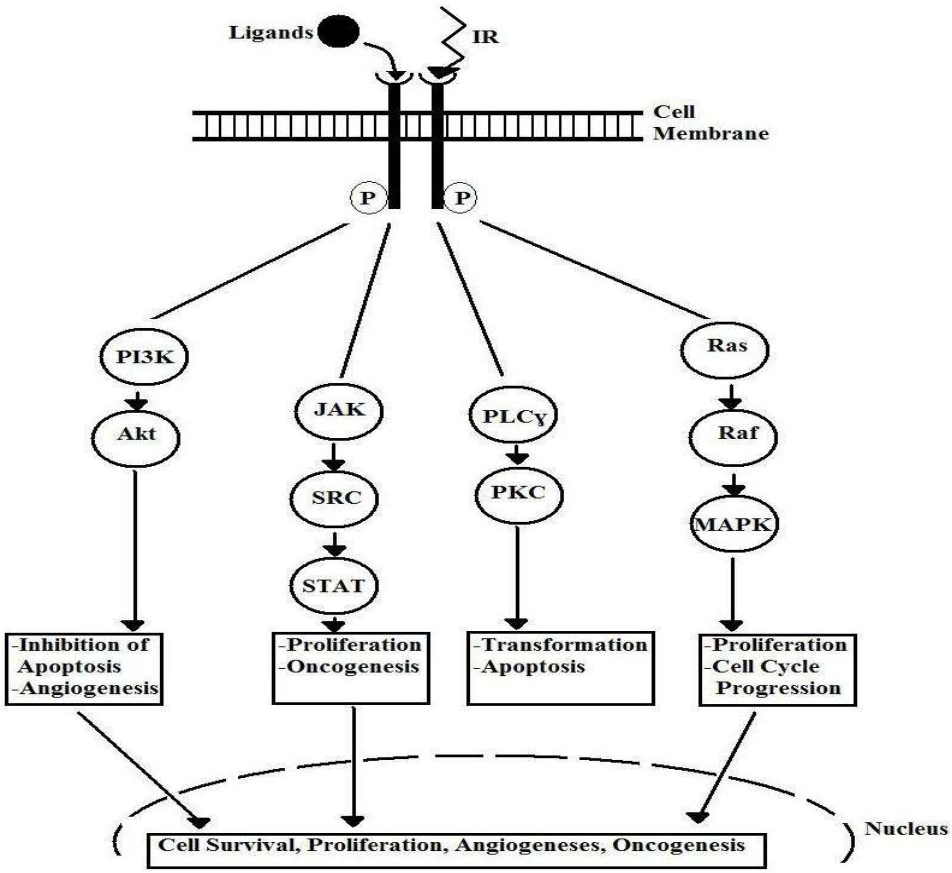

Fig. 1: Major Pathways of the EGFR. After activation and dimerization of the receptor, various downstream pathways are activated, ultimately promoting reduced apoptosis and an increased cell survival; in cancer cells oncogenesis is supported.

The fact that the EGFR supports cellular survival strategies indicates that the receptor can be highly important for malignant cells, with EGFR mediating downstream functions that can support an uncontrolled tumor progression and even help malignant cells to survive cancer treatments.

As shown in Fig. 1, the activation of EGFR can promote cell proliferation, inhibit apoptosis, support angiogenesis and invasion as well as metastasis in cancer cells (Herbst et al., 2003).
When activated, the EGFR activates PLC. Then, the activated PLC hydrolyses the membrane bound PIP2 to DAG and IP3. As a cofactor, DAG mediates the activation of PKC, which induces cell cycle progression as well as transformation and differentiation (Oliva et al., 2005). IP3 induces intracellular Ca^{2+} release, which can induce apoptosis.
Stimulated EGFR also induces RAS, which together with Raf activates ERK and MEK kinases. These kinases act as transcription factors in the nucleus and ultimately lead to increased cell growth (Sebolt-Leopold et al., 2004).

Another pathway activated via EGFR is the PI3K-Akt cascade. EGFR mediates the activation of PI3K which then activates Akt. Akt has the ability to inhibit apoptosis (Takeuchi et al., 2004). In addition, Akt induced cascades support angiogenesis (Hennessy et al., 2005).
The JAK-STAT cascade is activated via EGFR as well. The activated STAT proteins act as transcription factors and ultimately support cell survival and proliferation, as well as oncogenesis (Bowman et al., 2000).

The functions that are mediated via the activated EGFR are vital for normal cells to sustain their cellular integrity and normal growth. Thus, in normal tissues, the EGFR is necessary to support tissue regeneration and to mediate tissue responses to changing environmental situations.
However, when the EGFR is expressed by cancer cells, the very same receptor becomes a driving force of tumor growth and progression. Upon activation, the EGFR can increase the resistance of cancer cells to anti-tumor treatments, and therefore cause a reduced treatment efficacy.

2.5. The EGFR in Malignancies

The EGFR is regularly expressed on non-malignant epithelial cells. But due to its multitude of prosurvival functions, the EGFR also plays a major role in tumor etiology and their progression (Jorissen et al., 2003). The EGFR is overexpressed in a variety of cancers, such as head and neck squamous cell carcinomas (HNSCC) and glioblastomas (Barker et al., 2001; Gibson et al., 2003), as well as in a high percentage of non-small cell lung cancers (NSCLC) (Herbst et al., 2004). Once activated, the downstream pathways of the EGFR induce cellular responses that are not only of importance in normal cells, but the very same responses can be beneficial for cancer growth and cancer cell survival. Therefore, overexpression as well as dysregulation of the EGFR can support oncogenesis (Salomon et al., 1995).

2.6. Mutations of the EGFR

Besides the activation of the EGFR via ligands and irradiation, there are various genomic alterations of the EGFR that can lead to a dysregulated functioning and increase the activation of the downstream pathways.

The expression of a variety of EGFR mutations has been associated with an increased response to inhibitory strategies against the receptor itself. In NSCLC, the presence of a mutated EGFR is associated with clinical tumor responses to EGFR inhibition using the tyrosine kinase inhibitor (TKI) gefitinib (Lynch et al., 2004).

There are various EGFR mutations that are associated with drug sensitivity. For example, deletion in exon 19 of the EGFR gene (del 746-750), which is also present in PC-9 NSCLC cells, is connected to an increased responsiveness to EGFR inhibitors. The L858R mutation in exon 21 of the EGFR gene, for example found in the H3255 lung cancer cell line, also predisposes cells to EGFR inhibiting treatments (Paez et al., 2004).

Another mutation that has been shown to mediate treatment responses to EGFR inhibitors is the expression of the mutated EGFRvIII. This mutation causes the EGFR to be consecutively activated, without being dependent on extracellular stimuli (Moscatello et al., 1996). As a result, the downstream functions mediated by the EGFR continuously support cell survival. This has been associated with an increased resistance of cancer cells harboring EGFRvIII to irradiation (Lammering et al., 2004). As a result of the activated EGFRvIII, the cells show a reduced inactivation after anti-tumor therapies, and inhibition of the EGFR can greatly reduce survival capabilities in those cells. Cancer cells carrying EGFRvIII were shown to respond to EGFR inhibiting treatment with the TKI erlotinib (Akita et al., 2003).

An amplification of gene copies of the EGFR genes can lead to the overexpression of the EGFR, causing an increased sensibility of the overexpressing cells to EGFR activating signals. An increase in EGFR gene copies can often be found in lung cancers, and those cancers that inherit more gene copies also proved to inherit more somatic mutations of the EGFR as well (Ono et al., 2006). NSCLC often overexpress the EGFR, and with increasing EGFR expression, there is also an increased presence of mutant EGFR. However, even in the absence of EGFR mutations, NSCLC overexpressing wild-type EGFR can still respond to EGFR inhibition (Takano et al., 2005).

2.7. Radiation-Induced EGFR Activation

Besides its ligand dependent activation, the EGFR has the capability to autophosphorylate and become activated in response to irradiation (Schmidt-Ullrich et al, 1996; Contessa et al., 2002). This is of special interest for treatment of malignancies with ionizing radiation, as it is commonly the case in NSCLC. As a consequence of the ligand independent activation of the EGFR by irradiation, the downstream pathways of the EGFR may be activated, thereby promoting proliferation as well as anti-apoptotic effects (Dent et al., 1999).

It was hypothesized that one mechanism leading to autophosphorylation after irradiation depends on the generation of reactive oxygen species (ROS) (Kamata et al., 2000). ROS are generated through the irradiation of the cell and are thought to increase the phosphorylation of the EGFR tyrosine kinase domain by reacting with phosphatases of the cell, inactivating their enzymatic abilities and thereby inhibiting the dephosphorylation of the EGFR (Leach et al., 2001; Mikkelsen et al., 2003). The transient activation of the EGFR within minutes after irradiation with clinical relevant dose levels has been demonstrated for various cell lines (Schmidt-Ulrich et al., 1997, Sturla et al., 2005).

Following this first EGFR activation within minutes after irradiation, a second phase of EGFR activation has been identified in A431 squamous and MDA-MB-231 mammary carcinoma cells (Dent et al., 1999), as well as in DU145 prostate carcinoma cells (Hagan et al., 2000). These publications suggest that the second EGFR activation takes place within hours after irradiation and is thought to be mediated by an autocrine loop. Possibly via the RAS-MAPK cascade, irradiated cells produce ligands of the EGF receptor, presumably TGFα or amphiregulin, which then are released and lead to an autocrine EGFR stimulation (Dent et al., 1999, Hagan et al., 2000).

2.8. EGFR Inhibition Strategies

In malignancies, the activation of EGFR mediates cellular functions that mainly support cell survival and proliferation and it is evident that these effects are counteracting the effectiveness of therapeutic anti-tumor strategies. As a consequence, the EGFR inhibition is an important strategy to influence the uncontrolled proliferation of malignancies (Mendelsohn et al., 2000). It has been shown that, if the EGFR in cancer cells is overexpressed or inherits an activating mutation, cancers may be relatively resistant to chemo- as well as radiotherapy (Liang et al., 2003; Baumann et al. 2004). Strategies for EGFR inhibition to cause increased tumor sensitivity to chemotherapy and irradiation include two different approaches, involving tyrosine kinase inhibitors (TKI) and monoclonal antibodies (mAbs).

Monoclonal antibodies inhibit the EGFR by blocking the extracellular ligand binding domain of the EGFR. When the monoclonal antibodies bind to the EGFR, the receptor is blocked from binding extracellular ligands and the ligand dependent activation of EGFR is disrupted. Additionally, due to the molecular size and properties of the antibodies, the dimerization of the EGFR, which is required for activation of the intracellular tyrosine kinase domain, is inhibited. As a consequence, radiation-induced EGFR activation is impaired as well. In addition to the direct inhibition of the EGFR, monoclonal antibodies are thought to induce downregulation of the inhibited EGFR, leading to reduced EGFR expression (Jutten et al., 2009). It is also possible that the binding of monoclonal antibodies to the EGFR causes an immunomodulatory effect by homing immune cells and their cytotoxic immunologic reactions onto the antibody marked cancer cells. A drawback of EGFR inhibition via monoclonal antibodies is the required parenteral application, while due to their long half-life, the antibodies can be administered on a weekly basis during treatment (Guarino et al., 2009).
A commonly used monoclonal antibody for EGFR inhibition is cetuximab. Various studies were performed to asses the effectiveness of cetuximab as an EGFR inhibitor. Radiosensitization was achieved in vitro as well as in xenografts using cetuximab with cancer cells overexpressing the EGFR, including lung cancer cell lines (Raben et al., 2005; Krause et al., 2005).

In contrast to monoclonal antibodies, small molecule tyrosine kinase inhibitors (TKI) such as erlotinib act in the intracellular space, directly binding to the tyrosine kinase domain of the EGFR and blocking its catalytic domain. The blocking of the tyrosine kinase domain inhibits

the activation of the downstream pathways of the EGFR, blocking the EGFR mediated effects. Tyrosine kinase inhibitors were shown to radiosensitize various cell lines (Bianco et al., 2002, Solomon et al., 2003).
The tyrosine kinase inhibitors are small molecules that can be administered orally, but their short half life requires a daily application. Also, adverse effect of tyrosine kinase inhibitors are more pronounced, limiting their clinical dosing (Shin et al., 2001). Tyrosine kinase inhibitors also lack the effect of EGFR downregulation (Pollack et al., 1999) and might not be able to induce immunologic effects as possibly achieved by monoclonal antibodies.

Even though the aforementioned strategies of EGFR inhibition seem to cause similar effects with regard to the disruption of downstream pathways, their effects in a given cell line or tumor may be quite different (Krause et al., 2005).
In addition, the response to EGFR inhibition is very heterogeneous. Especially when tyrosine kinase inhibitors are used, the EGFR inhibition effects are inconsistent between various cell lines, in vivo as well as in vitro (Baumann et al., 2003; Giocanti et al., 2004). As a consequence, it is of great importance to identify markers that can predict the effectiveness of a certain EGFR inhibition strategy in each individual case to optimize cancer therapy.

2.9. KRAS and its Role in Radiotherapy

The RAS protein is an integral part of the Ras-Raf-MAPK downstream pathway of the EGFR (Adjei et al., 2001). When activated, the EGFR dimerizes and its tyrosine kinase domain becomes activated (see Fig. 1). As one of the important cascades that are activated, RAS activates the MAPK, which in turn promotes pro survival signals.
The RAS protein family consists of three proteins with GTPase activity, HRAS, KRAS and MRAS (Johnson et al., 2001). Because of its prominent role in the downstream cascades, RAS and its mutations have been studied in context of their effects on radioresistance of cancer cells. Mutations of the RAS genes occur in about 30% of tumors (Downward et al., 2003), making mutant RAS a common mutation in cancer. The mutation of RAS leads to its continuous activation, and this activation is thought to correlate with radioresistance (Cengel et al., 2005), implying that a mutation in the RAS protein could be of prognostic value for the efficacy of radiation therapy.

Recent studies tried to shed light on the importance of a mutated KRAS (mtKRAS) protein and its influence on radioresistance of cancer cells.
The mutated KRAS is thought to continuously activate its downstream pathways, leading to the activation of the MAPK. It was shown that via this pathway, the cellular production of EGFR ligands such as TGFα and amphiregulin can be increased, and the release of those ligands may lead to the autocrine activation of the EGFR (Schulze et al., 2001).
This is of special interest in cancers treated with radiation therapy, where the increased EGFR activation is reducing the anti-tumor effects of the irradiation. The presence of a KRAS mutation was hypothesized to cause an increased production of EGFR ligands, which in turn may mediate a consecutive activation of the EGFR. It was thus proposed that the autocrine EGFR activation leads to pro-survival signaling mediated through the PI3K-AKT cascade (Toulany et al., 2006). The PI3K-AKT pathway induces various pro survival effects, such as inhibition of apoptosis, but also has been linked to an increased DNA repair capacity (Toulany et al., 2006; Brognard et al., 2001). This activation of the PI3K-AKT pathways is not induced by the mtKRAS itself, but rather through the autocrine loop generated via ligand production induced by the RAS protein (Toulany et al., 2005).

Therefore, the KRAS mutation is thought to lead to an increased ligand synthesis, which then establishes an autocrine loop to activate the EGFR. As a consequence, the PI3K-AKT pathway is activated and mediates pro-survival signals that can lead to radioresistance. It has been demonstrated that cancer cell lines carrying a mutant KRAS can be radiosensitized, while other cancer cell lines with the wild type KRAS protein showed no radiosensitizing effect when treated with an EGFR inhibitor (Toulany et al., 2007). These results require validation, because in these studies KRAS mutant NSCLC A549 cells were compared with the KRAS wild type pharyngeal squamous cell carcinoma cell-line FaDu (Toulany et al., 2007). Due to their different epithelial origins the two cell lines are expected to carry multiple additional genetic differences that could influence the effect of EGFR inhibition on their signaling networks.
Importantly, if a KRAS mutation is generally associated with an increased response to a combination of EGFR inhibition and radiation, its presence could possibly be used as a predictive biomarker for the responsiveness of a given tumor to radiation and EGFR inhibition. This notion is distinct from emerging evidence that implicates mutant KRAS as a negative predictor of response to EGFR inhibition alone (Garassino et al., 2009).

To clarify the value of a mutant KRAS as a predictive marker for radiosensitization by EGFR inhibitors, further studies with additional cell lines of a given cancer type are needed. To address this question, the following experiments were conducted.

3. Material and Methods

3.1. Cell Lines

The experiments were conducted using A549, NCI-H460, Calu-6 and PC-9 cell lines. The PC-9 cells are a human pulmonary adenocarcinoma cell line, carrying an activating mutation in the EGFR kinase domain and wild-type KRAS. A549, NCI-H460 and Calu-6 cell lines are non-small cell lung cancer (NSCLC) cell lines harboring wild-type EGFR and mutant KRAS. See Section 3.6 for a list of all materials used in this project. All cell lines used in these experiments have been tested mycoplasma free.

3.2. Cell Culture

All cells were cultured using T25 or T75 cell culture flasks. NCI-H460 and PC-9 cells were kept in RPMI 1640 medium, Calu-6 cells were kept in Eagle's Minimum Essential Medium and A549 cells in DMEM media. All media were supplemented with 10% BGS (bovine growth serum), 1xHEPES buffer, 2 mmol/L L-Glutamine and 10,000 units/mL Penicillin-Streptomycin antibiotics. Cells were incubated at 37°C/5%CO_2 in a humidified atmosphere.

Handling of cells was always performed in a sterile environment of a cell culture hood. For plating of cells in experiments and during general passaging, old media was discharged from the flasks by suction. Flasks were then washed with 5ml 1xPBS. To detach cells, 0.7ml of 1xTrypsin was added to T25 flasks, and 2ml 1xTrypsin was used for T75 flasks. Flasks were incubated for 5 minutes to allow for cell detachment. After confirming cell detachment under a light microscope, the Trypsin was inactivated by adding 5 ml of medium to a T25 and 10 ml of medium to a T75 flask.

Cell suspensions were generated and cell numbers were obtained manually using a hemacytometer.

For routine passaging of cells, T25 flasks required a minimum cell amount of $5x10^5$ cells/flask and T75 flasks needed a minimum cell amount of $1x10^6$ cells/flask to ensure cells were maintained in exponential growth phase. Cells were grown to 70% confluence before trypsinization and replating. Cells were passed only up to passage 20 and then being replaced with freshly thawed cells. For frozen storage, $1x10^6$ cells were diluted into 1ml of medium

containing 5%DMSO, filled into 1ml cryogenic vials, placed into a methanol filled freezing container and frozen at -70°C. After 24 hours at -70°C, cryogenic vials were transferred to a liquid nitrogen tank.
To reconstitute frozen cells, cryogenic vials were thawed in a 37°C water bath. The thawed cell solution was then diluted into 5ml of medium and centrifuged for 5 minutes at 1,800 rpm. The DMSO-containing medium was discharged, the cell pellet reconstituted with 5ml medium, the cell solution placed into a T25 flask and incubated for cell growth.

3.3. Short-Term Cell Proliferation Assays

In order to investigate the influence of radiation and the effects of a treatment with 2µM erlotinib (kind gift from Dr. Jeff Settleman) combined with irradiation, A549, NCI H460 and Calu-6 cell lines were used for short-term proliferation assays.
For short-term proliferation assays, a T75 flask with cells at 70% confluence was trypsinized. A cell solution was created and the cell amount per ml solution was determined (Fig. 3.-1.). A density of $1x10^5$ cells was plated into T25 flasks, in order to reach 20% confluence at the end of the incubation (Fig. 3.-2.). For each cell proliferation assay, four T25 flasks with the same cell densities were plated and designated as follows:

Untreated	= Control flask without further treatment
IR	= Irradiation with 2 Gy
Erlotinib	= 2µM erlotinib
Erlotinib+IR	= 2µM erlotinib 45 minutes prior to IR with 2 Gy

After 6 hours to allow for cell attachment, the labeled T25 flasks received medium supplemented with 2 µM erlotinib working concentration, while cells in the other flasks were kept in regular medium. 45 minutes after erlotinib treatment started, the designated T25 flasks were irradiated with 2 Gy (Fig. 3.-3.). Irradiation was performed using a Siemens Stabilipan 2 X-ray generator operated at 250 kVp and 12 mA, at a dose rate of 1.98 Gy/min. After irradiation, the flasks were incubated for 72 hours (Fig. 3.-4.). After 72 hours, the medium of each T25 flask was removed and cells were washed with 5ml 1xPBS and trypsinized using 0.7ml Trypsin. After trypsinization, medium was added to a total volume of 4ml cell solution

per T25 flask. The total cell number present in the flask was then calculated by manual counting (Fig. 3.-5.).

Relative survival rates for single treatments with erlotinib or irradiation were calculated by dividing the total cell count of the treated flasks by the total cell count of the untreated flask.

The relative survival rate for the combined treatment with irradiation and erlotinib was calculated by dividing the total cell count of the combined treatment flask by the total cell count of the untreated flask and then dividing by the relative survival of the erlotinib treated flask.

For each cell line, short-term proliferation assays were done in triplicate. Related graphs were created using GraphPad Prism software (GraphPad Prism 4.03, GraphPad Software, San Diego, CA, USA).

The radiation dose of 2 Gy was chosen based on preliminary experiments with A549 cells. The cells were plated at low densities as described above and then received radiation with 2 Gy compared to 10 Gy. Due to the fact that at 10 Gy, cell proliferation was heavily suppressed while at 2 Gy (data not shown), cells showed a sufficient reduction of cell proliferation while still allowing enough room for erlotinib to take a visible influence on proliferation, 2 Gy was chosen to be the dose of radiation applied during the experiments.

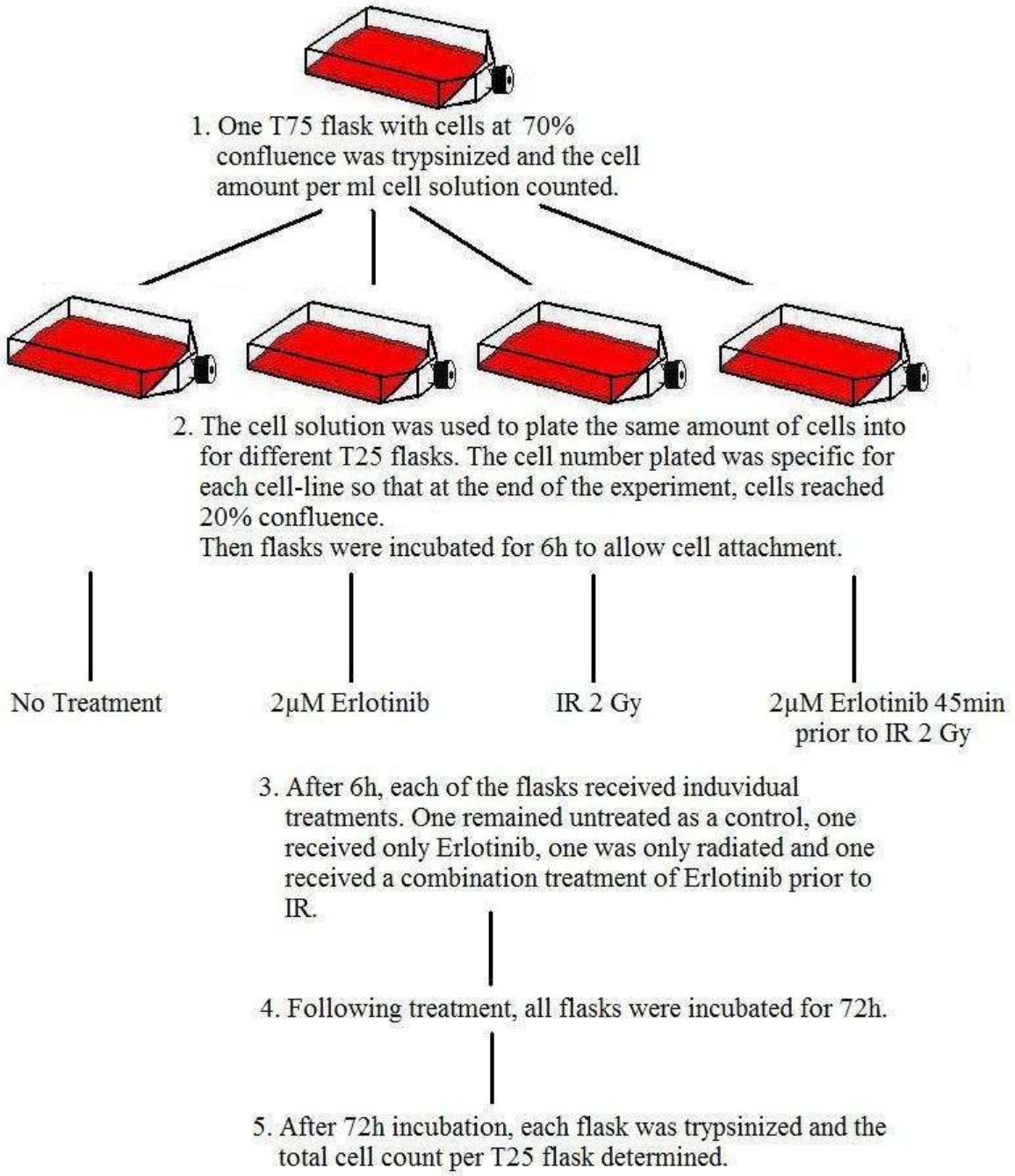

Fig. 3: Chart visualizing the steps taken during short-term proliferation assays. IR, ionizing radiation; h, hours.

3.4. Colony Formation Assays

In order to determine the cellular sensitivity to ionizing radiation (IR) and the tyrosine kinase inhibitor erlotinib, colony formation assays using A549, NCI H460 and Calu-6 cell lines were

performed. Two different assay versions were conducted, with the cells being plated either before or after receiving treatments.

For colony formation assays, cells were kept in T25 flasks for treatments. Cells were plated at two different cell densities per X-ray dose, allowing formation of 20 to 300 colonies per flask. Appropriate densities were determined in preliminary experiments. For “plating first” assays, cells were incubated for 16 hours prior to treatment. For “treatment first” assays, the treatment was conducted with cells at 70% confluence, followed by 5 hours of incubation prior to plating.

Cells were irradiated with doses up to 8 Gy. In parallel experiments, cells received 2 µM erlotinib 45 minutes prior to IR with doses up to 8 Gy.

Therefore, for each dose point two T25 flasks with two different cell densities were plated, one with cells only being irradiated, the other flasks with cells that received a combined treatment of radiation and 2µM erlotinib.

After treatment, the flasks were incubated to allow for 5-6 doublings of surviving cells. Since cell lines did not show a uniform growth rate, the duration of incubation prior to counting was cell line specific, with A549 cells needing 21 days of incubation, NCI H460 needing 14 days and Calu-6 cells requiring 18 days of incubation to produce viable colonies containing at least 50 cells.

At the end of the incubation time, the medium of the T25 flasks was aspirated. The flasks were washed with 1xPBS and colonies were fixed with methanol. The methanol was discharged and the colonies were stained for 30 minutes with methylene blue. After staining, the flasks were washed three times with tab water and dried overnight at room temperature.

For each flask, stained colonies with at least 50 cells per colony were manually counted using a light table and a microscope. Plating efficiencies were calculated as colonies per number of cells plated and surviving fractions as ratios of plating efficiencies for irradiated and unirradiated cells.

The dose enhancement factor (DEF) at the 10% survival level was calculated by dividing the dose needed to achieve 10% survival with irradiation alone by the dose needed to achieve 10% survival with the combination treatment of irradiation and 2µM erlotinib. Related cell survival curves were created by describing the relationship between the radiation dose (Gy) and the survival fraction (SF) of the plated cells with the equation:

$$SF = \frac{\text{Plating Efficiency (PE) of treated cells}}{\text{PE of control cells}}$$

In the equation above, plating efficiency (PE) was determined by:

$$PE = \frac{\text{Colonies counted}}{\text{Colonies seeded}}$$

The cell survival curves were graphically displayed using GraphPad Prism software while the statistical analysis was performed by two sided F-test using the same software.
All experiments consisted of 3 independent repeats.

3.5. Western Blotting

In order to investigate the activation of the EGFR in relation to treatment with radiation alone or a combination of erlotinib and irradiation, Western blotting was used with A549, NCI-H460, Calu-6 and PC-9 cell lines.

A549 cells and PC-9 cells were used as positive controls because their phospho-EGFR signal has been studied previously. NCI-H460 and Calu-6 cell lines were investigated for the pattern of EGFR phosphorylation after radiation therapy and the modulating effects of erlotinib treatment on phospho-EGFR after irradiation.

3.5.1. Cell Culture for Western Blotting

Cells for Western blotting were cultured by plating a specific cell amount into d60 60x15mm tissue culture dishes (see below). Medium was added so that each d60 dish contained 4ml total volume.
The amount of cells placed into the d60 dishes was specific for each cell line since their growth behavior was divergent. For A549 cells, $5x10^5$ cells/4ml were plated into each d60 dish. In case of NCI H460 and Calu-6 cells, $7x10^5$ cells/4ml were plated per d60.
Due to their growth behavior, PC-9 cells required $8x10^5$ cells/4ml to be plated into each d60. After the d60 dishes were plated, dishes were incubated until the cells reached 70% confluence. While growing to 70% confluence, cells were cultured in regular medium with 10% BGS. After reaching 70% confluence, the medium was removed from all d60 dishes. In exchange, the dishes received 4ml 0.5% BGS-medium. The reduction of bovine growth serum in the medium was necessary because the BGS itself might contain a variety of EGFR ligands that could interact with the EGFR, causing an interfering background phosphorylation of EGFR.

Complete serum starvation with media containing 0%BGS were attempted as well, but for general purposes 0.5%BGS containing media were preferred due the fact that 0%BGS-medium slowed down cell growth relatively more that 0.5%BGS-medium, indicating that cells might need a basal growth serum mediated stimulation for regular functioning (data not shown). To avoid possible disturbances of cell function by complete serum depletion, ultimately 0.5%BGS containing media were chosen for most experiments.
After 24 hours incubation in BGS-reduced cell-specific medium, the cells in the d60 dishes were ready for the next step in sample generation.

3.5.2. Treatment of Cells

Depending on the experiment setup, the dishes received different treatment types. Generally, one d60 dish remained untreated as a control.
Appropriate samples were irradiated with doses according to the experiment setup. The d60 dishes were then incubated for a specific time to allow the EGFR to be phosphorylated. Other samples were treated with 2µM erlotinib containing medium 45 minutes prior to being irradiated. As a control, other samples were solely treated with 2µM erlotinib 45 minutes prior to further handling.
To test the activity of the EGFR, samples were treated with 100ng/ml EGF for 15 minutes prior to further processing. Parallel dishes received a combination treatment of 2µM erlotinib 45 minutes prior to EGF stimulation to test the ability of erlotinib to inhibit EGFR phosphorylation.
After receiving their specific treatments, the d60 dishes were incubated to allow the generation and/or inhibition of possible phospho-EGFR signals.

3.5.3. Creating Total Cell Lysates

At the end of the incubation period, the d60 dishes were removed from the incubator and directly placed on ice. The medium was removed from the dishes and the cells washed three times with 5ml ice-cold 1xPBS. Placing the dishes on ice and washing them tree times with ice-cold PBS provided the necessary cooling of the cells to stop ongoing cell processes, thus conserving the phospho-EGFR.

Shock freezing the cells on dry ice before washing did not improve the signal quality and was not further pursued (data not shown).
The 1xPBS of the last washing cycle was removed thoroughly and 25µl cell lysis buffer were added twice onto the cell layer in the d60 dishes, each time incubating the lysis buffer for 2 minutes and scraping the cells off the d60 dishes using a disposable cell lifter. The lysis buffer was prepared freshly using 1ml Cell Extraction Buffer, 50µl protease inhibitor and 5µl PMSF. During lysing, the lysis buffer was kept on ice.
Initially, the lysis buffer used was created by using 1ml RIPA-Buffer and adding 10µl of protease inhibitor, 5µl PMSF and 5µl Na_3VO_4. This lysis buffer did not provide effective lysing and was discontinued.
After each scraping cycle, the cell-lysis buffer solution was transferred into a 1.5ml Eppendorf tube and placed on ice. The scraped cell-lysis buffer solution was incubated on ice for 30 minutes, vortexing the sample every 10 minutes.
Then, the samples were centrifuged at 14,000g for 20 minutes at 4°C. The protein containing supernatant was transferred into a new 1.5ml Eppendorf tube. At this point, the protein sample was either processed or stored at -70°C.

3.5.4. Protein Concentration Measurement

The protein concentration was measured using a GeneQuant Pro photometer (GeneQuant Pro, Amersham Biosciences) at 595nm wavelength. The photometer was calibrated using standardized samples containing bovine serum albumin at known concentrations.
After calibration, photometric samples were prepared using 798µl ddH_2O, 200µl protein dye and 2µl lysate. The solution was mixed, transferred into photometric cuvettes and the protein concentration was measured. The readings were recorded and based on the concentrations, Western blot samples with equal protein contents were created.

3.5.5. Immunoprecipitation of EGFR

Immunoprecipitation was performed to reduce the background while using specific pTyr-Antibodies. Since the immunoprecipitation as described below did not improve the pEGFR

signal, immunoprecipitation and the use of tyrosine specific antibodies were discontinued (data not shown).

To pre-clear the lysate, agarose beads were mixed with cell lysate into 1.5ml Eppendorf tubes for 30 minutes at 4°C. The solution was centrifuged and the supernatant moved to a fresh 1.5ml Eppendorf tube. An equal amount of protein of each sample was mixed with a total-EGFR antibody and cell extraction buffer was added until each sample had the same volume. The samples were incubated for one hour on ice. After incubation, beads were added to the samples and the mixture was incubated for 30 minutes at 4°C. After incubation, the beads were washed two times and reconstituted with 20µl 4x Sample Buffer (Invitrogen), 8µl Reducing Agent (Invitrogen) and 12µl ddH_2O. The solution was denaturated for 10 minutes at 70°C, centrifuged and the supernatant used as Western blot samples, loading equal amounts into each lane.

3.5.6. Creating Western Blot Samples

Samples were generated by mixing 2.5µl 10x Reducing Agent (Invitrogen), 6.25µl 4x Sample Buffer (Invitrogen) and a maximum of 16.3µl protein sample. Western Blots were run with a protein amount of 80µg per sample, and in case this protein amount was present in less then 16.3µl, the difference was filled with ddH_2O. During handling, all substances were kept on ice to conserve the phospho-EGFR signal.

Samples were denatured at 70°C for 10 minutes to inhibit the effects of possible protein interactions upon the phosphorylation status of the EGFR. Then, samples were placed on ice for 1 minute and the fluids were recollected by pulse spinning the samples 20 seconds at 12,000rpm. After the samples were collected at the bottom of the Eppendorf tubes, they could either be frozen at -70°C or blotted right away.

3.5.7. Running Western Blot Gels

To run the Western blot samples, the gel box was assembled and, if two gels were run at the same time, placed on ice. 1mm x 10wells 4-12%Bis-Tris gels were placed in the gel box, the outer chamber filled with 800ml and the inner chamber filled with 200ml 1xMOPS running buffer (50ml 20xMOPS running buffer + 950ml ddH_2O=1L 1xMOPS running buffer). In

addition, 500µl of antioxidant (Invitrogen) were added to the inner chamber to protect gels and samples from oxidative reagents.
The left and right outermost lanes of the gel as well as all free lanes that were not loaded with samples or weight standards were filled with 25µl 1x sample buffer. All samples, with a volume of 25µl each, were loaded into the lanes of the gel and the molecular weight standards with a volume of 17.5µl per lane were also loaded. The gel box was closed, connected to the power source and the gel ran for 100 minutes at 200V.

3.5.8. Transferring Western Blot Gels

After 100 minutes, the gel box was disassembled, the gel taken out of its protective case and placed into chilled transfer buffer (940ml ddH_2O + 10ml 10% SDS solution + 50ml 20x Transfer Buffer). The PVDF membrane was prepared by first washing in methanol, then ddH_2O and then in chilled transfer buffer. The transfer cassette was prepared, placing the membrane onto the gel, between two filter papers and two sponges soaked in transfer buffer. The sandwich cassette was closed and placed into the transfer box together with a cooling block and then filled with transfer buffer. The transfer box was connected to the power source and the transfer ran 70 minutes at 100V.
After the transfer, the transfer cassette was disassembled, and the gel placed in Coomassie while being rocked for 30 minutes on a shaker to stain for residual proteins in the gel.
The membrane was rinsed in 0.1% TBS-T (100ml TBS-T + 900ml ddH_2O) and blocked for 1 hour at room temperature on a shaker. For blocking, 10ml of a 5% milk solution were used, created by mixing 0.5g non-fat dry milk with 0.1% TBS-T.
Initially, a 5% BSA solution (0.5g BSA + 10ml 0.1%TBS-T) was used for blocking. Due to the high background noise present in pictures of membranes blocked with BSA instead of milk, 5% milk solution was eventually chosen for all blocking steps.

3.5.9. Blotting for EGFR

The membranes were blotted with antibodies specific for the protein of interest. Blotting was done by placing the membrane with the protein carrying side onto a paraffin film with 1.5ml 5% milk solution, containing the primary antibody at a 1:500 ratio (3µl of primary antibody in

1.5ml). The membrane was blotted over night in a cold room at 4°C. Then, the membrane was washed three times for 15 minutes with 0.1% TBS-T on a shaker.
Other methods, such as blotting the membrane for 2 hours at room temperature on a rocker or blotting over night at 4°C on a rocking table did not improve the quality of the Western blot and were discontinued (data not shown).
As primary antibodies for total phosphorylated EGFR, two antibodies were used. The 4G10® Platinum mouse antibody proved to be inferior to the PY-20 mouse and ultimately, PY-20, which binds to all phosphorylated tyrosine sites of the EGFR, was used as primary antibody for all blots.

Two specific phospho-tyrosine antibodies were used. The pY992 rabbit antibody as well as the pY1068 rabbit antibody did not show a better phospho-EGFR signal in blots compared to the PY-20 pan-phospho-tyrosine antibody and were discontinued (data not shown).
To test for total EGFR as a loading control, the H9B4 mouse antibody was used.

Next, the membrane was blotted with a horseradish peroxidase (HRP) linked secondary antibody specifically reacting against the primary antibody, thus marking the desired proteins with HRP. For mouse primary antibodies, an anti-mouse IgG secondary antibody and for rabbit primary antibodies, an anti-rabbit IgG secondary antibody was used.
The secondary blotting was done with 10ml of 5% milk containing the secondary antibody at a 1:20000 ratio (0.5µl antibody in 10ml blotting solution). After one hour blotting on a shaker at room temperature, the membrane was washed three times for 15 minutes with 0.1% TBS-T.

3.5.10. Visualizing the Blotted Proteins

To picture the proteins of interest, the two WestPico Supersignal ECL reagents were mixed at a ratio of 1:1, dispersed over the membrane and incubated for 5 minutes. Then, the membrane was placed into a radiation therapy cassette, the chemiluminescence film placed on the membrane in a dark room and after the appropriate exposure time required by each blot to show a signal, the film was developed in a developing machine.
Initially, BostonBioproducts ECL reagents were used, but proved to be too insensitive for detection of the blotted signals and were stopped in favor of the WestPico Supersignal ECL-reagents.

The pictures were digitalized using a Camera (Fuji) and a light table for better photographic imaging, or a scanner (Epson). If needed, Western blot pictures were adjusted for contrast and brightness using GIMP.

3.6. Complete List of all Materials

Cell Lines

Cell Line	Cell Line Specifications
A549	- NSCLC Cell Line - Cultivation in DMEM Medium - Provided by Settleman Lab, MGH Cancer Center, Boston, MA, U.S.A.
NCI-H460	- NSCLC Cell Line - Cultivation in RPMI Medium - Provided by Settleman Lab, MGH Cancer Center, Boston, MA, U.S.A.
Calu-6	- NSCLC Cell Line - Cultivation in MEM-Medium - Purchased through ATCC (American Type Culture Collection)
PC-9	- Human Pulmonary Adenocarcinoma Cell Line - Cultivation in RPMI Medium - Provided by Settleman Lab, MGH Cancer Center, Boston, MA, U.S.A.

Media for Cell Culture

Media Base	Additives Added for Cell Culture
RPMI 1640 (SIGMA)	- all media completed by addition of:
DMEM (SIGMA)	- 50ml BGS (HyClone; 10% total BGS/500ml medium)
MEM (SIGMA)	- 5ml L-Glutamine - 5ml 1x HEPES (Siena) - 5ml Penicillin-Streptomycin (CELLGRO)

Further Materials used to keep Cells in Culture

Material	Material Specifications
1x PBS	
1x Trypsin	
T25 Culture Flask	- 25cm^2 flask (BD Falcon)
T75 Culture Flask	- 75cm^2 flask (BD Falcon)
Incubator	- FORMA Scientific 37°C/5%CO_2
Hood	- STERIL Guard HOOD (Baker Company, Inc.)
Microscope	- Nikon Eclipse TS100
Counting Grid	- Reichert Bright-Line Hemacytometer
Sterilizer	- Harvey Sterile Max

Freezing Cells

Material	Specifications
50ml/15ml Tubes	- BlueMax (BD Falcon)
DMSO **(**Dimethyl sulfoxide)	- SIGMA
Centrifuge	- Beckman Coulter Microfuge 18 and Microfuge R
-70°C Freezer	- VWR Scientific
Liquid Nitrogen Tank	- CRYOMED Forma Scientific

Treatment of Cells

Material	Specifications
X-Ray Generator	- Siemens Sabilipan 2280KVp, 1.98Gy/min
EGF	- Epidermal Growth Factor, human (SIGMA)
Erlotinib	- Tarceva® (Roche), provided by Settleman Lab, MGH Cancer Center, Boston, MA, U.S.A.

Materials for Colony Formation Assays

Material	Specifications
1x PBS	
Methanol	- Fisher Scientific
Methylene Blue	- SIGMA
Microscope	- Stereomaster (Fisher Scientific)
Light Table	- Apollo Portable Light Box (Listel)

Materials for Cell Lysing

Material	Specifications
Tissue Culture Dish	- 60x15mm, Beckton Dickinson (FALCON)
1x PBS	- Cooled on ice
Styrofoam Box	- Filled with Ice
RIPA Buffer	- Boston BioProducts
Cell Extraction Buffer	- Invitrogen
Protease Inhibitor Cocktail	- SIGMA
NA_3VO_4	
PMSF	
Celllifter	- Fisherbrand Disposable CellLifter (Fisher Scientific)
Vortexer	- Vortex Genie (Scientific Industries, Inc.)
Centrifuge	- Microfuge®R Centrifuge (Beckman Coulter)
Photometer	- GeneQuant Pro Photometer (Amersham Biosciences)
Calibration Solution	- 1mg/ml BSA stock solution, Albumin Bovine Serum (SIGMA)
Protein Dye	- Protein Assay Dye Reagent Concentrate (BioRad)
ddH_2O Machine	- MILLIPORE MILLI-Q (Continental Water Systems)

Materials for Immunoprecipitation of EGFR

Material	Specifications
Beads	- Protein G Plus/Protein A-Agarose (Calibiochem)
20% DTT	- Bead Reconstitution Reagent

Materials for Western Blot

Material	Specifications
Gels	- NuPAGE 4-12% Bis-Tris Gel, 1mmx 10wells (Invitrogen)
Western Blot Box	- Novex Mini-Cell and XCell Surelock lid (Invitrogen)
Powersource	- Powerpac 200 (Bio-Rad)
Antioxidant	- NuPAGE Antioxidant (Invitrogen)
Running Buffer	- NuPAGE MOPS SDS Running Buffer (20x) (Invitrogen)
Molecular Weight Ladders	- Novex Sharp PreStained Protein Standards (Invitrogen) - PrecisionPlus Protein Kaleidoscope Standard (Bio-Rad)

Materials for Gel Transfers

Material	Specifications
Transfer Cell	- Mini Trans-Blot Cell (Bio-Rad)
Transfer Sandwich	- Mini PROTEAN 3Cell Sandwich (Bio-Rad)
Powersource	- PowerPac 200 (Bio-Rad)
Transfer Membranes	- PVDF Membrane Filter Paper Sandwich, 0.2µm Pore Size (Invitrogen)

Material	Specifications
Transfer Buffer Solution	- NuPAGE Transfer Buffer (20x) (Invitrogen) - 10% SDS-Solution ultraPURE (GIBCO, Invitrogen)
Gel Staining Dye	- SimplyBlue SafeStain (Invitrogen)
Membrane Blocking	- 0.1% TBS-T - Blotting grade Non Fat Dry Milk (Bio-Rad) - BSA Albumin Bovine Serum (SIGMA)
Knife	- GelKnife (Invitrogen)
Scale	- TL-104 (Denver Instrument Company)
Stirring Plate	- Type 1000 Thermolyne (Sybron Cooperation)
Shaker	- Hybri Shake (ThomasScientific)

Blotting Antibodies

Antibody Type	Specifications
Total p-Tyr Abs	- Anti-Phosphotyrosine (Clone PY-20), Mouse IgG_{2b}, (ImmunO, MP Biomedicals, LLC) - Anti-Phosphotyrosine, 4G10® Platinum Millipore (Upstate, Temecula California)
Total EGFR Ab	- Anti-Human EGFR, Mouse mAb (Clone H9B4), (Biosource)
Specific p-Tyr Abs	- Rabbit Anti-EGFR [pY^{992}] phosphospecific Antibody (Biosource) - p-EGFR [pY^{1068}] phosphospecific Antibody (Abcam)
Additional Loading Control Ab	- Monoclonal Mouse Anti-ß-Actin Antibody (Clone AC-15) (SIGMA)
Secondary Abs	- Goat Anti-Mouse IgG HRP-Linked ImmunoPure Antibody (ThermoScientific) - Anti-Rabbit IgG, HRP-Linked (Cell Signaling)

Materials for Membrane Exposure

Material	Specifications
ECL-Reagents	- SuperSignal WestPico Chemiluminescent Substrate (ThermoScientific) - Luminol and Oxidizing Solutions (Boston BioProducts)
Stripping Buffers	- Cell Extraction Buffer (Invitrogen) - Glycine (FisherScientific) - Tween 20 (FisherScientific)
pH-Meter	- pH Meter 430 (CORNING)
Membrane Exposure	- Radiation Therapy Cassette 25x30cm (DUPONT CRONEX) - Chemiluminescence BioMax Light Film 13x18cm (KODAK)
Film Developing Machine	- X-OMAT 2000 Processor (KODAK)
Digital Camera	- Fuji Finepix E900
Scanner	- Perfection 2480 PHOTO (EPSON)
Imaging Software	- The GIMP - Adobe Photoshop

4. Results

The EGFR plays an important role in the cellular response to irradiation. Irradiation can activate EGFR and its various downstream pathways in a variety of cell systems, leading to a broad range of pro-survival effects (Yarden et al., 2001). Inhibition of EGFR in combination with irradiation has been shown to increase the cytotoxic effects of radiation treatment (Huang et al., 1999; Huang et al., 2002; Milas et al., 2000; Bianco et al., 2002). Activating mutations in the KRAS proto-oncogene have been suggested as a positive predictive marker for radiosensitization by EGFR inhibitors, mainly based on the comparison of KRAS mutant A549 NSCLC cells with KRAS wild-type FaDu hypopharynx squamous cell carcinoma cells (Toulany et al., 2006). In this project, three KRAS mutant NSCLC cell lines, namely A549, NCI-H460 and Calu-6, were used to investigate the radiosensitizing effects of the EGFR TKI erlotinib (Tarceva®, Genentech).

4.1. Antibody Validation for Detection of Phosphorylated EGFR (pEGFR)

First, we sought to verify the ability of radiation to activate EGFR by phosphorylation of its tyrosine kinase domain. For validation of the anti-phospho-tyrosine antibody (PY-20), we utilized the PC-9 NSCLC cell line, which carries an activating mutation in the EGFR kinase domain (while KRAS is wild-type). This mutation leads to constitutive activation of EGFR which can be further increased by preincubation with EGF ligand.

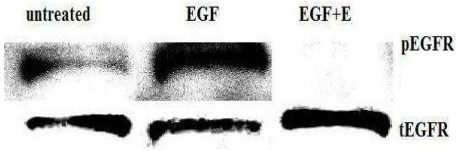

Fig. 1: Visualization of phosphorylated EGFR (pEGFR) in PC-9 cells. Three differently treated PC-9 Western blot samples were generated, one derived from untreated cells (untreated), one after stimulation with 100 ng/ml EGF 15 minutes prior to lysing (EGF), and one after treatment with 2 µM erlotinib 45 minutes prior to EGF treatment (EGF+E). Cells were maintained in medium supplemented with 0.5% BGS for 24 hours prior to treatment. pEGFR was detected with the PY-20 primary Ab. Total EGFR (tEGFR) was used as loading control.

The Western Blot in Fig. 1 visualizes the basal activation status of PC-9 cells, the influence of EGF treatment on EGFR phosphorylation (pEGFR) and the effects of erlotinib.

To test the samples for pEGFR, the pan-phospho-tyrosine antibody PY-20 was used. The pEGFR band was identified by its presence at the expected molecular weight of 170 kDa, confirmed by usage of molecular weight markers during blotting (not shown). Total EGFR protein (tEGFR) was used as a loading control and identified by blotting with a monoclonal tEGFR antibody.

The untreated PC-9 cells showed basal phosphorylation of the EGFR that was independent of external stimulation (untreated, Fig. 1). When the PC-9 cells were treated with 100ng/ml EGF 15 minutes prior to lysate generation, EGFR phosphorylation was enhanced (EGF, Fig. 1). This pEGFR signal was completely abrogated by pretreatment of PC-9 cells with 2 µM erlotinib 45 minutes prior to EGF treatment, thereby verifying the ability of erlotinib to inhibit EGFR phosphorylation (EGF+E, Fig. 1).

In conclusion, Figure 1 shows that the total phospho-tyrosine antibody PY-20 is suitable for detecting changes in the phosphorylation of EGFR. It also demonstrates that the EGF as well as the erlotinib used in this and the experiments below were functional.

4.2. Presence of Early EGFR Phosphorylation in A549 Cells

The A549 cell line has previously been studied and a radiation-induced increase in EGFR phosphorylation was demonstrated (Tanaka et al., 2008). To confirm this observation and to further study the kinetics of pEGFR in A549 cells, Western blots were performed.

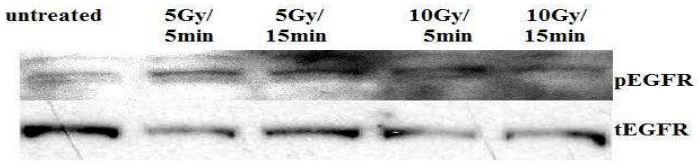

Fig. 2: Ionizing radiation **(**IR)-induced EGFR activation in A549 cells. Various lysates were generated: an untreated control (untreated), samples receiving 5 Gy irradiation and 5 minutes or 15 minutes incubation prior to lysing (5Gy/5min and 5Gy/15min), and samples receiving 10 Gy irradiation and 5 minutes or 15 minutes incubation prior to lysing (10Gy/5min and 10Gy/15min); cells were kept in 0.5% BGS-medium for 24 hours prior to treatment; pEGFR blotted with PY-20 primary Ab; loading control total EGFR (tEGFR).

The irradiation-induced changes of EGFR phosphorylation in A549 cells are shown in Fig. 2. Cells were irradiated with various doses and incubated for either 5 or 15 minutes prior to lysing.

Cells that did not receive irradiation showed a basal phosphorylation of the EGFR (untreated, Fig. 2).

Upon irradiation with 5 Gy followed by 5 minutes incubation (5Gy/5min, Fig 2), the pEGFR signals showed a strong increase from basal levels. Increasing the incubation time after 5 Gy IR to 15 minutes (5Gy/15min, Fig.2) did not further increase the signal strength of the pEGFR band.

When cells were irradiated with 10 Gy and incubated for 5 minutes prior to lysing (10Gy/5min, Fig. 2), the induced pEGFR signal strength was weaker than with 5 Gy of irradiation, and the pEGFR signal levels further decreased after 15 minutes of incubation (10Gy/15min, Fig. 2).

Therefore, the blot shows a basal pEGFR signal and a signal increase after irradiation. This increase is strongest with 5 Gy and 5 minutes incubation prior to lysing (5Gy/5min, Fig. 2), and slowly decreases with the lowest signal strength at 10 Gy and 15 minutes incubation (10Gy/15min, Fig. 2).

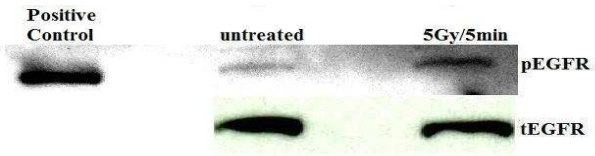

Fig. 3: IR-induced EGFR activation in A549 cells. Two samples were generated: an untreated control (untreated), and a sample receiving 5 Gy irradiation and 5 minutes incubation prior to lysing (5Gy/5min); cells were kept in 0 % BGS-medium for 24 hours prior to treatment; pEGFR blotted with PY-20 primary Ab; loading control total EGFR (tEGFR).

To confirm these findings, an independent repeat experiment is shown in Fig. 3. Cells were maintained in serum-free medium (0% BGS) for 24 hours prior to treatment. This complete serum starvation was performed to test if growth factor free medium influences the phosphorylation of EGFR.

Similar to Fig. 2, the untreated sample (untreated) showed a baseline activated EGFR. The sample that received 5 Gy irradiation followed by 5 minutes incubation prior to lysing (5Gy/5min, Fig. 3) showed a much stronger pEGFR band when compared to the untreated sample.

Taken together, both Western Blots in Fig. 2 and Fig. 3 showed an increase of the pEGFR signal when cells were irradiated, the strongest increase to be found at 5 Gy of irradiation followed by 5 minutes of incubation prior to lysate generation.

The presence of the IR-induced phospho-EGFR signal at 5 minutes after irradiation represents an important confirmation of published results that EGFR can be activated by irradiation (Tanaka et al., 2008). Also, the complete serum starvation performed with samples pictured in Fig. 3 did not alter the pEGFR signal when compared to the samples in Fig. 2, for which cells were kept in 0.5%BGS containing medium prior to lysing.

4.3. No Late Phase EGFR Phosphorylation Present in A549 Cells

The presence of an activating KRAS mutation has been suggested to be a predictor for radiosensitization by EGFR inhibition (Toulany et al., 2005). This function may be mediated through autocrine stimulation of EGFR. Published data show that about 120 to 180 minutes after irradiation, a second, ligand-dependent autocrine EGFR phosphorylation may take place (Dent et al., 1999). To study whether the presence of a KRAS mutation leads to a delayed EGFR phosphorylation via EGFR ligand secretion hours after irradiation, Western blots of irradiated A549 cells were performed.

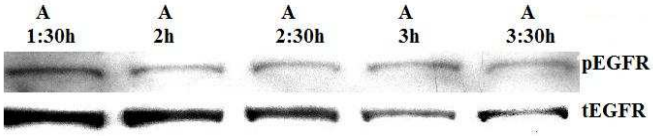

Fig. 4: IR-induced late phase p-EGFR in A549 cells. All samples were irradiated with 5 Gy and incubated for various lengths prior to lysate generation: 1:30 hours (A1:30h), 2 hours (A2h), 2:30 hours (A2:30h), 3 hours (A3h) and 3:30 hours (A3:30h); cells were kept in 0.5% BGS-medium 24h prior to treatment; pEGFR blotted with PY-20 primary Ab; loading control total EGFR (tEGFR).

To investigate a possible late phase EGFR phosphorylation, A549 cells were irradiated with 5 Gy and incubated for increasing lengths prior to lysing, as seen in Fig. 4. There was no appreciable increase in pEGFR at any time point (taking into account slightly more protein loading at the 1:30 hour time point). Especially between 2 hours and 3 hours of incubation, where the previously published EGFR phosphorylation took place (Dent et al., 1999), no increase of the phospho-EGFR signal was present.

4.4. Decreased Clonogenic Survival of A549 Cells after Adding Erlotinib to Irradiation

Next, we assessed the ability of erlotinib to radiosensitize A549 cells using colony formation assays, plating the cells prior to treatment. Cells were plated at appropriate densities and 16 hours later, 2 µM erlotinib was added. After 45 minutes of erlotinib incubation, cells were irradiated at doses of 2 to 8 Gy and incubated for colony formation.

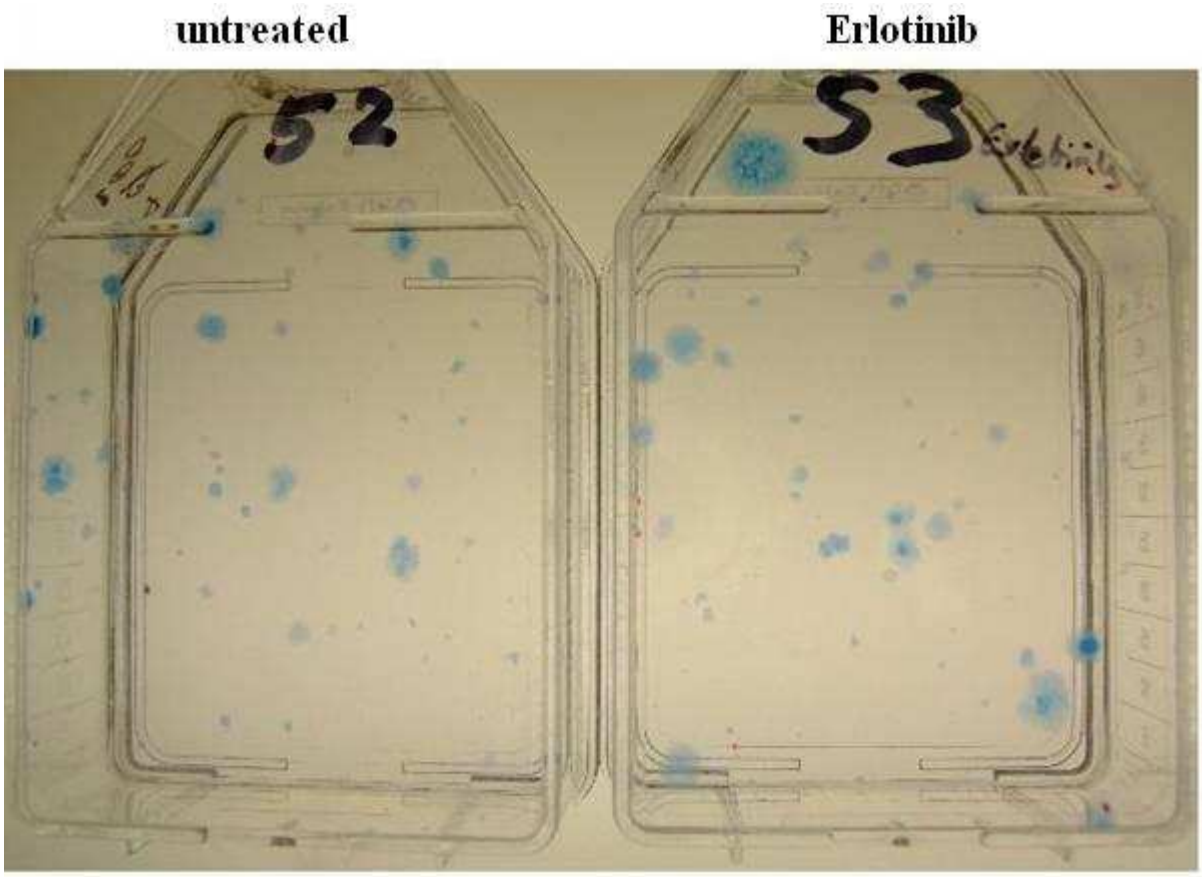

Fig. 5: A459 Colony Formation Assays-Effect of erlotinib alone. Two flasks of a representative colony formation experiment seen in Fig. 6: Left: A549 cells plated without treatment (untreated), 52 colonies; Right: A549 cells plated with 2µM erlotinib (Erlotinib), 53 colonies; erlotinib alone did not reduce average colony size compared to untreated control.

Fig. 5 shows two flasks of a representative colony formation experiment. One flask of cells remained untreated (untreated, Fig. 5). Cells in the second flask in Fig. 5 (Erlotinib, Fig. 5) were maintained in medium containing 2 µM erlotinib during colony formation. The total colony count of both flasks was almost identical, with 52 colonies in the untreated flask and 53 colonies in the flask that received erlotinib only.

It is also evident that the treatment with erlotinib did not influence the size of formed colonies, with both flasks having similar distributions of colony sizes.

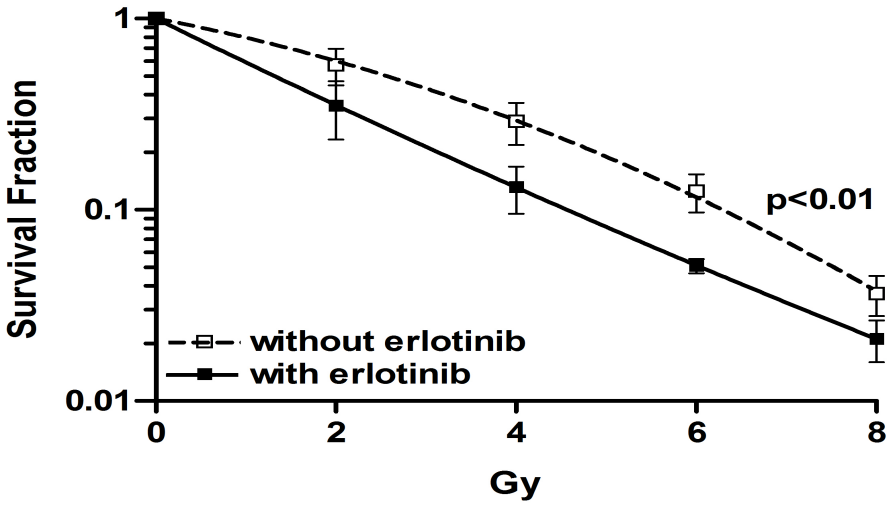

Fig. 6: A459 Colony Formation Assays. A549 cells were plated at different densities for each dose point and after 16 hours, one set of flasks received radiation (IR) only, while the other set was treated with 2 µM erlotinib 45 minutes prior to irradiation (--- = IR only; ── = erlotinib +IR); Statistical comparison by F-test, two-sided.

As shown in Fig. 6, colony formation assays for the A549 cell line were performed by plating cells for colony formation prior to treatment.

These experiments showed that pretreatment with 2 µM erlotinib 45 minutes prior to irradiation led to a clear radioenhancing effect over the entire dose range. The dose enhancement factor (DEF) at the 10% survival level was 1.4, confirming that A549 cells can be radiosensitized by erlotinib (Tanaka et al., 2008).

4.5. Erlotinib Does Not Radiosensitize NCI-H460 Cells in Colony Formation Assays

For the two KRAS mutant NCI-H460 and the Calu-6 cell lines, colony formation assays were performed in two assay variations, plating the cells for colony formation prior to treatment ("plating first") or treating the cells at 70% confluence prior to plating ("treatment first"). By treatment of cells at 70% confluence, the intent was to achieve a sufficient cell density to maximize any underlying ligand excretion that could influence EGFR phosphorylation.

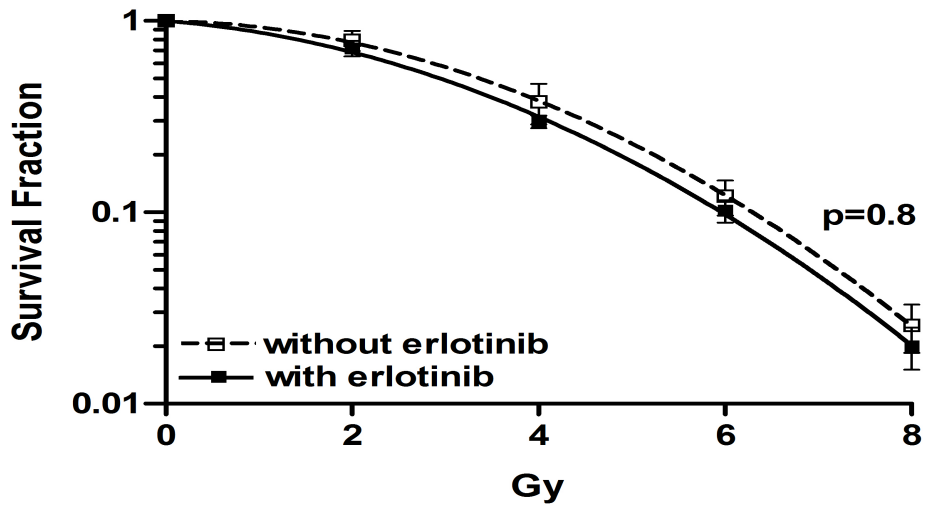

Fig. 7: NCI-H460 Colony Formation Assay – Plating First. H460 cells were plated at different densities for each dose point to allow colony formation, and after cell attachment, one set of flasks received IR only, while the other set was treated with 2 µM erlotinib 45 minutes prior to irradiation (--- = IR only; ── = erlotinib +IR); Statistical comparison by F-test, two-sided.

Using the "plating first" experimental setup, the colony formation assays for NCI-H460 cells did not show statistically significant radiosensitization, as shown in Fig. 7. Over the entire dose range, the flasks pretreated with erlotinib 45 minutes prior to irradiation (solid line, Fig. 7), showed a non-significant reduction of the survival fraction compared to the flasks that received irradiation only (dotted line, Fig. 7).

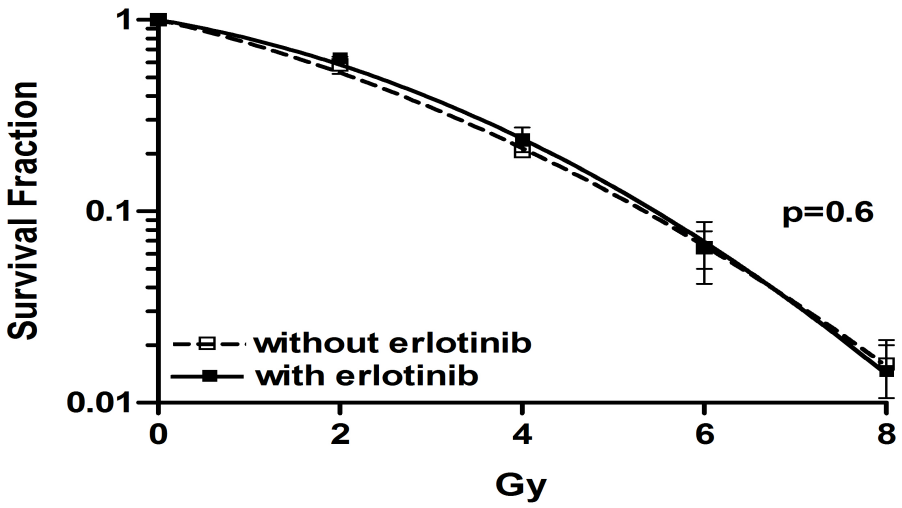

Fig. 8: NCI-H460 Colony Formation Assay – Treatment First. H460 cells were treated at 70% confluence either with IR only or with 2 µM erlotinib 45 minutes prior to irradiation. After 5h of incubation, for each dose and treatment type, two cell densities were plated for colony formation (--- = IR only; ── = erlotinib +IR); Statistical comparison by F-test, two-sided.

In the second set of colony formation assays, NCI-H460 cells were treated at 70% confluence prior to plating for colony formation. Using this "treatment first" experimental setup, the NCI-H460 cells were not radiosensitized by erlotinib, as seen in Fig. 8.

Over the entire dose range, the cells treated with 2µM erlotinib prior to irradiation (solid line, Fig. 8) had about the same surviving fractions as the cells that received IR treatment only (dotted line, Fig. 8).

In summary, radiosensitization by 2 µM erlotinib was not achieved in the NCI-H460 cell line. Neither the "plating first" setup nor the "treatment first" experiments with possibly improved cellular interactions induced radiosensitization.

4.6. Erlotinib Does Not Radiosensitize Calu-6 Cells in Colony Formation Assays

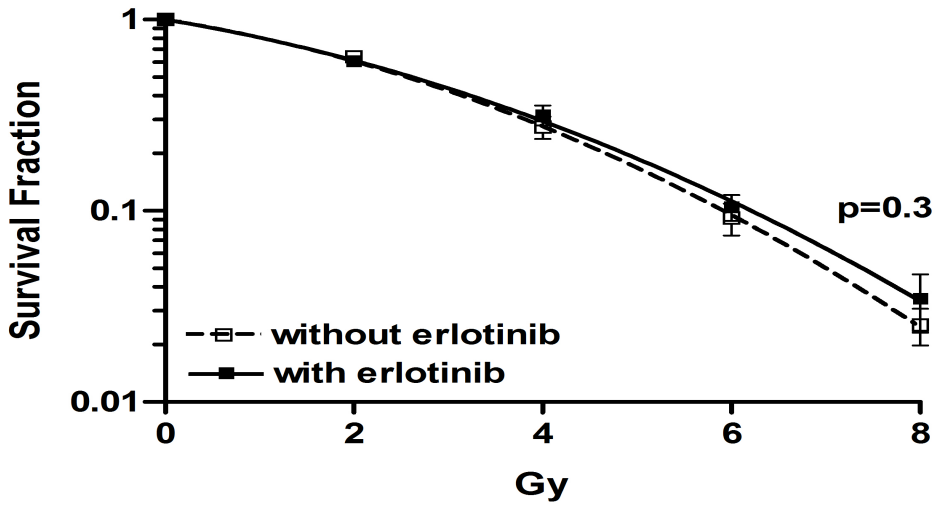

Fig. 9: Calu-6 Colony Formation Assay – Plating First. Calu-6 cells were plated at different densities for each dose-point to allow colony formation, and after cell attachment, one set of flasks received IR only, while the other set was treated with 2 µM erlotinib 45 minutes prior to irradiation (--- = IR only; ── = erlotinib +IR); Statistical comparison by F-test, two-sided .

Colony formation assays for the Calu-6 cells were performed by plating the cells prior to treatment (Fig. 9).

No statistically significant radiosensitization of Calu-6 cells was achieved in this assay. The flasks treated with 2µM erlotinib 45 minutes prior to irradiation (solid line, Fig. 9) showed no reduction in cell survival. Instead, the erlotinib pretreated cells showed slightly increased survival fractions at doses from 4 Gy to 8 Gy when compared to the cells treated with irradiation only (dotted line Fig. 9).

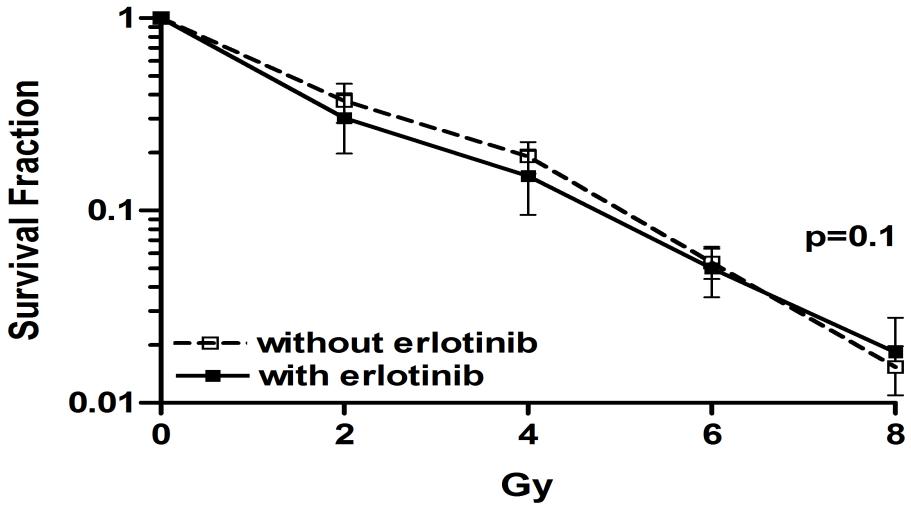

Fig. 10: Calu-6 Colony Formation Assay – Treatment First. Calu-6 cells were treated at 70% confluence either with IR only or with 2 µM erlotinib 45 minutes prior to irradiation. After 5h of incubation, for each dose and treatment type, two cell densities were plated for colony formation (--- = IR only; ── = erlotinib +IR); Statistical comparison by F-test, two-sided.

Next, colony formation assays for the Calu-6 cell line were performed by plating the cells for colony formation after treatment.

With treatment of the cells prior to plating, no radiosensitization of Calu-6 cells with erlotinib was achieved, as visualized in Fig. 10. The erlotinib pretreated cells (solid line, Fig. 10) showed a slightly reduced surviving fraction at doses of 2 Gy and 4 Gy when compared to the irradiated-only cells (dotted line, Fig. 10). At a dose of 6 Gy, both curves met up, and at 8 Gy the surviving fraction of erlotinib pretreated cells was slightly higher than with cells that were only irradiated (solid line, Fig. 10).

Taken together, neither the “plating first” nor the “treatment first” experimental setup demonstrated any radiosensitizing effect of 2 µM erlotinib in colony formation assays with Calu-6 cells.

4.7. Lack of IR Induced EGFR Phosphorylation in NCI-H460 Cells

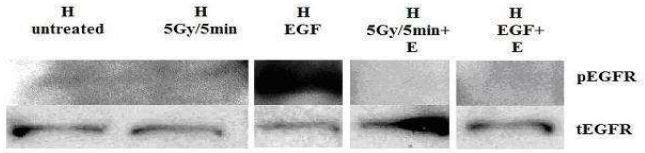

Fig. 11: IR- and EGF-induced EGFR activation in NCI-H460 cells. Various samples were generated: an untreated control (untreated), a sample receiving 5 Gy irradiation and 5 minutes incubation prior to lysing (5Gy/5min), a sample treated with 100ng/ml EGF 15 minutes prior to lysing (EGF), and two samples receiving 2 µM erlotinib 45 minutes prior to EGF treatment (EGF+E) or irradiation (5Gy/5min+E); cells were kept in 0.5% BGS-medium 24h prior to treatment; pEGFR blotted with PY-20 primary Ab; loading control total EGFR (tEGFR); gel was cut to allow intuitive sample order.

The Western Blot in Fig. 11 was done to investigate the activation of EGFR in response to different treatments of NCI-H460 cells.

The untreated H460 sample (untreated, Fig. 11) showed no basal pEGFR signal. No activation of the EGFR was achieved with 5 Gy irradiation followed by 5 minutes incubation prior to lysing (5Gy/5min, Fig. 11). In comparison, an overwhelmingly strong pEGFR signal was induced by treatment of H460 cells with 100ng/ml EGF (EGF, Fig. 11). This pEGFR signal was completely abrogated when cells were pretreated with 2 µM erlotinib 45 minutes prior to EGF stimulation (EGF+E, Fig. 11). The sample treated with erlotinib prior to IR (5Gy/5min+E, Fig. 11) did not show a pEGFR signal either.

Therefore, irradiation did not induce EGFR phosphorylation in the NCI-H460 cell line, consistent with lack of radiosensitization in the colony formation assay, and in contrast to irradiation-induced phosphorylation of EGFR in A549 cells.

4.8. Lack of IR Induced EGFR Phosphorylation in Calu-6 Cells

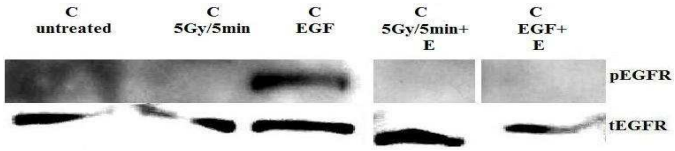

Fig. 12: IR- and EGF-induced EGFR activation in Calu-6 cells. Various lysates were generated: an untreated control (untreated), a sample receiving 5 Gy irradiation and 5 minutes incubation prior to lysing (5Gy/5min), a sample treated with 100ng/ml EGF 15 minutes prior to lysing (EGF), and two samples receiving 2 µM erlotinib 45 minutes prior to EGF treatment (EGF+E) or irradiation (5Gy/5min+E);cells were kept in 0.5% BGS-medium 24h prior to treatment; p-EGFR blotted with PY-20 primary Ab; loading control total EGFR (tEGFR); gel was cut to allow intuitive sample order.

The Western Blot in Fig. 12 was done to investigate the activation of EGFR in response to different treatments of Calu-6 cells.

The untreated Calu-6 sample (untreated, Fig. 12) showed no basal pEGFR signal. No activation of the EGFR was achieved with 5 Gy irradiation followed by 5 minutes incubation prior to lysing (5Gy/5min, Fig. 12). In comparison, a strong pEGFR signal was induced by treatment of Calu-6 cells with 100ng/ml EGF (EGF, Fig. 12). This pEGFR signal was completely abrogated when pretreating cells with 2 µM erlotinib 45 minutes prior to EGF stimulation (EGF+E, Fig. 12). The sample treated with erlotinib prior to IR (5Gy/5min+E, Fig. 12) did not show a pEGFR signal either.

Therefore, irradiation does not induce EGFR phosphorylation in the Calu-6 cell line, consistent with lack of radiosensitization in the colony formation assay, and in contrast to IR-induced phosphorylation of EGFR in A549 cells.

In summary, irradiation with 5 Gy followed by 5 minutes of incubation prior to lysate generation did not induce an increased pEGFR signal compared to the untreated sample. The phosphorylation of EGFR was greatly increased by stimulation with EGF, which was abrogated by pretreatment with erlotinib. No pEGFR signal was present in the erlotinib pretreated and irradiated sample.

Therefore, irradiation was not effective at inducing EGFR activation in the Calu-6 cell line.

4.9. Short-Term Proliferation Assays – IR-Dose Determination

Treatment of cells with erlotinib may impact cell proliferation and induce apoptosis through EGFR inhibition. Induction of apoptosis was suggested by the observation of a steeper survival curve caused by erlotinib in Fig. 6. Therefore, we utilized short-term proliferation assays to asses the effects of erlotinib treatment on the various cell lines. The hypothesis was that short-term assays correlate with the results of the colony formation assays.

First, to determine an appropriate dose of irradiation to be used for short-term proliferation assays, NCI-H460 cells plated at low concentrations were irradiated with different doses.

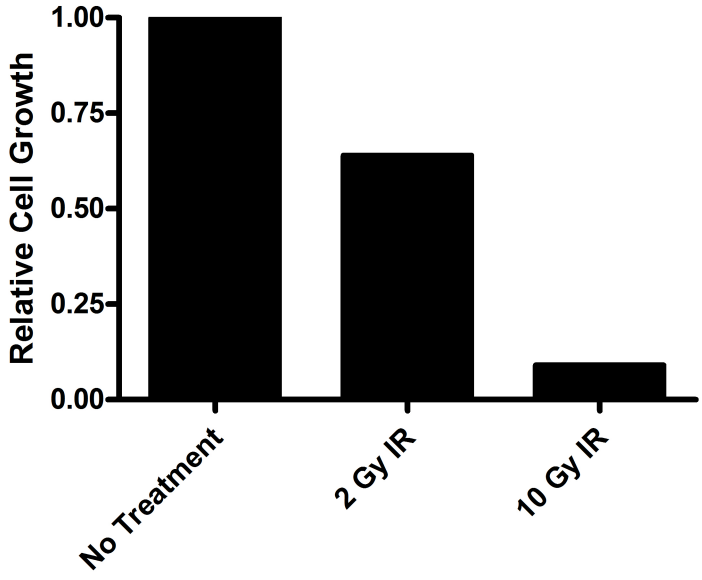

Fig. 13: Short-Term Proliferation Assay – IR-Dose tests with NCI-H460. To determine the right dose of IR for Short-Term Proliferation Assays, H460 cells plated at low densities were either not treated, or irradiated with 2 Gy or 10 Gy. After 72h of incubation, the total cell count and relative cell growth determined.

Fig. 13 shows the results of an initial experiment using short-term proliferation as an endpoint. To determine the best dose of irradiation at which a possible radiosensitization through erlotinib treatment could be detected, H460 cells were either irradiated with 2 Gy, 10 Gy, or remained untreated. After 72 hours of incubation, total cell counts were obtained and the fraction of surviving cells relative to untreated controls calculated.

10 Gy of irradiation led to a considerable reduction of cell growth, reducing the relative growth by 90% compared to the untreated control. At 2 Gy of irradiation, the growth rate was less impaired, with cell growth reaching about 70% of growth of the unirradiated cells.

For the further experiments, a dose of radiation was needed that did not impair cell growth to such a degree that any additional effects of erlotinib would be difficult to detect. With the use of 2 Gy, irradiation reduced the cell growth to only 70% of the control, thus leaving enough cells to demonstrate effects of erlotinib treatment in the following experiments.
Therefore, the clinically relevant dose of 2 Gy was chosen for the short-term survival assays.

4.10. No Radiosensitization of Calu-6 and NCI-H460 in Short-Term Proliferation Assays

Short-Term Proliferation Assays were performed to asses the effects of erlotinib on cell proliferation. Cells were plated at low densities of $1x10^5$ cells into T25 flasks, and following cell attachment, cells were irradiated with 2 Gy, either with or without receiving 2 µM erlotinib 45 minutes prior to irradiation. As controls, one flask remained untreated while another only received 2 µM erlotinib. After 72 hours, total cell counts for each flask were determined.

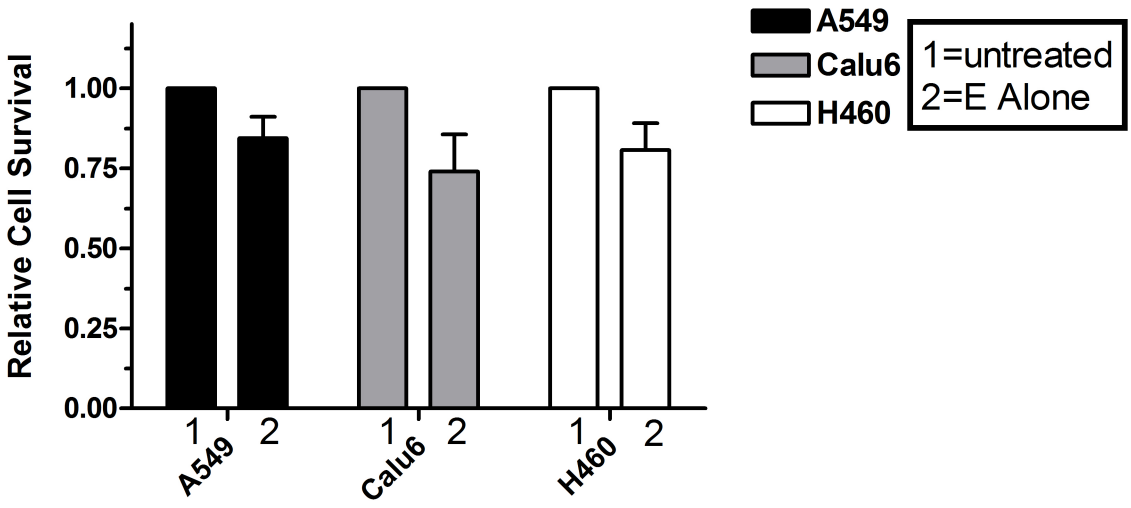

Fig. 14: Effect of erlotinib alone. Cells remained untreated (1), or received 2µM erlotinib (2). After 72h incubation, total cell count was determined and relative cell survival for (1) and (2) calculated.

Fig. 14 demonstrates an only mild impairment of cell proliferation by erlotinib alone in all three cell lines.
In the A 549 cells, the treatment with 2µM erlotinib [A549 (2), Fig. 14], slightly reduced the proliferation rate when compared to the untreated control [A549 (1), Fig. 14].

For the Calu-6 cell-line, 2µM erlotinib [Calu-6 (2), Fig. 14] reduced the cell proliferation compared to the untreated cells [Calu-6 (1), Fig. 14].

The NCI-H460 cells also showed a reduced proliferation of cells treated with 2µM erlotinib [H460 (2), Fig. 14] when compared to the untreated control [H460 (1), Fig. 14].

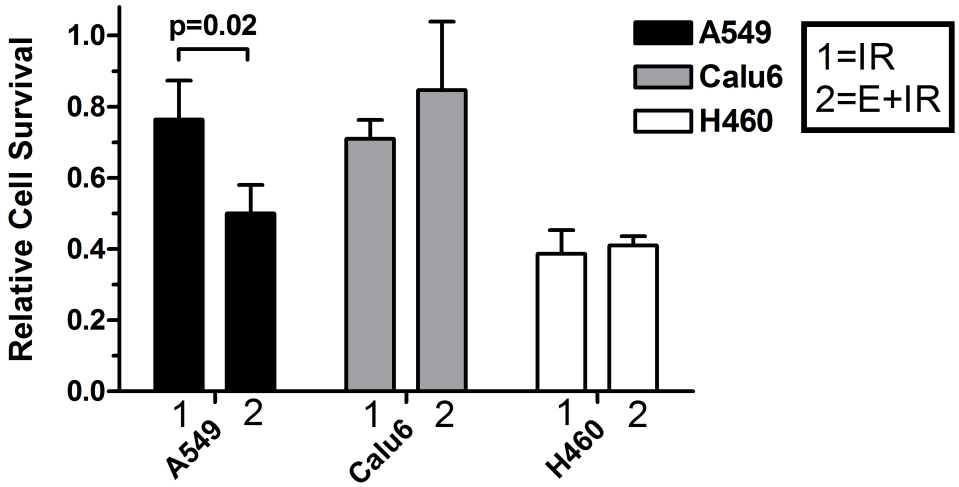

Fig. 15: Effects of combined treatment with IR and erlotinib. Cells were irradiated with 2 Gy (1), or received 2 µM erlotinib 45 minutes prior to IR (2). After 72 hours of incubation, total cell count was determined and relative cell survival for (1) and (2) calculated. Bars represent means with upper standard error, based on at least three independent repeat experiments. p-value, paired T-test, two sided.

For the A549 cell line, a radiosensitizing effect of EGFR inhibition by tyrosine kinase inhibitors (TKI) in colony formation assays has previously been published (Toulany et al., 2005). However, it was unknown whether TKI also affected short-term cell proliferation behavior.

Fig. 15 shows that treatment of cells with 2 µM erlotinib 45 minutes prior to irradiation caused a statistically significant radiosensitization in A549 cells. After 72 hours, the number of cells was markedly reduced by the combined treatment [A549 (2), Fig. 15] regime compared to cells treated with 2 Gy irradiation alone [A549 (2), Fig. 15].

In contrast to A549 cells, the Calu-6 cell line did not show a radiosensitization in the short-term proliferation assays. Cells that received the combined treatment of erlotinib prior to irradiation [Calu-6 (2), Fig. 15] did not show decreased cell numbers at 72 hours when compared to the cells treated with irradiation only [Calu-6 (1), Fig. 15].

In case of the NCI-H460 cell line, no radiosensitization in the short-term proliferation assays was present either. When comparing the combined treatment [H460 (2), Fig. 15] with the IR only treatment [H460 (1), Fig. 15], there was no significant decrease of cell numbers in the combined treatment. Cell counts at 72 hours were equally impaired by both treatment regimes.

In conclusion, A549 cells were radiosensitized, as predicted, by pretreatment with erlotinib prior to irradiation. For the other two cell lines, Calu-6 and NCI-H460, no radiosensitization was present in the short-term proliferation assays. These results were consistent with the results of the colony formation assays.

5. Discussion

Lung cancer is the leading cause of cancer deaths worldwide, having a great impact on personal and socioeconomic health. Current treatment regimes still leave a great percentage of patients with a poor outcome. To improve the outcome of patients by increasing the efficacy of radiation therapy, which represents a major component of cancer treatment, especially in late stage disease, new treatment strategies such as inhibition of the epidermal growth factor receptor (EGFR) are currently in development (Ansari et al., 2009; Sangha et al., 2009). While the EGFR inhibition is a promising strategy, not all patients are expected to benefit from a supporting inhibitory therapy in addition to irradiation. This indicates the need for predictive markers that can be evaluated for each cancer patient to initiate EGFR inhibitory treatment only in those for whom it will be beneficial. To study the value of a KRAS mutation in NSCLC cell lines as a predictive marker for response to a combined treatment with the tyrosine kinase inhibitor (TKI) erlotinib and irradiation, the presented experiments were conducted.

5.1. IR Induced pEGFR in A549 Cells and Radiosensitization by Erlotinib

First, the conducted experiments were able to prove that the TKI erlotinib has the ability to abrogate the phosphorylation of the EGFR (Fig.1). In the KRAS wild type (KRASwt) NSCLC cell line PC-9, which carries a EGFR mutation causing constitutive EGFR activation, erlotinib blocked the baseline pEGFR signal as well as the EGFR phosphorylation induced by EGF treatment. This corresponded well with published results on pEGFR induction by EGF and its abrogation through the TKI gefitinib (Ono et al., 2004). The PC-9 cell line, which due to its activating EGFR mutation is heavily depending on EGFR signaling, is also well known for its responsiveness to EGFR inhibitory strategies using various EGFR inhibitors such as gefitinib (Nishimura et al., 2008), supporting the experimental findings.

Following the validation experiments, the radiation-induced phosphorylation of EGFR in the A549 cell line was explored. By using various radiation doses and different incubation times after irradiation prior to cell processing, the experiments demonstrated an early EGFR phosphorylation (Fig. 2) that was most striking using 5 Gy of radiation followed by 5minutes of incubation. This observation demonstrated the ability of radiation to cause the activation of

EGFR within minutes of irradiation. The early phosphorylation response observed during these experiments was consistent with published data, implying an early phase phosphorylation of EGFR caused by irradiation (Toulany et al., 2007).
The early EGFR phosphorylation after irradiation was also detected in A549 cells that were previously cultured in growth factor depleted medium (Fig.3). These findings demonstrated that the early pEGFR signaling induced by irradiation in A549 cells is independent of supplemented growth factors and is caused by irradiation, independent of the culture media microenvironment.

After identifying the presence of an early pEGFR signal induced by irradiation, the phosphorylation kinetics of EGFR in the A549 cell line hours after irradiation was explored. Using the same conditions that induced the early EGFR phosphorylation, no late pEGFR signals were detected within 1.5 to 3.5 hours after irradiation (Fig. 4). The fact that no late phase EGFR phosphorylation was detected contradicted the assumption that the presence of a KRAS mutation always leads to a ligand dependent late pEGFR signal within two to three hours after irradiation (Grana et al., 2003). The experimental findings seen in Fig. 4 also questioned the idea that a KRAS mutation always induces a late phase pEGFR after irradiation, thereby making KRASmt cell lines generally susceptible for EGFR inhibition strategies (Toulany et al., 2007; Dent 1999).

While the early phosphorylation of EGFR by irradiation in the A549 cell line was demonstrated, the postulated presence of a late phase pEGFR signal after irradiation was not confirmed. The early pEGFR signal (Fig. 2, Fig. 3) was easily identified due to the marked increase of phosphorylated EGFR after irradiation compared to baseline levels. It is possible that a much more subtle increase might have been present in the late phase after irradiation, which could have escaped the sensitivity of Western blotting. Therefore, in the presented experiments of Fig. 4, there is a possibility that a late pEGFR signal change was not detected due to very low overall signal strength changes.
In future studies, to address the limitations of the conducted experiments, the late phase pEGFR signaling after irradiation could be studied in more depth. By sampling the cells in shorter time intervals as well as by using immunoprecipitation for phosphorylated EGFR, a potentially low signal increase hours after irradiation might be detected.

The fact that A549 cells showed no late phase pEGFR signaling after irradiation but were radiosensitized by EGFR inhibitors (Bianco et al., 2002) implied the possibility that the late EGFR phosphorylation might not be the most important signaling to be blocked by EGFR inhibitors. While the late phase pEGFR was absent, the early pEGFR signaling was clearly demonstrated. Thus, instead of the late phase pEGFR, the early activation of EGFR within minutes of irradiation could be the major target for inhibitory strategies of the EGFR to induce radiosensitization.

If the early activation of EGFR after irradiation is the mechanism responsible for cellular radioresistance, an important clinical implication would be to administer EGFR inhibitors such as erlotinib prior to irradiation, rather than administering drug treatment after irradiation with the aim of only blocking a possible second pEGFR signaling wave hours after irradiation. The pEGFR signaling behavior of the A549 cell line supported the hypothesis of this thesis to investigate other mutant KRAS cell lines for a similar EGFR signaling profile and by chance a similar response to EGFR inhibitor treatments.

After confirming the ability of irradiation to induce a phosphorylation of the EGFR in the A549 cell line, colony formation experiments were conducted to study the effects of erlotinib treatment prior to irradiation (Fig. 6).

The treatment of cells with erlotinib followed by irradiation led to a significant decrease in cellular proliferation in colony formation assays. The TKI erlotinib caused a radioenhancing effect which covered the entire dose range of the experiment, thus demonstrating that targeting EGFR with inhibitors such as erlotinib can cause radiosensitization of the A549 cell line. By blocking the phosphorylation of EGFR induced by irradiation and consequently abrogating the prosurvival signaling downstream of EGFR, the A549 cell line was markedly impaired in its ability to cope with radiation-induced cellular stress. In consequence, this led to a decrease in cell survival and proliferation as pictured by the colony formation experiments (Fig. 6). Interestingly, treatment with the TKI erlotinib alone did not have a strong impact on colony formation (Fig. 5), consistent with the reported association of mutant KRAS status with resistance to EGFR TKI. Comparing the untreated control with the erlotinib-treated colonies, the number and size of A549 colonies were almost identical. This finding implies that EGFR signaling alone does not significantly affect normal cell growth in A549 cells, and blocking EGFR for a certain period as done by erlotinib treatment in colony formation assays does not influence colony growth. In contrast, the pEGFR signaling after irradiation does significantly contribute to cell survival and proliferation, because the

combined erlotinib and irradiation treatment was able to reduce colony formation when compared to colonies that were irradiated only. These findings imply that pEGFR signaling in A549 cells is especially important to increase cell survival after irradiation, while under untreated growth conditions, EGFR is not the only driving force of cellular growth and proliferation.

The results of the colony formation experiments were consistent with published data on the radiosensitization achieved in colony formation of A549 cells using the EGFR tyrosine kinase inhibitor BIBX1382BS (Toulany et al., 2005; Toulany et al., 2006). The susceptibility of A549 cells to combined EGFR inhibitor and irradiation treatment was also demonstrated using the TKI gefitinib (Iressa) (Bianco et al., 2002). In addition to radiosensitizing effects caused by tyrosine kinase inhibition, the effectiveness of the monoclonal antibody cetuximab to block EGFR phosphorylation and to cause radioenhancement has also been published (Dittmann et al., 2005a,b), possibly by impairing DNA repair via the DNA-PK. Thus, the various published results on the responsiveness of the A549 cell line to combined TKI and irradiation treatment support the findings presented in Fig. 6.

Considering the findings on the EGFR phosphorylation after irradiation and the impact of combined EGFR inhibition and irradiation on colony formation of the A549 cell line, the KRAS mutation seemed to be less important for radioenhancement as previously postulated (Toulany et al., 2007). The late phase EGFR phosphorylation hours after irradiation was thought to be prominent in KRAS mutant cell lines, caused by increased EGFR ligand production and possibly leading to EGFR signaling driving radioresistance.

In contrast to this theory, no prominent late phase EGFR phosphorylation was seen in A549 cells (Fig. 4), while the A549 cell line was clearly radiosensitized by erlotinib (Fig. 6). The fact that A549 cells radiosensitized despite the lack of a late pEGFR signaling questioned the role of a KRAS mutation in radioresistance. In addition, KRAS mutant Calu-6 and NCI-H460 cell lines could not be radiosensitized by erlotinib. A KRAS mutation might not always lead to ligand mediated late pEGFR signaling after irradiation, and such a signaling might not be the cause of radioresistance. Based on the experimental findings of Fig. 4 and Fig. 6, the presence of an early phase EGFR phosphorylation and its abrogation by erlotinib might be of importance in achieving radiosensitization with combined treatment regimes.

The presented experiments confirmed that the A549 cell line is responsive to combined TKI and irradiation therapy to reduce cell survival. For future clinical practice, NSCLC cell lines

should be screened for similarities with cancer cells responding to EGFR inhibitory treatment to filter for those cases of cancer patients that will benefit from combined treatment regimes. Based on the conducted experiments, the early phosphorylation of EGFR following irradiation might also be of importance for successful radiosensitization by EGFR inhibition.

5.2. No Radiosensitization by Erlotinib and Lack of IR Induced pEGFR in NCI-H460 and Calu-6 Cells

To further investigate the predictive value of a KRAS mutation as a marker for responsiveness to EGFR inhibition combined with irradiation, the two NSCLC cell lines NCI-H460 and Calu-6, both carrying a KRAS mutation, were studied. To assess the ability of the combined treatment regime to impair cell growth, colony formation experiments for both cell lines were conducted in two assay setups, receiving treatment prior or after plating cells for colony growth.

Despite the finding of the KRAS mutant A549 cell line to be radiosensitized, no radiosensitization in colony formation experiments was demonstrated for the NCI-H460 (Fig. 7, Fig. 8) and the Calu-6 cell line (Fig. 9, Fig. 10). These findings suggested that a KRAS mutation does not predispose cells to radiosensitize when treated with EGFR inhibitors. The lack of a radiosensitization in NCI-H460 and Calu-6 colony formation experiments was not consistent with the idea that the presence of a mutant KRAS is a positive predictive marker for response to EGFR inhibition and radiation. It also questioned the role of KRAS mediated EGFR ligand production in radioresistance. If KRAS dependent ligands were to cause EGFR activation leading to radioresistance after irradiation, blocking this signaling cascade in the NCI-H460 and Calu-6 cells would have led to a reduced cell growth in colony formation assays. Because the NCI-H460 and Calu-6 cell lines were not radiosensitized by erlotinib, the conducted experiments questioned the assumption that a mutant KRAS, via ligand mediated EGFR stimulation, always leads to pro survival signaling that could be targeted with EGFR inhibitors to cause radiosensitization (Toulany et al., 2007). Thus, the idea of an indirect interaction between KRAS and EGFR via ligands was not supported. The presented colony formation experiments rather suggested that KRAS dependent ligand production is either not present in all KRAS mutant cell lines or that KRAS not only interacts indirectly, but under certain circumstances directly with EGFR and its downstream pathways. While the A549, NCI-H460 and Calu-6 cell lines all carry a KRAS mutation, those mutations are located at

different codons. In A549 cells, which radiosensitized during colony formation, mutations are present at c.34G>A. In contrast NCI-H460 carries a c.183A>T mutation and in Calu-6 cells, the two mutations c.180_181TC>CA and c.181C>A are present (COSMIC Catalogue Of Somatic Mutations In Cancer, http://www.sanger.ac.uk). The fact that A549 cells inherit a different KRAS mutation than NCI-H460 and Calu-6 cells might influence KRAS function and consequently the cellular response to irradiation. In case of the NCI-H460 and Calu-6 cell line, the KRAS mutations present in those cells might enable a direct KRAS interaction with EGFR downstream pathways, leading to a ligand independent downstream signaling that would cause radioresistance even if cells were treated with the TKI erlotinib. Therefore, experiments are needed to evaluate the impact of various KRAS mutations on EGFR and its downstream signaling.

In conclusion, the performed colony formation experiments of NCI-H460 and Calu-6 cells did not support the hypothesis of a KRAS mutation being responsible for radioresistance and making KRAS mutant cell lines candidates for successful combined EGFR inhibitor and irradiation treatment regimes (Toulany et al., 2006). In this paper, Toulany et al. presented their findings that the KRAS mutant NSCLC cell line A549 was radiosensitized using the EGFR inhibitor BIBX1382BS. Comparing the inhibitor response of A549 cells with the non responding KRAS wild type squamous cell carcinoma cell line FaDu, Toulany et al. proposed that a KRAS mutation could generally be predictive of EGFR inhibitor effectiveness. While the findings of the presented experiments in this thesis seemed to contradict this assumption, the fact that Toulany et al., using A549 and FaDu cells, compared two cell lines of different origin questions general implications drawn from this paper. Instead, the KRAS role in EGFR inhibitor responsiveness should be studied using cell lines of similar origin. This approach would rule out the chance of different cellular responses caused by greatly varying cellular properties as imaginable with cells from different origins.

Interestingly, published results on whether or not EGFR inhibition has radiosensitizing effects vary for NCI-H460 and Calu-6. Using nimotuzumab, a monoclonal antibody against EGFR, no radiosensitization in clonogenic survival assays was achieved in NCI-H460 cells (Akashi et al., 2008), supporting the experimental data presented in Fig. 7 and Fig. 8. In contrast, a growth inhibitory effect on the Calu-6 cell line using a combined treatment of 50 cGy and the TKI gefitinib has been reported (Bianco et al., 2002). The use of 50 cGy represents a very small irradiation dose compared to the more clinical relevant doses between 2 Gy and 8 Gy used in the presented experiments. Also, the TKI gefitinib was applied to cells consecutive from day 1 to day 5 after irradiation, compared to the single application of erlotinib used in

the presented colony formation assays. Therefore, conditions in which growth inhibition for Calu-6 was achieved by Bianco et al. differ greatly from the assay setup used to conduct the colony formation experiments of this thesis. With a dose range for colony formation of 2 Gy to 6 Gy, the experiment setup used by Akashi et al., the published results on NCI-H460 lacking radiosensitization are more comparable with the findings on NCI-H460 of Fig. 7 and Fig. 8.

Based on the presented experiments in Fig. 7 through 10, NSCLC cell lines carrying a KRAS mutation cannot generally be expected to radiosensitize using the TKI erlotinib. The fact that experiments relied solely on the TKI erlotinib as EGFR inhibitor raised the question if cell lines would response differently to other inhibitors. Generally, varying inhibitor effectiveness can be expected, considering that different TKI might target different protein sites of the tyrosine kinase domain. Based on the different mechanism of action, TKI and monoclonal antibodies might also cause varying radiosensitization in the same cell line.
For future experiments, various tyrosine kinase inhibitors as well as monoclonal antibodies targeting EGFR should be employed. By evaluating NSCLC cell lines such as NCI-H460 and Calu-6 for response to a range of EGFR inhibitors, experiments could clearly identify cell lines that do not respond to EGFR inhibitors at all, as well as those cells that might be radiosensitized by a special inhibitor only.
Another consideration is that the conducted experiments studied the response of cell lines to combined TKI and irradiation under in vitro conditions. Future xenograft studies could complement the data from in vitro experiments. Under clinically more relevant in vivo conditions, the studied cell lines might respond differently to EGFR inhibition.
Considering the data presented on colony formation of NCI-H460 and Calu-6 in context with the colony formation experiments of the A549 cell line, a KRAS mutation present in NSCLC cell lines might not be a useful positive predictive marker to identify those cancers that will respond to a combined treatment with the TKI erlotinib and irradiation. Since NSCLC were so heterogeneous in their cellular responses to treatment, screening for other positive predictive markers than KRAS mutation should be continued to allow for a highly individualized cancer treatment regime.

After having identified the lack of radiosensitization of NCI-H460 and Calu-6 cells by erlotinib in colony formation assays, experiments were conducted to explore the irradiation-induced EGFR phosphorylation in those cell lines. While using the same experimental

condition as applied to A549 Western blots, no early phase pEGFR signal was detected for both NCI-H460 (Fig. 11) and Calu-6 (Fig. 12). When treated with EGF, a phosphorylation of EGFR was achieved in both cell lines, proving the general ability of EGFR to become phosphorylated in NCI-H460 and Calu-6 cells. These EGF induced pEGFR signals were completely abrogated by erlotinib, underlining the effectiveness of the TKI erlotinib to block pEGFR signaling in those cells. Considering that no early pEGFR signal was induced within minutes after irradiation, the experiments imply that the pEGFR response to irradiation might be variable between various cell lines.

Another explanation for the lack of early pEGFR signaling in NCI-H460 and Calu-6 cells could be the fact that the induced pEGFR signal in those two cell lines might be very weak. Such a marginal pEGFR signal increase might not have been detected with the Western blots seen in Fig. 11 and Fig. 12. To rule out the chance of a missed pEGFR signal increase, follow up experiments should use immunoprecipitation for NCI-H460 and Calu-6 cells to isolate the EGFR after irradiation to visualize the possible presence of a very faint pEGFR signaling. In addition, cell lines should be examined for downstream signaling of EGFR after irradiation to identify the influence of irradiation on the diverse EGFR downstream signaling pathways.

The finding that NCI-H460 as well as Calu-6 cells did not show a pEGFR signal induction within minutes after irradiation is contradicting published data on irradiation-induced pEGFR. Various papers have suggested that irradiation causes release of cellular reactive oxygen species (ROS), which due to their high reactive potential inhibit pEGFR inactivating proteases, thus leading to an increased pEGFR signaling (Leach et al., 2002; Kamata et al., 2000). Because an irradiation-induced pEGFR signal could not be seen in NCI-H460 and Calu-6 cells, the physical and chemical reactions related to ROS might not be universal to all cell lines. Maybe cellular properties of NCI-H460 and Calu-6 cells quickly inactivate irradiation-induced ROS, ultimately not causing a pEGFR increase.

Therefore, in contrast to the idea that irradiation always induces pEGFR signaling (Schmidt-Ulrich et al., 1997), NCI-H460 and Calu-6 might be cell lines in which EGFR signaling cannot be caused by irradiation.

While the A549 cell line was radiosensitized by erlotinib, no such effect was detected in NCI-H460 and Calu-6 cells. Even though all three cell lines carry a KRAS mutation previously thought to cause pEGFR activation via ligand production, radiosensitization by EGFR inhibition was not achieved in all of the cell lines. Based on these findings, a KRAS mutation in NSCLC cell lines cannot be used as a positive predictive marker for cellular response to EGFR inhibition with erlotinib. For the future application of individualized and precisely

targeted EGFR inhibition strategies added to radiation therapy in NSCLC patients, the identification of novel predictive biomarkers is needed.

In this context, the conducted experiments imply that the presence of an early pEGFR signaling after irradiation might be an important marker for EGFR inhibitor response. In A549 cells, in which irradiation caused an early pEGFR signal (Fig.2, Fig. 3), pEGFR inhibition with the TKI erlotinib induced radiosensitization in colony formation assays (Fig. 6). In contrast, no early IR induced pEGFR signaling was seen in NCI-H460 (Fig. 11) and Calu-6 (Fig. 12) cells, and erlotinib did not cause radiosensitization neither in NCI-H460 (Fig. 7, Fig.8) nor in Calu-6 (Fig. 9, Fig. 10). Studies with more cell lines should be conducted to further evaluate the relation between the presence of an irradiation-induced early EGFR phosphorylation and the cellular responsiveness to EGFR inhibitors.

5.3. Only A549 Cells Radiosensitize in Short-Term Proliferation Assays

In addition to colony formation assays, short-term proliferation assays of A549, NCI-H460 and Calu-6 cells were used to examine the influence of a combined erlotinib and irradiation treatment on cellular proliferation. At first, the clinical relevant dose of 2 Gy irradiation was determined to be most useful for the following proliferation assays (Fig. 13). At 2 Gy, cellular proliferation was markedly reduced but not lowered to a degree at which possible additional effects of erlotinib would be masked by proliferative inhibition caused by irradiation. When all three cell lines were treated with erlotinib alone (Fig.14) in proliferation assays, erlotinib caused a slight decrease of cellular proliferation in all three cell lines. This implies that EGFR inhibition alone had a relatively low anti-proliferative effect similar in all three cell lines. Due to the diverse range of growth signaling taking place in cells, other growth promoting signaling cascades might drive cellular proliferation when EGFR is blocked in unstressed cells, thus explaining the small anti-proliferative effect of erlotinib alone.

In short-term proliferation assays, only A549 cells showed a radiosensitizing effect by the combined erlotinib and irradiation treatment, while NCI-H460 and Calu-6 cells were not radiosensitized (Fig. 15). These finding strengthened the assumption that a KRAS mutation does not necessarily lead to EGFR inhibitor response in NSCLC cell lines. Apparently, in A549 cells, the EGFR signaling network induced after irradiation is a major driving force of

cellular survival and proliferation. By abrogating the pEGFR signaling with erlotinib and eliminating this signaling pathway after irradiation, A549 cells were radiosensitized and markedly impaired in proliferation, as seen in Fig. 15. On the other hand, NCI-H460 and Calu-6 cells, which did not show pEGFR signaling to be induced by irradiation (Fig. 11, Fig. 12), did not respond with reduced proliferation to erlotinib treatment.

Even though the short-term proliferation assays were in line with the findings of colony formation assays, the mechanism by which proliferation was influenced by combined erlotinib and irradiation treatment was not assessed. In future experiments, the cellular response to treatment should be studied to determine whether cells went into senescence, transiently arrested in G1or G2 phase or became apoptotic. Also, short-term proliferation assays are generally thought not to correlate with colony formation assays (Brown et al., 1999). This was mainly due to the assumption that apoptotic effects, which take place rather quickly, would especially influence short-term proliferation assays. In colony formation assays, an initial apoptotic effect was then thought to be masked by long term proliferation of surviving cells. As with the presented experiments, there might be conditions in which proliferation assays and colony formation assays are correlating. The strong anti-proliferative effects seen for A549 cells in colony formation and proliferation assays could be the result of the combined erlotinib and irradiation treatment influencing various survival signals, cell proliferation and DNA repair rather than only causing apoptosis. This long term decrease in cellular proliferation would explain the analogy of the performed short-term proliferation and colony formation assays.

These short-term proliferation assays strengthened the idea that early pEGFR signaling might be the target for successful EGFR inhibition, underlining the necessity for follow up experiments studying irradiation-induced pEGFR in more cell lines and their response to EGFR inhibition. Also the conducted experiments show that a mutant KRAS cannot be used as a positive predictive marker for cancer cell response to EGFR inhibition strategies and screening for other predictive markers has to continue.

6. Summary

This thesis project studied the use of a KRAS mutation present in three NSCLC cell lines, A549, NCI-H460 and Calu-6, as a positive predictive marker for achieving radiosensitization by a combined treatment with the tyrosine kinase inhibitor (TKI) erlotinib followed by irradiation. After validating the method using the KRAS wild type PC-9 cell line, Western blots for phosphorylated EGFR were performed for the three cell lines. In A549 cells, Western blots revealed an early pEGFR signal 5 minutes after irradiation with 5 Gy, but within 1.5 and 3.5 hours after irradiation, no late pEGFR signal was present. In contrast, both the NCI-H460 and Calu-6 cell line did not show a pEGFR signal 5 minutes after irradiation with 5 Gy. In colony formation assays performed for A549, NCI-H460 and Calu-6 cell lines, radiosensitization by treatment with the TKI erlotinib prior to irradiation was only achieved in A549 cells, reaching a dose enhancement factor (DEF) of 1.4 at the 10% survival level. For NCI-H460 and Calu-6 cells, no radioenhancement was detected in colony formation assays. In addition, short-term proliferation assays of the three cell lines were conducted. While erlotinib alone compared to the untreated control had only a small impact on cell proliferation, the combined treatment of erlotinib followed by 2 Gy of irradiation showed a clear anti-proliferative effect on the A549 cell line. Corresponding with the findings of the colony formation assays, the proliferation of NCI-H460 and Calu-6 cells was not significantly decreased by the combined treatment regime.

Even though the KRAS mutant A549 cell line was radiosensitized in colony formation and proliferation assays, the other two KRAS mutant cell lines NCI-H460 and Calu-6 did not respond to EGFR inhibition. Taken together, the presence of a KRAS mutation in those three NSCLC cell lines was not predictive for cellular response to EGFR inhibition combined with irradiation. Therefore, a KRAS mutation present in NSCLC cell lines cannot be used as a general positive predictive marker for achieving radiosensitization with the TKI erlotinib.

Also, the presence of a KRAS mutation in A549 cells was not associated with a late phase pEGFR signal after irradiation, questioning whether a KRAS mutation always leads to an increased pEGFR signaling via ligand production. Furthermore, the experimental data showing that a pEGFR signaling within minutes after irradiation was only present in A549, but not in NCI-H460 and Calu-6 cell lines implies that irradiation-induced pEGFR might not be a universal phenomenon, but rather influenced by yet unknown cellular properties. The fact that an early pEGFR signaling was only present in A549 cells, which also was the only cell line to be radiosensitized by erlotinib, implies that the early generation of a pEGFR signal

after irradiation might be important for cellular radioresistance and therefore being an important target for EGFR inhibitory strategies. While the conducted experiments demonstrated that the KRAS mutation present in the NSCLC cell lines A549, NCI-H460 and Calu-6 was not predictive for the cells response to erlotinib and irradiation treatment, the early pEGFR signaling after irradiation might be of predictive value. For future individualized therapies of NSCLC, further studies are needed to possibly find a cellular marker predicting the response to combined EGFR inhibition and irradiation, ultimately providing an easy and fast screening method of cancer patients to identify those who will benefit of adding EGFR inhibitors to their irradiation treatment regime.

7. Literature

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10. Statement of Originality

I herewith declare that I have performed the work for this thesis independently and without improper help. This work does not contain any material written or published by another person except where acknowledged. References in word or content are stated with edition, year, volume and page. I have listed all persons who directly participated in the process of this thesis. This body of work has not previously been submitted for a degree at this or any other university.

Signature: ……..