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Radiosensitization of bronchial carcinoma cells by Cetuximab

Dissertation

Thesis to earn a Doctoral Degree of Medicine

At the Faculty of Medicine of the University of Hamburg

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Hamburg

Hamburg June 17, 2012

Accepted by the Faculty of Medicine
of the University of Hamburg on:

Published with the authority of
the Faculty of Medicine of the University of Hamburg

Examination Board, Chairman: Prof. Dr. E. Dikomey / Exp. Radioonkologie

Examination Board, 2nd Consultant: Prof. Dr. C. Petersen / Radioonkologie

Examination Board, 3rd Consultant: Prof. Dr. W. Fiedler / Onkologie und Hämatologie

Contents

1 Hypothesis

Lung cancer is the number one cause of death in both men and women. About 80% of these cancers are non small cell lung carcinomas (NSCLC). Of these NSCLC approximately 80% overexpress the epidermal growth factor receptor EGFR. This receptor can be activated by the ligands EGF, TGFα, amphiregulin, ionizing radiation as well as other stressors to the cell [Chen2007]. EGFR is involved in a variety of pro-survival signals. A strong activation of the receptor leads to increased cell survival, proliferation, angiogenesis, metastasis and decreased apoptosis, which could explain why many malignancies are resistant to ionizing radiation alone.

The EGFR has been a target for a variety of inhibiting treatment approaches with the aim of radiosensitizing NSCLC as well as other carcinomas. Several inhibitors and antibodies are already being clinically used, but they don't sensitize all carcinomas. The specific mutations of EGFR seem to have some predictive value, but so far there is no reliable screening tool to predict which NSCLC can be sensitized by EGFR inhibition.

Recently a novel link between EGFR activation and DNA repair was shown [Dittmann2005/I]. Dittmann focuses on the amount of residual DSB 24h after IR where he sees an increase in the EGFR inhibitor treated group. He links this finding to the interaction of the EGFR with DNA-PK and thus impaired DSB repair.

Cetuximab has been shown to radiosensitize several NSCLC. The underlying mechanism is not fully understood yet.

The objective of this thesis is to investigate the influence of EGFR inhibition by the monoclonal antibody Cetuximab on the induction and repair of double strand breaks (DSB). So far the effect of Cetuximab has only been tested extensively on a small number cell lines and most work groups have mainly looked at the levels of DSB at late time points.

In addition to the repair kinetics most previous publications focused on, these experiments will also investiate the early effects of the antibody. One point of interest is the induction of DSBs. Three different NSCLC cell lines with different EGFR levels and KRAS mutation status are included in the experiments. The A549 cells with wit a K-RAS mutation but p53 and EGFR wildtype can be sensitized by Cetuximab. While the NCI H460 cells with K-RAS mutation, p53 and EGFR wildtype and the Calu6 cells with K-RAS mutation, p53 null and EGFR wildtype are not influenced in the same way by Cetuximab. The amount of DBS will be visualized by using the γ-H2AX-foci technique. One objective of this thesis is to find out wether the amount of DSBs shown by γ-H2AX-foci varies between sensitized and non-sensitized cell lines. This would mean that these foci can be used as a biomarker for radiosensitzation. In order to find out more about the mechanism of the radiosensitization the effect of Cetuximab on Non Homologous End Joining (NHEJ), the most important DSB repair pathway is also investigated. Additionally the effect of DNA-PK inhibition on DSB repair will be looked into.

2 Introduction

2.1 The ErbB family

The epidermal growth factor receptor belongs to the ErbB family, a group of four receptor tyrosine kinases. These closely related transmembrane receptors include EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). Each member of the family has an intracellular tyrosine kinase domain, a transmembrane domain and an extracellular domain [Bazley2005]. They are very similar in structure and have their main differences at their ligand binding site [Harari2007]. These receptor proteins play an important role in regulating many cellular processes. Among them are growth, development, tissue turnover and wound healing.

2.2 The Epidermal Growth Factor Receptor

The EGFR was the first out of this family to be characterized and sequenced (Cohen at al., 1975; Ullrich et al., 1984). It has a molecular weight of 170kD and can be found on the surface of various cells of epithelial, mesenchymal and neuronal origin [Wells1999]. Being an ErbB receptor it shares the main features with the other receptors of this family. EGFR consists of three different structural domains, the cystin rich extracellular

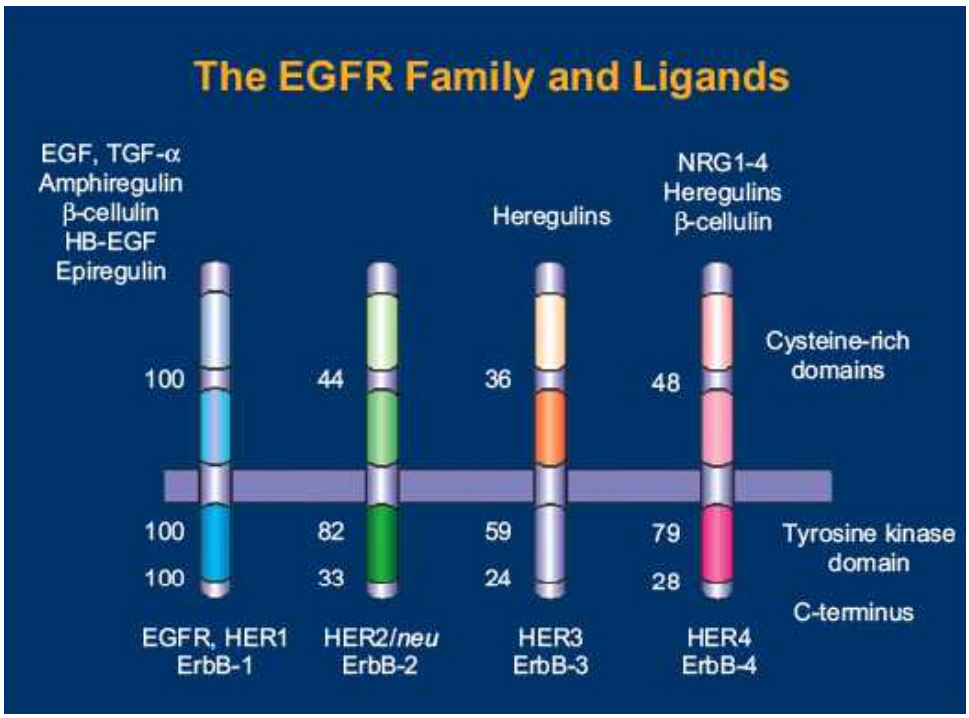


Figure 1: The ErbB family and its activating ligands, source: Medscape Images

domain (621 amino acids), the short hydrophobic transmembrane domain (23 amino acids), and the cytoplasmatic tyrosine kinase domain (542 amino acids) [Nyati2006].

While inactive as monomer the receptor is evenly distributed over the cell surface. Ligand binding leads to dimerisation and in consequence activation. Once activated the receptor conducts the extracellular stimulus to the intracellular compartment, the tyrosine kinase domain being the signal transducer. The receptor can be activated by the binding of various ligands as well as physical stress to the cell.

Even though EGFR is present in a variety of different tissues its activation or expression is altered in a large portion of carcinomas. EGFR plays an important role in tumor progression and etiology making it a target of increasing interest in cancer treatment

regimes.

2.3 Activation of the EGFR

The extracellular domain of the receptor is the binding site for ligands. Today there are more than 11 different ligands known to stimulate the EGFR [Hynes2005], [Yarden2001]. The receptor can be activated by the binding of epidermal growth factor, TGF-α, amphiregulin, β-Celulin, heparin binding EGF, and epiregulin to the extracellular domain [Nyati2006]. In addition to that ionizing radiation and other stressors to the cell such as heat shock, H_2O_2, and cisplatin trigger an EGFR answer [Chen2007]. Activation leads to dimerisation and in consequence autophosphorylation of the tyrosine kinase domains. The phosphorylated residues of the cytoplasmatic domain serve as docking sites for signaling molecules involved in the regulation of different signaling cascades within the cell [Olayioye2000]).

EGFR can either form homodimers with a second EGFR, or heterodimers with other members of the ErbB family [Bowers2001].

The heterodimerization is influenced by the binding ligand, its affinity to the receptor, pH stability of the complex [Beerli1996], [French1995], as well as the microenvironment of the cell. The different possible ligands and the variability of dimerisation put the activation of the receptor into a divers system. In addition to the variations in activation EGFR also has a variety of downstream pathways it can activate. Among these are the PI3K, Ras/MAP, STAT and PLCγ pathways [Lemmon1994], [Yarden2001]. This complex pattern of activation and interaction makes EGFR signaling a complex and interconnected network.

2.4 EGFR Mediated Pathways and Functions

The activation of the EGFR initiates a wide spectrum of cellular pathways. It has been linked to cell proliferation, survival, growth, migration, angiogenesis, metastasis, tumor cell invasion, and inhibition of apoptosis [Chen2007], [Herbst2003].

The EGFR plays an important role in various cell survival pathways. Taking the

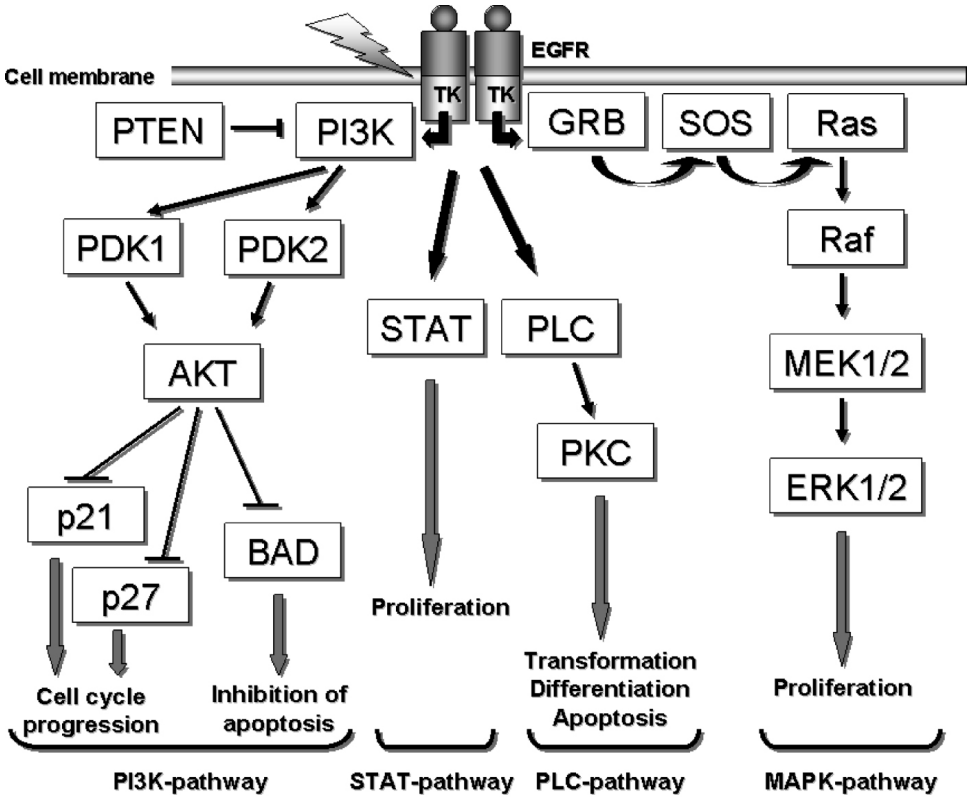


Figure 2: Major pathways of EGFR signaling promoting decreased apoptosis, cell proliferation, differentiation. [Rodemann2007]

signaling pathways it is involved in account it becomes easy to understand why the overexpression of EGFR or its constant activation by mutation can lead to uncontrolled tumor progression and even to resistance to certain treatment regimes.

One of the signaling effects is the activation of phospholipase C-gamma (PLCγ). This enzyme catalyses the hydrolysation of phosphatidylinisitol 4, 5-bisphophate into the second messenger molecules 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 then provokes an increase in intracellular Ca2+ which can induce apoptosis. DAG activates protein kinase C. PKC is an inducer of cell cycle progression.

The EGFR also activates phosphoinositide 3-kinase (PI3K) a kinase that has the serine-threonine kinase Akt as a downstream effector. Akt phosphorylates various substrates involved in the regulation of cellular functions such as cell growth, survival, glucose metabolism and protein translation. It inhibits the proapoptotic proteins BAD and caspase 9 [Paez2002].

Activated Ras recruits Raf which then phosphorylates MEK and this in turn ERK allowing it to translocate into the nucleus where it activates several transcriptional factors [Garcia-Echeverria2008]. This pathway plays a central role in the regulation of proliferation and survival of normal cells as well as tumor cells [Granas2006].

Another pathway activated by EGFR is the JAK-STAT cascade. Upon activation STAT dimerizes and translocates into the nucleus where acts as a transcription factor for genes promoting proliferation and cell survival [Mukherjee2005]

In addition to cascade mediated pathways the EGF receptor itself has been shown to translocate into the nucleus upon ionizing radiation and act as transcription factor itself [Lin2001]. Recently it has been shown that it also interacts directly with DNA-PK one of the key components of the repair complex for double strand breaks [Dittmann2005/I].

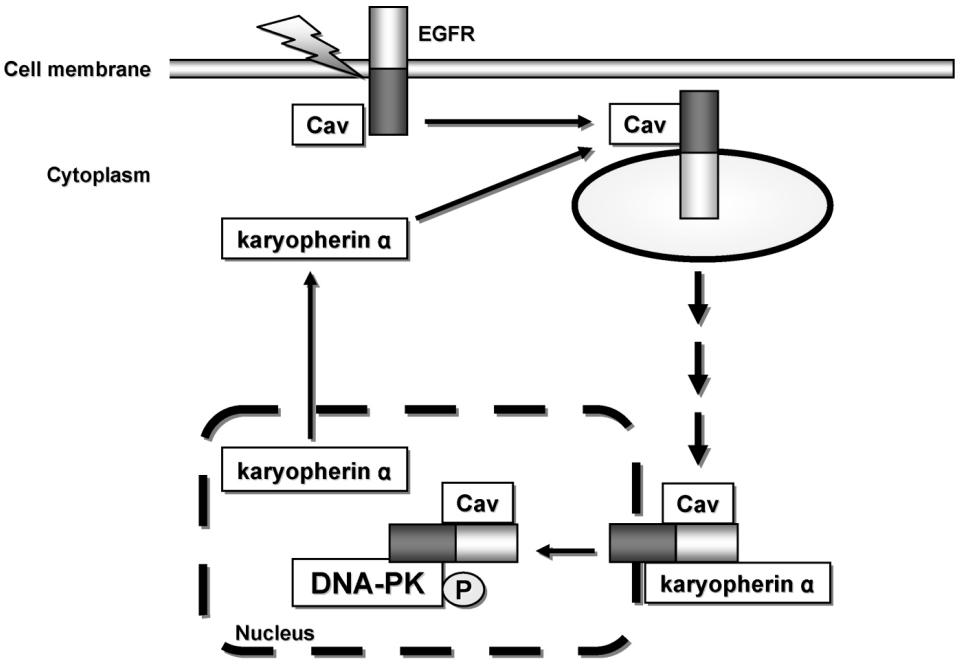


Figure 3: Radiation induced translocation of EGFR via caveolin and in a karyopherin-driven process. In the nucleus EGFR activates DNA-PK [Rodemann2007]

2.5 EGFR in malignancies

EGFR is expressed on normal human cells and the functions are important to maintain cellular integrity. Tissues rely on these pathways to ensure regeneration and to react to changes in the environment [Maas-Szabowski2003].

EGFR hyperactivation in tumor cells on the other hand leads to uncontrolled growth, increased mobility of the cancer cells, decreased apoptosis, and stimulation of angiogenesis. Thus making EGFR a major factor for tumor progression. In the clinic it has also been associated with resistance to chemo- and radiotherapy and in consequence poor prognosis [Bartlett1996], [Grandis1998], [Chow1997], [Volm1998], [Maurizi1996].

The majority of non small cell lung cancers and other solid tumors such as glioblas-

tomas, carcinomas of the colon, breast and head and neck cancers show a hyperactivation of EGFR [Mendelsohn2000]. This increased EGFR signaling gives the cancer cells a clear growth advantage in vivo [Clarke2001]. Higher levels of activity have also been linked with increased malignancy.

There are several mechanisms that can lead to an altered level of receptor activity. Chromosome or gene amplification causes an overexpression of the receptor which makes the cell more sensitive to low levels of activating signals and can also lead to more spontaneous dimerization. Another reason for strong EGFR signaling is the autocrine overproduction of ligands as a result of KRAS mutation [Milano2008]. Mostly these cells produce EGF or TGFα, a mechanism that renders them independent of ligand concentration in the vicinity. Deregulation of EGFR can be due to mutations. The most common alteration of the EGFR gene is a deletion of exons 2-5. This leads to EGFRvIII, a consecutively active form of the receptor that doesn't depend on extracellular stimuli any more. The cells with this variant are resistant to irradiation [Fujiwara2007].

2.6 EGFR mediated resistance to ionizing radiation

The majority of lung cancers are detected at late stages of the disease [Schuurbiers2009]. These carcinomas are often inoperable and IR is one of the major components of treatment. IR inflicts a variety of damage to the DNA. Among them are single stranded breaks, complex double stranded breaks and damage to single bases. DSBs being the most severe defect. When not repaired properly DSBs can result in chromosomal aberration, apoptosis or further mutation [Hagen1972].

While full tumor control or a decrease in tumor size is the intended outcome of the treatment those cancers with altered levels of EGFR activity are often resistant to

IR. This is partially due to the fact that the increased EGFR signaling leads to radioprotection. As mentioned above EGFR is involved in a variety of pro-survival and anti-apoptotic pathways [Ang2002].

EGFR overexpression is found in a variety of malignancies. In the case of gene amplification and overexpression one of the relevant mechanisms for the radioresistance is increased DSB repair. In mouse experiments it has been shown that the amount of EGFR a cell expresses correlates with the level of resistance [Akimoto1999], [Kasten-Pisula2011].

The increased repair capacity is in part due to the constitutively active PI3K/Akt and RAS-mitogen activated pathways, which transduces hyperactivation of DNA-PK [Schuurbiers2009]. This enzyme is one of the key components of the repair complex for DSB. In addition it is described that EGFR translocates into the nucleus upon activation by IR. In the nucleus the receptor activates DNA-PK and increases the repair of DNA damage [Dittmann2005/II].

The truncated variant III of the EGFR (EGFRvIII) is mainly found in gliomas, but does occur in other types 3 too [Kuan2001]. In comparison to wild type EGFR this constantly active version of the receptor gives the cells a survival advantage in stressful situations or in an environment with limited nutrients. This results in potential selection of the resistant clone and tumor growth in vivo [Theys2009]. EGFRvIII continuously supports survival and seems to be acting as an oncogene. The increased proliferation ability leads to a more rapid growth of the tumor.

In the case of EGFRvIII the radioresistance is mediated through the PI3K/Akt, Ras/ MAPK pathway, but the EGFRvIII doesn't have the ability to translocate into the nucleus.

There may be additional mechanisms behind the hyperactivation of EGFR, but in the previously described alterations of the receptor irradiation of the cell mediates an

activation.

One further theory concerning the underlying mechanism of radioresistance is that IR leads to reactive oxygen species in the cell which in turn induce autophosphorylation of the receptor. In addition to that ROS inactivate phosphatases and this might lead to delayed dephosphorylation of the EGFR [Leach2001], [Mikkelsen2003].

2.7 Treatment regimes

The activation of the EGFR leads to proliferation and progression of carcinomas. This means that the tumor is able to grow fast and aggressively. As a consequence a lot of cancers are detected at a late stage. This is often the reason for inoperability leaving irradiation and chemotherapy as the only therapeutic possibilities. The EGFR signaling helps the cells to repair damage faster and to decrease apoptotic signaling, consequently carcinomas with altered levels of EGFR activity are often resistant to these treatments [Ogawa1993], [Etienne1999]. While there has been a lot of progress in treatment strategies within the last decades the overall survival still remains unsatisfactory especially when single agent treatment is applied [Baumann2004].

Even though the overactivation of EGFR in general is a negative prognostic marker it also leads to a new rationale for treatment. The cells depend on a high level of EGFR signaling and inhibition of the receptor disrupts the signals promoting survival and proliferation. This affects the tumor cells more than cells of normal tissue making it a specific approach. The interference with the downstream EGFR-dependent signaling pathways makes the cells more sensitive to chemotherapy or irradiation because their ability to repair quickly and repress anti-apoptotic signaling is reduced. In short they lose their growth advantage.

In addition to their specific target the EGFR inhibitors show low toxicity to normal tissue.

Today there are two different groups of EGFR inhibitors in use, namely tyrosine kinase inhibitors (TKI) and monoclonal antibodies (mAbs). TKIs such as Erlotinib and Gefitinib are small molecules that act in the intracellular compartment and bind to the kinase domain of the receptor hereby blocking its catalytic function. The following disruption of the signal cascades blocks EGFR mediated downstream effects such as proliferation, angiogenesis and metastasis. TKIs have anti-proliferative and anti-invasive effects on different carcinomas. They have been shown to inhibit growth when applied as a single agent. This is due to the suppressed EGFR signaling and the decreased activation of the MAPK pathway. Some evidence suggests that inhibition leads to an increase in apoptosis via the regulation of the proapoptotic cytochrome C [Lui2008].

In combination with cytotoxic agents such as platin derivates TKIs show a cooperative antiproliferate and proapoptotic effect [Ciardiello2000]. The same holds true for the combination of IR and the inhibitor. While TKIs alone show a modest inhibition of tumor growth the combined treatments result in marked regression in tumor growth [She2003], [Bianco2002].

The monoclonal human-mouse chimeric antibody Cetuximab has a five-fold greater affinity to the extracellular domain of the EGFR than the natural ligands. It inhibits dimerisation and by doing so ligand or IR induced activation of the receptor. In addition to that Cetuximab mediates internalization of the receptor by removing it from the cell surface and from potential interaction with ligands. It has also been shown that the immunoglobulin construct mediates an antibody dependent cellular toxicity against some tumor types [Hanna2006]. A radiosensitizing effect of Cetuximab has been shown in vivo and in vitro [Mendelsohn1997]. The underlying mechanisms are the same as the TKI

mediated inhibitions of downstream pathways promoting proliferation and DSB repair.

It is described that mainly cells with a KRAS mutation are radiosensitized by the inhibitors. KRAS is a part of the Ras/Raf/MAPK downstream pathway of EGFR [Adjei2001]. It promotes pro-survival signaling and is mutated in 30% of tumors [Downward2003]. The mutated KRAS results in constantly active protein. Through the MAPK pathway it leads to the production of the EGFR ligands TGFα and amphiregulin. Their release in turn leads to an autocrine activation loop of EGFR [Schulze2001]. By breaking this loop via EGFR inhibition cells with a certain KRAS mutation can be radiosensitized [Rodemann2007].

A lot of in vitro experiments showed good results for tumor growth delay, but it is not quite clear yet to what extent they actually control the tumor [Baumann2004]. So far actual control of the disease has been shown for mAb but not for TKIs. The reason for that is not yet understood.

2.8 Rationale for biomarkers

The use of molecular targeted therapies leads towards a personalized medicine. Present data shows that the combined treatment of EGFR inhibition and irradiation has very promising potential. In contrast to the results of in vitro experiments the results from clinical trials have been very heterogeneous and partially disappointing. Especially the effects of TKIs vary a lot between different cell lines in culture, xenografts and tumor models [Baumann2003], [Giocanti2004].

The main problem is that even the xenograft models do not adequately mimic the exact disease setting. The actual tumor is much more complicated and integrated into a very complex system of influential factors. Thus a straight translation of the in vitro

results is often not possible. Neither is a prediction of treatment success.

While phase II studies in general have shown encouraging results, the phase III studies so far failed to reproduce these. There was no significant clinical benefit detectable for combined treatment. Only 10%-20% of patients with NSCLC that received treatment with EGFR inhibitors achieved clinical response to the treatment. There are several characteristics that are correlated with increased sensitivity to these agents. Among them are female gender, Asian descent, patients that have never smoked, patients with an adeno- or bronchioalveolar carcinoma and with tumors that show polysomy or amplification of EGFR. These factors show a tendency towards a group of patients that might benefit, however they have no predictive value since patients from other subgroups also tend to respond to treatment [Tsao2005].

One theory is that mainly those tumors that truly depend on the EGFR signaling will respond with radiosensitization and growth inhibition when treated with the inhibitory drugs. In order to determine those patients that will benefit from EGFR inhibition it is important to find an individual screening method.

Molecular markers such as EGFR mutation, and amplification, or K-RAS mutation have been taken into consideration and were tested extensively. So far one of the few clear correlation between mutation and benefit is an EGFR mutation in exons 18-21 and response to TKI monotherapy. Some KRAS mutations lead to cell mutants that can be radiosensitzed while others don't. Right now the problem of how to screen patients for responsiveness to EGFR inhibition alone or in combined treatment schemes needs to be faced.

The pretreatment screening is important because patients should not suffer from the side effects of a treatment they will not benefit from. Or even worse that leads to a progression of the disease because the tumor is not responsive to the applied regime.

Most screening tools that are currently being used are molecular and not functional markers. And even though they show some predictive value, so far one cannot fore say the cellular response based on them. Tumor cells host more than one mutation and the behavior of the cell depends on the interaction of different pathways.

In order to give each patient the best treatment possible it is necessary to distinguish between those tumors that are susceptible to EGFR inhibition and those that are not. For this task it is important to have a biomarker that gives us an idea of the complex tissue response. There are certain expectations that such a marker has to meet. First of all it has to be sensitive and specific, in addition to that it needs to be developed for high throughput, as well as cost-efficient.

There are several approaches to this problem, one of them evaluates the efficiency of DNA repair based on the level of DSB in the genome. In this case the DSB are being used as a functional biomarker for the cellular response to treatment.

2.9 Induction and repair of double strand breaks

Most treatment regimes include agents that cause DSB. In the case of IR they occur directly, in the case of replication fork stalling by chemotherapeutics an indirect mechanism is causing the damage. In both cases the result is a potentially lethal damage to the cell. DBSs are the most dangerous lesions to the DNA because they can result in severe consequences for cell survival and genomic stability. This is due to the fact that they can lead to lethal chromosomal aberation. False repair can cause chromosomal aberration and the initiation of carcinogenesis [Ismail2008]. The rationale behind the application of DSB causing agents is to damage the tumor cells so severely that they undergo mitotic catastrophy because of chromosomal aberrations, or apoptosis.

One way of determining whether a cell can be radiosensitized by drug treatment is to measure the amount of residual double strand breaks. DSBs can be quantified by using the γ-H2AX- foci technique. Higher eukaryotic cells have two main mechanisms to repair DSBs, homology directed repair (HDR) and non homologous end joining(NHEJ). Primarily they rely on NHEJ. During NHEJ the DNA ends are processed and nonligatable nucleotids are removed before rejoining them. This repair pathway is error prone because unlike HDR it doesn't use a template to repair precisely. On the other hand it can take place in all phases of the cell cycle. HDR is only possible in S- and G2-phase when there is an identical sister chromatin present [vanGent2007].

NHEJ has a fast and a slow component working at half times of 10-30 min and 2-10 hours [Iliakis2004]. The repair complex for NHEJ starts with the binding of the DNA dependent protein kinase subunits Ku70 and Ku80 to the lose DNA ends. These attract and activate the catalytic subunit DNA-PKcs which then phosphorylates a number of targets. Among them is DNA-PK itself and H2AX. The rejoining of the double strand break is catalyzed by a core complex consisting of DNA-PK, XRCC4, DNA ligase 4 and Artemis [Chen2007].

2.10 γ-H2AX- foci as a marker for double strand breaks

Eukaryotic DNA is organized into a higher-order structure. The basic unit of the chromatin is the nucleosome. It consists of 147 base pairs that are wrapped 3-fold around an octamer of histones. Each octamer is made of two copies of the highly preserved histones H2A, H2B, H3 and H4. Between the single nucleosomes there is a short linker section (20-28bp). The organization into higher levels of condensation is mediated by other proteins as well as interaction between core histones of different nucleosomes [Kinner2008].

Because of this compaction chromatin is usually not accessible to enzymes. Before repair is possible it has to undergo relaxation. This process is initiated by changes in the histone code. There are at least eight modifications known to belong to this code, among them are phosphorylation, acetylation and methylation of specific domains of the histones. The pattern of those posttranslational changes then determines the many different chromatin states [Ayoub2009], [Strahl2000].

Several histone modifications have been described upon the occurrence of DSBs. One that has been of particular interest is the phosphorylation of H2AX. Four variants of H2A have been reported so far H2AZ, H2AX, macroH2A, and H2ABbd [Ausio2002], [Redon2002], [FernandezCapetillo2004].

H2AX makes up about 5%-25% of the total cellular H2A depending on the cell type and is distributed evenly over the DNA. In response to DSB H2AX is phophorylated at Ser^{139} by the phophatases ATM, DNA-PK, and ATR. The phosphorylated form is called γ-H2AX. This modification appears only in the vicinity of a break sites and is detectable within minutes following the damage reaching its maximum after half an hour. Approximately 2000 γ-H2AX are formed per DSB [Kinner2008]. This strong signal amplification at the site of the break is important for the recruitment of further repair proteins to the damage and for the induction of chromatin remodeling [Ismail2008], [Srivastava2009]. It has been shown that γ-H2AX serves as a regulator of checkpoint signaling and or repair itself [vanAttikum2009]. In H2AX null cells the recruitment of repair proteins to radiation induced breaks is impaired [Ayoub2009].

Once the DSB is repaired the γ-H2AX-focus disappears. Some phosphatases involved in the dephosphorylation of the histone variant are known, but the underlying mechanism for the removal is not fully understood.

The measurement of γ-H2AX-foci is an indirect method of detecting DSBs. While

the breaks are the actual marker of DNA damage γ-H2AX-foci is the tool that is being used for detection. There are several reasons that make γ-H2AX-foci so interesting in terms of a potential screening tool for radiosensitisation. First of all H2AX phosphorylation is one of the first responses to DSBs, it takes place within an area of 2kbp or more around the DSB and is detectable with a specific antibody. Second the foci cannot be induced by any other lesion than double strand breaks, making it a sensitive method [Kinner2008]. When compared to other methods of DSB detection is was shown that the number of foci correlates with the number of breaks [Sedelnikova2002], [Cai 2009]. Physical methods for DSB detection such as pulsed-field-electrophoresis require doses well above the clinically used dose of irradiation as well as DNA that has been freed of histones. When using γ-H2AX-foci cells in culture or biopsies can be worked with and doses aplied that are also being used in clinical treatments. By doing so, two factors for potential errors are eliminated.

One disadvantage of using foci for the detection of DNA damage is that they take a certain time to form and also their removal lags behind. These delayed kinetics of γ-H2AX-foci have no influence on the adequacy of the marker but they need to be considered when interpreting the results.

It has been shown that the results in foci analysis correlate with the findings in clonogenic survival.

Because of reasons mentioned above γ-H2AX has the potential of becoming a screening tool for the repair capacity of tumors. Decreased repair capacity in treated cells would speak for treatment induced radiosensitization.

3 Material and methods

3.1 Cell Lines

The experiments were conducted using A549, NCI H460 and Calu6 cell lines. These are human non small cell lung cancer cell lines with wild type EGFR and mutant KRAS. A549 and NCI H460 are p53 wild type while Calu6 is a cell line that is p53 null.

All cell lines have been tested mycoplasma free.

3.2 Cell Culture

All cell lines were grown until they formed a confluent monolayer. The cultivation took place in T25, T75 or T175 flasks.

The A549 were kept in DMEM, H460 in RPMI 1640 medium, Calu6 grew in Eagles α-Minimum Essential Medium. All media were supplemented with 10% bovine growth serum, 2mmol/L L-Glutamine and 10,000 units/ml Penicillin-Streptomycin antibiotics.

They were incubated in 37°C/ 5%CO2 and 100% humidity.

For plating and general passaging the old medium was discharged from the flasks the cells were then washed with 1xPBS which was sucked out after. In order to detach the

cells 1ml of 1xTrypsin was added to T25 flask, 1.5ml to T75 flask, and 4ml to T175 flask. Afterwards the flasks were incubated at 37°C for 5min.

After checking cell detachment under a microscope the trypsin was inactivated by adding 4ml of serum containing medium to a T25, 8ml to a T75, and 16ml to a T175 flask.

The manual counting took place under a microscope using a counting grid, then the volume of cell suspension needed was calculated and the cells plated. The minimum number of cells to ensure optimal growth conditions was $5x10e^5$ cells/flask for T25, $1x10e^6$ cells/flask for T75, $2.3x10e^6$ cells/flask for T175 flasks.

All cell lines were grown until they formed a confluent monolayer before trypsinizing and replating them again. This was necessary every second day for A549 and every third day for NCI H460 and Calu6. Each cell line was only passed up to passage 20 before being replaced with a freshly thawed batch.

For storage $1x10e^6$ cells were diluted into 1ml of medium containing 5% DMSO, filled into labeled cryogenic vials and then placed into a methanol containing freezing container. They were first frozen at -70°C for 24h and then transferred into the liquid nitrogen tank where they were stored at -196°C.

For thawing the vials were transferred into a 37°C water bath. The cell solution was then diluted into 5ml of the respective medium and centrifuged at 1,500rpm. The DMSO containing medium was discharged and the cell pellet resuspended in 5ml of full medium. The solution was then plated in a T25 flask and incubated.

3.3 Irradiation of Cells

At the time of irradiation the cells were in the plateau phase. They were irradiated with 1Gy or 2Gy (0.883 Gy/min).

3.4 Detection of DSBs

3.4.1 Cell Culture for Staining

The cells were trypsinized and counted as described before. Then a suspension with a concentration of 8x10e^4 cells/ml was produced and 0.5ml of this dilution plated in the chambers of 8 well plates. Those cells were then allowed to settle over night.

3.4.2 Pretreatment of Cells

The next day all media was disposed off by suction and the chambers on right side of the slide were treated with 0.5ml full medium containing 100nM of the monoclonal EGFR antibody Cetuximab (C225) while the left half received 0.5ml fresh full media. After one hour all but the zero hour control slide were irradiated with 1Gy as described before. After irradiation the non irradiated 0h slide was fixed right away, the other slides were put back into the incubator and then fixed at 15min, 30min, 3h, 6h, and 24h.

The same experiment was later conducted with variation in treatment, first using 2Gy, then using 2μM of the tyrosine kinase inhibitor Erlotinib.

In order to have a closer look at the role of DNA-PK inhibition on the results the set up was repeated comparing 100nM C225, 10μM of the DNA-PK inhibitor NU7026, a combination of the two inhibitors, and no treatment.

In the next set of experiments Erlotinib and C225 were applied right after irradiation and the slides then fixed after 1.5h in order to allow the inhibitor to influence the cells for the same time as the 0.5h time point

3.4.3 Fixing and Staining of Cells

At the respective time points the media was removed and the cells washed with 1xPBS. The following steps took place at room temperature (RT). In order to make the membrane permeable for the antibodies 100μl of permeabilization buffer was added to each well and left on the cells for 5min. The permeabilizer was removed and in order to avoid washing off of cells during further steps 100μl fixing buffer was added for 30min.
After half an hour the buffer was removed and the cells washed with 1xPBS three times. In order to prevent too much background signals because of antibody binding at unspecific sites 200μl blocking buffer was added and cells were allowed to sit over night at 4°C.

The next day all chambers were washed with 1xPBS again. The primary antibody, an anti phospho γ-H2AX antibody, was diluted 1:200 in 2% BSA / 0.1% Triton X/ PBS and 100μl of the solution was added to each well and allowed to incubate in a humidified chambers for 3 hours at 37°C. From now on all washing steps were performed with 1xPBS / 0.1% Triton X. To remove all primary antibody that wasn't bound to specific foci, cells were washed three times for 5min each. They were then incubated with the fluorescent secondary antibody diluted at 1:1000 in 2% BSA/ 0.1% Triton X/ PBS for one hour at RT. The secondary antibody has its absorption maximum at 493nm and its maximum emission at 520nm. From this step on light exposure had to be avoided to prevent fading of fluorescence signal. The incubation took place in a dark chamber.

After washing 3 times for 5min the cells were incubated with DAPI to counterstain the DNA [1mg/ml] 1:1000 in double distilled water. DAPI is a fluorescent stain that binds strongly to DNA. It is excited with ultraviolet light. When bound to double-stranded DNA its absorption maximum is at 358nm and its emission maximum at 461nm. It appears brightly blue under the microscope when the UV/DAPI filter is used. The cells were then washed twice for 2min before detaching the plastic chambers walls from the silde. Now the samples were carefully dried by removing all excess liquid with a pasteur pipette attached to a suction unit without actually touching the cell covered area of the slides. After adding one drop of antifade the slides were sealed with cover slips and nail polish.

3.4.4 Counting γ-H2AX-foci

The counting was conducted manually under a fluorescent microscope using the 100x oil immersion objective. Fluorescent microscopes are capable of imaging the distribution of a molecular species based on the properties of its fluorescence emission. By using the DAPI filter alternating with the filter for green fluorescence it was possible to detect nuclei as well as counting foci.

Not all nuclei are visible at a single optical plane. Some might be in focus, while others appear only as a blur. It is necessary to slowly scroll through the whole nucleus counting every nuclei that comes in and out of focus. Because scanning through the nucleus manually and counting the foci by eye is only possible up to a certain amount of foci per cell the limit for counting was set at 20 foci per cell. Per former well 100 cells were counted and the number of foci per nucleus noted.

For A549 five repeats of the assay were performed, for NCI H460 and Calu6 three

repeats. The related graphs were created using GraphPad Prism software.

3.5 Cell survival / Colony formation assay

3.5.1 Plating

In order to determine the efficacy of the DNA-PK inhibitor NU7026 a colony formation assay was performed with A549.

For the colony formation assay the cells were kept in T25 flasks until they formed a confluent layer. The cells were trypsinized and counted before plating as previously described. The cells were plated at specific densities and allowed to settle over night (16h). The next day they were irradiated with doses from 2Gy-8Gy. For each dose two T25 flasks were plated at three different densities. One receiving only irradiation, the other was pretreated with 10μM NU7026 and then irradiated one hour later. The cells were then allowed to grow for 20 days before fixing and staining.

3.5.2 Fixing and Staining

The medium was discharged and the flasks washed with 1xPBS and fixed with methanol. After discharging the methanol the colonies were stained with methylene blue for 30min. The flasks were then washed three times with tap water and allowed to dry at RT over night.

Only colonies consisting of more than 50 cells were counted manually using a light table and a microscope. The plating efficiency was calculated as colonies per number of cells plated and the surviving fractions as ratios of plating efficiencies of irradiated

and unirradiated cells. The related graphs were created and statistical analysis with 2-sided F-test was performed using GraphPad Prism software. The experiment consisted of three independent repeats.

3.6 Non Homologous End Joining (NHEJ) assay

3.6.1 Plasmid Amplification

pEJ and EGFPNI, two different green fluorescent plasmids with a GFP backbone, and the active cutting enzyme I SCE 3xNLS (3 nuclear localization sequences) as well as the inactivated form SCE-I- were amplified using the QIAGEN Maxi Kit for plasmid amplification.

The substrate pEJ contains two SCE cutting sites in the 5' untranslated region of the GFP transcript. Between those two sides an artificial start codon has been inserted out of frame with the original open reading frame thus preventing GFP from being translated.

First electro competent bacteria and the DNA were thawed on ice while the electroporation cuvette was being chilled at -20°C. 10μl of the bacteria were pipetted into the chilled cuvette and .5μl of the respective DNA with an unknown concentration added. The cuvette was then put into the electropulser and the samples run at Ec1 program, the pulsing took

6.0ms for pEJ

5.2ms for EGFPNI

5.1ms for SCE-I-

5.2ms for I SCE 3xNLS

5.6ms for the transfection control PUC

Immediately after the transfection 1ml of S.O.C medium was added, the bacteria resupended and then transferred into a 14ml tube. The tube was put into the 37°C bacterial shaker for 60min while the LB-antibacterial plates were warmed up in the incubator.

GFP contains a gene promoting resistance to kanamycin, for that reason pEJ and EGFPNI were grown on selective plates with 0.05ng/ml kanamycin. The plasmids for the cutting enzymes made the transfected bacteria resistant to ampicillin consequently they were grown on 0.1mg/ml ampicillin plates. For each plasmid 5μl, 10μl, 20μl, 50μl, 100μl of the bacterial solution was striped onto the LB-antibacterial plates. The plates then incubated over night at 37°C to allow colonies to form.

Since only successfully transfected bacteria were able to form colonies on the plates any colony could be chosen the next day. A single colony from a not too crowded plate was picked with a pipette tip and inserted into a tube with 5ml of LB-medium with the same concentration of the respective antibiotic. This tube was put into the bacterial shaker and shaken at 37°C and 250rpm for 8 hours. Then 1ml of the solution was put into 250ml of the same medium and left in the bacterial shaker over night. The next day the over all density was measured to determine the cell mass in 250ml. After that the 250ml were spun down at 4°C and 6,300rpm (6,000 RCF) for 15min and the supernatant carefully disposed off.

3.6.2 DNA Purification

The plasmid DNA was isolated from their E.coli construct using the Qiagen Maxi Kit. First each pellet was resuspended in 10ml of chilled Qiagen buffer P1 already containing

LyseBlue and RNAse. Next 10ml of buffer P2 were added, the tube inverted vigorously until the entire sample turned homogeneously blue, and left to incubate at RT for 5min.

Last 10ml of the chilled buffer P3 was added to the tube which was again inverted 4-6 times until the samples were full of cloudy precipitates. The whole lysate was then poured into the filter cartridges with the nozzles still closed and left to incubate at RT for 10min. The precipitates started to float on top. Meanwhile the filter tip was equilibrated with 10ml of QBT buffer and the column allowed to empty by gravity flow.

After removing the cap from the filter cartridges the plunger was inserted and the clear content (containing the plasmid) was filtered into the previously equilibrated filter tip where it was allowed to drip through into the waste collection flask by gravity flow. The plasmid was adsorbed to the filter tip which was washed twice with 30ml of buffer QC in order to eliminate all impurifications. Then 15ml of buffer QF was pipetteted onto the column and the eluate now containing the plasmids collected in a 50ml tube. In order to precipitate the DNA 10.5ml of room temperature isopropanol was added to the eluated DNA and mixed by inverting. The tubes were centrifuged at 15,000G for 30min at 4°C. Afterwards the supernatant was decanted carefully before washing the pellet in 5ml room temperature 70% ethanol and centrifuging at 15000G for 10min at 4°C. After the last spin the supernatant was again carefully disposed off and the pellet air dried for 5min-10min before resuspending it in 200μl TE 10nM Tris/1nM EDTA buffer.

After preparing samples of 5μl plasmid solution and 495μl double distilled water the DNA was quantitated and the purity detected via 260nm/280nm ratio.

pEJ:	3.435μg/μl	260/280:	1.808
EGFNI:	2.390μg/μl	260/280:	1.846
SCE-I-:	1.950μg/μl	260/280:	1.822
I-SCE 3NLS:	1.270μg/μl	260/280:	1.886

3.6.3 Restriction Digest and Gel Electrophoresis

In order to confirm that the right plasmids had been amplified a restriction digest was performed. Restriction enzymes cut DNA at very specific recognition sequences. The DNA fragments were then run on an agarose gel in order to separate the target molecules. The gel consists of a cross linked polymer to which some ethidium bromide was added. After loading the gel and subjecting it to an electric field the negatively loaded DNA fragments will travel depending on their charge and size. The ethidium bromide intercalates with the DNA and will later allow visualizing the fragments in UV light.

Knowing the number of cutting sites an enzyme has on a length of DNA as well as the size of the respective DNA fragment this still doesn't prove the presence of the right plasmid, but makes it very likely.

The type II site-specific deoxyribonuclease restriction enzyme HINDIII was used. It is a restriction enzyme isolated from Haemophilus influenzae and hydrolyzes the palindromic DNA sequence AACGTT in the presence of the cofactor Mge^{2+}.

First all plasmid samples were diluted to 1μg/μl in TE buffer and 3μl of HINDIII [100 units/μl] was prepared to avoid over digestion and star activity. After adding 2μl of the restriction buffer, 15μl double distilled H_2O, 0.5μl [1μg/μl] plasmid solution, 0.5μl HINDIII, and 2μl bovine serum albumin [1mg/ml] into a 1.5ml tube the restriction mix was allowed to incubate for 1 hour at 37°C. Meanwhile 500ml 1xTris-acetate-EDTA (TAE) was prepared. 100ml of the buffer were heated in the microwave with .8g agarose

for 2min to dissolve the powder. The hot liquid was then poured into the mold of the tank and the comb inserted. Before allowing the gel to cool down and harden .5μl of ethidium bromide was added and swirled in carefully with a pipette tip. Ethidium bromide is an intercalating agent that can be used as a fluorescent tag. After exposure to ultraviolet light it emits an orange colour which intensifies about 20-fold after binding to DNA.

The gel was left to cool and harden at RT for 30 min, then the rest of the TAE buffer was poured over the gel until it was fully covered before the comb was carefully extracted.

Before loading the chambers two loading samples for each plasmid were prepared. One of them received the cutting enzyme HINDIII. After digestion 4μl of 6x loading dye was added. Next to every cut sample 0.25μl of the uncut DNA with 4.5μl ddH_2O and 1μl loading dye was pipetted into the chamber.
Loading order on the gel:

1. 15μl ladder
2. pEJ uncut
3. pEJ cut
4. EGFPNI uncut
5. EGFPNI cut
6. SCE-I- uncut
7. SCE-I- cut
8. I SCE 3xNLS uncut
9. I SCE 3xNLS cut

The gel was run at 80V for 2.5 hours. While running the gel was closely watched and the progress of the DNA fragments frequently checked with a UV camera to prevent the

DNA from running off the gel.

	Plasmid length	no of cutting sites
pEJ	4838bp	1
EGFPNI	4700bp	1
SCE-I-	5755bp	2
I-SCE 3xNLS	5452bp	1

3.6.4 Transient Transfection

Transfection is a non-viral method of introducing foreign DNA into a cell. If this is done transiently the DNA can be lost at any time due to environmental factors, but will be lost during mitosis by the latest. As a transfection reagent Metafectene Pro was applied to the cells. It uses repulsive membrane acidolysis to transfer the DNA into the cells and is less toxic than its predecessors.

Before using the Metafectene Pro for the NHEJ assay it had to be optimized to find the optimal ratio of DNA and the reagent. The highest transfection efficiency was found with 6μg of DNA and 18μl of Metafectene Pro.

The cells were trypsinized and counted as described before. For the transfection eight T25 flasks with 5x10e^5 cells each were plated and allowed to attach over night. The next morning four of the flasks were pretreated with 100nM Cetuximab in full medium for one hour. During this time the eight samples 300μl of DMEM without the supplementary antibiotics were mixed with 18μl of Metafectene Pro in 1.5ml tubes. In a different set of tubes the DNA samples were prepared. Two of each of the following were mixed into another set of 1.5ml tubes with 300μl of antibiotic free DMEM:

- 6μl SCE-I- negative control

- 6μl EGFPNI positive control

- 1μl I-SCE 3 NLS + 5μl pEJ NHEJ sample of interest

- 1μl SCE-I- + 5μl pEJ determination of background

Each of the DNA samples was mixed with one of the Metafectene mixes and allowed to incubate for 20min at room temperature before adding it into the labelled T25 flasks. Immediately after transfection the cells were irradiated with 2Gy.

After that the cells were incubated for 24 or 48 hours.

3.6.5 Flow Cytometry

The cells were trypsinized as described previously and in addition the flasks were rinsed with PBS to make sure most of the cells were transferred into the 15ml tube. But instead of counting and plating them into new flasks they were spun down at 1,000rpm for 4min. The supernatant was removed with a suction unit before adding 10ml of fresh PBS and the cells were spun down again. The PBS was carefully sucked out.

The fixing of the samples was done by adding .5ml of 4% paraformaldehyde (PFA) drop by drop while slowly vortexing the sample in order to get a good single cell solution. The cells were then left to fix for 2 hours at room temperature. After that they were spun down, washed and spun down again. Last they were resuspended in 2% fetal bovine serum (FBS), dispensed through a cell strainer and placed on ice until they were run on the FACS machine. The experiment was performed 4 times and the results from the FACS machine were analysed using FlowJo analysis software.

3.7 Complete Listing of Material

Cell lines

cell line	Specification
A549	NSCLC Cell Line
Adenocarcinoma	Cultivation in DMEM Medium
K-RAS mut / p53 wt	Provided by Settleman Lab, MGH Cancer
EGFR wt	Center, Boston, MA, U.S.A.
NCI H460	NSCLC Cell Line
Large cell carcinoma	Cultivation in RPMI Medium
K-RAS mut / p53 wt	Provided by Settleman Lab, MGH Cancer
EGFR wt	Center, Boston, MA, U.S.A.
Calu6	NSCLC Cell Line
Adenocarcinoma	Cultivation in MEM-Medium
K-RAS mut / p53 null	Purchased through ATCC (American Type
EGFR wt	Culture Collection)

Media for Cell Cultures

Base	Supplements for all media
DMEM	50ml BGS (10% total BGS/500ml medium)
RPMI 1640	5ml L-Glutamine (Cellgro)
α MEM (SIGMA)	5ml 1x HEPES (Sigma)
	5ml Penicillin-Streptomycin (Cellgro)

Further Material used for cell culture

Material	Specification
1xPBS	137mM NaCl, 2.7mM KCl
	12mM Phospate (HPO_4^{2-}, $H_2PO_4^-$)
1xTrypsin	Sigma
T25 Culture Flask	$25cm^2$ (BD Falcon)
T75 Culture Flask	$75cm^2$ (BD Falcon)
T175 Culture Flask	$175cm^2$ (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	Specification
50ml/15ml Tubes	BlueMax (BD Falcon)
DMSO	Sigma
Centrifuge	Sorvall
-70°C Freezer	VWR Scientific
Liquid Nitrogen Tank	CRYOMED Forma Scientific

Treatment

Material	Specification
8 well chamber slides	BD Falcon
X-Ray Generator	Siemens Sabilipan 2280KVp, 1.98Gy/min
Cetuximab	Erbitux® (Bristol-Myers Squibb Company)
Erlotinib	Tacreva® (Roche), provided by Settleman Lab, MGH Cancer Center, Boston, MA, U.S.A.
NU7026	Sigma Aldrich

Fixing and Staining

Material	Specification
1xPBS	137mM NaCl, 2.7mM KCl
	12mM Phospate (HPO_4^{2-}, $H_2PO_4^-$)
Fixing buffer concentrations:	0.5% Triton-X
	20mM HEPES, pH 7.9
	50mM NaCl
	3mM KCl
	300mM Sucrose
Permeabilizing buffer concentrations:	3% PFA
	2% Sucrose
	1xPBS
Blocking buffer concentrations:	0.5% NP40
	10% BGS
	0.3% NaN3
	1xPBS
Bovine serum albumin 2% (BSA)	Sigma
Primary antibody	Mouse antibody to gamma H2AX [2F3] (phospho 139)
	100 ?g (0.5 mg/ml)
	Ab18311 - 100
	Lot: 537186
	(Abcam)
Secondary antibody	Goat anti-mouse IgG, IgM
	A10680: Alexa Fluor® 488 (H+L) 2mg/ml
	(Invitrogen)
4'-6-Diamidino-2-phenylindole (DAPI)	Sigma
Vectashield antifade mounting media	Vector Laboratories Inc
Glass cover slips	Fisher scientific
Top coat	New York Colour Cosmetics
Fluorescent microscope	Olympus

Colony Formation

Material	Specification
T25 Culture flasks	25cm^2 (BD Falcon)
1xPBS	137mM NaCl, 2.7mM KCl
	12mM Phospate (HPO_4^{2-}, $H_2PO_4^-$)
Methanol	Fisher Scientific
Methylene Blue	SIGMA
Microscope	Stereomaster (Fisher Scientific)
Light Table	Apollo Portable Light Box (Listel)

Non homologous end joining

Plasmid amplification

Material	Specification
EGFP-NI	Invitrogen
pEJ	Jochen Dahm-Daphi
I-SCEI 3xNLS	Maria Jasin
SCE-I -	John Settleman
PUC	Invitrogen
Electro MAX DH10TM Cells	Invitrogen 18290-015
Electroporation Cuvette, 1mm gap	Fisher Biotech
Micropulser	BioRad
S.O.C. media	Invitrogen
Round-bottom tubes	14ml BD Falcon Round-Bottom Polypropylene Tubes
	50ml BD Graduated Tubes (Fisher)
LBmedia	Containing .1mg/ml Ampicillin / .05ng/ml Kanamycin
Bacterial inoculation loops	Fisher Scientific
Bacterial incubator	PRECISION
Bacterial shaker	HI-LabOne
Overall densitiy Spectrophotometer	Perkin-Elmer
Centrifuge	RC 5C Plus Centrifuge, Sorvall
SLA-600TC Rotor	Sorvall
SLA-500 Rotor	Sorvall
Plasmid Purification Kit	QIAFilter Plasmid Maxi-Kit, QIAGEN

Material	Specification
50ml Falcon round-bottom tubes	Fisher
TE buffer	10nM Tris (Tris-(hydroxymethyl)-aminomethane)
	10nM EDTA ehtylenediaminetetraacetic acid
1.5ml microcentrifuge tubes	Fisher
DdH_2O	MILLIPORE MILLI-Q (Continental Water Systems)
disposable plastic cuvettes	Fisher Scientific
pipettes	Fisher
pipette aid	Drummond scientific
GeneQuant pro	GE Healthcare (Amersham Biosciences)
BSA	Sigma
HIND III	New England Biolabs
Agarose gel	0.8% agarose
TAE buffer	1x Tris-acetate-EDTA (TAE)
Ethidiumbromide	Sigma
Loading dye	BIOPLUS
Molecular ladder	BioLabs
Gene flash	Syngene

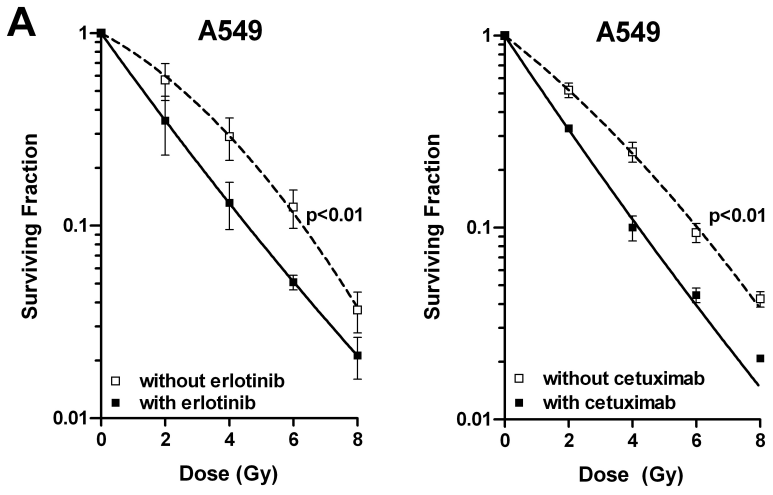
Transient Transfection

Material	Specification
Plasmids in TE buffer	pEJ, EGFP-NI, I-SCE-I 3xNLS, SCE-I-
Metafectene Pro	Biontech
T25 Culture flasks	25cm^2 (BD Falcon)
DMEM	antibiotic free
Styrofoam box	filled with ice
1xPBS	137mM NaCl, 2.7mM KCl
	12mM Phospate (HPO_4^{2-}, $H_2PO_4^-$)
Allegra Centrifuge	Beckmann Coulter
PFA (paraformaldehyde) 4%	Boston Bioproducts
FBS (fetal bovine serum) 2%	Sigma
Round-bottom falcon tube, cell strainer cap	BD biosciences
FACS Calibur flow cytometer	Becton Dickinson
Flow cytometry analysis software	FlowJo

4 Results

Other workgroups have previously demonstrated that a combined treatment of EGFR inhibition by monoclonal antibodies and irradiation increases the sensitivity of initially radioresistant cell lines [Dittmann2005/I]. In experiments conducted earlier in our workgroup it has been shown that the specific tyrosine kinase inhibitor Erlotinib radiosensitizes A549 but not Calu6 and NCI H460 cells [Nanda2008].

This project focuses on the effect the monoclonal antibody Cetuximab has on the level of double strand breaks in these 3 cell lines. In addition a NHEJ assay was conducted to investigate the influence of Cetuximab on this specific DNA repair mechanism.



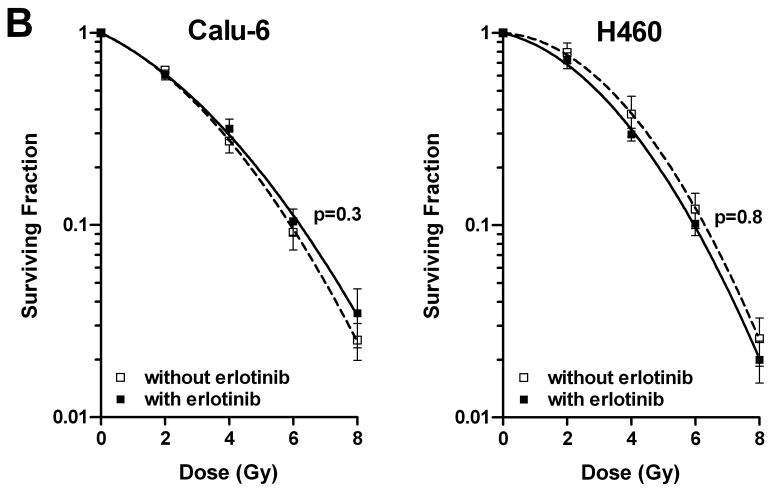


Figure 4: Cell survival of A549, Calu6 amd NCI H460 cells after EGFR inhibition by TKI Erlotinib and mAb Cetuximab. Cells were plated at different densities. 16h later one set received one hour of pretreatment with 2μM Erlotinib or 100nM Cetuximab then the pretreated and the control group were irradiated with 1Gy. Statistical comparison by F-test, two-sided.[Nanda2008]

4.1 Effect of Cetuximab on number of DSBs

Since it has been reported, that Cetuximab decreases the DSB repair capacity [Dittmann2005/II] The amount of γ-H2AX-foci in A549 cells at different time points was first investigated. The cells were pretreated with Cetuximab and irradiated with 1Gy one hour later. They were then fixed and stained at the indicated time points. The slides were counted under a fluorescent microscope and foci per nucleus recorded. The experiment was repeated 5 times. Cetuximab results in an elevated number of foci at all time points after irradiation with 1Gy (figure 5 and 6)

While the initial foci determined 0.5h after irradiation are little round spots within the nucleus, the foci detected 24h after IR appear larger (figure 5D)

Treated as well as untreated cells show an increase in the number of γ-H2AX-foci after

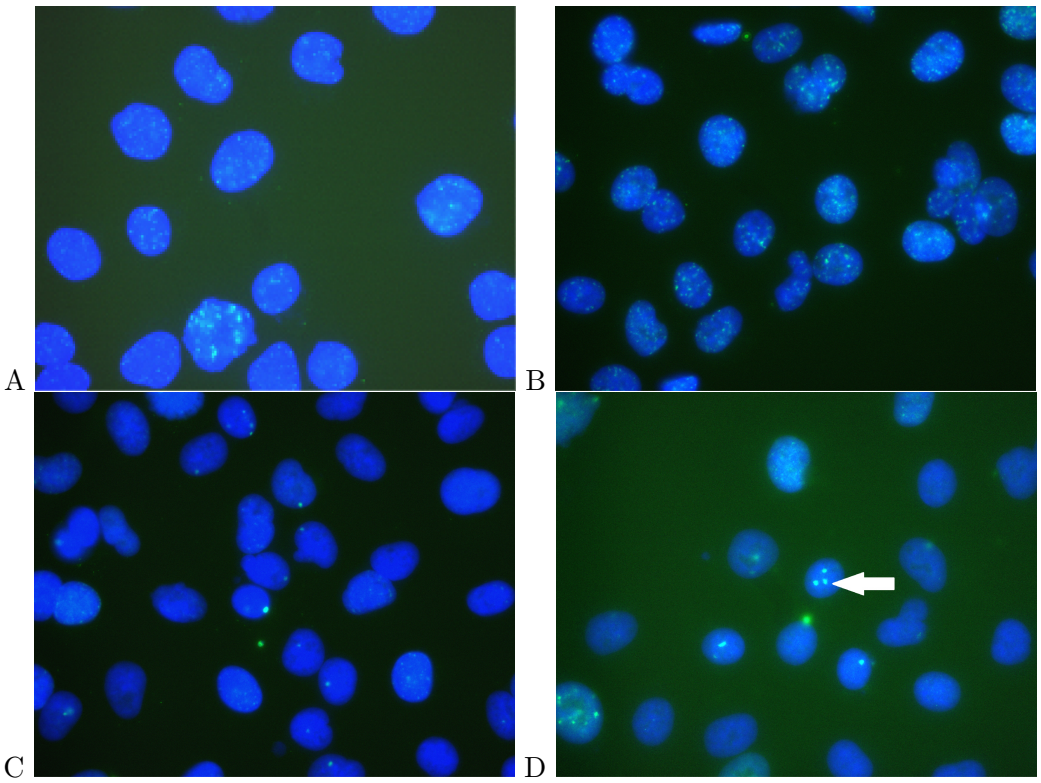


Figure 5: γ-H2AX-foci in A549 cells after EGFR inhibition and irradiation. Representative images of foci in nuclei after 1Gy. Arrow shows γ-H2AX-cluster. Blue: DAPI staining. Magnification 100x.
A 0.5h untreated
B 0.5h 100nM Cetuximab
C 24h untreated
D 24h 100nM Cetuximab

irradiation. While the untreated ones present with the maximum of damage in the range of 11 to 20 foci per cell, Cetuximab increases the percentage of cells with 20 or more foci from 32.0% to 49.9% (figure 6). This divergence in γ-H2AX-foci per nucleus can be seen at all time points.

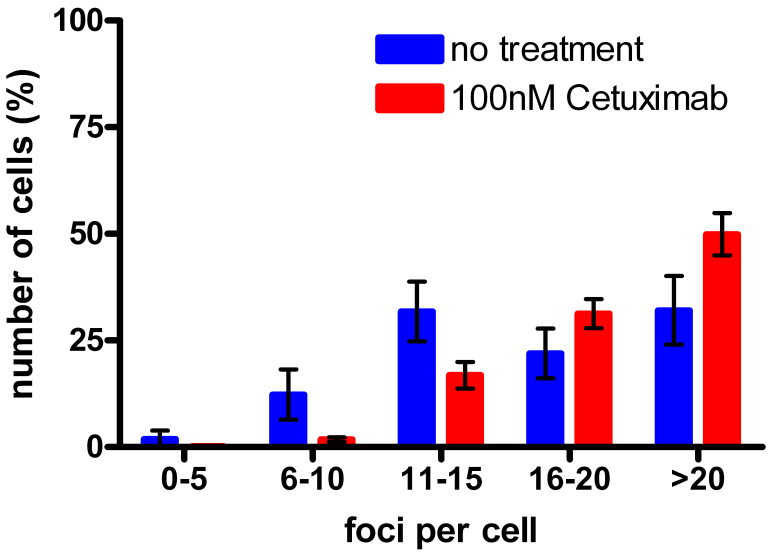


Figure 6: Induction of γ-H2AX-foci in A549 cells. Cells were pretreated with or without 100nM Cetuximab one hour prior to irradiation with 1Gy. All were fixed and stained 0.5h post IR. 200 cells per slide were counted. Bars represent the number of γ-H2AX-foci per nucleus with standard two sided error based on 5 independent repeats.

24 hours after IR most of the cells have repaired the majority of their DSB. There is a small group of cells that still have 20 or more γ-H2AX-foci and Cetuximab increases this fraction (figure 7). Surprisingly irradiation with 2Gy doesn't further increase the amount of cells with more than 20 foci.

Figure 8 shows the DSB repair kinetics in A549 cells by using γ-H2AX-foci as a tool for detection. For visualisation the 20+ cut off is demonstrated. For both gropus a decrease in γ-H2AX-foci can be seen with time. But treatment with Cetuximab increases the percentage of cells with 20 or more foci in the sample that was fixed 30 minutes after irradiation from 32% to 50% of all cells counted. When comparing the samples 24h post IR a difference of 2% (untreated) to 8% (treated) can be seen.

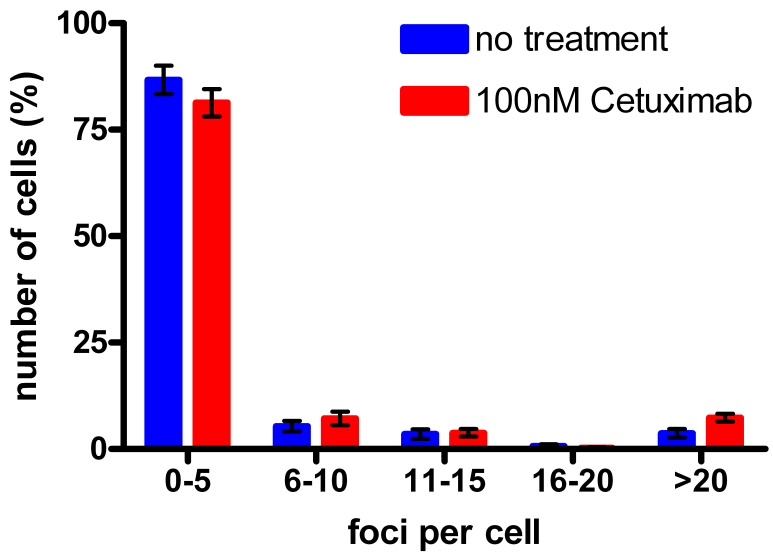


Figure 7: Repair of γ-H2AX-foci. Cells were pretreated with or without 100nM Cetuximab 1 hour prior to irradiation with 1Gy (A). All were fixed and stained 24h post IR. Bars represent the percentage of cells with the respective number of γ-H2AX-foci per nucleus with standard error based on 5 independent repeats.

4.2 Effect of Cetuximab on NHEJ activity

4.2.1 Plasmid validation

The DNA for the NHEJ assay was amplified using electrocompetent E. coli. All plasmids contained either the information for resistance to ampicillin or kanamycin. Since the bacteria were grown on selective plates and in media with the respective antibiotic only those bacteria that had internalized the plasmid were able to proliferate. In order to verify that the correct plasmids had been amplified a restriction digest was performed. The exact size and number of cutting sites is specific for each DNA and known for all four plasmids. First the previously purified DNA was cut using the restriction endonuclease HINDIII. This enzyme cuts SCE-I- at two different sites while all others have one cutting

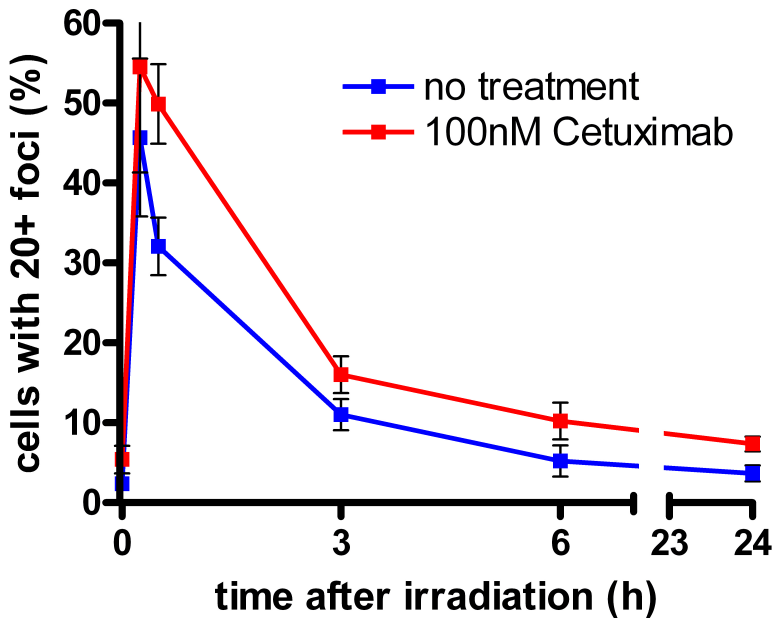


Figure 8: Repair kinetics of A549 cells after treatment with Cetuximab and IR. The cells were treated with or without 100nM Cetuximab 1h before irradiation with 1Gy. They were then fixed and stained at the indicated time points. γ-H2AX-foci per nucleus were counted and the fraction of cells with 20 foci or more calculated, thus demontrating the fraction of highly damaged cells. The graph demonstrates the average percentage of this group over time with two sided standard error based on 5 independent repeats.

site. After incubation the samples were run on an agarose gel containing ethidium bromide.

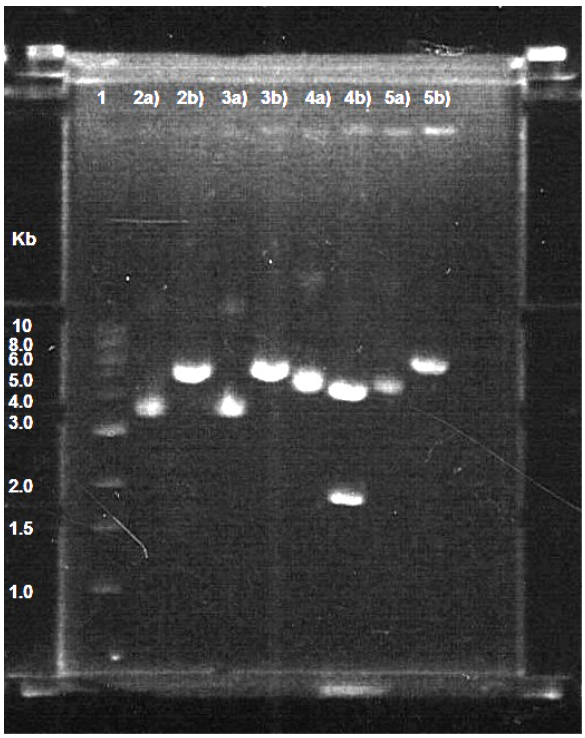


Figure 9: Control of correct plasmid internalization. Visualisation of EtBr intercalated with DNA bands after 2.5h at 80V via electroporesis

1	ladder	3a	EGFPNI uncut	4b	SCE-I- cut
2a	pEJ uncut	3b	EGFPNI cut	5a	I-SCE 3xNLS uncut
2b	pEJ cut	4a	SCE-I- uncut	5b	I-SCE 3xNLS cut

As can be seen in figure 9, the uncut samples travelled faster than the cut ones. The speed the DNA migrates with depends on its size and charge. Since the plasmids used are double stranded DNA in a ring structure they tend to fold up into complex shapes that migrate in a more complicated matter according to their tertiary structure.

The uncut plasmids are circular while the cut ones are linear. Circular fragments of DNA tend to travel more rapidly, this is due to the fact that they form supercoiled forms and nicked circles. These shapes are smaller and thus faster than the linearized DNA. This also means that only the bands of the cut plasmids can be used to determine

the size properly, because here the speed is relative to the size.

When comparing the bands to the molecular ladder it can be seen that all "cut"' bands are were they are expected them to be. pEJ is 4838bp long(2b), EGFPNI 4700bp (3b), I-SCE 3xNLS 5452bp (3b) these three plasmids have one cutting site. SCE-I- size is 5755bp and has two cutting sites for HINDIII, here two bands are visible, one with a size of 1800bp and the other with 3900bp (4b). These results make it very likely, that the correct plasmids were amplified.

4.2.2 Transient transfection

Two flasks of A549 cells were transiently transfected with SCE-I- as negative control. In this sample there shouldn't be any fluorescence besides the small amount of autofluorescence every cell shows. The next group was transfected with only EGFPNI as a positive control. Here every cell that expresses the plasmid should have a fluorescent signal. This makes the sample a good mean of determining how effective the transfection was.

Figure 10A shows the transfection effiency after 24h transfection time. Further 24h do not the amount of succesfully transfected cells, because of that only pictures of cells that were harvested after 1 day are shown. All samples were run on the FACS Calibur and the data analyzed using the FlowJo analysis program.

The transfection efficiency was determined measuring the signal of the enhanced green fluorescent protein EGFP. Compared to the wildtype GFP it emits a 5-10 fold enhanced fluorescent light when exposed to blue light. The transfection worked in 57.9% of the cells.

Figure 10B shows the negative control. In this group the cells were transfected with the inactive cutting enzyme SCE-I- only. Here a glowing fraction of 0.61% of the cells

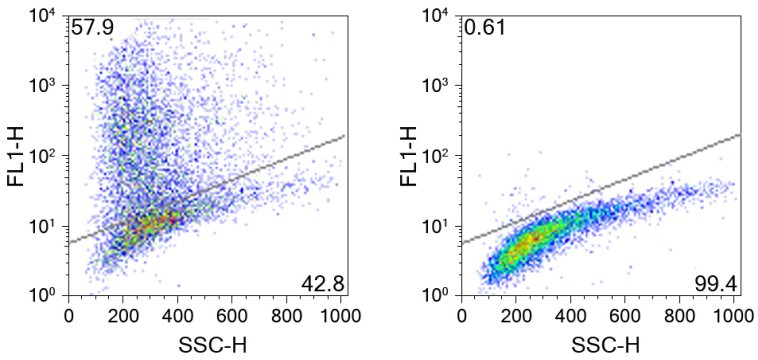


Figure 10: Transfection effiency of A549 cells) (Flow cytometry of GFP signalling A, EGFPNI transfection efficiency B, SCE-I- negative control, transient transfection 24h, 2Gy

can be seen.

4.2.3 NHEJ activity in A549 cells after Cetuximab treatment

Two flasks each were transfected with pEJ and I-SCE 3xNLS, as well as pEJ and SCE-I-. PEJ has an off frame start codon inserted between its two SCE cutting sites. In theory this prevents the GFP from being translated. But it is possible, that a small portion of the cells will still have a GFP signal. This is due to unspecific damage to the plasmid during transfection or radiation that results in loss of the artificial start codon and thus leading to a fluorescent signal.

The actual sample of interest is the one containing pEJ and I-SCE 3xNLS. Here the cells contain the plasmid and the active cutting enzyme. Every cell that internalized both plasmids should express pEJ and the functioning enzyme. Once both are expressed I-SCE 3xNLS will cut the GFP variant at the two cutting sites and free it off the out of

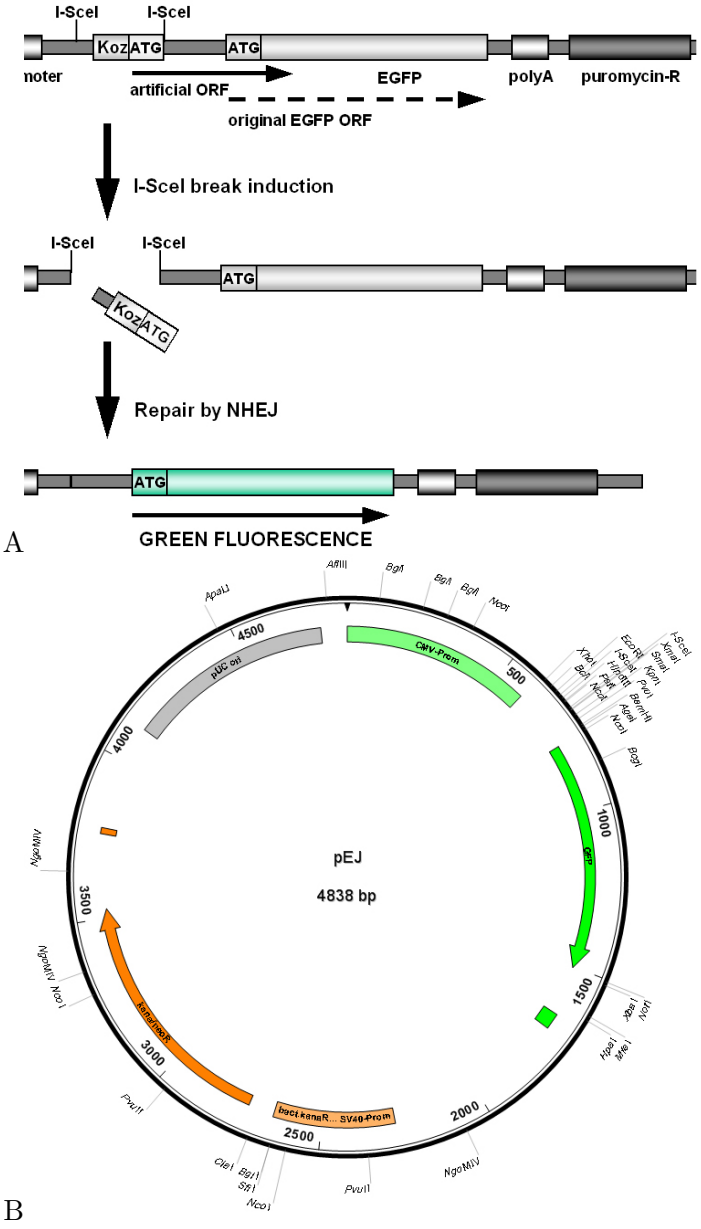


Figure 11: pEJ plasmid constructA, NHEJ GFP assay B, circular plasmid map of pEJ

frame start codon. Since the promoter is in the 5' untranslated region of the eliminated area the plasmid has to be repaired via NHEJ before GFP can be expressed. Imme-

diately after transfection one out of each pair of samples received 100nM Cetuximab. One hour later all eight flasks were irradiated with 2Gy. Irradiation causes simple and complex DSB as well as single strand breaks and other damage to the DNA. A cutting enzyme on the other hand only inflicts simple DSB on the DNA. The irradiation was done in order to ensure the conditions for DNA repair were similar to the ones in the γ-H2AX-foci experiments.

The Cetuximab treatment had the purpose of showing us whether the treated cells would repair slower. According to previously mentioned papers that link EGFR inhibition to impaired repair the inhibited cells should have a lower percentage of glowing cells than the untreated cells.

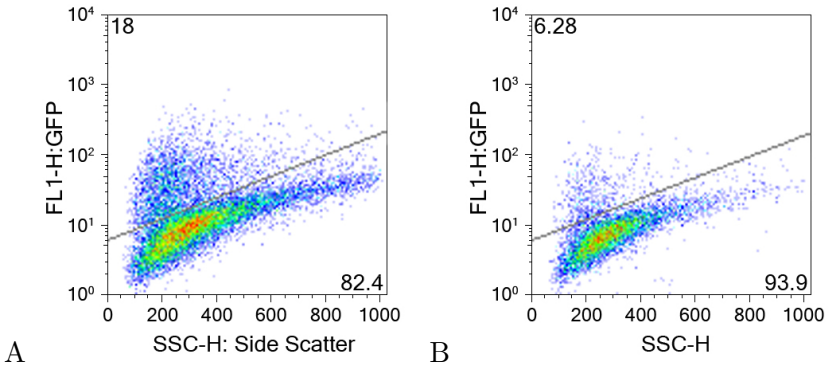


Figure 12: NHEJ visualized by GFP signaling, transient transfection 24h, 2Gy
A, pEJ + I-SCE 3xNLS B, peJ + I-SCE, flow cytometry done on FACS Calibur, analysed with FlowJo analysis software.

Figure 12A shows one representative example for the cotransfection with pEJ and the active cutting enzyme SCE-I 3NLS. Figure 12B is an image of the unspecific background in the SCE-I- and pEJ cotransfection. Both samples were not treated with Cetuximab. The unspecific background leads to a fraction of 6.3% glowing cells. In double trans-

fected and specifically cut sample this fraction makes up 18% of all living cells. After subtracting the background there are 11.7% of cells left with a successful double transfection, cutting and rejoining of DNA in untreated cells.

In order to determine the actual effect of an EGFR inhibition by Cetuximab on NHEJ

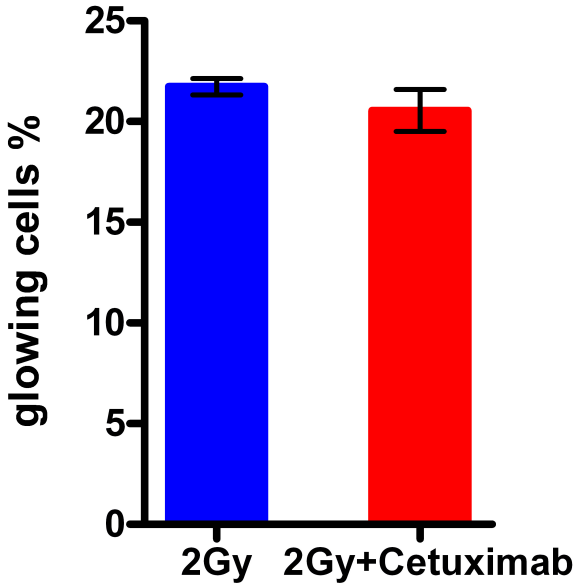


Figure 13: Effect of Cetuximab on NHEJ capacity
A549 pretreated with or without 100nM Cetuximab 1h prior to cotransfection, immediate irradiation with 2Gy, corrected for transfection efficiency, the bars represent the percentage of glowing cells in these groups with two sided standard error based on 3 independent repeats

activity the background in the treated and untreated group had to be subtracted. After taking the percentage of glowing cells in the pEJ + SCE-I- transfected cells into calculation the treated samples had a fraction of 10.75% glowing cells and the untreated ones contained 11.72 The green fluorescent cells represent the positiv repair events.

The experiment was repeated 3 times and results were corrected with the transfection efficiency using GraphPad Prism software. The results only differed 2.4% [1.87;

2.93].

As can be seen in figure 13 pretreatment with Cetuximab doesn't impair the activity of NHEJ on the DSB breaks induced by the cutting enzyme. Both samples have similar levels of glowing cells. According to literature the main result of EGFR inhibition is decreased DNA-PK activity [Dittmann2005/II]. If this was indeed the most important effect there should be a bigger difference in properly repaired and thus glowing cells between both groups.

The results of this experiment fortify the theory that EGFR inhibition by Cetuximab has effects on DNA repair that are independent of interference of NHEJ.

4.3 Effect of EGFR inhibition on DSB induction

4.3.1 γ-H2AX-foci in A549 with Cetuximab and Erlotinib treatment after irradiation

It has previously been reported that decreased repair capacity by EGFR inhibition increases residual foci 24h after irradiation [Huang2000], [Dittmann2005/I]. In addition to that the previously described experiments already show a difference in the number of foci 15 minutes after IR. Linking Cetuximab to impaired DSB repair could explain the elevated levels of severely damaged cells at late time points after irradiation. However even the fast component of NHEJ with it's half time of 10-30min seems to be too slow to be held accountable for the difference in foci at 15min.

So if this increase in foci is not due to the impaired repair of DBS, EGFR inhibition must have additional effects. The first possible explanation discussed was that EGFR signaling effects the induction of DSBs via alteration of chromatin compaction.

The new theory was that EGFR signaling has other or additional effects on the DSBs than DNA-PK interaction, the inhibition of which has previously been argued to be the reason for the increased toxicity of the combination of IR and EGFR blockage [Huang1999]. Our first aim was to determine whether the difference in foci at early time points is a result of something other than impaired DSB repair. If this actually is the case, then EGFR signaling must have additional and yet unknown effects on the DNA. When abrogated these make the DNA more vulnerable for ionizing radiation. The next step was trying to narrow down the mechanism for the previously described results. For this the incubation scheme was changed. Now the cells were first irradiated with 1Gy and then treated with Cetuximab right after. Afterwards they were incubated for 1.5h in order to have the same Cetuximab exposure time as in the 0.5h time point of the previous experiments. Here the samples were pretreated for one hour and then fixed 30min post IR. In the previous experiments the biggest difference in foci had been visible after half an hour.

According to the theory that attributed the radiosensitizing effect of Cetuximab to impaired repair of DSB changing the order of treatment should lead to the same difference between treated and untreated cells as seen in the previous experiment.

If it is true that Cetuximab influences the vulnerability of the DNA to irradiation there should be no difference in the treated and untreated cells now, because the inhibitor didn't have the time to sensitize them prior to irradiation.

And indeed in figure 14 the treated cells and the control have the same amount of foci.

The EGFR is said to translocate into the nucleus after IR. Since Cetuximab was applied after irradiation it is possible that this process has already taken place. In this case the antibody which only reaches the receptor on the outer membrane of the cell

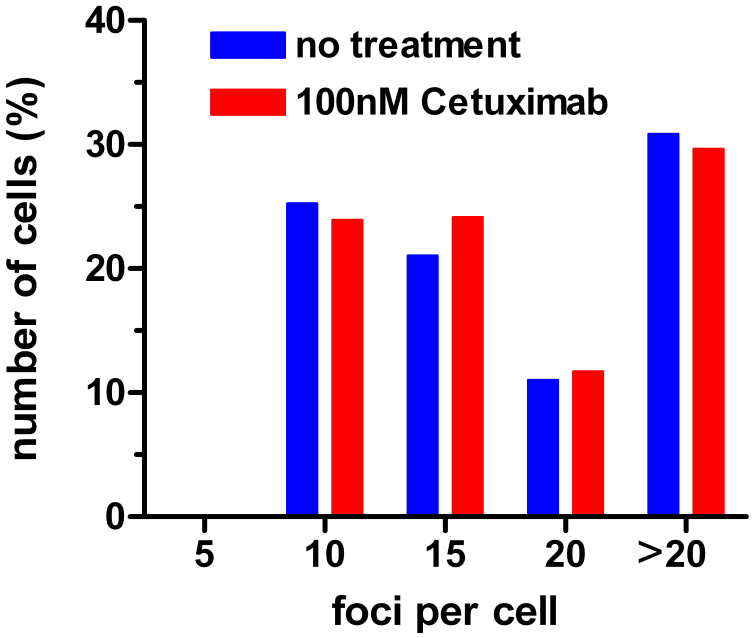


Figure 14: induction of γ-H2AX-foci; A 549 irradiated with 1Gy and treated with or without 100nM Cetuximab right after IR. Cells were then fixed after 1.5h of incubation. The bars represent the percentage of cells with the respective number of foci

cannot influence the signaling any more. To further strengthen our theory the setting of the experiment needed to be changed in order to rule out this possible mistake.

Erlotinib is a small molecule that binds to the intracellular adenosine trisphosphate binding site of the receptor and by doing so blocks signal transmission. Exchanging Cetuximab with Erlotinib an inhibitor was used that works inside the cell. This eliminates the problem of the quick translocation possibly distorting the results.

As can be seen in figure 4 the TKI Erlotinib leads to an increase in foci A549 as well. The repair kinetics after combined treatment of Erlotinib 1h prior to irradiation with 1Gy are similar to the ones in the Cetuximab treated cells. In both treatmente we see differences as early as 30 min post IR in the number of foci when comparing treated

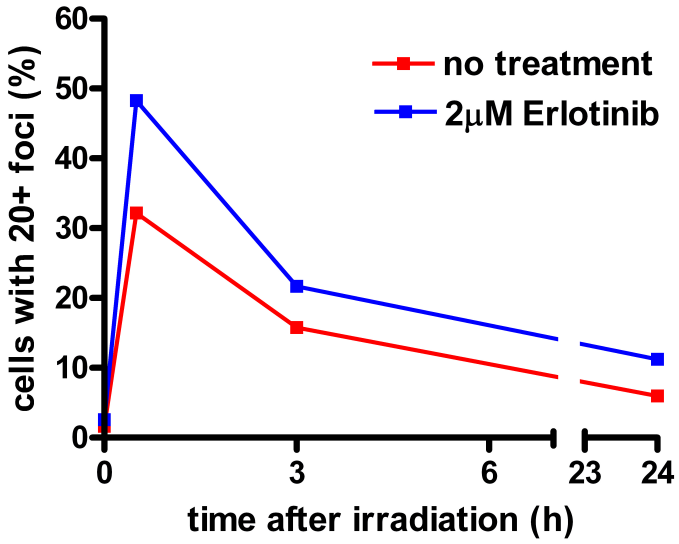


Figure 15: repair kinetics of A549
pretreatment with 2μM Erlotinib one hour before irradiation with 1Gy. Cells were then fixed and stained at indicated time points and percentage of cells with 20 or more foci calculated

and untreated cells. Figure 15 shows, that in addition to higher levels of cells with a lot of DSB 24h after irradiation Erlotinib induces an increase in foci at the early time points too. Having demonstrated that it causes the same effects as Cetuximab as far as γ-H2AX-foci kinetics are concerned, it is legitimate to use Erlotinib for further investigation of the described findings.

Figure 16 shows that treatment with Erlotinib after irradiation has no effect on the number of double strand breaks induced by 1Gy either. This supports the theory that the EGFR inhibition by antibody or TKI indeed influences the vulnerability of the DNA and needs to be added prior to IR in order to induce a radiosensitizing effect.

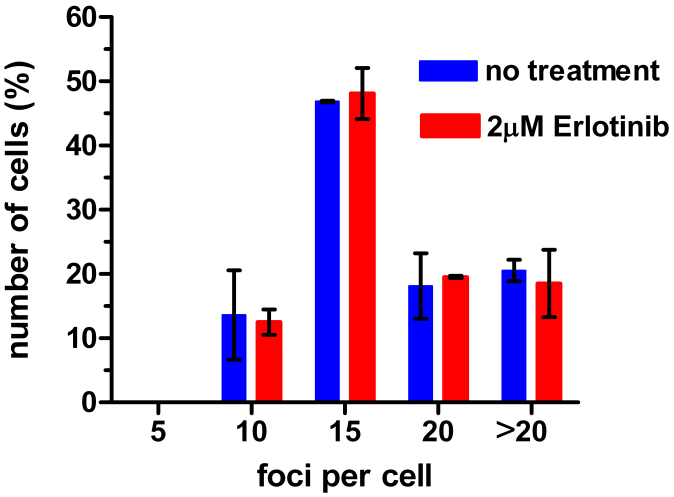


Figure 16: Repair of γ-H2AX-foci; A549 irradiated with 1Gy and treated with or without 2μM Erlotinib, fixed and stained after 1.5 h of incubation. Bars represent the percentage of cells with the respective number of foci with standard error based on 2 independent repeats

4.3.2 DSB repair after direct inhibition of DNA-PK / NHEJ

Other work groups stated that the increase in residual γ-H2AX-foci is due to decreased activation of DNA-PK and thus decreased NHEJ. If this is the main effect EGFR inhibition has on DNA repair and integrity, treatment with the DNA-PK inhibitor NU7026 should show the same repair kinetics over time as Cetuximab and Erlotinib.
The previous set-up was repeated with a variation in treatment. This time A549 cells received either fresh media, 100nM Cetuximab, 10μM DNA-PK inhibitor NU7026 or combined treatment of Cetuximab and NU7026 one hour prior to irradiation with 1Gy.

The results of this experiment further strengthen the point that EGFR signaling doesn't only result in activation of DNA-PK as shown by [Dittmann2005/I], [Dittmann2005/II], but has further influences on the response of A549 to irradiation as well. Figure 17 shows the previously described increase of the fraction of cells with 20 or more γ-H2AX-foci.

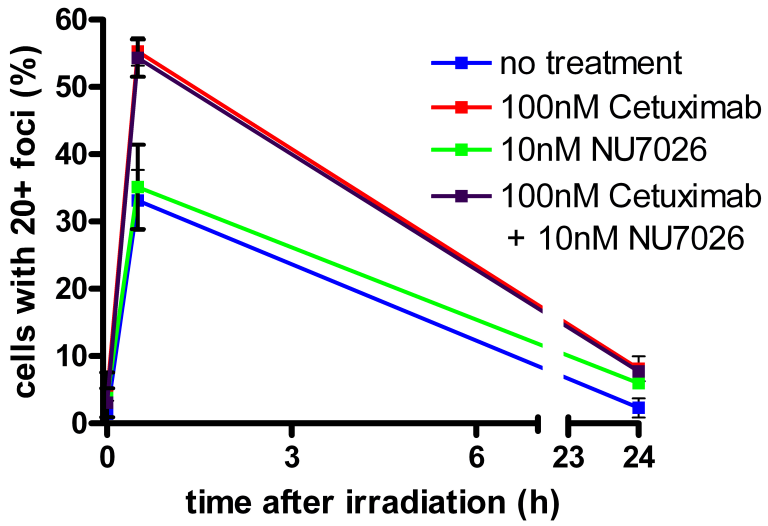


Figure 17: Repair kinetics of A549
1h pretreatment with 100nM Cetuximab, 10μM NU7026, the combination of both or simply fresh media 1 hour before irradiation with 1Gy. The cells were then fixed and stained at the indicated time points. The graphs represent the percentage of cells with 20 or more γ-H2AX-foci at each time point, two sided standard error based on 3 independent repeats.

This phenomenon is visible 30 minutes after irradiation in the Cetuximab treated cells and in the samples receiving a combined treatment of Cetuximab and NU7026, but not in the cells that were only treated with NU7026 or in the control group. This supports the assumption that pretreatment with Cetuximab has effect on the induction of DSBs that are independent of DNA-PK inhibition.

The NU7026 treated cells show no difference to the control group at 0.5 h, but at 24h an increase in residual foci becomes visible. Since DNA-PK is one of the most important proteins for DSB, inhibition of this complex should lead to more residual damage because of a repair inhibition. The fact that NU7026 doesn't increase the amount of cells with 20 or more foci 30min after IR makes it very likely that the effect of the EGFR

antibody at these time points is a result of a different mechanism than the EGFR dependent decreased activation of DNA-PK that has widely been described in literature.

When comparing the Cetuximab and NU7026 treated cells there is not a big difference in the number of cells with 20 or more γ-H2AX-foci 24h.

In order to address the question whether the DNA-PK inhibitor does work properly at the applied concentration three sets of survival assays were plated. The cells were plated at different concentrations one day prior to treatment in order to allow attachment over night. One group of flasks was treated with 10μM NU7026 one hour before irradiation. The control group received fresh media instead. Both groups were then irradiated with doses of 2Gy - 8Gy and incubated for 20 days before fixing and staining. The colony formation assay seen in figure 18 shows that NU7026 is active and radiosensitizes A549 at a concentration of 10μM.

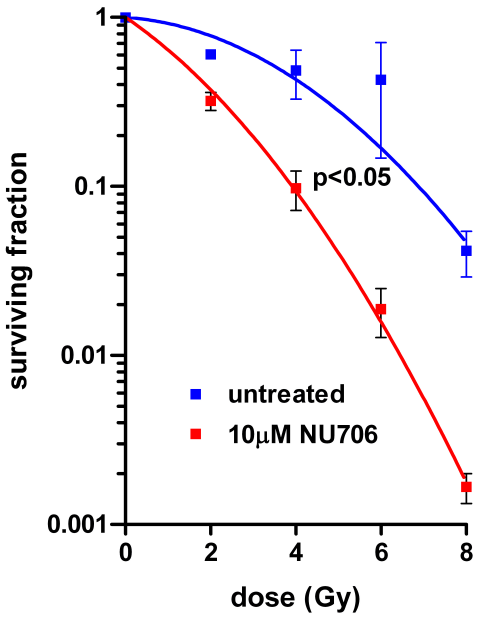


Figure 18: Radiosensitisation of A549 by NU7026 on cell survival; A549 colony survival essay treatment with 10μM NU7026 one hour prior to irradiation with different doses; Statistical comparison by F-test, two-sided

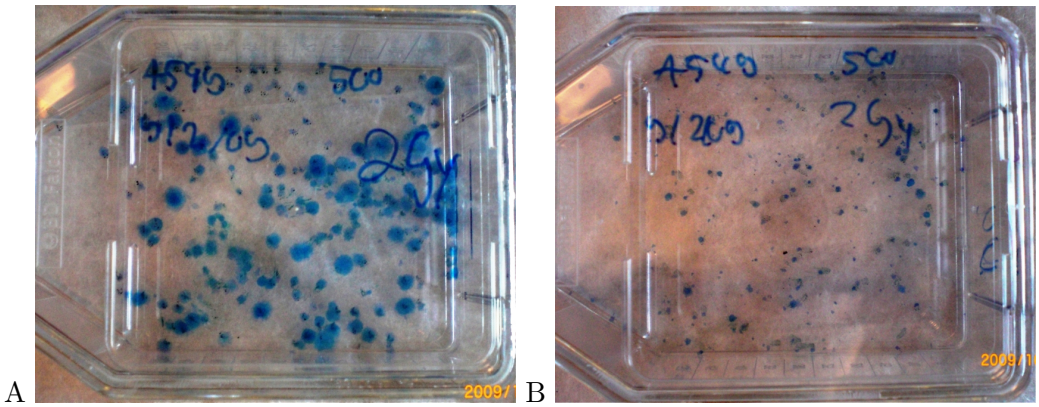


Figure 19: Effect of NU7026 on cell survival, representative images; A549 colony formation assay after IR with 2Gy and incubation for 20 days A, control group B, 10μM NU7026, colonies with more than 50 cells were counted.

Figure 19 shows two representative flasks of a colony formation experiment. One flask (A) remained untreated the other (B) received 10μM NU7026 one hour before irradiation and was maintained in media with the DNA-PK inhibitor while incubating. The treatment decreased the number and size of colonies greatly. The distribution of colonies in the flasks was the same in treated and control experiment.

4.4 γ-H2AX-foci in Calu6 and NCI H460 cells after combined treatment with Cetuximab and irradiation

Since one of the skopes of this research project was to find out whether γ-H2AX-foci are a valuable tool for the detection of radiosensitization through EGFR inhibition, the amount of γ-H2AX-foci after treatment was also determined in Calu6 and NCI H460 cells. Both cell lines are known not to be sensitized by Cetuximab. So if the increase in DSBs at eary time points after Cetuximab application is the underlying mechanism for increased sensitivity to irradiation and the γ-H2AX antibody shows these breaks it could be expected to see no increase in foci after treatment in these two cell lines.
In figure 20 a peak value of about 50% of cells with 20 or more DSBs can be seen at 0.5h. After that the amount of γ-H2AX-foci is gradually decreasing. In the A459 cells this fraction made up 50% in the treated cells and 32% in the untreated ones.

Cetuximab treatment doesn't increase the percentage of cells with 20 or more γ-H2AX-foci in NCI H460 cells. There is no detectable difference between the untreated (dotted line) and the treated (solid line)cells at any time point. EGFR inhibition doesn't affect the amount of DSB. The same holds true for Calu6 cells.

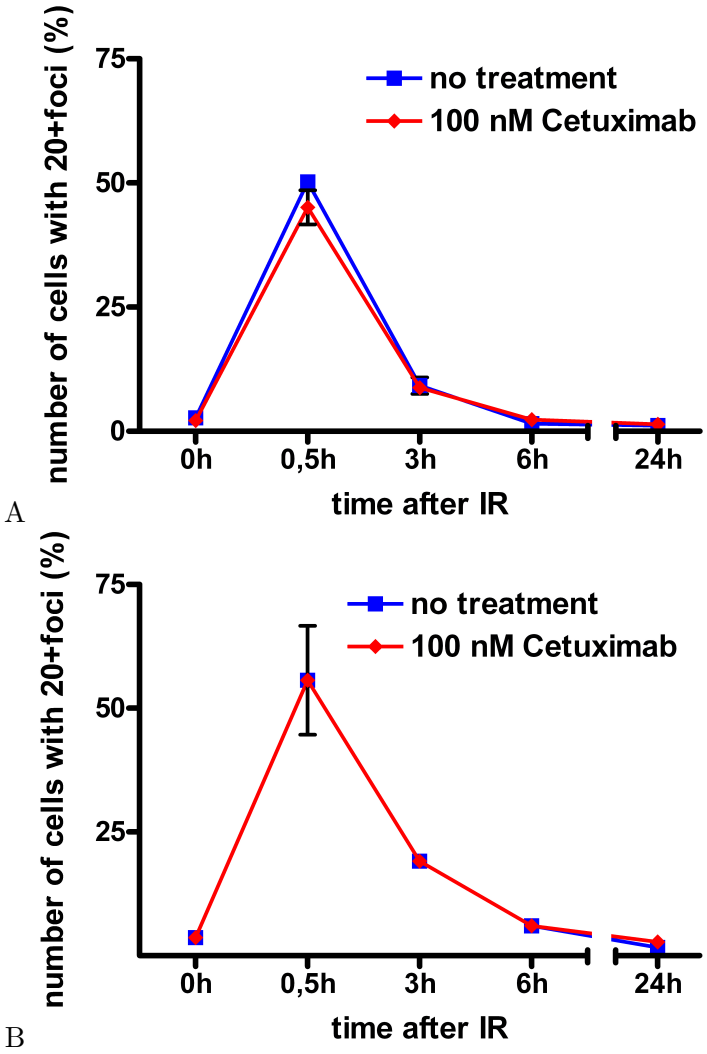


Figure 20: Repair kinetics in NCI H460 and Calu6 cells; Fraction of highly damaged cells (20 or more γ-H2AX-foci per nucleus). The cells were treated with or without 100nM Cetuximab 1h prior to IR with 1Gy. They were then fixed and stained at the indicated time points. γ-H2AX-foci per nucleus were counted and the faction of cells with 20 foci or more calculated. The graphs demonstrate the average percentage of this group with two sided standard error based on 3 independent repeats. A, NCI H460 and B, Calu6

5 Discussion

Due to its high incidence and mortality lung cancer is the carcinoma causing the most death worldwide [WHO2009]. Even though there has been a lot of progress in treatment regimes within the last decades, these still leave a fairly big percentage of patients with a poor outcome. This is partially due to late detection of the disease, but also to resistance to radiation, one of the major components especially in late stage cancer treatment. New drugs with very specific targets have been developed and are already being used in clinical trials as well as routine treatments. One strategy to render cancer cells more sensitive to radiation is the inhibition of the epidermal growth factor receptor. EGFR is a transmembrane receptor that is overexpressed or mutated in the majority of lung cancers. While some patients benefit greatly from the combined treatment of EGFR inhibition and radiation, others don't [Harari2006], [Bonner2006].

This leads to the question of how to identify those cancers that are susceptible for the combination of irradiation and supporting treatment. It is known that carcinomas with an EGFR mutation in exons 18 to 21 benefit from TKI treatment alone [Qin2005]. Other bronchial carcinoma cell lines are radiosensitized by EGFR inhibition. In vitro experiments as well as clinical trials lead to the conclusion that tumors that don't posses such a mutation can also respond to the inhibition. However promising the combined treatment is in theory, it only leads to tumor control in a small fraction of patients. So far there is no reliable predictive marker to asses for which patients inhibitors like

Cetuximab, Erlotinib, and Gefitinib will improve the outcome.

Irradiation causes a wide range of lesions to the DNA, double strand breaks being the most dangerous for the cell. Incorrectly repaired DSB can lead to chromosomal aberrations, death as well as further mutation. The cell survival after IR depends on a variety of repair mechanisms, pro-survival and anti-apoptotic signaling. The epidermal growth factor receptor plays a role in numerous of these pathways. Several work groups have published that EGFR is involved in DSB repair through activation of DNA-PK, one of the most important proteins in the repair process [Chen2007], [Dittmann2005/I]. Upon activation the receptor undergoes nuclear translocation and activates DNA-PK via direct interaction. In accordance with that it has been shown that EGFR inhibition causes an increase in residual DSB when measured 24h after irradiation. This is said to be due to a repair delay in the inhibitor treated cells [Toulany2006].

The aim of the present study was to investigate the underlying mechanism of radiosensitization by EGFR inhibition. Previous publications indicate that the reason for the repair delay is a decrease in DNA-PK activation. The hypothesis of this project is that impaired NHEJ is not the only reason for an increase in DSBs, but rather that EGFR inhibition has other, earlier effects that modulate the cellular reaction to DNA damage as well.

5.1 EGFR inhibition by Cetuximab treatment in A549 cells

It has been shown that the monoclonal EGFR antibody Cetuximab radiosensitizes the NSCLC cell line A549 but not the cell lines Calu6 and NCI H460 [Steiner2007]. However the underlying mechanism isn't yet understood.

The first experiments demonstrate that Cetuximab increases the number of severely

damaged cells with more than 20 γ-H2AX-foci in A549 cells after irradiation at early and late time points (figure 2 and figure 4). The effect of Cetuximab on A549 cells has previously been investigated intensively [Dittmann2005/I] [Dittmann2005/II]. Dittman and his group focused on colony formation, the activation of DNA-PK, as well as the amount of γ-H2AX-foci at late time points after irradiation. They reported an increase in residual DSBs 24h after IR. Additionally they found that EGFR inhibition by the monoclonal antibody blocks the irradiation induced nuclear translocation and leads to decreased DNA-PK activation. They suggested that the increase in residual γ-H2AX-foci is a result of impaired NHEJ due to an inactive repair complex.

In correspondence with already published work the increase in residual foci was reproducable. This effect has been described with an average difference of 6 foci per nucleus when comparing treated (15 foci per cell) and untreated cells (9 foci per cell) 24 hours after irradiation with 2Gy [Dittmann2005/I].

This thesis project also focuses on the early effects of EGFR inhibition and includes the repair kinetics using γ-H2AX-foci as a detection tool for the DSBs caused by ionizing radiation.

However looking at the distribution of foci over time, the average of DSB per cell doesn't seem to be an accurate way of describing the difference of combined treatment or irradiation alone. Especially at the early time points and with a high dose of IR the cells show a high level of damage. Without confocal microscopy that allows to scan through each nucleus with focal planes of defined thickness the counting of nuclei becomes inaccurate with increasing numbers of DSBs. Because of this reason up to 20 foci per cell were counted, and all cells with more DSBs noted as having more than 20 γ-H2AX-foci per nucleus.

So far it is not completely clear what the mechanism for the radiosensitizing effect of

EGFR inhibition is. Comparing the nuclei at early time points with the ones 24 hours after irradiation a change in the morphology of γ-H2AX-foci is detectable. They increase in size over time, figure 5D shows a cell with those large foci. It is not understood what causes this enlargement in size. Different workgroups reported that while most of the foci are already repaired 24h post IR some of them cluster together and form large γ-H2AX-foci that have lost their round shape [Desai2005]. It is also not known which cells are damaged more severely, the ones with a high level of smaller foci 24h after IR or the cells with few large cluster of foci. So far the augmentation of cells with high levels of foci has been referred to when talking about the radiosensitizing effect of various treatments.

Believing that highly damaged DNA and impaired repair leads to decreased survival shifted the focus towards those cells with foci when determining radiosensitization. Especially at early time points after irradiation the percentage of cells with more than 20 DSB is large in both treated and untreated cells. This makes the calculation of an average inaccurate. Under these conditions more than 20 γ-H2AX-foci could mean any number of γ-H2AX-foci per nucleus higher than the cut off. Since the exact number of foci per cell was not counted it is impossible to calculate the average. In order to get a more comprehensive view on the results the cells where sorted into groups according to their number of DSBs after counting.

Over time most damage is repaired and there are only few cells left in the groups of 11-15 and 16-20 foci per cell. While in most cells the number of γ-H2AX-foci is reduced to 0-5 foci per nucleus there is a small group that still has more than 20 γ-H2AX-foci 24 hours after irradiation. Using the average would imply that the distribution is a Gaussian one. Especially when looking at the 3 or 6 hour time points the curve looks more like a u-shape.

By using the median of foci per cell the problem of not knowing the exact number of foci per cell in the group with 20 or more foci could be avoided. On the other hand this way of illustration doesn't show the difference in treated and untreated cells 24 hours after IR very well. The reason for that being the very small fraction of cells with a lot of damage while the majority has already repaired most of its DSB. By plotting the median the subgroup of cells with a high level of residual damage is lost.

According to our opinion the most accurate way of showing the increase of DSB after treatment was graphing the percentage of cells with 20 or more foci as seen in figure 8. Here a previously not described effect can be seen. While the increase at 24 hours has been published by different workgroups and linked to impaired DSB repair the changes at early time points after irradiation have not been investigated before. Interestingly, here the biggest difference between treated and untreated cells is detected.

When looking at the amount of highly damaged cells a yet undescribed effect of EGFR inhibition can be seen. A549 cells already show quite a big increase in the fraction of cells with more than 20 γ-H2AX-foci per cell 15 minutes after irradiation when comparing treated and untreated cells.

Cells repair DSB through two main repair mechanisms. One of them, namely NHEJ is more error prone than homologous recombination but it can take place in all phases of the cell cycle. Because of that NHEJ is the dominant pathway most cells rely on for DSB repair. It has a fast and a slow component, the fast one working with a half time of 10-30 minutes, the slow one with of 2-10h [Iliakis2004].

The increase of residual γ-H2AX-foci in the EGFR antibody treated cells 24 hours after irradiation has previously been linked to impaired DNA repair due to a decrease in DNA-PK activation and thus impaired NHEJ. Seeing the effect Cetuximab has already on cells 15 minutes after IR makes it hard to believe that this is also a consequence of

impaired DNA repair alone. Even holding the fast component of NHEJ accountable, 15 minutes seem like a very short time to already see an effect of decreased DSB repair in the treated cells.

To test if there are additional mechanisms of sensitization besides DSB repair inhibition the experimental settings were changed with the aim of excluding decreased repair as the underlying mechanism. Now the cells first received IR and the inhibitor was added right after. Conducting this experiment with Cetuximab as well as with Erlotinib did indeed negate the difference between treated and untreated cells. As figure 14 and figure 15 demonstrate reversing the order of treatment leads to the same amount of foci in both groups. According to the theory that EGFR inhibition leads to a repair delay and thus to more residual foci these changes in the order of treatment shouldn't have change the previously detected difference. This means that it is important for the radiosensitizing effect of the inhibitors that they are applied before irradiation and accordingly that a repair delay is not the only consequence EGFR inhibition has on the cells.

One problem not yet taken into consideration is the fact that Cetuximab is an antibody that only inhibits the EGFR on the outer membrane of the cell. EGFR gets activated and translocates into the nucleus right after irradiation. It is possible that the translocation already takes place during irradiation. If the translocation was that fast, applying the antibody immediately after transferring them from the X-ray machine into the sterile cell culture hood wouldn't be quick enough and the receptor would already be out of reach for the antibody. In this case the findings wouldn't really prove the intended point. Obviously there would be no difference in treated and untreated cells if the inhibitor was added to late and couldn't reach its target any more. The experiment with Cetuximab therefore doesn't really help to determine the reason for the increase in foci. Erlotinib is a small molecule that targets also the receptors inside the cell. Even

after a quick translocation it would still inhibit the EGFR. Repeating the experiment with Erlotinib doesn't change the fact that the reverse order of treatment negates the increase in foci.

This indicates that the underlying mechanism is not a repair delay alone in the treated cells, but that EGFR signaling must have additional effects on the cells that are not know off yet. Possibly their abrogation by EGFR inhibition leads to more irradiation induced damage to the DNA.

After having shown that neither the monoclonal antibody Cetuximab nor the small molecule Erlotinib increase the number of γ-H2AX-foci when applied directly after irradiation another step towards showing that DNA-PK inhibition is not the explanation for the increase in DSBs at early time points was taken.

First a colony formation assay was performed in order to demonstrate that the DNA-PK inhibitor NU7026 does radiosensitize A549 at the applied concentration. The reduced survival of the pretreated cells can be seen in figure 18.

The next target of interest was the number of γ-H2AX-foci in 4 parallel set ups for the A549 cells. Just like before one group of cells received Cetuximab an hour prior to irradiation, one full media, and this time one group was treated with the DNA-PK inhibitor NU7026, the fourth group received NU7026 and Cetuximab. Assuming that decreased DNA-PK activation is the underlying mechanism for the increase in foci in Cetuximab treated cells comparing the NU7026, the Cetuximab, and the double treated group of cells should show the same repair kinetics. However, figure 17 shows that these two inhibitors influence the cells in different ways. While NU7026 indeed only causes a repair delay with an increase in cells with more than 20 DSB 24h after IR, Cetuximab shows the additional effect of an increase in this subgroup of cells at the early time points when compared to the untreated, and the NU7026 treated cells.

It could still very well be that Cetuximab causes a repair delay and that this or a combined effect is the explanation for the increase in cells with more than 20 γ-H2AX-foci at the late time points. But now it has to be considered that the EGFR inhibitor causes more breaks at the early time points when applied one hour prior to irradiation. This is not a result of DNA-PK inhibition otherwise it would have occurred in the NU7026 treated cells as well. With all results presented it has to be assumed that impaired NHEJ is not the only reason for the Cetuximab induced radiosensitization of A549.

In order to further narrow down the mechanisms behind the difference in the fraction of cells with more than 20 γ-H2AX-foci caused by EGFR inhibition at the early time points more experiments are necessary. One question worth looking into is whether EGFR inhibition leads to a change in chromatin compaction thus making the treated cells more vulnerable. Here a Micrococcal nuclease assay could be helpful. The assay is a valid method of detecting chromatin compaction. The denser the DNA is organized the longer it takes the Micrococcal nuclease to digest it.

It is not known if the reason for the increase in DSB is a change in compaction or something completely different.

Another explanation for the increased number of DSB could be that ionizing irradiation creates reactive oxygen species in the cell. These free radicals are highly reactive and can cause damage to cell structures and reduce cell survival. Cells use a variety of enzymes to protect themselves from the effects of ROS. One of these enzymes is the glutathione peroxidase. EGFR activation is shown to increase the activity of this enzyme peroxidase [Okamura2009]. So if EGFR inhibition leads to decreased levels of glutathione one would expect the cell to be more susceptible to IR, since it is lacking its defense mechanisms against ROS.

However the reason leading to the increase in γ-H2AX-foci at the early time points after IR is still unknown.

At present there is a lot of speculation involved and a lot of research still needed in order to find the underlying mechanisms.

5.2 NHEJ activity after EGFR inhibition

In accordance to literature the original hypothesis was that the reason for radiosensitization by Cetuximab is the impairing effect it has on NHEJ. In order to test this repair pathway specifically a plasmid construct was used to investigate the influence of the inhibitor on the repair of DSBs inflicted on the DNA by the cutting enzyme I-SCE 3NLS. This experiment allows quantification of the correct cutting and rejoining of the pEJ plasmid via GFP fluorescence as a marker for the possiv repair event. The cells that were pretreated with Cetuximab don't show lower levels of fluorescence. This means there was no impaired NHEJ in the group treated with the EGFR inhibitor. These findings go well in line with our findings that inhibited NHEJ is not the only impact Cetuximab has. However it must be taken into consideration that the DSB induced by the cutting enzyme I-SCE 3NLS are simple breaks while irradiation causes a variety of simple as well as complex DSB in addition to other damages. Irradiation also leads to free radicals in the cell that can cause further damage if they are not eliminated by antioxidant defense mechanisms. So it could well be that the underlying mechanism for the repair of these clean cut breaks is not quite the same as the repair of damage caused by irradiation. In contrast this assay doens't induce an activation of EGFR like for example IR does, this might be one reason why EGFR can not be inhibited. On the other hand this assay is frequently being used to investgate this specific repair pathway.

5.3 EGFR inhibition by Cetuximab in Calu6 and NCI H460 cells

Having shown that γ-H2AX-foci indeed do have different levels in the treated and untreated A549 cells the next step was to prove that this was not a general effect of the inhibitor, but a predictive screening tool for radiosensitization. The same experiment was performed on Calu6 and NCI H460, two cell lines previously reported not to be radiosensitized by Cetuximab [Steiner2007]. These two cell lines don't show an increase in DSB at any time point after IR when comparing the treated and untreated cells. Figure 20 shows that the percentage of cells with more than 20 γ-H2AX-foci is the same in both lines of treatment at each timepoint. This holds true for any other cut off or mean of visualization for the distribution of DSBs as well. The two cell lines also don't show an increase in lower numbers of foci. This confirmes our theory that the the underlying mechanism for the radiosensitization of A549 is indeed connected to the increase in foci and γ-H2AX-foci can be used as a marker for the sensitizaion. However the remaining question is why only A549 react with higher levels of DSB while the others don't. First of all out of these cell lines A 549 cells are the only ones overexpressing EGFR. It is likely that they depend on EGFR signaling much stronger than other cell lines for survival. The receptor is involved in numerous pathways mediating repair and cell survival. It has been suggested before that it's interaction with DNA-PK and thus with DNA repair is the reason why certain cells can be sensitized by EGFR inhibition. According to this theory treatment with Cetuximab leads to impaired repair of irradiation induced damage and to decreased survival of the cells.

Comparing the amount of DSBs in the three cell lines used here of which the behavior to EGFR inhibition and irradiation is known supports the theory that the radiosensitizing effect in different cell lines can be predicted by measuring their increase in γ-H2AX-foci after Cetuximab treatment and irradiation. Having shown that these foci

especially at early time points are predictive in these three cell lines the next step would be to include more cell lines in order to see if the reliability of the screening tool also holds true for them. All experiments were performed under in vitro conditions. In order to gain more clinically relevant data it would be necessary to include in vivo material and test xenografts as well as material from biopsies to see if the marker still predicts radiosensitization in the complex environment of living tumor tissue.

6 Summary

This thesis addressed the reliability of γ-H2AX-foci as an indicator for radiosensitization in lung cancer cell lines obtained by EGFR inhibition. For this 3 cell lines were compared: A549 that are susceptible for the combined treatment, NCI H460 and Calu6 that are not.

Effect of EGFR-inhibition on the number of induced DSBs was studies using γ-H2AX-foci as a sensitive marker for DSBs.

For A549 cells there was a clear increase of γ-H2AX-foci at all time points after irradiation. This was not seen for the other two cell lines.

This goes well in accordance with the fact that A549 cells can be radiosensitized by the EGFR antibody Cetuximab while the other two cell lines can't.

When looking at the early effects of the EGFR inhibition the sensitizing effect doesn't seem to be a result of repair delay, it rather seems that the abrogated EGFR signaling makes the cells more vulnerable for ionizing radiation.

Against the reasoning of the whole effect being due to slower NHEJ speaks the fact that a difference in foci can be seen as quickly as 15 minutes after irradiation. Even the fast component of the repair mechanism isn't fast enough to generate such an early alteration.

With all the results gathered the possibility was discussed that EGFR inhibition by antibody or small molecule also influences the induction of DSB in certain cell lines.

Supportive to this alternative theory is the fact that the inhibitors Cetuximab and Erlotinib don't increase the amount of foci per cell when applied right after irradiation. Apparently the inhibitor needs to be applied before in order to sensitize the cell for irradiation.

This argument is supported by the observation that the DNA-PK inhibitor NU7026 leads to completely different foci kinetics. In this case there is no early difference in the number of foci, but only the increase in residual foci 24 hours after IR that has been linked to a repair delay. Since DNA-PK inhibition doesn't cause the early increase in breaks the mechanism behind it can scarcely be decreased DNA-PK activation due to EGFR inhibition. In line with these results the NHEJ construct that specifically tested the NHEJ activity after defined induction of DSB didn't show impaired repair capacity in the Cetuximab treated cells.

In search of a different mechanism causing the increase in DSB at early time points various ideas were taken into consideration. So far it is known that EGFR is involved in various pathways, but it has not been linked to chromatin compaction or other mechanisms that could lead to increased vulnerability to DNA damage. Further investigation is necessary to determine the reason for the early post IR increase in foci in A549 cells.

In summary all these findings suggest that γ-H2AX-foci especially at the early time points might be a reliable screening tool for radiosensitization. While the reason for the sensitization is most likely an increase in DBSs, the underlying molecular mechanism is not completely understood yet and needs further investigation.

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7 Acknowledgements

The experimental work for this thesis was conducted in the laboratories of the Department of Radiation Oncology, Massachusetts General Hospital, Boston, MA, USA. I want to thank Dr. med. Henning Willers for the support and time, and for giving me the opportunity to work in his laboratory. I am very grateful for the help he provided during the experimental work.

Additionally I have to thank the complete staff of the workgroup for their support, most of all Liliana Gheorghiu and Chaké.

I further want to thank PD Dr. med. J. Dahm-Daphi for mediating the contacts and enabling me to conduct the research abroad. Special thanks goes to Dr. U. Kasten-Pisula for her time and patience during the writing process as well as the correction.

Finally I'd like to thank my parents for their motivation and interest in everything I do. And grandma, I wish you could read this too. Thank you so much for giving me the financial opportunity to take this chance.

8 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.

Date _____

Signature _____