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The Role of p53 in Cetuximab Induced Radiosensitization of A549 Cells and the Mechanisms Involved

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Table of Contents

1. Hypothesis

The majority of non small cell lung cancers (NSCLC) exhibit overexpression of the epidermal growth factor receptor (EGFR), resulting in rapid growth and resistance to most therapeutics. Much of the resistance to therapy is due to anti-apoptotic or proliferative signaling, e.g. overexpression of EGFR. So the interception of EGFR signaling made its way from bench to clinical use and is now part of the treatment of many malignancies. Treatment options may now include a combined therapeutic approach of EGFR inhibition and other therapeutic options, as the blockage of the EGFR pathway can result in a more pronounced response to established therapy, for example radiotherapy. Thus EGFR inhibiting therapeutics can act as a radiosensitizer, but only a subset of the treated tumors shows this kind of radiosensitization.

So far, there are no biomarkers or screening tests for gene mutations in routine clinical use which could predict the efficacy of combined EGFR inhibition and radiotherapy. There is evidence that a KRAS wild-type is beneficial for the response to this kind of treatment, but this only holds true for certain cell lines, while others seem unaffected, even though they show a KRAS wild-type. So it could be that EGFR, and the linked pathway, are key players in this context.

Another frequent mutation in cancer concerns the p53 gene, which has been studied extensively and is linked to poor response to treatment. It is not clear, to date, whether the p53 status is crucial for the response to combined treatment of EGFR inhibition and radiation therapy.

The A549 cell line is a long established and well characterized cell line. It is known to respond favorably to the combined treatment of EGFR inhibition and radiation therapy. Additionally, it features a KRAS mutation and a p53 wild-type, making it a good candidate to investigate whether a p53 wild-type is a prerequisite for efficient EGFR inhibition.

The aim of this thesis is to demonstrate the importance of a functional p53 for the radiosensitization of A549 cells and the implication for the cell, when subjected to the EGFR inhibitor Cetuximab (monoclonal antibody). This was achieved by introducing a mutated p53 gene into the A549 cell line and comparing the reaction to Cetuximab and IR of unaltered cells.

2. Introduction

2.1. Lung Cancer

Lung Cancer is the leading cause of cancer related deaths in the United States. With death numbers exceeding the sum of the other three leading causes in cancer death, namely: breast, colon and prostate.

The connection between smoking and lung cancer has already been known since Sir Richard Doll observed the smoking habits and cancer related deaths of fellow physicians, starting in the 50s. This prospective study showed quickly, that the mortality rate of smokers versus non-smokers was nearly 15fold higher and also dropped significantly as soon as people stopped smoking. (Doll et al. 1994) Those findings have been confirmed in various studies, albeit with different attributable risks, but always with a significant indication that smoking has an important contribution to the most common cancer related death. (Thun t al. 1995) So the most effective way to lower the numbers of lung cancer related deaths would be the reduction of smoking in the community.

The majority of patients (85%) present already advanced disease, at which point the 5yr survival rate is below 15% (Nesbitt et al. 1995). So efforts were made to develop a screening method that could detect the disease at early stages, at which the 5yr survival rate can be as high as 70% (Nesbitt et al. 1995). Low-dose spiral CT have a high rate of detecting noncalcified nodules, that have a chance of becoming malignant (Henschke et al. 1999), but newer reports of the false positive rate go from as low as 8% (Pedersen JH, 2009)to over 30% (Lopes Pegna et al. 2009). Another approach is sputum cytology, which has the benefit of not exposing the patient to radiation, but has a poor predictive value (Kennedy et al. 1996). Although the idea of early lung cancer detection has been around for a long time, there are no

tests available for the clinical routine. As a result efforts right now focus on prevention and dealing with late stage patients.

2.2 Mutations in lung cancer

2.2.1 P53

The p53 tumor suppressor gene is the most commonly mutated gene found in cancer. A mutation in the p53 gene is found in 50% of NSCLC and about 70% in small cell lung cancer (SCLC). (Levine et al. 1991)

The p53 protein is sometimes referred to as the "guardian angel of the genome", as the activation of the p53 gene is induced by DNA damaging agents, e.g. radiation. To protect the organism from accumulating mutations in the DNA, it can then induce cell-cycle delay, e.g. for repairing the damage to the DNA, apoptosis, the programmed cell death or senescence (Greenblatt et al. 1994). The p53 gene is located on the 17p chromosome and is composed of 393 amino acids.

The key to understanding how the p53 protein works, is the way it is activated and interacts with the DNA. There are three important elements to the p53 protein. At first there is the transactivation domain at the N-terminus of the protein. Upon activation, e.g. DNA damage, the protein changes its three dimensional configuration, preparing it to oligomerize with three other p53 molecules. For this purpose the C-terminus contains an oligomerization domain, which allows the four p53 proteins to bind to one another. To bind to the DNA, the p53 protein contains a DNA binding side, allowing the four conjoined proteins to work as a transcription factor.

P53 acts as a tumour suppressor gene, which is normally present at low protein concentration in the normal cell. This low level is maintained by a negative feedback loop involving another protein called MDM2. MDM2 has two means of interacting with p53. It blocks the trans-activation domain and works as an ubiquitin ligase, which causes degradation of the p53 protein. Additionally p53 is a transcription factor for MDM2, so low levels of p53 will result in low levels of MDM2, allowing more p53 to accumulate. (Appella et al. 2001)

However this negative loop can be interrupted by stress factors, e.g. radiation, which leads to high levels of p53 in the nucleus. Here it serves as a transcription factor for protein cascades leading to repair, apoptosis or senescence. (Chen et al. 1996)

How a cell will respond to stress is both determined by its type, e.g. fibroblast, and the kind of stress the cell is undergoing. Basically there are promoters for genes that generate growth arrest and those that lead to apoptosis. Activated p53 usually has a high affinity to genes associated with growth arrest, so that low concentrations are sufficient for expression of the down-stream genes. The proteins associated with apoptosis exhibit a low affinity to activated p53 and need a higher concentration of p53 to be activated. This explains the finding that slightly mutated p53, which exhibits a low tendency to bind to its targets, can still lead to growth arrest, but not to apoptosis. (Friedlander et al. 1996) (Ludwig et al. 1996)

A mutation of the p53 gene may result in an inability of the p53 protein to properly bind to its DNA binding site.(Weinberg 1991) This results in low concentration of cell cycle inhibiting factors like p21(El-Deiryet al. 1993) or pro-apoptotic bax (Prives 1998). Mutations most frequently occur within the p53 core domain (residues 100-300) at so called hot spots, most commonly associated with changes from the base pair G:C to T:A. This specific mutation is mostly associated with polycyclic aromatic hydrocarbons, like benzopyrene, a compound in cigarette smoke.(Hainaut et al. 2001) These mutations are most commonly found at the codons 175, 248 and 273 (along with other hot spots described by different authors

(Vahakangas et al. 2001)), suggesting a preference for certain genetic sites by mutagens derived from cigarette smoke. Interestingly some authors suggest that a mutated p53 also may be endowed with gain-of-function activities, like NF-kB activation (Weisz et al. 2007) and promoting tumor invasion by affecting integrin and EGFR trafficking (Muller et al. 2009).

Novel therapies for targeting mutated p53 have the goal to restore the dysfunctional protein to a working molecular structure. Those chemicals have been found in large screening studies, under which substances like PRIMA1 have been discovered. It has been shown that this increases levels of p53 effector proteins, like p21, Bax and PUMA (Bykov et al. 2002), and has anti-tumor effectivity in vivo (Rao et al. 2008).

So from the first description of p53 in 1979, and being declared the "protein of the of the year" in 1993, to this day, research on p53 has come a long way, but still new developments are made daily, making p53 one of the most interesting proteins for cancer researchers.

2.2.2 EGFR

For a human cell it is crucial that cell growth and differentiation is tightly regulated by intrinsic and extrinsic factors. One of the main ways to communicate with other cells is via excreting soluble mediators, e.g. growth factors. The most prominent among those is the epidermal growth factor (EGF) and its according receptor (EGFR) (Scaltriti and Baselga 2006).

The EGFR belongs to a growth factor receptor family, also known as the ErbB or HER family, which comprises four closely related receptors, HER-1 through 4. EGFR (HER-1, ERBB1) and HER-2 play an important role in cancer treatment, as over-expression or a deregulation in the related pathway result in poor outcome for patients. (Hemming et al. 1992)

The ErbB family shares a lot of common traits. They are all composed of an extracellular region (ectodomain) a hydrophobic transmembrane region and a cytoplasmatic tyrosine kinase domain (endodomain). (Yarden and Sliwkowski 2001) While the ectodomain defines the receptor affinity to a certain growth factor, the endodomain translates the signal into the cell's interior and helps with hetero- und homodimerization, a crucial feature for EGFR activation (Press and Lenz 2007).

In human cancer various ways of abnormal receptor activations have been described. One of the most common mechanisms is the overexpression of EGFR, for example by gene duplication. Other ways include ligand independent activation of the tyrosine kinase domain to activate the consecutive pathways (Burgess et al. 2003).

The EGFR pathway is involved in a multitude of crucial cell cycle controlling pathways and its activation can promote mitosis, angiogenesis or inhibit apoptosis. (Bianco et al. 2007) Physiologically EGFR signaling is tightly controlled. However in tumor cells, aberrant expression or overexpression of EGFR and its ligands is observed, resulting in increased tyrosine kinase activation, which leads to uncontrolled cell proliferation, inhibition of apoptosis and angiogenesis. These mechanisms support tumor growth and cancer development. (Arteaga 2001) (Baselga. 2002)

The inhibition of EGFR signaling showed a stop of cell cycle progression, inhibition of the production of pro-angiogenic factors and induction of apoptosis in numerous in vitro and xenograft models. (Fan et al. 1993) (Karnes et al. 1998)

There are two major classes for EGFR-targeted therapies: monoclonal antibodies targeting specifically the receptors, e.g. Cetuximab, and specific tyrosine kinase inhibitors (TKIs), like Erlotinib. Besides having the same end-point, namely inhibiting EGFR signaling, the mechanisms behind those drugs differ. The monoclonal antibodies target the ectodomain of

the EGFR and by this preventing dimerization and tyrosine kinase activation, and subsequently blocking EGFR signaling. The TKIs on the other hand are able to pass the cell membrane and block the phosphorylation site of the EGFR endodomain, resulting in the blocking of further signaling (Baselga 2002).

The first monoclonal antibody approved, and by this one of the more extensively studied ones, is Cetuximab. After being approved by the FDA in 2004, for single treatment or combined treatment with Irinocetan, it has been shown that it does not only promote cell death via suppressing EGFR signaling, but also by antibody-dependent cytotoxicity (Bleeker WK et al. 2004).

Unfortunately the majority of patients did not show a therapeutic response or developed resistances during treatment, like constitutive activation of downstream signaling pathways or receptor mutations (Camp et al 2005).

As there is likely just a sub-population of patients responding sufficiently to the treatment with Cetuximab and its related drugs, there is a need for predictive factors, which could lead to a more targeted form of therapy, or combined therapy. There has already been a focus on the KRAS status of cancer cells, indicating that patients with wild-type KRAS benefit more pronounced from a treatment with Cetuximab than those with a mutated KRAS gene (De Roock et al. 2008). However a recent study has shown that DNA repair of double strand breaks is inhibited by EGFR inhibitors regardless of mutations in KRAS. (Myllynen et al. 2011)

Currently, research on EGFR related cancer treatment is concerned with improving the patient's benefit from EGFR inhibiting drugs. One approach is combining EGFR treatment with other therapeutics, which could enhance the drug's impact beyond additive effects, e.g. irradiation or antagonists for PI3-kinases. (Geng et al. 2004) Another approach is to uncover

the underlying molecular and genetic differences, which could explain why some patients respond well to the treatment and others do not. This research could prove useful in selecting patients who would benefit from the treatment, resulting in a more personalized medicine.

3 Material and Methods

3.1 Cell Culture

A549 is a lung cancer cell line obtained from a 58 yr old Caucasian male and has been classified as a non small cell lung cancer with epithelial appearance (Lieber 1976).

For A549 52 genes have been completely sequenced of which 3 show relevant somatic mutations in CDKN2A/p16, KRAS and STK11. According to the Sanger institute database the p53 gene in A549 was categorized as wildtype.

The cells were cultivated in T25, T75, T175 polystyrene flasks with Dulbeccos Modified Eagle Medium to which 10%BGS, 1xHEPES buffer, 2mmol/l L-glutamine and 10,000 units/mL Penicillin/Streptomycin antibiotic mixture were added.

The incubation environment contained 5 % CO_2 in humidified air at 37 °C.

Cell culture handling was always performed in a sterile environment under a working hood with laminar vertical flow and surfaces and handling tools presterilized with 70 % iso-propanol.

Cells were stored in liquid nitrogen in cryogenic vials containing 10^6 cells with full medium containing 5 %DMSO.

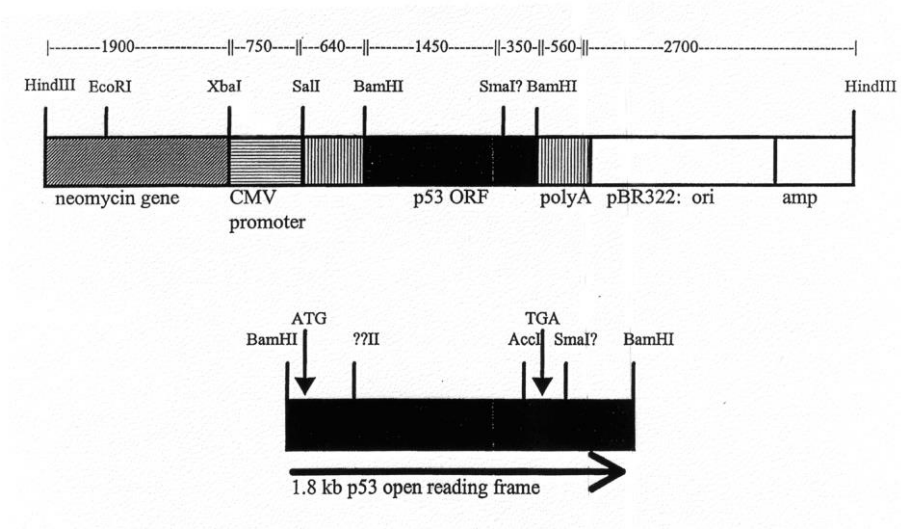
For thawing the vials were left in a 37°C waterbath and then transferred in 4 ml full medium and spun down at 1,5000 rpm for 5 minutes. Excess medium was removed to get rid of DMSO in cell culture and the cell pellet was resuspended in 5ml full medium and incubated in a T25 flask over night or until proper growth was observed.

For cell passaging, cell culture grade 1 x Trypsin was used and after 3 minutes incubation time of neutralized with full medium before reseeding.

Cell numbers were determined by manually counting using a hemocytometer. A549 cells were seeded at appropriate densities for and allowed to grow to 70-80 % confluency, before passaging. Passage numbers did not exceed 20.

3.2 Plasmid

For transient and stable transfection the following plasmid was used:



Picture 1 Plasmid Vector used for inserting p53-mutant gene

Two altered p53 genes were inserted into this plasmid. One has a mutation at location 179 which changes the amino acid to Glutamine (p53-179Q) and the other at 273 changing the amino acid to Leucine (p53-273L). Additionally a plasmid with no inserts was used (cDNA)

3.3 Plasmid Propagation

Plasmid propagation was conducted using electrocompetent Escherichia coli (ElectroMAX DH10B Cells by Invitrogen).

For the transformation 1 µl of plasmid solution was mixed well with 10 µl of the bacteria. Those were transferred to an electropuration cuvette and pulsed for 5.5ms. Then the bacteria were incubated in 5 ml S.O.C. medium on a pre-heated bacterial shaker for 45 minutes at 250 rpm. The bacteria were plated at different concentrations on agar-plates containing 0.1 mg/ml Ampicillin and were left to incubate over night in a bacterial incubator at 37 °C.

One colony was picked and transferred to 5 ml LB Medium containing Ampicillin at a concentration of 0.1 mg/ml. This was left in the bacterial shaker at 250 rpm for 7 hours.

From this stock 1ml was transferred to a 500 ml Erlenmeyer flask containing 250 ml of LB-Medium with Ampicillin at 0.1 mg/ml and left over night on the bacterial shaker.

For plasmid purification the QIAGEN® Plasmid Purification Maxi Kit was used.

The bacteria grew over night and then spun down in a cooled centrifuge at 6,000 x g for 15 minutes.

The precipitate was resuspended in 10 ml of P1 Buffer and 10 ml of P2 lysis Buffer was added and mixed by inverting 5times. The mixture was incubated at room temperature for 5 minutes. Chilled P3 buffer was added and incubated on ice for 20 minutes after vigorous shaking.

The precipitate was collected at the bottom of the tube after spinning it down for 30 minutes at 20,000 x g at 4 °C. The collecting tip was equilibrated by applying the QBT Buffer and then loaded with the supernatant containing the plasmid DNA. The column tip was washed twice with the QBS buffer and the DNA was eluted using the QF Buffer.

After adding isopropanol, a pellet was formed, after centrifuging it for 30 minutes at 15,000 x g at 4 °C. The supernatant was discarded and the pellet was resuspended in 70 % ethanol and

spun down again at 15,000 x g at 4 °C for 10 minutes. Again the supernatant was discarded and the pellet was left to air dry and then resuspended in TE buffer.

DNA concentration was measured using GeneQuant and then diluted down to a DNA concentration of 1 µg/ µl.

3.4 Plasmid Digestion

For the digestion of the plasmids cDNA, p53-179Q and p53-273L 2 µl of 10 X NEBuffer 2, 2 µl of 10 X BSA (1 mg/ml), 0.5 µl of the restriction Enzyme HindIII and 0.5 µl DNA (1 µg/ µl) were mixed with purified water to a final volume of 20 µl. The mix was incubated for an hour at 37 °C.

3.5 DNA Electrophoresis

For the gel 0.8 g of agarose was dissolved in 500 ml TAE buffer and with 10 µl ethidiumbromide added to the mix.

The loading is performed by adding 4 µl 6 X loading dye to each restriction reaction, which are then loaded onto the prepared gel together, along with uncut DNA (0.25 µl uncut DNA + 4.5 µl H2O + 1 µl 6 X loading dye). A molecular ladder was added to the gel for weight comparison.

The gel was run at 80 Volt (V) for one hour and photographed on an UV light transilluminator.

3.6 Transient Transfection

Tranfection was performed using Metafectene®, which is a poly-cationic transfection reagent based on liposome technology, allowing it to form a complex with the plasmids (cDNA, p53-179Q, p53-273L) and to penetrate the cell membrane. After that the endosome like complex

gets dissolved in the acidic environment of the endosome releasing free DNA to be transcribed in the nucleus.

For transfection a T75 flask was seeded with 10^6 cells they day before to allow cell attachment and growth. The transfection reagent was created by adding 300 µl serum free medium containing 10ug DNA to 300 µl serum free medium containing 60 µl Metafectene and letting it incubate for 15 minutes at room temperature. The mixture was then added to the flask containing fresh full medium and incubated over night.

3.7 Stable Transfection

For stable Transfection the same protocol was initially followed as described under Transient Transfection, with the exception that the plasmids were linearized (see 3.4) beforehand, using the enzyme HindIII.

Afterwards the same protocol as described under 3.6 was applied to transfect the cells. The transfected cells were plated in serial, or logarithmic, dilutions, reducing the cell concentration by a factor of 10 after every step. The cells were incubated in G418 enriched medium (500 µg/ml).

After visible colonies grew, single colonies were micro-trypsinized and expanded into growing cultures. To check for clones, bearing a p53 over expression, a western blot using anti-p53 anti-bodies was performed

3.8 Short Term Proliferation Assay

To evaluate the radiosensitizing effect of cetuximab and erlotinib 10^5 cells of unaltered A549, A549-cDNA, A549-p53-179Q or A549-p53-273-L cells were seeded into T25 flasks and were incubated for 5hrs to allows for attachment. Thereafter, the medium was either replaced with medium containing the drug (100 nM, 300 nM Cetuximab or 2 µM Erlotinib) or fresh full medium and irradiated one hour later. After 72 hours the cell number was determined manually and was documented in total cell numbers per flasks. Each assay was repeated 3 times.

3.9 Colony Formation Assay

To determine the long-term effects of irradiation and Cetuximab, a colony formation assay was performed.

For colony formation cells were seeded at low densities in T25 flasks appropriate for each treatment and irradiation dose. The optimal densities were determined in preliminary experiments. Treatment was performed 18 h later.

Colonies were allowed to form for at least 14 days. After colony formation was completed the medium was removed, cells were washed with PBS and fixated with methanol. Colonies were stained methylene blue at room temperature for 30 minutes, washed with H_2O and counted using a table with a light source and a magnifying glass.

A colony was defined as an assembly of cells with at least 50 individual cells. Plating efficiencies (PE) were calculated as Number of colonies divided by number of cells plated. The survival fraction (SF) was calculated as PE of the treated cells divided by the PE of the untreated cells.

All experiments were repeated three times from different passage numbers.

Statistical analysis and graphics was performed with commercially available software (GraphPad Prism 5.0). Results were compared using student's two sided t-test for unpaired samples.

3.10 Western Blotting

3.10.1 Western Blot samples

To get sufficient amounts of cells accessible for protein lysates, ~$5x10^5$ cells/4 ml were plated in d60 dishes and et attach and grow over night so they would be at about 70 % visual density.

The dishes were treated accordingly with 100 nm Cetuximab, 2 Gy/8 Gy Irradiation or combined treatment. For comparison with basic protein levels an untreated dish was always established for every experiment. For combined treatment Cetuximab was added one hour prior to irradiation.

The medium was removed from the dishes and the cells washed three times with 5ml ice-cold 1 x PBS. The 1 x PBS of the last washing cycle was removed thoroughly and 25 µl cell lysis buffer (Cell extraction buffer (Invitrogen) + 25 µl protease inhibitor + 5 µl PMSF) were added twice onto the cell layer in the d60 dishes, each time incubating the lysis buffer for ~3 minutes and scraping the cells off the d60 dishes using a disposable cell lifter.

After each scraping cycle, the cell-lysis buffer solution was transferred into a 1.5 ml Eppendorf tube and placed on ice. The scraped cell-lysis buffer solution was incubated on ice for 30 minutes, vortexing the sample every 10 minutes.

Then, the samples were centrifuged at 14,000 x g for 20 minutes at 4 °C. The protein containing supernatant was transferred to a 1.5ml Eppendorf tube. At this point, the protein sample was either processed or stored at -70 °C.

The protein concentration of 2 µl lysate in 798 µl ddH_2O and 200µl protein dye was measured using a GeneQuant pro photometer at 595 nm wavelength. The photometer was calibrated using standardized samples containing bovine serum albumin.

Samples were generated by mixing 2.5 µl 10 x reducing agent (Invitrogen), 6.25 µl 4 x sample buffer (Invitrogen) and a maximum of 16.3 µl of the protein sample. Western blots were run with a protein amount of 50 µg per sample, and in case this protein amount was present in less than 16.3 µl, the difference was filled with ddH_2O. During handling, all substances were kept on ice.

Samples were denaturated at 70 °C for 10 minutes. Then, samples were placed on ice for ~1 minute and spun down for 20 seconds at 12,000 rpm and either directly used or deep frozen at -70°C.

3.10.2 Running Western Blot Gels and PVDF membrane transferral

1mm x 10 wells 4-12 % Bis-Tris gels were placed in the gel box and the outer chamber filled with ~800 ml and the inner chamber filled with ~200 ml 1 x MES SDS running buffer (50 ml 20 x MES SDS running buffer + 950 ml ddH_2O=1 L 1 x MES SDS running buffer).

All samples, with a volume of 25 µl each, were loaded into the lanes of the gel and the molecular weight standards with a volume of 17.5 µl per lane were also loaded. The gel ran for 300 minutes at 100 V or an appropriate length of time for specific proteins.

Afterwards the gel was placed in chilled transfer buffer (840 ml ddH_2O + 10 ml 10 % SDS solution + 50 ml 20 x transfer buffer + 100 ml methanol). The PVDF membrane was prepared by first washing in methanol, then ddH_2O and then in chilled transfer buffer. The transfer cassette was prepared, placing the membrane onto the gel, between two filter papers and two sponges soaked in transfer buffer. The sandwich cassette was placed into the transfer box ran 70 minutes at 100 V.

After the transfer, the gel was checked for residual proteins with Coomassie Blue.

The membrane was rinsed in 0.1 % TBS-T (100 ml TBS-T + 900 ml ddH_2O) and blocked for 1 hour at room temperature on a shaker. For blocking, 10 ml of a 5 % milk solution were used, created by mixing 0.5 g non-fat dry milk with 0.1 % TBS-T.

3.10.3. Blotting for proteins

The membranes were blotted with antibodies specific for the protein of interest. Blotting was done by placing the membrane with the protein carrying side onto a paraffin film with 1 ml 5 % milk solution, containing the primary antibody at the following ratios: Anti-filamin mouse 1:1000, Anti-BAX rabbit 1:500, Anti-p53 sheep 1:1000, Anti-p21 mouse 1:500, Anti-PUMA rabbit 1:500, Anti-bcl2-proper rabbit 1:500. The membrane was blotted over night in a cold room at 4 °C. Then, the membrane was washed three times for ~15 minutes with 0.1 % TBS-T on a shaker.

To detect the primary antibody, secondary antibodies linked with horseradish peroxidase (HRP) were used. Those were diluted in 10 ml 5 % milk blocking solution in the following ratios: goat anti-rabbit 1:10,000, goat anti-mouse 1:10,000, donkey anti-sheep 1:10,000.

And the membrane was incubated on a shaker for one hour and washed three times for ~15 minutes with 0.1 % TBS-T before detecting the antibodies.

To detect the antibodies SuperSignal WestPico Chemiluminescent Substrate from ThermoScientific and HRP Chemiluminescent Substrate Reagent Kit from Novex were used. They were mixed at a ratio of 1:1 and equally dispersed over the membrane and incubated for 5 minutes before placing the membrane in the developer cassette in protective foil and transferred to the dark room in that.

3. 11 Nuclear Fragmentation Assay

To look for apoptotic nuclei the treated cells were plated in coverslip chambers and then treated accordingly. After being treated and the appropriate incubation time after treatment, e.g. 48 hours, has passed, the coverslips were taken out of the incubator and the protocol for DAPI staining was followed.

This included a two times rinse with PBS to get rid of excess medium and one rinse with a prepared fixative containing methanol and acetic acid in a respective ratio of 3 to 1. The cells were then incubated in the fixative for 10 minutes. The samples were stained with DAPI in a concentration of 1 µg/ml for 2 minutes and sealed with Antifade and nail polish.

To look nuclear fragmentation the slides were portrayed under the microscope and the stained cells were counted manually.

3.12 Flow Cytometry

Propidium Iodide is an intercalating molecule that can be used to determine DNA levels in single cells. The molecule can be excited at wavelength of 488 nm and emits detectable red fluorescence.

The cells are prepared by trypsinizing a previously prepared T75 flask containing about 10^5 cells grown over night and the cells trypsinized.

The cells were transferred to a 15 ml centrifuge tube and spun down at 15,000 rpm in a cell centrifuge, and resuspended in PBS and spun down again. The pellet was then resuspended with ice-cold 100 % ethanol as a fixative and then left over night or at least one hour before further processing.

To prepare the samples for flow analysis, the flow cytometry tubes were wrapped in aluminium foil. The samples were spun down at 15,000 rpm and the supernatant was discarded, this step was repeated twice. The pellet was then gently resuspended in 200 µl PBS and then mixed with 500 µl staining solution, which contained Igepal CA-630 (with a working concentration of 0.1 %), RNAse (working concentration 1 mg/ml) and Propidium Iodide (working concentration 20 µg/ml).

The mix was then transferred to the flow cytometry tubes and the samples were run on the FACS Calibur.

Data collection and analysis were performed using the commercially available FlowJo software.

3.13 Complete Listing of All Materials

Cell Lines

Cell Line	Cell Line Specifications
A549	- NSCLC Cell Line - Cultivation in DMEM Medium - Provided by Settleman Lab, MGH Cancer Center, Boston, Massachusetts, U.S.A.

Cell Culture Material

Material	Material Specifications
1x PBS	- Phosphate Buffered Saline (NaCl: 8.01 g/l, KCl: 0.2 g/l, $Na_2HPO_4 \bullet 2\ H_2O$: 1.78 g/l, KH_2PO_4: 0.27 g/l; pH: 7.4)
1x Trypsin	- Sigma-Aldrich, St. Louis, Missouri, U.S.A.
T25 Culture Flask	- 25cm^2 Falcon flask (BD, Franklin Lakes, New Jersey, U.S.A.)
T75 Culture Flask	- 75cm^2 Falcon flask (BD, Franklin Lakes, New Jersey, U.S.A.)
Incubator	- FORMA Scientific 37°C/5%CO_2 (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)

Hood	- STERIL Guard HOOD (Baker Company, Inc., Sanford, Maine, U.S.A.)
Microscope	- Nikon Eclipse TS100 (Nikon, Tokio, Japan)
Couting Grid	- Reichert Bright-Line Hemacytometer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
Sterilizer	- Harvey Sterile Max (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)

Material	Specifications
50ml/15ml Tubes	- BlueMax (BD, Franklin Lakes, New Jersey, U.S.A.)
DMSO	- Sigma-Aldrich, St. Louis, Missouri, U.S.A.
Centrifuge	- Sorvall (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
-70°C Freezer	- VWR Scientific, Radnor, Pennsylvania, U.S.A.
Liquid Nitrogen Tank	- CRYOMED Forma Scientific (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)

Treatment of Cells

Material	Specifications
X-Ray Generator	- Siemens Sabilipan 2280KVp, 1.98Gy/min (Siemens, Forchheim, Bavaria, Germany)
Cetuximab	- Erbitux® (Bristol-Myers Squibb Company, New York City, U.S.A.)
Erlotinib	- Tarceva® (Roche, Basel, Switzerland)

Materials for Colony Formation Assays

Material	Specifications
Methanol	- Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.
Methylene Blue	- Sigma-Aldrich, St. Louis, Missouri, U.S.A.
Microscope	- Stereomaster (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
Light Table	- Apollo Portable Light Box (Listel)

Materials for Western Blot

Material	Specifications
Tissue Culture Dish	- 60x15mm, Beckton Dickinson (BD, Franklin Lakes, New Jersey, U.S.A.)
Cell Extraction Buffer	- Invitrogen, Carlsbad, California, U.S.A.
Protease Inhibitor Cocktail	- Sigma-Aldrich, St. Louis, Missouri, U.S.A.
PMSF	- Sigma-Aldrich, St. Louis, Missouri, U.S.A.
Celllifter	- Fisherbrand Disposable CellLifter (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
Vortexer	- Vortex Genie (Scientific Industries, Bohemia, New York, U.S.A.)
Centrifuge	- Microfuge®R Centrifuge (Beckman Coulter, Brea, California, U.S.A.)
Photometer	- GeneQuant pro Photometer, (Amersham Biosciences, Amersham, United Kingdom)
Calibration Solution	- 1mg/ml BSA stock solution, Albumin Bovine Serum (Sigma-Aldrich, St. Louis, Missouri, U.S.A.)
Protein Dye	- Protein Assay Dye Reagent Concentrate

	(BioRad, Hercules, California, U.S.A.)
ddH2O Machine	- MILLIPORE MILLI-Q (Continental Water Systems, New South Wales, Australia)

Material	Specifications
Gels	- NuPAGE 4-12% Bis-Tris Gel, 1mmx 10wells, (Invitrogen, Carlsbad, California, U.S.A.)
Western Blot Box	- Novex Mini-Cell and XCell Surelock lid (Invitrogen, Carlsbad, California, U.S.A.)
Powersource	- Powerpac 200 (Bio-Rad, Hercules, California, U.S.A.)
Running Buffer	- NuPAGE MES SDS Running Buffer(20x), (Invitrogen, Carlsbad, California, U.S.A.)
Molecular Weight Ladders	- Novex Sharp PreStained Protein Standards, (Invitrogen, Carlsbad, California, U.S.A.)
Transfer Cell	- Mini Trans-Blot Cell, (Bio-Rad, Hercules, California, U.S.A.)
Transfer Sandwich	- Mini PROTEAN 3Cell Sandwich, (Bio-Rad, Hercules, California, U.S.A.)

Powersource	- PowerPac 200, (Bio-Rad, Hercules, California, U.S.A.)
Transfer Membranes	- PVDF Membrane Filter Paper Sandwich, 0.2µm Pore Size, (Invitrogen, Carlsbad, California, U.S.A.)
Transfer Buffer Solution	- NuPAGE Transfer Buffer(20x), (Invitrogen, Carlsbad, California, U.S.A.) - 10% SDS-Solution ultraPURE, (Invitrogen, Carlsbad, California, U.S.A.) -10% Methanol (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
Gel Staining Dye	- SimplyBlue SafeStain, (Invitrogen, Carlsbad, California, U.S.A.)
Membrane Blocking	- 0.1% Tris-buffered-saline with Tween , (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) - Blotting grade Non Fat Dry Milk, (Bio-Rad, Hercules, California, U.S.A.) - BSA Albumin Bovine Serum, (Sigma-Aldrich, St. Louis, Missouri, U.S.A.)
Knife	- GelKnife, (Invitrogen, Carlsbad, California, U.S.A.)
Scale	- TL-104, (Denver Instrument Company)

Stirring Plate	- Type 1000 Thermolyne, (Sybron Cooperation)
Shaker	- Hybrid Shake (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)

Blotting Antibodies

Antibody Type	Specifications
Anti-p53	-Monoclonal Sheep anti-p53 Antibody (Calbiochem/Merck, Darmstadt, Germany)
Anti-p21	-Monoclonal Mouse anti-p21 Antibody (Calbiochem/Merck, Darmstadt, Germany)
Anti-bcl-2 family	-Monoclonal Rabbit anti -Bax -PUMA -bcl-2-proper (Cell Signaling, Beverly, Massachusetts, U.S.A)
Additional Loading Control Antibody	- Monoclonal Mouse Anti-ß-Actin Antibody (Clone AC-15), (Sigma-Aldrich, St. Louis, Missouri, U.S.A.)
Secondary Antibodies	- Goat Anti-Mouse IgG HRP-Linked

	ImmunoPure Antibody, (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) - Anti-Rabbit IgG, HRP-Linked, (Cell Signaling, Beverly, Massachusetts, U.S.A)

Materials for Membrane Exposure

Material	Specifications
ECL-Reagents	- SuperSignal WestPico Chemiluminescent - Novex ECL; HRP Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
Stripping Buffers	- Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
Membrane Exposure	- Radiation Therapy Cassette 25x30cm, DUPONT CRONEX(Sigma-Aldrich, St. Louis, Missouri, U.S.A) - Chemiluminescence BioMax Light Film 13 x 18 cm

	(Kodak, Rochester, New York, U.S.A.)
Film Developing Machine	- X-OMAT 2000 Processor (Kodak, Rochester, New York, U.S.A.)
Digital Camera	- Fuji Finepix E900 (Fuji, Minato, Tokio, Japan)
Scanner	- Perfection 2480 PHOTO, (Shinjuku, Tokio, Japan)
Imaging Software	- Adobe Photoshop (Adobe Systems, San Jose, California, U.S.A.)

Plasmid Propagation

Material	Specifications
Micropulser	- BioRad Micropulser (Bio-Rad, Hercules, California, U.S.A.)
Electropuration Cuvette	- 1mm Gap (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
Electrocompetent E.coli	- ElectroMAX DH10B Cells (Invitrogen, Carlsbad, California, U.S.A.)
S.O.C. Medium	- Invitrogen, Carlsbad, California, U.S.A.
Falcon Tubes	- 14ml BD Falcon Round-Bottom Polypropylene Tubes

	- 50ml BD Graduated Tubes (BD, Franklin Lakes, New Jersey, U.S.A.)
LB Plate	- Containing 0.1mg/ml Ampicillin (Sigma-Aldrich, St. Louis, Missouri, U.S.A)
LB Medium	- Containing 0.1mg/ml Ampicillin (Sigma-Aldrich, St. Louis, Missouri, U.S.A)
Inoculating Loop	- Disposable Rigid Inoculating Loop (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
Bacterial Shaker	- HI- LabOne
Bacterial Incubator	- PRECISION
Plasmid Purification Kit	- QIAFilter Plasmid Maxi-Kit - QIAGEN, Hilden, Northrhine-Westfalia, Germany
Centrifuge	- RC 5C Plus Centrifuge - Sorvall (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
SLA-600TC Rotor	- Sorvall (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)
SLA-500 Rotor	- Sorvall (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)

Microfuge 18 Centrifuge	- Beckman Coulter
1.5ml Eppendorf Tubes	BD, Franklin Lakes, New Jersey, U.S.A.
Ethanol	- 200 proof(absolute), Sigma-Aldrich, St. Louis, Missouri, U.S.A.)
ddH20	- MILLIPORE MILLI Q ((Continental Water Systems, New South Wales, Australia)
GeneQuant pro	Amersham Biosciences, Amersham, United Kingdom
Quartz and Glass Micro Cell	- Spectrophotometer vial (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.)

Dapi Stain

Material	Specifications
Chamber Slides	- Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.
PBS	- 8.00 g of NaCl, 0.20 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, ad 800ml H20
Fixative	- 300 ml Methanol and 100 ml Acetic Acid
DAPI solution	- DAPI Solution 1mg/ml (Sigma-Aldrich, St. Louis, Missouri, U.S.A.)
Antifade	- Invitrogen, Carlsbad, California, U.S.A.

FACS

Material	Material Specifications
100% Ethanol	- Sigma-Aldrich, St. Louis, Missouri, U.S.A.
Flow Cytometry Tubes	- BD, Franklin Lakes, New Jersey, U.S.A
RNAse	- QIAGEN, Hilden, Northrhine-Westfalia, Germany
Propidium Iodide	- AbCam, Cambridge, Masssachusetts, U.S.A.
Igepal CA 630	- Sigma-Aldrich, St. Louis, Missouri, U.S.A.
FACS Calibur	- Flow Cytometer (BD, Franklin Lakes, New Jersey, U.S.A.)

4. Results

4.1 A549 cells are equally radiosensitized at higher concentrations of Cetuximab or Erlotinib

Pilot experiments were performed to test whether an anti-EGFR antibody and a tyrosine kinase inhibitor may both modulate the cell proliferation after IR. A549 cells were pre-incubated with either Cetuximab (100nM and 300nM) or Erlotinib (2µM) for 1 h and then irradiated. The cell number was determined 72 h later and compared to untreated control dishes. Fig.1 shows that combined treatment with either EGFR inhibitor reduced the cell proliferation by 30% compared to radiation alone. There was no difference in growth inhibition between Cetuximab at different concentrations and Erlotinib. For the following experiments Cetuximab at 100nM was used.

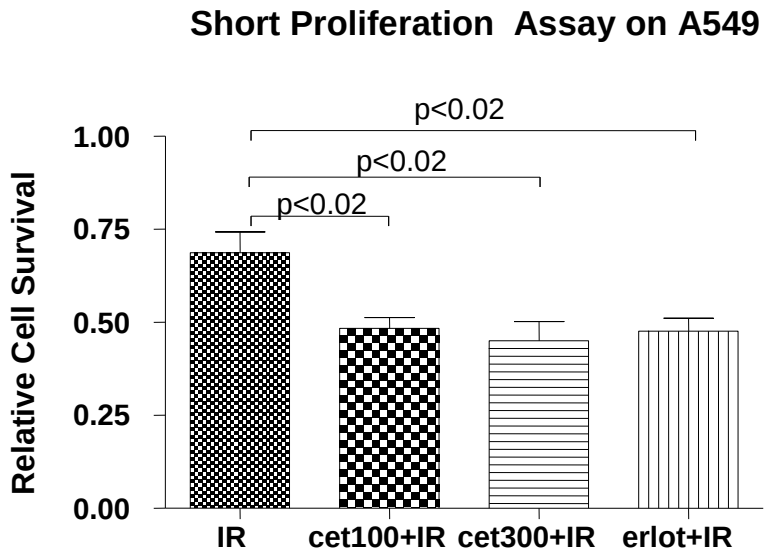


Figure 1: Short Proliferation Assay A549cells were plated at 100,000 cells per T25 flask 6 h before X-irradiation (2Gy). Cell number of individual flasks were determined 72h later. Relative cell survival corresponds to the cell number of treated samples normalized to those of untreated controls

4.2 Successful p53 Expression after Transient Transfection

To determine, whether introducing a mutant p53 gene in A549 cells would affect their response to the combined treatment of Cetuximab and IR, transient transfection was employed. Copies of an empty vector or a plasmid containing mutant p53 c-DNA (179Q or 273L, respectively) were transfected and tested for successful expression by western blotting. (Fig. 2) The cells were transfected and left to incubate for 48 hours. After this cell extracts were prepared and blotted. Transfection of the empty vector control showed no detectable signal corresponding to the endogenous wt p53 due to low endogenous p53 levels and short exposure time. In contrast, transfection of vectors coding for p53-179Q or p53-273L, respectively, resulted in strong p53 signals indicating robust overexpression of both mutant forms.

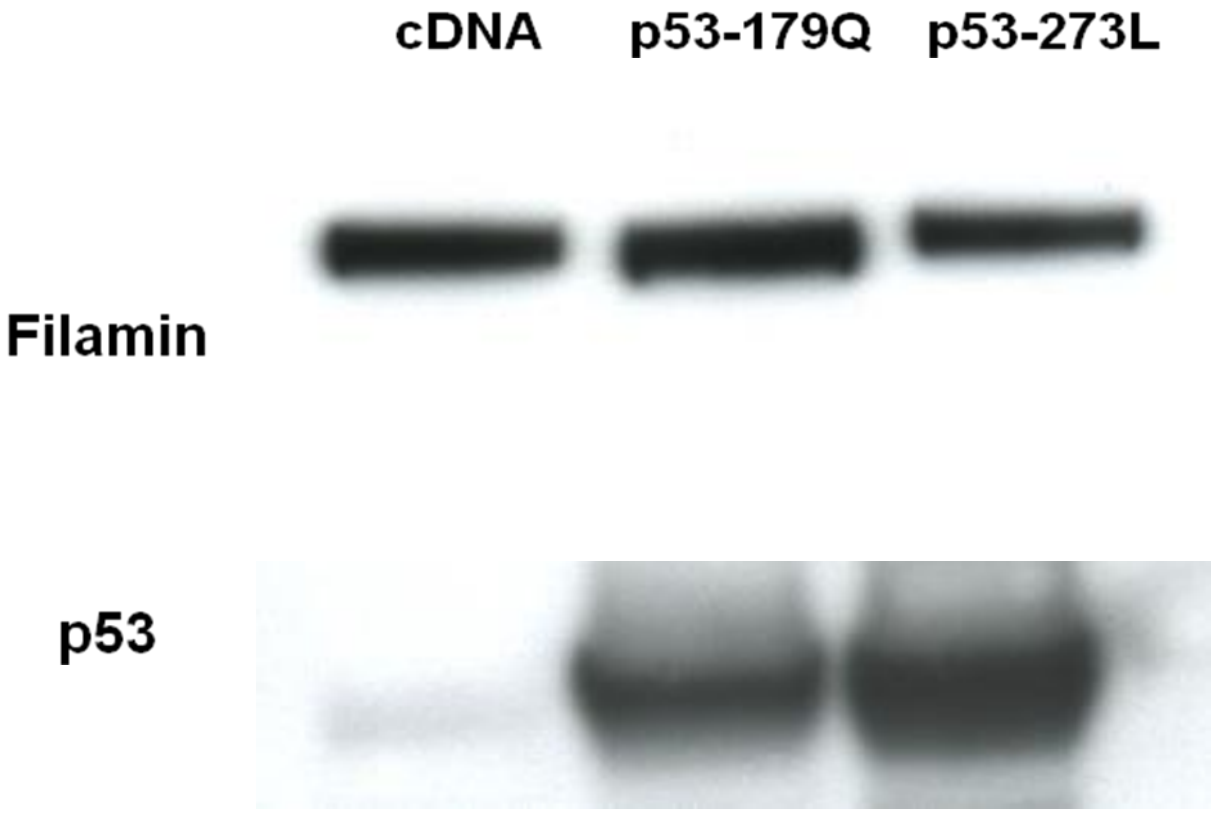


Figure 2 p53 Western Blot 48hrs after transfection The p53 expression plasmid or the empty control vector was transfected using Metafectene© , 48h later cells were lysed and whole cell proteins were immunoblotted for p53 expression. Filamin was detected as loading control.

4.3 Short Proliferation Assay after Transient Transfection

After transfecting the cells, a short proliferation assay was performed to test whether transient p53 expression might impact upon early cell growth.

Figure 3 shows that expression of mutant p53 slightly improved cell growth after 2 Gy of X-rays when compared to the empty vector alone. Addition of Cetuximab significantly reduced short term cell proliferation in cells that expressed only endogenous wt p53 but not in those expressing mutant p53.

This result suggests that wild-type p53 might be involved in radiosensitization of A549 cells.

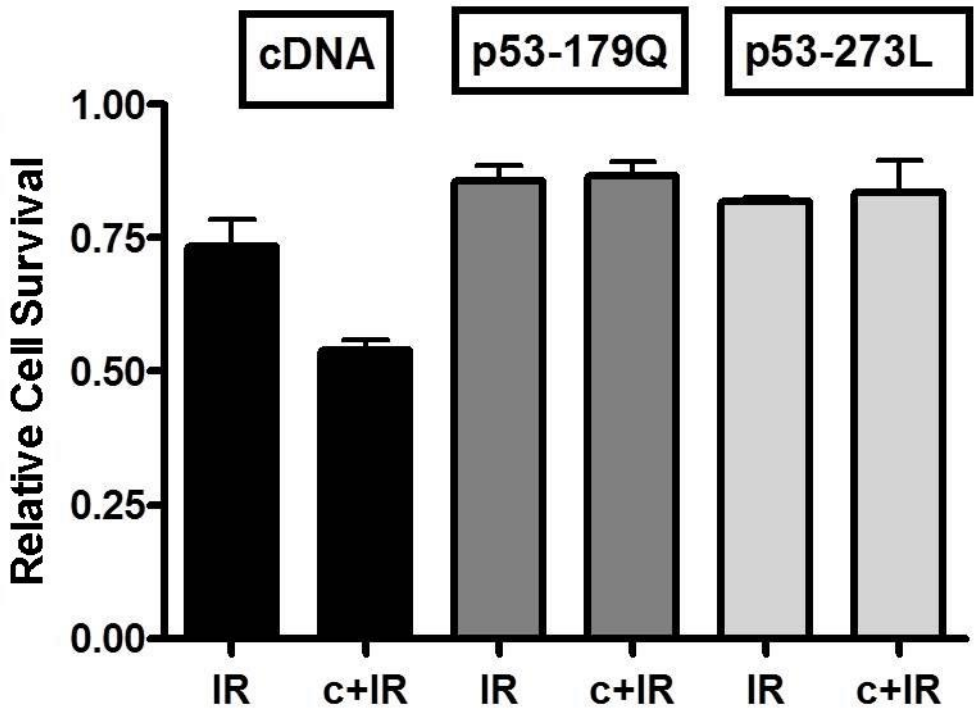


Figure 3 Short Proliferation assay with transient transfected p53-mutants Cells were transfected and treated 48 hrs after accordingly. Cell numbers were counted manually and relative cell survival calculated.

4.4 Stable Integration and p53 Expression

After confirming that p53 mutations affect short term proliferation in transiently transfected cells, a colony survivals assay was performed to also prove long-term p53 effects. Since transgenic proteins such as p53 are only expressed for a short period of time after transient transfection, the plasmids must be integrated into the cell's genome, to guarantee stable expression over the time period required for a colony survival assay.

For a stable transfection, it is necessary to linearize the plasmid by digesting it with a restriction enzyme, which cuts at a non-coding site, so that the all critical genes can be transcribed from the plasmid. Linearization with a restriction enzyme, here HindIII, prior to transfection, is a way to ensure that more DNA is available for integration, since circular plasmid DNA is less likely integrated in the cells genome.

To check for proper cleavage, samples were electrophorized on an ethidiumbromide containing agarose gel .

Figure 4 shows that the plasmids run according to their molecular weight, suitable for stable transfection.

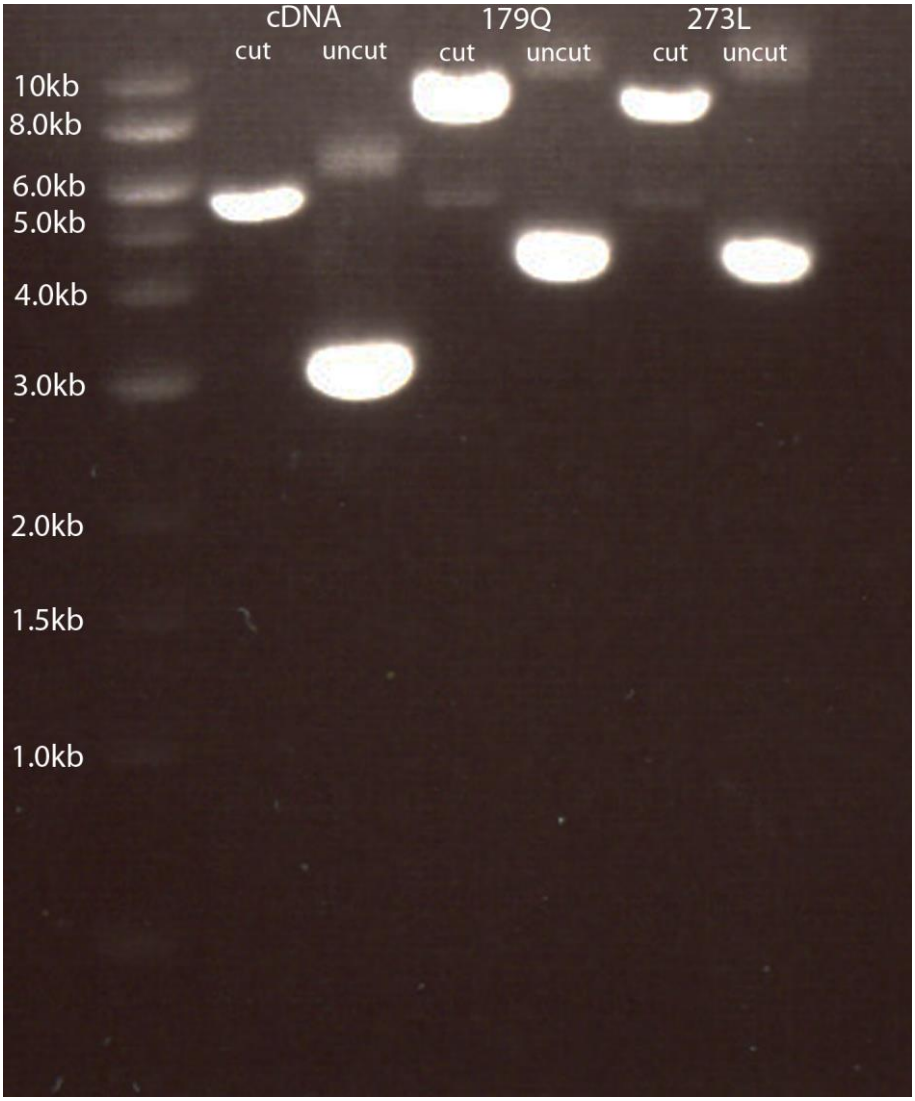


Figure 4: Plasmid digestion: Note that the cleaved DNA runs at the according height (cDNA=6550kb; 179Q/273L=8350kb), whereas the uncut DNA can be found further down the gel. This is due to circular DNA folding tightly in a three dimensional space named supercoiling which allows faster migration through a polymer gel compared to relaxed DNA.

4.5 Western Blot on Stable Transfected A549 Cells

After selecting the clones using antibiotics (G418) for selection pressure, the culture was expanded. To check those clones for stable p53 expression, a protein extract from a confluent T75 culture flask and was analysed using western blotting.

Figure 5 shows the result of the blot of individual clones, with strong p53 signals for clones p53-273L No. **III**, p53-179Q No. **II** and III. Those clones were selected for further experiments. A weak p53 signal was found for an empty vector clone which was employed as control (cDNA, No. I).

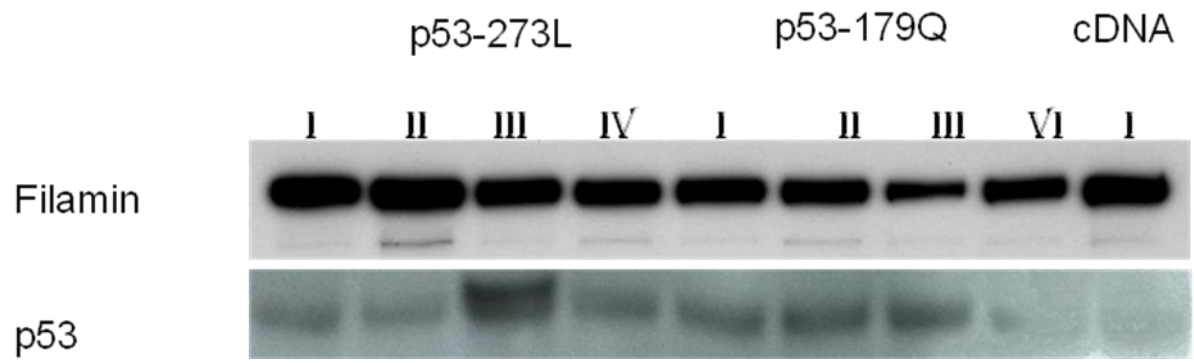


Figure 5 Western Blot for p53 on stable transfected clones The bands appear at the expected height, when compared with a protein standard (not shown). Filamin was probed for loading control

4.6 Radiosensitization with Cetuximab is wt-p53 dependent

To confirm the results from the short proliferation assay after transient transfection, the experiment was repeated with the stably transfected mutants. One reason for this was the possibility, that the results we obtained from the transient transfection assay, was not due to the effects of the mutant p53 inserted, but from the transfection procedure. Another reason was to test whether the cells not containg a mutated p53 still behave the same way after the many passages of long-term culture.

Figure 6 shows that the early relative cell proliferation for the empty vector (cDNA) resembles that of native A549 cells (figure 1) and equally shows radiosensitization by 25% upon cotreatment with Cetuximab. The stably transfected A549 expressing mutant p53 show no radiosensitization and higher relative cell survival for radiation and combined Cetuximab treatment than A549 containing the empty vector.

This indicates that the stable expression and transient transfection of mutant p53 have the same effect in short term proliferation assay.

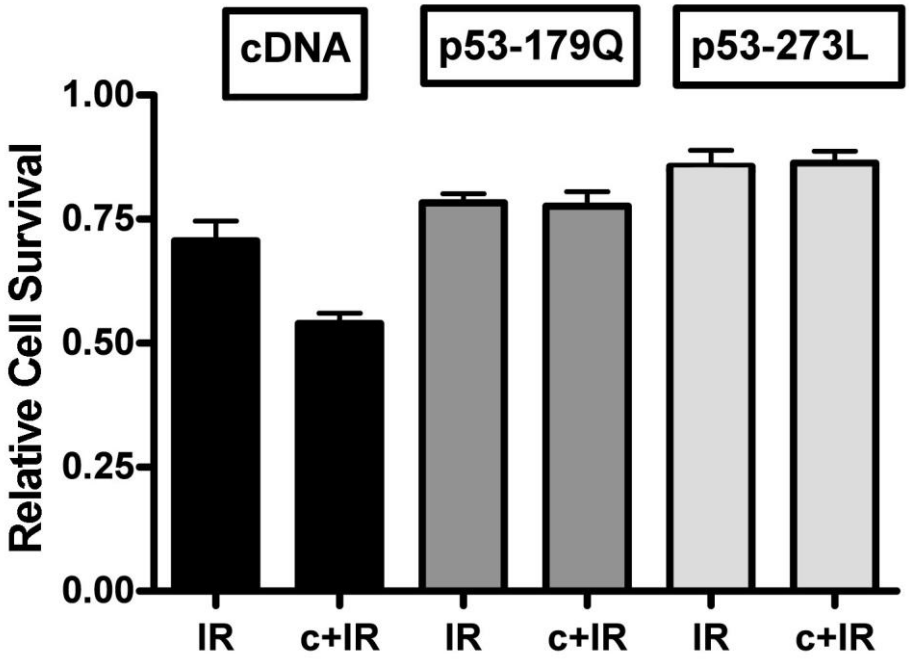


Figure 6. Short proliferation assay with stable transfected A549s The cells were treated with irradiation only (IR) or irradiation and Cetuximab (c+IR) and 6hours after plating and counted manually after 48hrs. Relative cell survival was calculated with untreated cells as a reference.

4.7 Colony Survival Assay with Stable Transfected Cells

To test whether the short term effects of IR and cetuximab upon cell growth also translates to colony formation for A549 cells with endogenous wild-type p53 gene and those expressing a dominant-negative mutant in addition, a colony survival assay was performed. Figure 7 shows examples of stained tissue culture flasks after colony formation had been completed. 7**a** and **b** include the clone containing the cDNA plasmid without specific treatment and with Cetuximab alone. The numbers in colonies is almost identical, but show a difference in colony size, with smaller colony size indicating slower cell growth after Cetuximab treatment and slowing down in cell growth. 7 **c, d** show the same for clones containing 273 L, but, as expected, show no difference in colony size after Cetuximab treatment. Surprisingly they show an overall smaller colony size than the cDNA clone shows. This can be attributed to the expression of the mutant p53 form which might have slowed down cell proliferation. The same is true for 179Q clones (not shown).

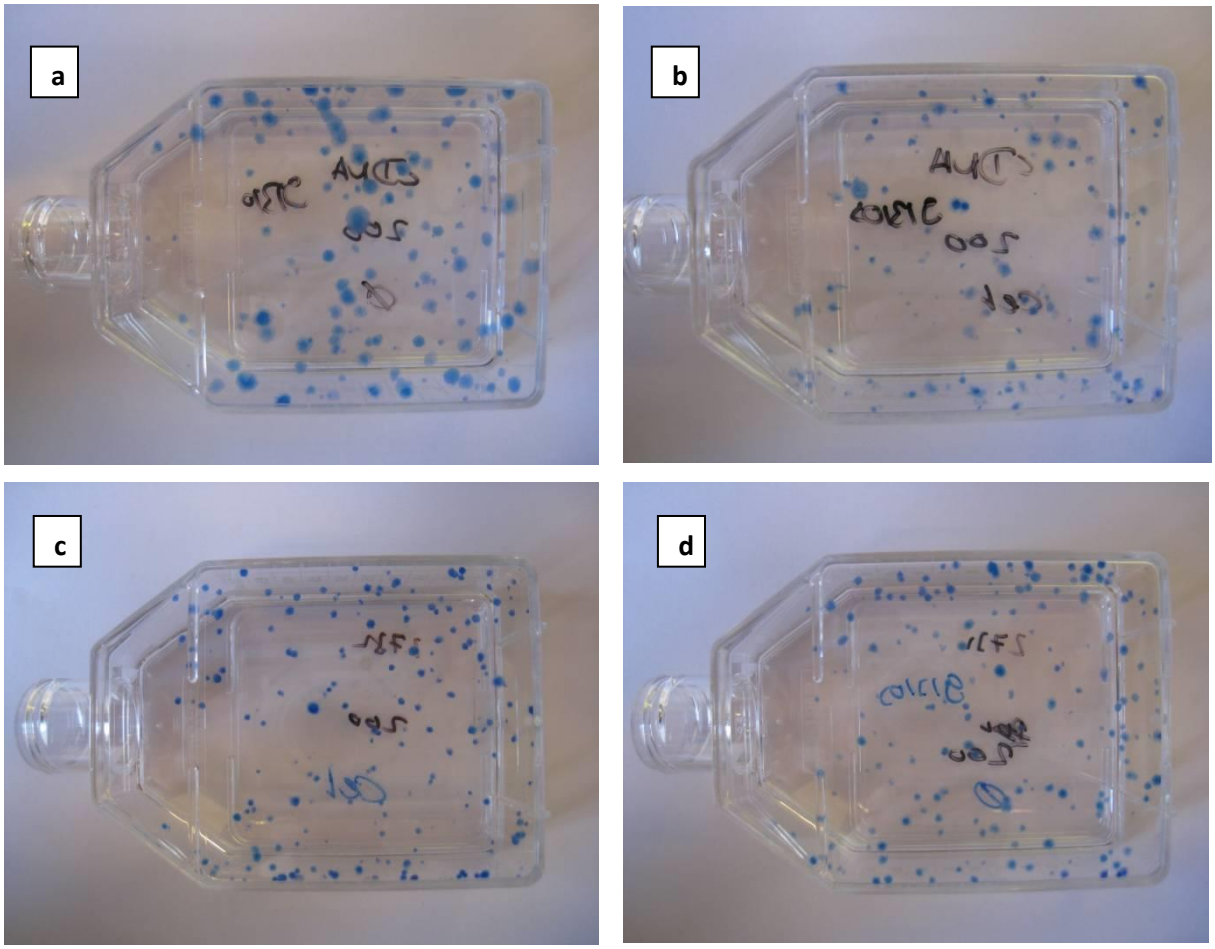


Figure 7 Selected Colony formation assay flasks Photographs of stained flasks after colony formation. Cells were treated with irradiation only (IR), or irradiation and Cetuximab (c+IR). After 14 days colonies were fixated , stained and counted manually.

Figure 8 shows the quantitative analysis of all colony formation assays.

Figure 8**a** shows the radiosensitizing effect of Cetuximab on A549 cells expressing endogenous wild-type p53 (empty vector control). The colony formation is equal to previous results obtained with naïve A549 cells (not shown) suggesting that integration of the empty c-DNA vector did not alter the response to irradiation or Cetuximab per se. Figure 8**b** and **c** show the results of the p53 mutant A549 cell lines.

In 8**b** the p53 protein containing the 179Q mutations is expressed. Colony formation with increasing radiation dose is almost similar compared to A549 cells with the empty vector. On the other hand there is only minimal radiosensitization upon Cetuximab treatment. This results in higher survival fraction for combined treatment especially with low irradiation doses and only a small radiosensitizing effect of higher radiation doses. Applied p value tests show it is not significant to the .05 level, suggesting that the 179Q mutation in p53 reduces the radiosensitizing effect.

This effect is even more clear in figure 8**c** showing the 273L mutation. This "hotspot" mutation is virtually suppressing the radiosensitizing effect of cetuximab in this cell line. The graph shows the same response to irradiation as p53-wildtype A549 cells, but no change in survival fraction when Cetuximab is used in combined treatment.

It can be concluded from these results that Cetuximab only radiosensitizes A549 cells when there is only p53-wildtype present.

Next we wish to elucidate the underlying mechanism for the reduced cell survival after the combined treatment and whether this is due to increased apoptosis.

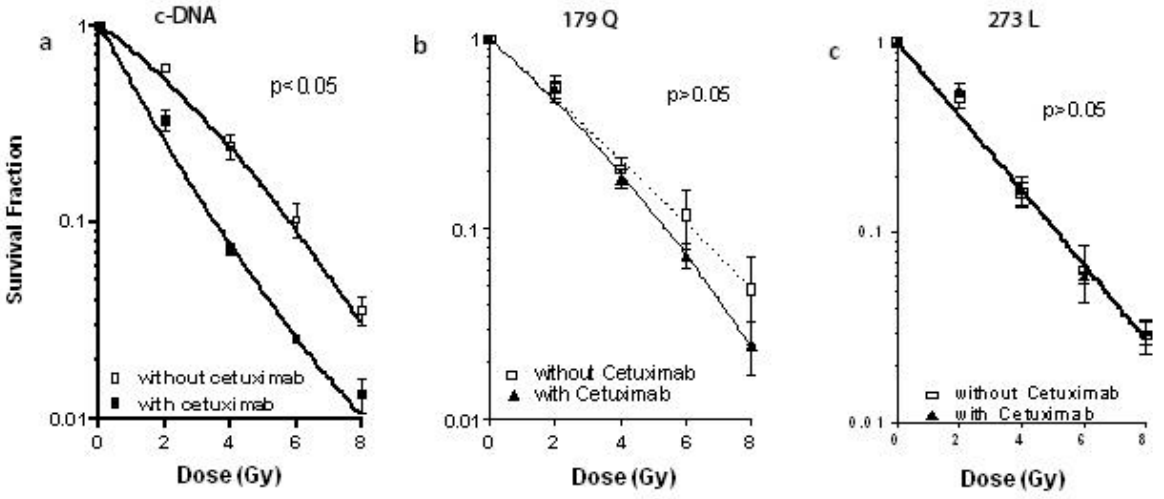


Figure 8 Colony survival with stable transfected A549 cells Survival curves were fitted using the linear quadratic equation and statistical analysis was carried out by use of the F test (2-sided) to test for significant difference between survival fractions.

4.8 Involvement of bcl2-family Proteins

To this end, proteins were investigated known to be involved in p53-mediated apoptosis, namely the bcl-2 related proteins BAX, BIM and PUMA (fig. 9).

Irradiation or Cetuximab only treatment had no effects on the expression of bcl-2 related proteins. However combined treatment showed elevated signals.

Western blots for BAX show an elevated signal after Cetuximab plus 8 Gy of X-rays compared to untreated or single treated cells. The BIM blot shows a substantial increase in signal for Cetuximab and 8Gy of X-Rays as compared to single treament. PUMA shows an increase in the beta fraction for Cetuximab and 2Gy, as well as for Cetuximab and 8Gy, while there was no detectable increase compared to single treatment alone.

These results suggest an involvement of p53 dependent pro-apoptotic proteins.

To test whether there is significant apoptosis after IR and Cetuximab, three experiments were conducted.

Apoptosis was studied by three different methods:Firstly a western blot for Caspase-3 expression, a protein associated with apoptosis. Secondly a DAPI stain of treated cells, to check for nuclear morphology associated with apoptosis. And thirdly flow cytometry was performed to check for sub-G1 fractions, which are associated with apoptotic cells.

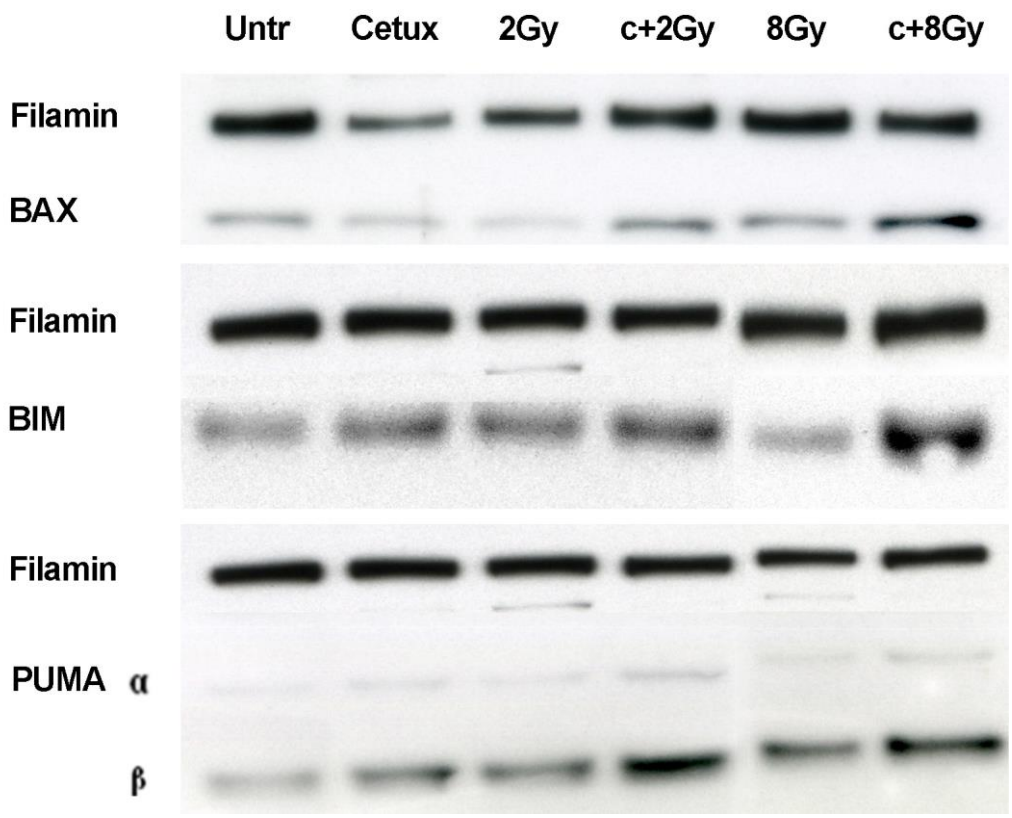


Figure 9 Western Blot for pro-apoptotic bcl-2 family proteins A549 cells were treated with Cetuximab (Cetux), 2 Gy, Cetuximab + 2Gy (c+2Gy), 8Gy, Cetuximab + 8Gy (c+8Gy) and an untreated control. These were blotted for the proteins BAX, BIM and PUMA 48hrs after treatment. Elevated protein levels were found in combined treatment opposed to radiation only treatment. Correct protein size was confirmed by a standard protein marker (not shown). Filamin was probed as a loading control.

4.9 Western Blot for Caspase-3

To determine whether apoptosis occurs in A549 cells after irradiation and/or combined treatment, a western blot to detect Caspase-3, an early apoptotic marker, was performed. If apoptosis occurs Caspase-3 would be cleaved into two smaller sub-units, which can both be detected by the antibody used.

However, in figure 10 only full-length caspase-3 was detected with no indication of cleavage activity suggesting that there is no apoptosis involving Caspase-3.

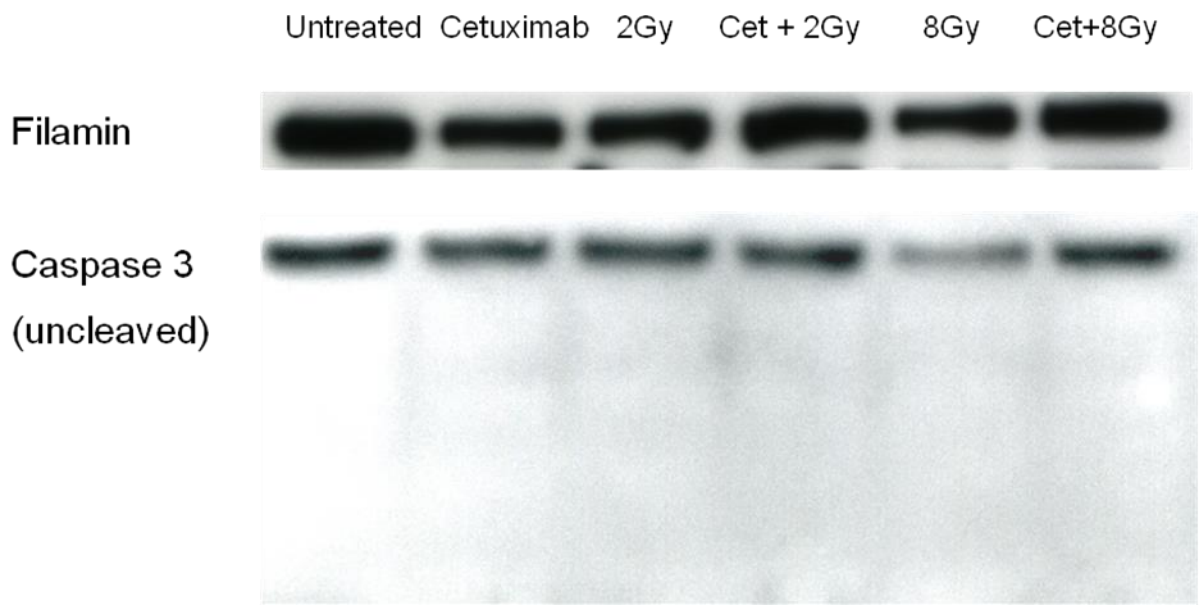


Figure 10 Western Blot for Caspase-3 The cells were treated with Cetuximab (Cetux), 2 Gy, Cetuximab + 2Gy (c+2Gy), 8Gy, Cetuximab + 8Gy (c+8Gy) and an untreated control. The cells were 48hrs later processed. The blot shows uncleaved Caspase-3 at the correct height, confirmed by a molecular ladder (not shown), but no cleaved protein downstream, indicating a lack of apoptosis.

4.10 DAPI Stain for Nuclear Fragmentation

To confirm the lack of apoptosis in A549 cells, DAPI stained nuclei were examined after IR and combined treatment with Cetuximab.

To check for apoptosis on a single cell level, the cells were subjected to the aforementioned treatments and stained with DAPI. This was done to check for nuclear fragmentation, which indicates ongoing apoptosis.

Figure 11 shows photographs of DAPIstained nuclei 48 hrs after treatment.

The overall numbers of apoptotic nuclei remain low for the treatments. There were 1.2% apoptotic nuclei for untreated cells, 0.9% for cells treated with Cetuximab only. Irradiation with 2 Gy of X-rays resulted in 1.9% apoptotic nuclei and 2.1% apoptotic nuclei for combined treatment. Irradiation with 8 Gy of X-rays resulted in 7.0 % apoptotic nuclei and combined treatment achieved 7.7% apoptotic nuclei. The results of apoptotic nuclei after IR is in accordance with previously published results for A549 cells. Figure 12 visualizes these results in a bar chart. It shows a steady increase in apoptotic nuclei with irradiation dose, but no significant difference in cells additionally treated with Cetuximab.

These findings indicate that there is no additional nuclear fragmentation due to Cetuximab pre-treatment. To confirm, that no relevant increase in apoptosis is present, flow cytometry was performed to detect loss of nuclear DNA a further hallmark of apoptosis, named "sub-G1" DNA content in the flow cytometer.

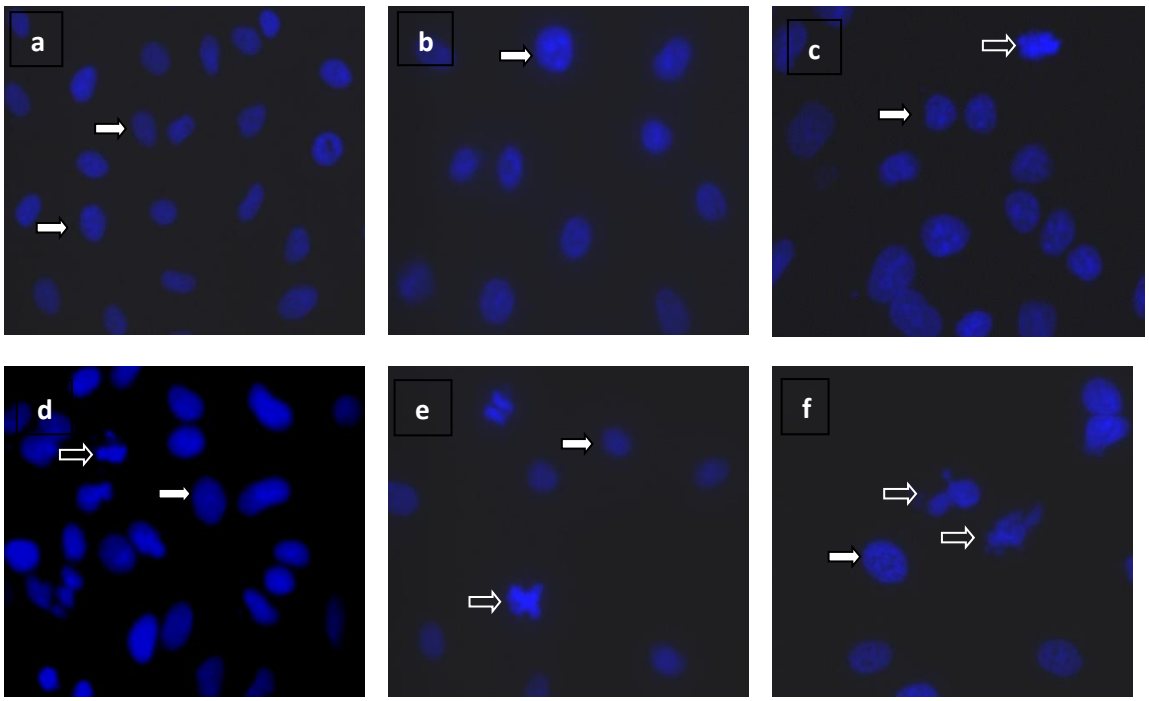


Figure 11 DAPI Staining for nuclear fragmentation Full arrows indicate normal nuclei and empty arrows point **at nuclei undergoing fragmentation.** There are no apoptotic nuclei for untreated (**a**) and Cetuximab only (**b**). There are low numbers for 2Gy only (**c**) and 8 Gy (**e**), as for combined treatments (**d,f**)

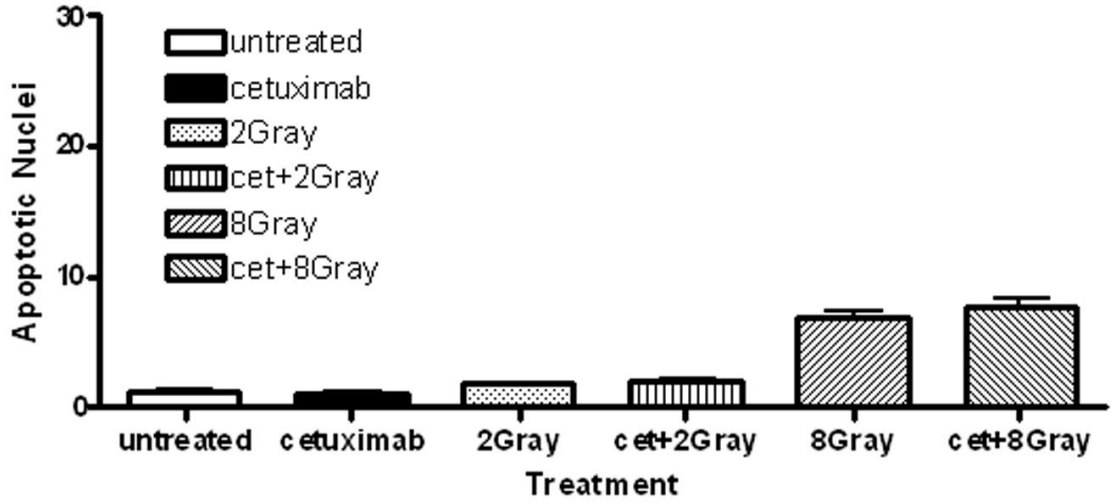


Figure 12 Nuclear Fragmentation Assay Bar chart representing percentage of apoptotic nuclei. Cells were treated with irradiation only (2 Gray, 8 Gray), or irradiation and Cetuximab (cet+2Gray cet+8Gray)

4.11 Flow Cytometry for sub-G1 Fraction

Figure 13 shows the distribution of the PI stained DNA with clear G1 and G2/M peaks indicating a normal cell cycle. However there was no sub-G1 cell population, which would be an indicator for apoptosis, neither for untreated cells (**a**) nor after IR (**c**) and/or cetuximab (**b, d**).

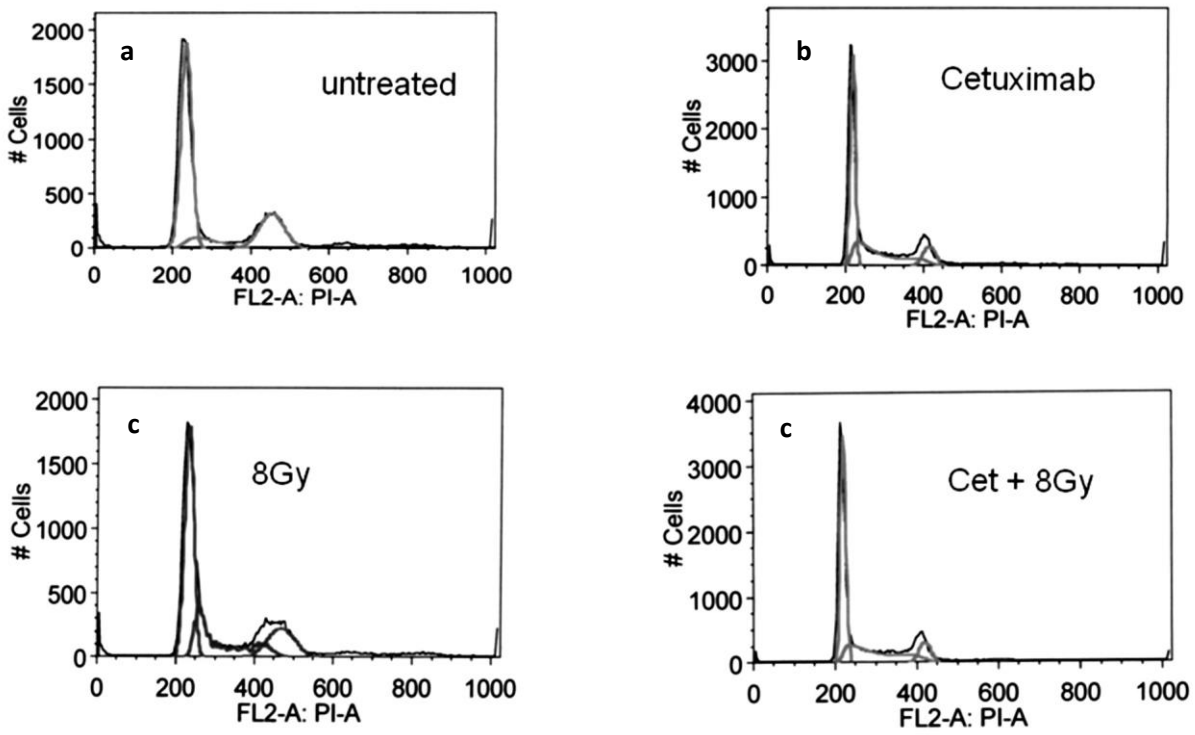


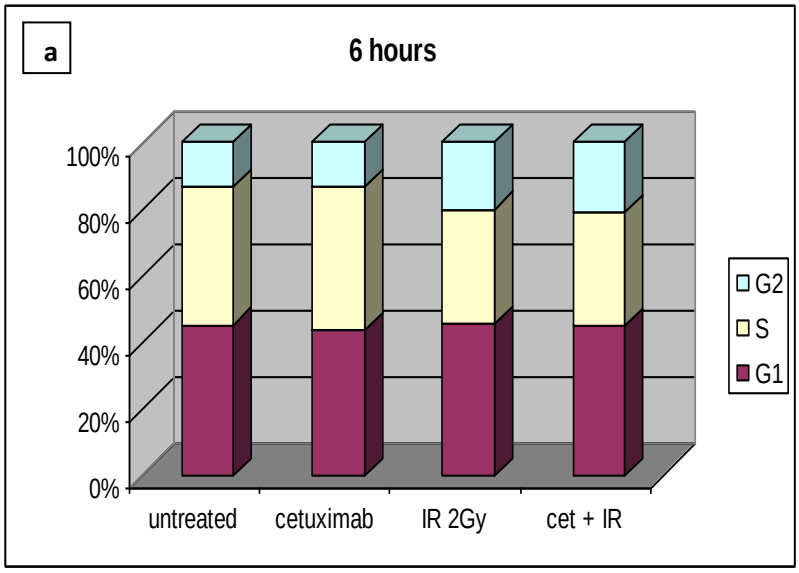
Figure 13 Flow Cytometry for cell cycle with PI staining Figures show the cell cycle of A549 cells 48hrs after the according treatment. Cells were left untreated (**a**), treated with Cetuximab only (**b**), irradiation only (8 Gray, **c**), or irradiation and Cetuximab (Cet+8Gray, **d**)

4.12 Flow Cytometrie for 72 hour Cell Cycle Analysis

To further investigate the nature of cell inactivation after the various treatment modalities cell cycle was followed for 72 h.

Figure 14a shows that the fraction of G2/M cells increased for the treatment groups that received either 2Gy alone or in combination with Cetuximab but not for the group that was treated with Cetuximab only. Figure 14b displays the difference in cell cycle distribution at 12 hours. There is a pronounced G2/M arrest for irradiated A549 cells and interestingly a G1 arrest with a reduction in S phase for combined treatment, indicating an altered cell cycle for Cetuximab pre-treated A549s. Figure 14c includes the 24 hour time point with an almost equal distribution in cell cycle. Most likely due to a return to normal cell cycle for unaffected cells and an "overgrow" of affected cells, as A549 have a doubling time of less than 16hrs. Figure 14d shows an increase in G1 phase for combined treatment, which is not observable for irradiation only treatment. The 48 hr (14e) and 72 (14f) time points show a return to normal cell cycle patterns.

These results indicate that, due to the treatment with Cetuximab before irradiation, a G1 arrest related mechanism plays a role in the radiosensitizing effect.



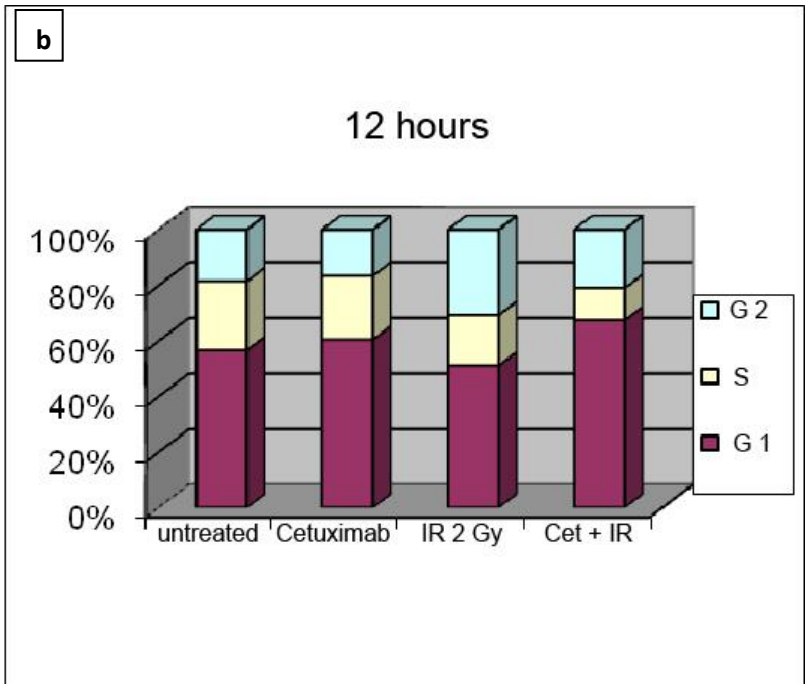
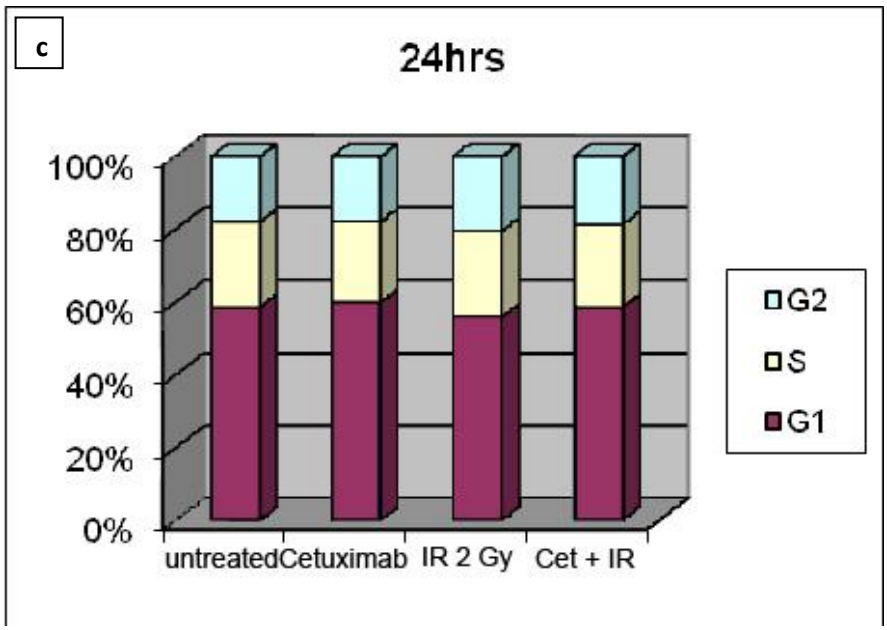


Figure 14 a/b: Cell cycle bar chart: Comparing the cell cycle distribution after 6 and 12 hrs. After 12 hours irradiation (IR 2Gy) shows a higher percentage in G2 phase, while combined treatment (Cet + IR) shows additionally an increase in G1 phase.



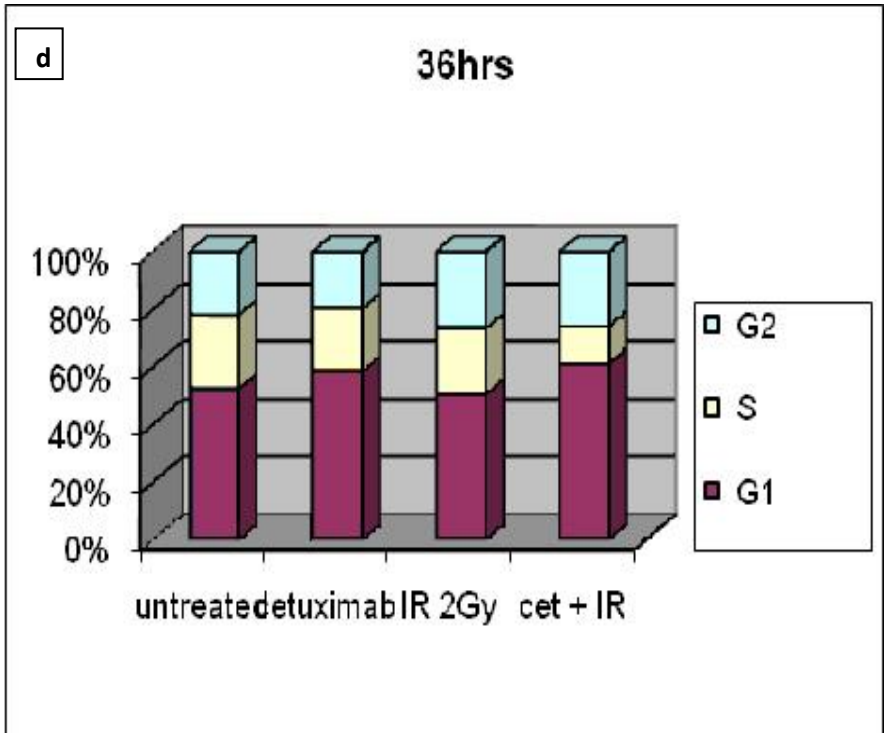


Figure 14 c/d: Cell cycle bar chart: Comparing the cell cycle distribution after 24 and 36 hrs.

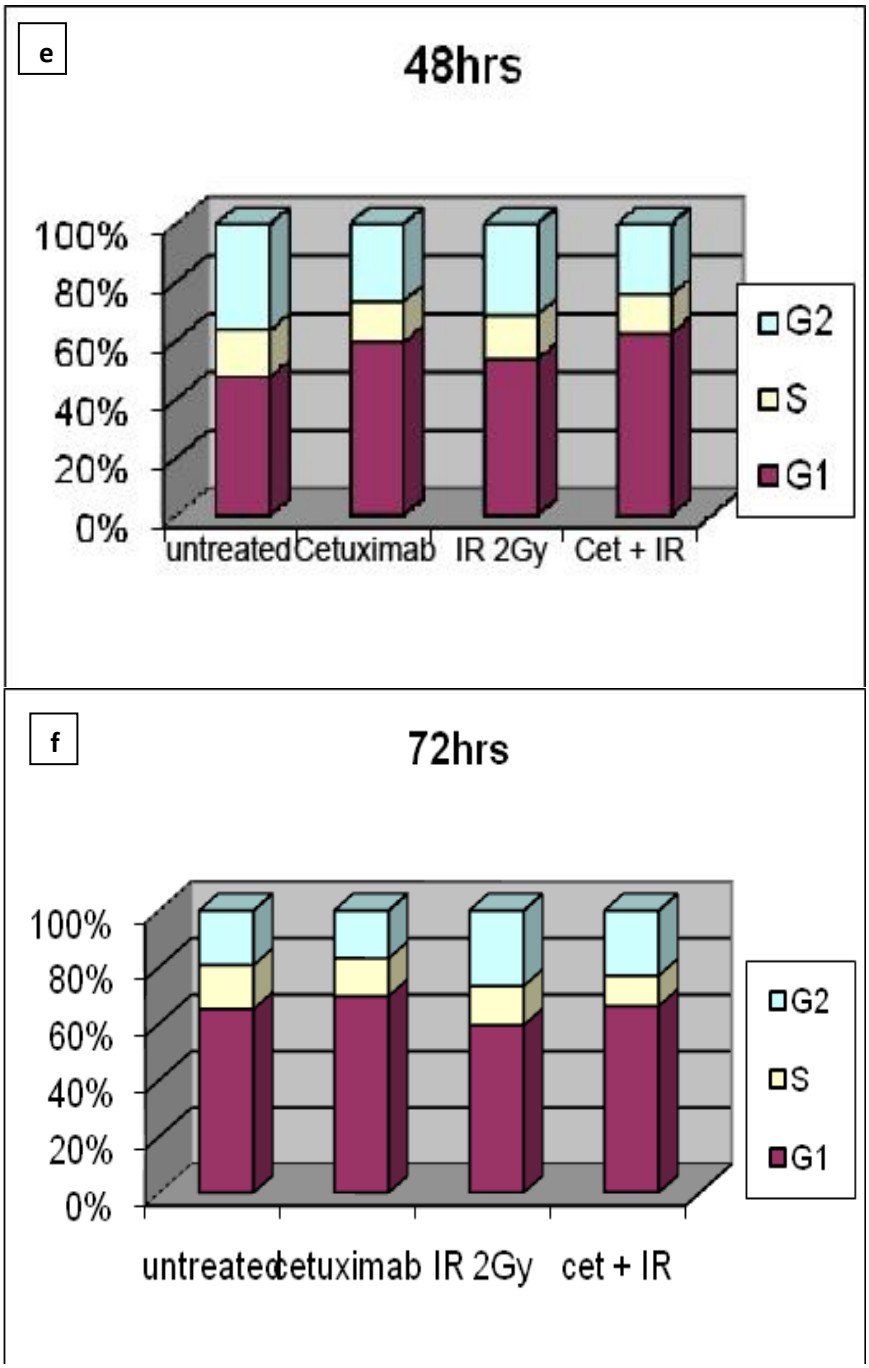


Figure 14 e/f Cell cycle bar chart: After 48 hours there was no observable difference between the irradiated group and the group with combined treatment, as well as after 72 hours.

5. Discussion

Due to lung cancer being the number one cause of cancer related death in the US there is constant need to find more effective treatment methods. (Nesbitt et al. 1995) The combination of established therapeutic regiments, like radiation, with new drugs, e.g. Cetuximab, might prolong survival of lung cancer patients. (Ready 2005) Although pilot experiments were promising, it became clear that success was not equally shared by all patients. Until now, it is not clear as to why some patients benefit from this combination of treatments and others do not.

P53 mutations appear in about 50% of all human tumors being a key factor in carcinogenesis. (Soussi and Wiman 2007) Our question was whether p53 status is related to the response to the combined treatment of Cetuximab and IR. We found that Cetuximab-mediated radiosensitization is only achieved with expression of wild-typ but not mutated variants of p53. In particular, we asked whether Cetuximab-associated radiosensitization was equally reflected by short term and long term cell survival experiments, and which mechanisms underly the enhanced radiosensitivity. As p53 is involved in major antiproliferative pathways, like apoptosis, cell cycle arrest and senescence, its role for efficient EGFR radiosensitization was investigated.

5.1. P53 is crucial for Radiosensitization in the A549 Cell Line

A short term survival assay with transiently transfected A549 cells showed a clear indication that the introduction of mutant p53 in the A549 cell line leads to a loss of radiosensitization (fig. 3).

The same was shown for long term survival in a colony formation assay. While the wild-type cell line containing the empty cDNA vector (fig. 8a) shows a significant radiosensitization, the A549 cell line containing the 179Q or 273L show that the radiosensitizing effect is largely diminished. Additionally the radiosensitization was not equally reduced for the various p53 mutants. A549 cells exhibiting the 179Q mutation showed a trend toward lower colony survival, indicating a slight radiosensitizing effect, but 273L bearing cells did not show any differences in colony survival at all when comparing those pretreated with Cetuximab to the untreated control. It has been described that mutations in lung cancer are not equally distributed and that there are some mutations more commonly found, hotspot mutations, in smokers than in non-smokers. (Hainaut et al. 2001) Greenblatt et al. have inferred already in 1994 how hotspot mutations, including Codon 273, play an important role in cancer genesis. (Greenblatt et al. 1994) The results in this thesis are in good accordance with the findings of Greenblatt et al. as the reduction of radiosensitization of the 273L p53 mutant was more pronounced as opposed to 179Q.

With these findings it can be concluded that a functional p53 protein is a prerequisite for a radiosensitizing effect in A549 cells.

5.2. Effects of Cetuximab on p53 related bcl-2 Proteins

As effects can already be seen in a short term assay (fig. 1), an immediate mechanism must apply, like apoptosis or cell cycle arrest, which could lead equally to reduced clonogenic survival, e.g. colony numbers. The bcl-2 family is tightly entwined with p53 activation and regulation. (Nakano and Vousden 2001) (Hoetelmans 2004) The western blots showed an increase in those Bcl-2 family proteins. For high doses of radiation these increases were very pronounced for BIM, while the increase for BAX known for its pro-apoptotic features was lower. (Wang et al. 2005) After low dose radiation the PUMA ß protein showed the highest elevation in protein levels.

This indicates that there is an activation of bcl-2 family members, which is specifically triggered after the combined treatment of Cetuximab and radiation. This further supports the notion, that Cetuximab induces a mechanism in A549 cells, which translates into cells being more susceptible to radiation therapy.

To further elaborate the mechanisms of radiosensitization, especially apoptosis, we looked into changes in the cell cycle of A549 after treatment.

5.3. Apoptosis or Senescence?

To elucidate whether the combined treatment results in apoptosis, three assays were performed, namely a western blot to detect for the apoptotic signaling protein Caspase 3, a nuclear fragmentation assay and flow cytometry to monitor a sub-G1 population.

The western blot for Caspase 3 (fig. 10) showed clear bands for intact Caspase, but signs of cleavage, which would indicate an ongoing apoptosis. We conclude that A549 cells do not undergo apoptosis after combined Cetuximab and radiation treatment, at least not the mode that is Caspase 3-dependent. However, this appears unlikely, since in general Caspase 3 dependent apoptosis has been described in A549 cells. (Sanli et al. 2011) A nuclear fragmentation assay confirmed the absence of apoptosis after single or combined treatment, which corroborates previous findings. (Das et al. 2006) The third assay to detect apoptotic cells was flow cytometry of PI-stained cells. There is no increase in the sub-G1 population, which further supports that the radiosensitizing effect of Cetuximab in A549 cells cannot be attributed to a significant level of apoptosis.

Hence, there must be another mechanism that might explain the radiosensitizing effect of Cetuximab. To monitor how the cell population reacts to the different treatment regimens, the propidium iodide stained flow cytometry assay was expanded to a 72 hour cell cycle analysis, which shed light upon the issue.

The cell cycle analysis shows how the cell cycle distribution shifts during 72 hours with different treatments. The cell cycle response started similiarly with a G2/M arrest for irradiated cells and those with combined treatment. This changed after 12 hrs where a higher G1 population for combined treatment was found, this was also true for the 36 hour time point. This indicates a G1-block for cells that either transitioned from the G2 block or cells that accumulated in G1 proportionally to the unaffected cells. However these results have the

limitation that the untreated control group did not show a consistent cell cycle distribution over the 72 hours. This G1 block could very well be a G0 arrest which cannot be differentiated in this assay from G1 arrest. The underlying mechanism for this accumulation of the G1/G0 subgroup could be cells going into senescence. The reduced number in colony formation would then be due to these dormant or senescent cells, as senescent cells do not form colonies. (Saretzki 2010) These results are supported by previous findings that described that EGFR inhibition can suppress the transition from G1 to S-phase in cells. (Ling et al. 2007)(Chinnayan et al. 2005) Furthermore Di Gennaro et al. have reported a G1 arrest and partial G2/M block in head and neck squamous carcinoma cells after treatment with tyrosine-kinase inhibitors. (Di Gennaro et al, 2003) This could relate to senescence, which is an irreversible cell-cycle arrest that suppresses proliferation of cells exposed to stress signals, e.g. radiation. (Schmitt 2007) Further work that has been performed in this laboratoratory in extension of the thesis has revealed that after combined treatment numereous cells stained positive for b-Gal indcating transition to a senescent status which proved then to be the underlying mechanism of radiosensitization by Cetuximab. (Wang et al. 2011) An increase in senescence-related transcription factor E2F1 was found, as well as trimethylated histone H3K9. This was also successfully proven in xenograft mouse models. Additionally senescence was determined to be the cause of radiosensitization in 5 other cell lines and was abrogated in two cell lines by the introduction of a mutant p53 (LU99B and A549). (Wang et al. 2011)

In non-cancerous cells the connection between p53 and G1-arrest as a response to radiation induced damage, e.g. double-strand breaks, has been shown. (Borgmann et al. 2004) Other works have shown how there are distinct mechanisms for p53-mediated senescence in response to ionizing radiation with p21 being the main senescence effector there. (Mirzayans et al. 2010) Additionally, ERK signalling, together with nuclear translocation of the EGFR

complex, is involved in the cell cycle progression and an overactivation due to EGFR overexpression in cancer cells can prevent cancer cells from entering senscence. (Chambard et al. 2007) Additonally it has been resported before by Hotta et al. that tyrosine-kinase inhibitors induce senescence in NSCLC cell lines, are activated predominantely over a p21 dependent pathway, rather than via activation of p16. (Hotta et al., 2007) These results were confirmed by Wang et al. where they showed that A549 cells harboring wildtype p53 showed increased p21 levels after combined treatment of Cetuximab and IR. (Wang et al., 2011) Di Gennaro et al. found similar results for head and neck squamous carcinoma cells treated with EGFR inhibitors. There G1 arrest and growth inhibition was related to a functional p21. (Di Gennaro et al., 2003)

We propose that Cetuximab induced radiosensitization in A549 cells is dependent on a functional p53 protein (fig. 3 and 8) and the mechanisms underlying the reduction in cell number (fig. 1 and 8) is unlikely apoptotis (fig. 10, 12, 13), but rather cell-cycle arrest and senescence (fig. 15-19). Our results imply for clinical pratice that NSCLC tumors should be screened for p53 mutations when the combined treatment of Cetuximab and irradiation is considered.

6. Summary

In this thesis the role of p53 for radiosensitization by EGFR-inhibition in the A549 cell line was investigated. A549 is an example of NSCLC cell lines presenting a p53 wildtype and is susceptible to radiosensitization with the anti-EGFR antibody Cetuximab.

To evaluate whether the effect of radiosensitization is dependent on the wildtype or mutant status of p53, dominant negative mutant p53 variants (273L and 179Q) were introduced via a plasmid vector into a p53 wildtype background. In the growth assay performed on cells after transient transfection, it was shown that Cetuximab induced growth inhibition is dependent on p53 wildtype. In colony formation the p53 wildtype A549 cells showed reduced colony counts, i.e. Cetuximab induced radiosensitization, while the cell lines with mutated p53 genes showed no radiosensitizing effects. These findings show that p53 wildtype is crucial for radiosensitization in A549 cell lines.

Caspase cleavage, nuclear morphology and flow-cytometry did not show any evidence of apoptosis as the underlying mechanism for radiosensitization.

Cell cycle analysis with flow cytometry revealed a shift in cell cycle distribution in favor of G1/G0, after the cells were treated with Cetuximab and IR. This could reflect a G0 arrest associated with senescence, which has been proven to be true in work conducted in extension of this thesis.

In conclusion, these results indicate that p53 wildtype is essential for successful Cetuximab induced radiosensitization in A549 cells and that the underlying mechanisms for this effect is not apoptosis, but cell cycle arrest in G0 presumably through senescence. These findings may be exploited for personalized cancer therapies based on screening of the p53 status in NSCLC tumors as a parameter that helps deciding whether Cetuximab should be used in combination with radiation therapy.

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10. Statement of Originality

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

Fabian Morsbach 21.04.2013, Zürich