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Radiosensitivity in lung cancer with focus on p53

BY

MICHAEL BERGQVIST

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ABSTRACT

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In Sweden approximately 2800 new lung cancer patients are diagnosed every year. Radiotherapy is used with curative intention in certain groups of patients. The aim of this thesis is to study the basis of differences in radioresistance and the possibility to predict response to radiotherapy.

In the first study we investigated, using the comet assay, four lung cancer cell lines with different sensitivity towards radiation. A clear dose-response relationship for radiation-induced DNA single strand and double strand breaks were found. All cell lines showed a remarkably efficient repair of both the DNA single strand and double strand breaks one hour after irradiation. However, further studies in one radioresistant and one radiosensitive cell line demonstrated that repair during the first 15 min had the best accordance with radiosensitivity measured as surviving fraction.

In the second and third study, sequencing studies of the p53 gene were performed on cell lines as well as on tumour material. Cell lines that were expressing a mutation in exon 7 were associated with increased radiosensitivity compared with tumor cell lines with mutations in other exons. In the clinical study, 10 patients were found to be mutated in the p53 gene whereas the other 10 patients were not. No correlation to clinical parameters could be drawn.

In the fourth study, serum from 67 patients with a confirmed diagnosis of non-small cell lung cancer was investigated for the presence of p53 antibodies. P53 antibodies in sera, taken prior to radiation treatment, were associated with increased survival.

The summary of this thesis indicates that the p53 gene has an impact on the effect of radiotherapy in lung cancer. The presence of p53 antibodies might be of clinical interest for predicting survival after radiotherapy. Further studies on the importance of the p53 gene on early repair are of interest.

Key words: Lung cancer, comet assay, p53, cDNA, p53 antibodies

Michael Bergqvist, Department of Oncology, University Hospital, SE-751 85 Uppsala Sweden

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”Optimisten har säkert lika ofta
fel som pessimisten men han har
roligare ”
Ordspråk från Blekinge

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals

I. Bergqvist M, Brattström D, Stålberg M, Brodin O and Hellman B. Evaluation of radiation-induced DNA damage and DNA repair in human lung cancer cell lines with different radiosensitivity using alkaline and neutral single cell gel electrophoresis
Cancer Letters 133 (1998): 9-18

II. **Bergqvist M**, Brattström D, Gullbo J, Hesselius P, Wagenius G and Brodin O. p53 status and its in vitro relation to radiosensitivity and chemosensitivity in lung cancer
Submitted

III. Brattström D, **Bergqvist M**, Lamberg K, Kraaz W, Scheibenflug L, Gustafsson G, Inganäs M, Wagenius G and Brodin O. Complete sequencing of the p53 gene in 20 patients with lung cancer: mutations, chemosensitivity and immunohistochemistry
Medical Oncology 15 (1998): 225-261

IV. **Bergqvist M**, Brattström D, Larsson A, Hesselius P, Wagenius G and Brodin O
p53 auto-antibodies in non-small cell lung cancer patients can predict increased life expectancy after radiotherapy
Anticancer Research 18 (1998): 1999-2002

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Abbreviations

ATM	Ataxia telangiectasia mutated gene product
ATR	Ataxia telangiectasia related gene product
BER	Base excision repair
CDK	Cyclin-dependent protein kinases
cDNA	Complementary DNA
CT	Computed tomography
Da	Dalton (international unit for molecular weight: 1 Da=1.657 x 10^{-24} grams)
DNA	Deoxyribonucleic acid
DSB	Double strand breaks
EM	Electromagnetic radiation
FADD	Fas-associated death domain
HRR	Homologous recombinational repair
IHC	Immunohistochemistry
IR	Ionizing radiation
LMDS	Locally multiple damaged sites
MMR	Mismatch repair
mRNA	Messenger RNA
MTSHI	Multi-target single-hit inactivation
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NSCLC	Non-small cell lung cancer
PET	Positron emission tomography
RB	Retinoblastoma protein
SCLC	Small cell lung cancer
SF2	Surviving fraction after 2 Gy
SSB	Single strand breaks
STSHI	Single-target single hit inactivation

Contents

INTRODUCTION

Lung cancer is one of the world's most lethal cancers, killing approximately 1 million people each year (Minna, 2002). Approximately 2800 new lung cancer cases are detected each year in Sweden (National board of health and welfare, 2000). Smoking is responsible for the majority of all lung cancer cases and if smoking is abolished, a presumed reduction of 90 % in the number of lung cancer cases is believed to occur (Boyle, 1997).

Suspicion of lung cancer is often based on chest X-ray examination of the lung, in combination with clinical symptoms. The diagnosis of lung cancer is based on microscopic examination of tumour material, often received through bronchoscopy. Much effort is put into determining the extent of the cancer and the reason for this is that proper staging is the foundation for treatment, expected survival, as well as the basis for research. CT is the most important tool for providing additional anatomic and morphologic information about lesions. However, its limitation is that it cannot distinguish between benign and malignant abnormalities. During recent years, PET has been shown to be of clinical usefulness as a more sensitive and specific examination for staging of lung cancer (Coleman, 1999) but this method has not yet come into routine use in Sweden. Lung tumours are staged according to a tumour-node-metastasis (TNM) system, in which T stands for tumour size, N for regional lymph nodes and M for metastasis (Bulzebruck et al., 1992).

Lung cancer is divided histologically into five main groups; squamous cell carcinoma, adenocarcinoma, large cell carcinoma and adenosquamous carcinoma (plus other less common entities). With respect to similarities in tumour biology and treatment, adenocarcinoma, squamous cell-, small cell-, and large cell carcinoma are often grouped together as non-small cell carcinoma (Travis WD, 1999). Surgical management of small cell lung cancer generally yields little benefit since these tumours disseminate early to regional lymph nodes and distant sites. However, this issue is not fully elucidated and some authors advocate that patients with very early stage tumours should be considered for a combined treatment modality including surgery and chemotherapy (Deslauriers, 1997). Chemotherapy is the backbone in the treatment of SCLC. Four-six courses of the combination of etoposide plus cis- or carboplatin is standard treatment in Sweden. However, recent data indicate that Irinotecan might be more efficient than Etoposide (Sandler, 2002). The role of chest irradiation is well documented and radiotherapy is an important addition for patients with limited stage disease. Patients in complete remission should receive prophylactic cranial irradiation in order to reduce the risk of brain metastases (Osterlind, 2001).

Non-small cell lung cancer patients (stage I+II), who are physically able to tolerate surgical intervention often have a good prognosis after surgical resection, with 5-year survival of 50-70% for T1 and T2 tumours (Luketich JD, 1996). Higher stages of disease and extended resections increase morbidity and mortality (Deslauriers et al., 1989). Trials aiming to explore the potential benefit of induction chemotherapy have shown a potential benefit in patients staged IIIA treated with cisplatin-based chemotherapy administered for three cycles prior to surgery (Rosell et al., 1999; Roth et al., 1998). These trials included only a small number of patients and are therefore, in Sweden, not considered conclusive, and larger studies are needed to fully elucidate this issue.

The role of induction radiotherapy in non-small cell lung cancer patients has not resulted in any improvement in survival (Shields et al., 1970). However, a subgroup of patients with superior sulcus tumours received preoperative radiotherapy and favourable results have been reported for these patients (Attar et al., 1998).

Postoperative radiotherapy for non-small cell lung cancer patients was studied in a meta-analysis including 2128 patients from 9 trials. The results demonstrated better local control but showed a significant adverse effect on survival with a 21% relative increase in the risk of death for patients receiving radiation (Meta-analysis Trialist Group PORT, 2000). However, criticism of the results of this study has been raised since many of the trials investigated in this study used outdated radiotherapy techniques, which might have caused increased risk of lethal toxicity.

A majority of trials investigating the potential role for adjuvant chemotherapy have reported that approximately 60% of the intended postoperative treatments were fulfilled due to toxicity (Pisters KMW, 2002). A meta-analysis enrolling 4357 patients presented in 1995 showed no improvement in survival for patients receiving adjuvant chemotherapy (Non-small Cell Lung Cancer Collaborative Group, 1995). However, the majority of patients in this meta-analysis received long-term low-dose alkylating agents. A subgroup analysis of patients receiving modern chemotherapy with a few courses of cisplatin-containing regimens, demonstrated a modestly improved survival. The conclusion from this meta-analysis was that it was necessary to perform more studies on adjuvant chemotherapy and a few large studies have been performed, the results of which are being awaited.

Unfortunately, approximately eighty percent of all cases of patients with non-small cell lung cancer at diagnosis are not candidates for surgery (Rudd, 1991). Stage III is a heterogeneous group, in which stage IIIB has a 5-year survival rate similar to, or only slightly better than, stage IV disease. Stage IIIA has a better prognosis with a five-year survival of more than 10 %. The majority of stage III patients in good or modest performance status are treated with radiotherapy and retrospective single institution data suggest approximately 5 % five-year survival in this group (Johnson DH, 1996). In a meta analysis, a modest but significant improvement of survival was found for chemotherapy given adjuvant to radiotherapy for these patients (Non-small Cell Lung Cancer Collaborative Group, 1995). There are data demonstrating a further improved local control and survival from concomitant radio-chemotherapy (Fietkau, 2001).

In a meta analysis in which the value of chemotherapy was investigated in patients with advanced NSCLC, it was found that patients with advanced malignancies had a small but significant prolongation of survival from cisplatin-containing combinations compared with best supportive care, and these data have been confirmed by other studies (le Chevalier, 1996).

Since the effectiveness of antiemetics has improved (Hainsworth et al., 1991) and randomised studies have demonstrated a prolonged survival and better quality of life for patients with advanced lung cancer, the number of these patients receiving chemotherapeutics has increased and is believed to increase further in the future.

In the past, radiation treatment was regarded as a palliative form of treatment instead of a curative indented treatment. The five-year survival rates are in general between 5-10 %, varying between different clinical trials, but local control rates are higher (Mornex, 1999). Several factors have been associated with increased responsiveness of radiation therapy. In a study performed by the Radiation Therapy Oncology Group trial for lung cancer, (RTOG), three different radiation doses of 40, 50 and 60 Gy, delivered with daily 2 Gy fractions were evaluated in terms of percentage of local control and survival (Perez et al., 1987). They found that 3-year survival rates were 6% after 40 Gy, 10 % after 50 Gy and 15 % after 60 Gy. Further, 60 Gy induced a better local control than 40 Gy, thus the probability of controlling a tumour increased with the higher doses.

The question concerning tumour size was investigated in 149 patients. This study showed that the risk of local relapse at five years was 38% for tumours less than 3 cm, 45 % for tumours between 3 and 5 cm and 68 % for

tumours larger than 5 cm (Morita et al., 1997). Repopulation has been addressed since a study published in 1988 proposed that proliferation might start 3 weeks after initiation of treatment, thus requiring an additional radiation dose to compensate for this increase in malignant clonogenic cells (Withers et al., 1988). These *in vitro* data were investigated in a clinical trial in which it was found that two-year survival rates dropped from 33 to 14 % if treatment was delayed more than five days (Cox et al., 1993).

Radiation therapy induces both acute and late complications and one of the major challenges is to find fractionation schedules that achieve a high rate of local control and low complication frequencies. Late complications are the ones most feared. These complications arise in slowly proliferating tissues and it has been shown that the most important parameter is fractionation size, not the time factor; thus large fractions increase the risk of late damage. Early acute effects, on the contrary, are found in rapidly proliferating tissues and depend merely on the daily radiation dose and the weekly radiation dose rather than the fractionation size (Mornex, 1999). To counteract the issues of repopulation and complications, the question concerning hyperfractionation/ accelerated hyperfractionation as well as the concomitant boost has been investigated in different clinical trials.

In a phase III study, 1.2 Gy administered twice daily, with a 4-hour interval, comprising 70 Gy, was compared with daily 2 Gy fractions with/ without concomitant chemotherapy. After a one-year follow-up, a significant advantage for patients treated by means of hyperfractionated radiotherapy was found (Sause, 1994). To further push the boundaries and address the issue concerning repopulation, the CHART study was performed. CHART stands for continuous hyperfractionated accelerated radiation therapy. The main concepts of this study were to complete treatment before the onset of repopulation or acute effects and to perform treatment without any gap, thus providing treatment including weekends. Radiation treatment was given in daily 1.5 Gy fractions, three times a day, during 12 days, comprising totally 54 Gy. These patients were compared with patients obtaining daily 2 Gy fractions during 6 weeks. Patients receiving CHART had a significantly increased survival after two years; 29 % in comparison with 20 % for patients receiving the conventional treatment schedule (Saunders et al., 1996).

To avoid the problem often found when accelerating a radiation schedule with esophageal mucosal reaction, a treatment interruption can be induced or a concomitant boost approach can be used. This approach is founded on the reduction of field size, only treating the tumour, and this has also been shown to have possible benefits (Schuster-Uitterhoeve et al., 1993).

In Sweden, the recommendations concerning radiation treatment are shown in **TABLE I** (Lungcancer nationellt vårdprogram 2001).

TABLE 1. Radiation treatment for patients with NSCLC and SCLC

Stage	**Recommendation**
I, II , IIB NCLC	Patients that are medically not operable, curative intended radiation treatment > 60 Gy
Tumours engaging the thoracic wall (T3, N0/N1, M0) (NSCLC)	Incomplete thoracic surgery, radiation treatment > 60 Gy in combination with chemotherapy
III A NSCLC	Radiation treatment >60 Gy, often in combination with chemotherapy. For some patients, surgery might be of supplementary value
III B NSCLC	Radiation treatment >60 Gy, often in combination with chemotherapy
IV NSCLC	Palliative radiotherapy
Vena cava superior syndrome	Mainly radiation treatment but in selected cases, other local treatments
I,II,III SCLC limited disease	Irradiation 45-50 Gy to the primary tumours
Mainly limited disease	Prophylactic brain irradiation 30-36 Gy

Radiobiology

The effect of radiation depends on the absorption of radiation. The absorption occurs by interaction with orbital electrons in the atoms that are being exposed. The type of radiation, and the energy of the radiation, decides the nature of the interaction with the orbital electrons (Kempner, 2001).

Radiation produces a variety of damage to macromolecular components. It alters DNA and RNA bases and sugars, induces DNA-DNA and DNA-proteins cross-links and further induces single and double DNA strand breaks (Höglund, 2000; McMillan and Peacock, 1994). Double-strand breaks (DSB) are the most critical effects of ionising radiation and these lesions are the determinants if eukaryotic cells are destined to die after treatment of ionising radiation (Frankenberg-Schwager, 1990; Iliakis, 1991).

DNA DSB and their correlation to radiosensitivity

The number of unrejoined DNA DSB in cells has been correlated to cell killing in eukaryotic cells (Frankenberg-Schwager, 1989; Iliakis, 1991). However, there are inconsistencies in available data since some studies have reported positive correlations (Cassoni et al., 1992; Kelland et al., 1988; Schwartz et al., 1988) whereas others have reported no correlation (McMillan et al., 1990; Olive et al., 1994; Woudstra et al., 1996).

In a study in which repair of DNA SSB and DNA DSB was studied, DNA single-strand break induction and repair was not found to be an important factor in the radiation response of human tumour and normal cell lines. Instead, the rate at which DNA double strand breaks were repaired was a critical factor underlying radioresistance (Schwartz et al., 1988).

Cellular repair of DNA damage

The repair of induced DNA damage is of vital importance for conserving the genome's integrity and avoiding carcinogenesis. In general, there are five major DNA repair systems: nucleotide excision repair, base excision repair, mismatch repair, homologous recombinational repair, and non-homologous end joining (Martin, 2001).

- Nucleotide excision repair, repairs DNA with helix-distorting damage due to adducts produced by alkylating chemotherapeutics and UV light (Wood, 1997).
- Base excision repair removes the majority of base and sugar-phosphate lesions induced by oxidative damaging reactions such as ionising radiation (Weinfeld et al., 2001).
- Mismatch repair is a post-replication correction system that removes induced damage as well as extra-helical loops and nucleotide mispairs (Marti et al., 2002).
- Homologous recombinational repair results in sequence information that is lost due to damage in one double-stranded DNA molecule being accurately replaced by physical exchange of a segment from an homologous intact DNA molecule (Thompson and Schild, 2001).
- Non-homologous end joining means that the induced DSB are aligned and ligated (Bernstein et al., 2002).

Inherent radiosensitivity

In 1956, Puck and Marcus published the first radiation survival curve for mammalian cells in culture. Survival in this study was determined as the ability to procreate colonies of cells (Marcus, 1956). The question concerning inherent radiosensitivity was further analysed by Fertil and Malaise, and they concluded that tumours more resistant to cure by radiotherapy produced cell lines that were more resistant to clinically relevant doses of

radiation (Deacon et al., 1984; Fertil and Malaise, 1981). Since then, several studies have established the concept of measuring intrinsic radiosensitivity as a parameter of predicting radiation responsiveness in the clinical situation. In stage I–III carcinoma of the cervix, a significant correlation between tumour SF2 and patient response to radiation therapy was shown (West et al., 1993). In this study, it was further shown that if the *in vitro* data expressed SF 2 > 0.40, these patients had a significantly increased rate of local recurrence and poorer survival at 3 years follow-up. Other studies in this area have involved head and neck cancers and preliminary data indicated that for a SF2 < 0.36, there was a trend towards higher local control and survival rates (Girinsky et al., 1993). However, an up-date of this work did not find a statistically significant association (Eschwege et al., 1997). In a Swedish study published in 1998, the authors successfully established growth in 110 out of 156 specimens from head and neck cancers. SF2 values were obtained from 99/156 specimens. Eighty-four of these patients underwent radical radiation treatment and the median SF2 value for these tumours was 0.40. At a mean follow-up time of 25 months, 14 patients had developed local recurrence and the median SF2 value from these tumours was 0.53, which was significantly higher than the median of 0.38 from those 70 patients without local recurrence. The authors concluded that tumour SF2 was a significant prognostic factor for local control but not for overall survival (Bjork-Eriksson et al., 1998).

The results from *in vitro* studies of SF2 in lung cancer reveal a wide range for each category (SCLC and NSCLC) of lung cancer (Brodin, 1990) but no data exist, to our knowledge, concerning predictive assays measuring SF2 prior to radiation therapy for these patients.

Important factors associated with radioresponsiveness are hypoxia, repopulation, and the redistribution of cells progressing through the cell cycle, but these issues won't be further discussed in this thesis.

The tumour suppressor gene, p53

In 1979, the discovery of a nuclear phosphoprotein was initially reported. This p53 protein was discovered in extracts from simian 40 transformed cells that formed an oligometric complex with the large T antigen, one of the two SV40 oncogene products (Lane and Crawford, 1979; Linzer and Levine, 1979). The p53 gene was localised to the short arm of chromosome 17. It had a coding region composed of 11 exons (Miller et al., 1986), and the corresponding protein of the p53 gene was a 53-kDa nuclear phosphoprotein consisting of 393 amino acids (Soussi and May, 1996)

The p53 protein can be divided according to functional domains, conserved domains and structural domains. These domains are further divided according to the following (May and May, 1999):

The functional domains: transcriptional activation domain (residues 1-42), sequence-specific DNA-binding domain (102-292). The C-terminal portion of the p53 contains an oligomerization domain (residues 323-356) and a regulatory domain (residues 360-393).

Conserved region: I (residues 13-23), II (residues 117-142), III (residues 171-181), IV (residues 234-250), V (residues 270-286).

Structural domains, L (=loops): Loop-sheet-helix structure (residues 112-141) and (residues 278-286). The first loop L1 (112-124), binds to DNA within the major groove. The second loop L2 (residues 163-195) binds to the minor groove within the DNA. The third loop L3 (236-251) packs against L1 and stabilizes it. A zinc atom tetrahedrally coordinated on amino acids Cys 176, His 179, Cys 238 and Cys 242 stabilizes the structure of the L2 and L3 loops.

The N-terminal region of p53 contains a transcriptional transactivating domain which can form a direct connection with several basal transcription factors such as, for example, the TBP-protein (TATA-binding protein) (Horikoshi et al., 1995). The amino terminal region also contains the binding site for mdm2 (Kubbutat et al., 1997). The transcriptional transactivating domain is linked to the central core by a prolin-rich region that is believed to be involved in growth-suppressor function of p53 (Ruaro et al., 1997). The core domain binds to DNA sequence specific regions and is believed to execute apoptosis, cell-cycle arrest, growth control and control of repair and replication (Dahm-Daphi, 2000). The carboxyl region is rich in basic residues and shows non-specific DNA/RNA binding, and these bindings can modulate the sequence specific DNA binding activity of the central core (Hupp et al., 1992). Three nuclear localization sequences and a nuclear export signal that regulates the sub-cellular localization of the p53 protein, are also located within the carboxyl-regional region (Shaulsky et al., 1990; Stommel et al., 1999).

Biological functions

Under normal circumstances, the levels of p53 protein are low and not detected by immunohistochemistry. However, if the cell is exposed to cellular stress, including DNA damage (Kastan et al., 1991), oncogene activation (Palmero et al., 1998), hypoxia (Graeber et al., 1996) as well as cytokine activation, viral infections, heat shock or metabolic changes (Eizenberg et al., 1995), this results in rapid elevation of the p53 protein. The activation of p53 protein often results in cell cycle arrest or cell death through apoptosis. p53 is also involved in DNA replication (Cox et al., 1995) and repair of DNA damage (Smith et al., 1995).

p53 functions as a tumour suppressor gene and this is performed through the ability of the protein to induce cell cycle arrest or apoptosis. However, the decision to induce growth arrest or apoptosis does not entirely depend on p53 functions. In the majority of cases, after DNA damage, normal diploid fibroblasts undergo cell cycle arrest (Di Leonardo et al., 1994), whereas most lymphoid and myeloid derived cell lines undergo apoptosis after irradiation (Clarke et al., 1993). Growth factors have also been proposed to protect against p53-induced apoptosis (Canman et al., 1995). The choice of the cell to induce cell cycle arrest or apoptosis has also been proposed to be due to the amount of over-expression of p53 (Chen et al., 1996). Further observations have, in addition, led to suggestions that the decision to induce apoptosis is made after cell cycle arrest has occurred (Guillouf et al., 1995).

The cell cycle is divided into four phases. Two key protein types are involved in the cell cycle control and these proteins are the cyclin-dependent protein kinases (CDK), which are enzymes that phosphorylate the serine and threonine amino acids of among others, the p53 gene, thus activating them. The other group of proteins are the cyclins, which bind to the CDK's, enabling them to act as enzymes. These proteins are destroyed and resynthesized during each cycle. During the progression through the cell cycle, different cyclins are activated (Bodis, 2001) **FIG 1+FIG 3.**

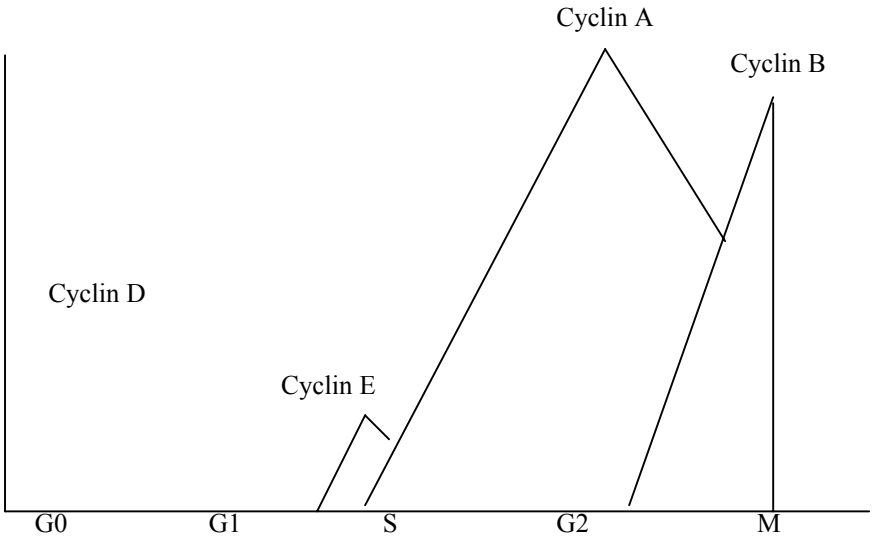

FIG 1.

The expression of cyclins during the cell cycle, the x-axis expressing the different phases of the cell cycle and the y-axis expressing the general levels of the cyclins.

During the progression of the cell through the cell cycle, the cell passes two checkpoints: the G1 and the G2/M checkpoint. Concerning growth arrest, the arrest at G1 and G2/M phases are reversible (Bates et al., 1999) and mediated by p21 $^{WAF1/CIP1}$ gene, which is a direct target of p53 transcriptional activation (el-Deiry et al., 1993). The p21 $^{WAF1/CIP1}$ gene is an inhibitor of cyclin-dependent kinase complexes (Harper et al., 1993) and also binds to proliferating cell nuclear antigen which directly inhibits DNA replication (Luo et al., 1995). p53 also mediates cell cycle arrest by interacting with the GADD45 gene. This gene (Growth Arrest and DNA Damage-inducible Genes) is associated with G1/S and G2/M arrest, often as a result of exposure to ionising radiation (Fornace et al., 1988).

Apoptosis, also called programmed cell death, is a series of morphological changes characterized by nuclear condensation, cell shrinkage, plasma membrane blebbing as well as DNA fragmentation. These cellular changes are performed by caspases (Salvesen and Dixit, 1997). Two different pathways can activate the caspases:

1) activation by the death receptors Fas/APO1 and/ or DR5 (Ashkenazi and Dixit, 1998),

2) activation of the caspases can also be induced by the adaptor protein, APAF-1 which is cytochrome c dependent.

p53 is directly linked to caspase activation through several transcriptional targets as bcl-2, a gene that can suppress apoptosis in a range of cell types (Jacobson et al., 1993). p53 is also linked to Bax, a gene that promotes apoptosis (Miyashita and Reed, 1995). When p53 induces Bax and inhibits Bcl 2, this result in mitochondrial release of cytochrome c. APAF-1 becomes activated and cleaves caspase 9, which activates downstream effector caspases. P53 induced genes seem to activate apoptosis through the production of reactive oxygen species (ROS), that also results in the release of cytochrome c (Polyak et al., 1996).

The death receptors can be activated by p53. Fas/APO1 belongs to the tumour necrosis factor receptor superfamily and when this receptor is activated, for example by its ligand FasL, it recruits FADD (Fas-associated death domain), which in turn recruits and activates caspase 8. This series of events results in the activation of effector caspases (**FIG 2**) (Burns and El-Deiry, 1999)

KILLER/DR5 is a member of TNF-related apoptosis-inducing ligand and, when overactivated, induces apoptosis through effector caspases (Ashkenazi and Dixit, 1998).

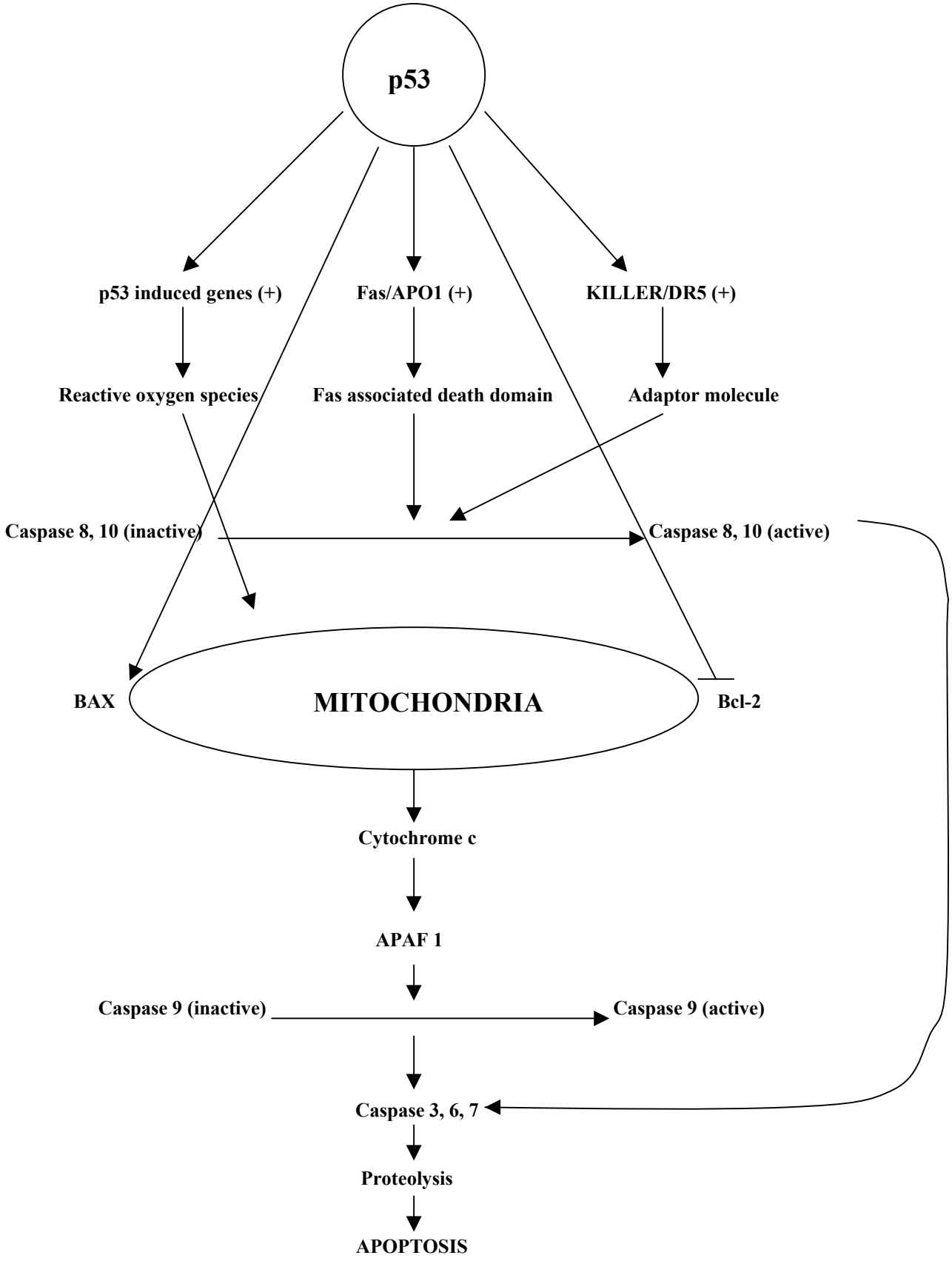

FIG 2. *p53 and its role in apoptosis*

Posttranslational regulation

The levels of endogenous p53 expression are maintained at low levels within the cells due to the rapid degradation by ubiquitin-dependent proteolysis. This process, ubiquitin-dependent proteolysis, is activated by a direct transcriptional target mdm2 (Haupt et al., 1997). mdm2 functions in two ways, firstly it binds to the activation domain of p53 and thus inhibits the ability of p53 to stimulated transcription (Momand et al., 1992). Secondly, it targets p53 for rapid degradation (Haupt et al., 1997). Cells that express mutant p53 lose this mechanism since only wild-type p53 and mdm2 protein exist in a tight regulatory feedback (Kubbutat and Vousden, 1998a). However, the stability and degradation of p53 is not entirely dependent on the mdm2 pathway. Other proteins, JNK (jun-N-terminal kinase) have been described to degradate p53 by ubiquitin-dependent proteolysis (Fuchs et al., 1998). Viral proteins, such as papillomaviruses E1B and E6, are also capable of inducing p53 degradation (Kubbutat and Vousden, 1998b).

The p53 protein can posttranscriptionally be modified by phosphorylation at serine residues within the amino- and carboxyl terminal region (Stommel et al., 1999) and further acetylated at lysine residues within the carboxyl terminal region (Prives, 1998). The stabilisation of p53 is induced by the ATM kinase, which phosphorylates the N-terminus of p53 in vitro stabilizing the p53 protein (Canman et al., 1998). The ATR kinase has the same functions and is closely correlated to the ATM kinase (Tibbetts et al., 1999). Stabilization of p53 is also induced by oncogenes such as Myc (Zindy et al., 1998). It is not only stabilisation of the p53 gene that elicits a response but also the conformation of the p53 protein has been suggested to be involved in the response. The C-terminal region of p53 might reciprocally regulate the ability of the p53 protein to bind DNA via the sequence specific DNA-binding core (Anderson et al., 1997). Further mechanisms such as point mutations, phosphorylation and acetylation that modifies the C-terminal region of p53, have been shown to activate DNA binding (Gu and Roeder, 1997; Marston et al., 1998; Prives and Hall, 1999).

The N-terminus is also involved in the modulation of p53 protein activation since phosphorylation at the N-terminal region, decreases p53's interactions with TFIID in virus-transformed cells (Pise-Masison et al., 1998). Phosphorylation of Ser 15 within the N-terminus increases the recruitment of CBP/p300 (Lambert et al., 1998).

P53 function can be modulated by control of subcellular localization. Translocation to the nucleus is fundamental for p53 function and the C-terminus contains three nuclear localization sequences and a nuclear export signal (Martinez et al., 1997; Shaulsky et al., 1991; Stommel et al., 1999).

The role of p53 mutations

P53 mutations are common in lung cancer. In SCLC approximately 70-90 % are mutated, whereas corresponding values for NSCLC were 45-75% (Chiba et al., 1990; D'Amico et al., 1992; Kishimoto et al., 1992; Takahashi et al., 1991). Squamous cell carcinomas are more frequently mutated (55-65 %) than adenocarcinoma (30-40 %) (Tammemagi et al., 2000). Japanese and American studies have reported higher incidence of mutations in SCLC (Coppola et al., 1996; Przygodzki et al., 1996) than European studies (Iggo et al., 1990; McLaren et al., 1992), thus epidemiological differences are possible. Further, specific mutations such as G:C to TT:AA transversions exist, and these have been shown to be associated with cigarette smoke (Hainaut et al., 1997)

Mutations in p53 can be divided into three broad classes according to their impact on the structure of the DNA – binding domain. Class I mutations affect residues such as Arg 248 and Arg 273, thus residues of the DNA-binding surface. Class II affects residues that are involved in the connections between the scaffold and the binding surface, such as Arg 175 and Arg 249. Class III mutations are located within the scaffold and disrupt the regulation of p53 protein flexibility (Hainaut et al., 1997).

The majority of human cancers are mutated in the p53 gene, but approximately 20 % of all mutations are located at five hot-spot codons. The majority of these mutations are further located within exon 7 as shown in **TABLE II**.

TABLE II. Localisation of hot-spot mutations and their corresponding exon

exon	Mutation (codon)
5	175
7	245
7	248
7	249
8	273

AIMS OF THE PRESENT STUDY:

I. To investigate if the "comet assay" is a proper method for characterization of radioresponsiveness of lung carcinoma cells and, further, to investigate if the intrinsic radioresponsiveness of lung carcinoma cells is due to DSB or the rejoining rate of the induced lesions.

II. To investigate, in human lung cancer cell lines, the impact of p53 mutations on intrinsic radiosensitivity and/or chemosensitivity.

III. To investigate the feasibility of p53 sequencing in tumours from patients operated for lung cancer and to correlate these data with clinical and immunohistochemical results.

IV. To investigate the impact of p53 antibodies in serum on prognosis in lung cancer patients after radiotherapy.

ARTICLES I-IV. MATERIALS and METHODS

Cell lines

U-1810 was established from a primary large-cell lung carcinoma. This cell line, which grows firmly attached to the bottom of a culture vessel, is characterised by its large epithelial cells with microvilli, cellular junctions and tonofilaments, and its ability to produce neurofilaments and keratin (Bergh et al., 1985).
U-1752 was established from a squamous cell lung carcinoma. This keratin-producing cell line also grows firmly attached to the bottom of a culture vessel as a monolayer with an *in vitro* morphology of an epithelial tumour with tonofilaments and desmosomes (Bergh et al., 1981).
U-1285 was established from a pleural effusion of a small-cell lung carcinoma. In contrast to the cell lines established from the non-small cell lung cancers, U-1285 grows in clusters mainly confined to the culture medium. It expresses the typical phenotypic characteristics of small-cell lung cancer cell lines grown *in vitro*, for example, by having dense core granules and by producing various polypeptide hormones and neurofilaments (Bergh et al., 1982).
U-1906E was established from a brain metastasis of a primary small-cell lung carcinoma. Although having the typical *in vitro* morphology of a small-cell lung cancer, U-1906E usually grows both attached to the bottom of the culture vessel, and as a suspension in the culture medium (Bergh et al., 1982).
U-1906L was established from U-1906E as a result of U-1906E changing certain phenotypic characteristics after 4-6 months of continuous culture (Brodin et al., 1995). This cell line grows more adherent to the bottom of the plastic dishes and has a higher plating efficiency.
U-1690 was established from a Caucasian male with small cell lung cancer. These cells grow in large and dense clusters, partly attached to the bottom of the plastic dishes (Bergh et al., 1985).
NCI-H23 was established from a 51-year-old man with adenocarcinoma, prior to surgery. This cell line grows adherent to the plastic dishes with a relatively slow growth rate (Brower et al., 1986).
NCI-H125 was established from a 61-year-old patient with adenosquamous carcinoma. This cell line also grows adherent to the plastic dishes (American Type Culture Collection).
NCI-H157 was established in 1979 from a patient with lung cancer squamous cell carcinoma, stage 3B, and grows attached to the bottom of the plastic dishes (American Type Culture Collection).

Cultivation of cells

The cells were cultivated at 37 ^{o}C in an atmosphere with 95% relative humidity and 5% CO_2 using the same type of medium (RPMI 1640 with 5% fetal calf serum, 5 % neonatal calf serum and 1% glutamate) for all cell lines. Because the different cell lines had different modes of growth, two different cultivation procedures had to be used. Cell lines growing attached to the bottom of the plastic dishes, were cultivated on Falcon plates (60x 15 mm, Becton Dickinson, N.J., USA) The cell lines growing in clusters/ or floating were cultivated in cultivation flasks (25 cm^2, Corning Costar Co., N.Y., USA); the cell lines were sub-cultivated twice a week.
To get free cell suspension cell lines growing firmly attached to the bottom of the plates, these cells were incubated with 0.25% trypsin in phosphate buffered saline (PBS, pH 7.4) for 2 – 3 minutes. The trypsination was

performed after rinsing the aggregates of cells with PBS containing 0.2% EDTA. Single cell suspensions of the cell lines growing as clusters or floating, were easily obtained by carefully pipetting the culture medium a couple of times.

Irradiation of cells

In **Article 1**, irradiation experiments were performed and the cell lines were irradiated with different doses using the γ-rays from a ^{60}Co source (0.55 Gy/min; Siemens Gammatron III).

The cell lines were irradiated both as single cell suspensions in Eppendorf tubes after resuspending for 2 – 3 minutes in phosphate-buffered saline (pH 7.4) containing 0.25% trypsin and 0.2% EDTA, and when still growing on the plates. The dishes were covered by a 1 cm bolus to allow sufficient build-up for relevant dose homogeneity. Immediately after irradiation, the cell suspensions in the Eppendorf tubes were kept on ice and in the dark until the single cell gel electrophoresis procedure was started. The cells growing in the vessels were incubated for another 60 min at 37 ^{o}C to allow for DNA repair before a single cell suspension was established as above. Two additional post-incubation times (15 and 30 min) were used in the experiments focusing on the repair kinetics of DNA double strand breaks in U-1810 and U-1285 irradiated at 2 Gy.

All experiments were performed on duplicates of cell cultures from each treatment, using non-irradiated cells (0 Gy) from both the Eppendorf tubes and the vessels as 'internal standards'. Each individual experiment (always including 1 radiosensitive and 1 radioresistant cell line) was repeated 3 times on different occasions. Cell viability, as determined by both the trypan blue exclusion technique and by the appearances of the comets in the fluorescence microscope (Hellman et al., 1995), was constantly found to be > 98% in all experiments.

In **Article II**, the generation of data on the survival fraction of 2 Gy (SF2) was obtained from the literature. The distribution of SF2 values ranged from 0.10-0.90. Data for the individual cell lines are shown in **TABLE III**.

Table III. Survival fraction of 2 Gy obtained from literature

Cell line	U-1690	U-1285	U-1906E	U-1906L	NCI-H23	NCI-H125	NCI-H157	U-1810	U-1752
SF2	0.10	0.25	0.43	0.60	0.20	0.37	0.80	0.88	0.90
	(Brodin)	(Brodin et al., 1991)	(Brodin et al., 1995)	(Brodin et al., 1995)	(Morstyn et al., 1984)	(Morstyn et al., 1984)	(Morstyn et al., 1984)	(Brodin et al., 1991)	(Brodin et al., 1991)

Single cell gel electrophoresis

In **Article I**, single cell gel electrophoresis was utilised. This procedure was performed as follows. Ten µl of single cell suspension ($1 – 5 \times 10^6$ cells/ml) was mixed with 70 µl 0.6% low-melting point agarose (International Biotechnologies Inc., New Haven, CT, USA) in Dulbecco's PBS (pH 7.4). This mixture was layered on top of an ordinary microscope slide precoated with low-melting point agarose, which had been allowed to dry (Vaghef et al., 1996).

Single cell gel electrophoresis under neutral conditions was performed essentially by following the procedure of Olive (Olive et al., 1991). After lysis for 1 hour at + 4^oC in a freshly prepared lysing solution (30 mM Na_2-EDTA, 0.5 % lauryl sulphate, pH 8.3), the microscope slides were transferred to horizontal plastic containers where the high concentration of salts was reduced by rinsing the slides with fresh electrophoresis buffer (90 mM Trizma base, 90 mM boric acid, 2 mM Na_2-EDTA). After transferring the slides to the electrophoresis unit (Sigma, Horizontal, Dual mode), fresh electrophoresis buffer was added sufficient to cover the slides. The electrophoresis was performed for 10 min using a field strength of 0.25 V/cm (10 V; 1-2 mA). After electrophoresis, the slides were rinsed with 0.4 M Trizma buffer (pH 7.5), dried at room temperature and kept in a sealed container until image analysis.

Single cell gel electrophoresis under alkaline conditions was performed essentially by following a modification of the procedure of Singh et al. (Singh et al., 1988; Vaghef et al., 1996). The alkaline procedure differed from the neutral version in that: 1. the cells were lysed for 1 hour in ice-cold 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Trizma base, 1 % Na-lauryl sarcosinate; 2. pH was adjusted to 10 with NaOH, with Triton X-100 and 10% DMSO added just before use; 3. that the cells were allowed 30 min of DNA unwinding in the electrophoresis buffer before the electrophoresis; 4. that no salt was removed before the electrophoresis; 5. that the electrophoresis was performed using another buffer (1 mM Na_2-EDTA, 300 mM NaOH); and 6. that the electrophoresis was performed for 5 min using a field strength of 0.7 V/cm (25 V; 300 mA).

The dried slides were stained with ethidium bromide (20 µg/ml; 40 µl/slide) and examined at x350 magnification with a fluorescence microscope (Olympus BX60F-3, Olympus Optical Co., Tokyo, Japan) equipped with an excitation filter 515-560 nm and a barrier filter 590 nm. The microscope was attached to a black and white CDD video camera (Model ICD42-E, type F/L, Ikegami Tsushinki Co., Tokyo, Japan) connected to a computer-based analytical system. The image analysis program Aequitas (1A, version 1.22, DDL Ltd., Cambridge, UK) with its special application for the 'comet assay' AutoCell (version 2.0/9E, Reppalon AB, Hägersten, Sweden) was used when evaluating the degree of DNA damage. The tail parameters that were used as indicators of DNA damage and the image analysis system, have previously been described in great detail elsewhere (Hellman et al., 1995). In each experiment, 30 – 35 comets/slide from 3 x 2 slides/treatment were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells and superimposed comets.

Sequenced-based analysis of p53:

In **Articles II+III**, cDNA based sequencing of the entire p53 gene has previously been described in detail (Sjogren et al., 1996). Briefly, RNA was extracted and purified from tumours under stringent conditions to avoid degradation followed by cDNA synthesis using murine leukaemia reverse trancriptase (200U, Pharmacia Biotech AB).

The polymerase chain reaction (PCR) was utilized to amplify 4 overlapping PCR fragments covering the complete protein-coding region of p53. Solid phase sequencing was performed separately for the 4 fragments followed by electrophoretic separation and detection of the sequence ladders, respectively, using ALF express (Pharmacia Biotech). Evaluation of p53 sequence data was done using internally developed prototype software for mutation assignment.

Fluorometric Microculture Cytotoxicity Assay (FMCA)

In **Articles II+III** the FMCA was utilized. This method has previously been described in detail (Larsson et al., 1992). Briefly, a cell suspension is prepared and then 10,000 cells/well were pipetted into a 96-well microtiter plate with cytotoxic drugs added in different concentrations. Each drug was tested at six different concentrations obtained by serial dilution and each concentration was tested in duplicate. The dishes were then incubated at 37 degrees Celsius for 72 hours. A dye (fluorescein diacetate) was added after 40 minutes incubation. The microtiter plate was measured in a Fluoroscan II system. Each experiment was repeated three times. A quotient between control and living cells was calculated from the dye-hydrolysis. Dose-response curves were plotted and IC_{50} levels (drug concentration leading to 50 % cell death) were calculated using the software Graph Pad Prism. Based on the individual IC_{50} each cell line was classified as having drug resistance that was low (lower IC_{50} than panel median), intermediate (IC_{50} within+ 1SD) or high (more than + 1 SD).

Immunohistochemistry (IHC)

In **Article III**, immunohistochemistry was performed. In general, paraffin-embedded tumour material was cut into 4 μm sections and deparaffined in xylene and rehydrated in ethanol and distilled water. Pre-treatment in a microwave oven at 750W (three times for 5 min each) enhanced antigen accessibility to antibodies (Cattoretti et al., 1993). The antibody used for p53 protein detection was DO7, diluted to 1:200. This antibody is a mouse monoclonal antibody that recognises both wild-type and mutant forms of p53 (Vojtesek et al., 1992). For Bcl-2 detection, a mouse monoclonal antibody from DAKO was used with a dilution factor of 1:40. For detection of BAX, a rabbit polyclonal antibody from Santa Cruz Tech. was used, with a dilution factor of 1:200. A diaminobenzidine (DAB) detection kit that includes biotin-labelled secondary antibodies directed at mouse immunoglobulins, avidin-labelled horseradish peroxidase, and DAB as the localisation reagents was used. For detection of Bcl-2 antigen, we used secondary antibodies that detect both mouse and rabbit immunoglobulins (Duet, DAKO). Positive control for p53 was a squamous epithelial carcinoma from a penis cancer and for Bax and Bcl-2 positive controls we used an endometrial carcinoma. Negative control IHC reactions were performed by omitting the primary antibody.

The results of the IHC were evaluated according to a four-degree scale for percentage of stained cells: 0= negative staining, 1= single cells are stained, 2=<50% and 3=>50%. For staining intensity, a two-degree scale was used (weak or strong). The evaluation was done without knowledge of clinical outcome and p53 mutation status.

For interpretation of the results, the scoring for percentage of stained cells was multiplied by a factor 2, and if weak staining intensity was present a subtraction of 1 was made.

To be judged as a significant staining, we used a cut-off at 5 for each antibody, in the arbitrary scale.

Patients

In **Article III,** solid tumour tissue samples from 20 consecutive patients undergoing surgery for primary NSCLC at Uppsala Akademiska Hospital were investigated. There were ten males and ten females and the ages varying between 31 and 80 years. In **Article IV,** 67 serum samples from patients with NSCLC admitted to the Department of Oncology in Uppsala have been consecutively collected and were analysed for expression of p53 antibodies. All patients gave informed consent prior to collection of blood samples and the samples were stored at -70° C until analysed.

p53 autoantibody investigation

Blood was collected in 7 ml serum tubes without additive (367609, Becton Dickinson, Rutherford, NJ).

P53 autoantibodies were measured by a sandwich ELISA (Dianova, Hamburg, Germany). Human recombinant p53 was bound to microtiter plates. Standards and samples were pipetted into the wells. After incubation and washing, a horseradish peroxidase conjugated polyclonal goat anti-human IgG was added. After incubation and washing, a chromogenic substrate was added and the colour intensity was measured at 450 nm in a Titertek Multiskan. A relative index for patient sera was calculated as follows:

(E450 (sample) -E450 (low control)) / (E450 (high control) - E450 (low control)). Samples with an E450 less than the low control were considered negative. The ELISA assays were performed without knowledge of clinical data.

Statistics

In **Article I**, pooled data from the entire cell populations of all experiments subjected to the same type of treatment were analysed using the non-parametric one-tailed Kolmogorov-Smirnov two sample test (Siegel, 1988). In the dose-response experiments, statistical significance was judged using linear regression analyses based on the median values obtained in the individual experiments. The level of significance was set at 5%.

In **Article II**, mutations in p53 could be anticipated in all of these cell lines, thus the primary hypothesis in that study was to investigate if a correlation existed between changed radiosensitivity and/or chemosensitivity and single exons within the p53 gene. A standard ordinary least square regression method was used to calculate if the localisation of the mutations (exon), contributed to radiosensitivity. We assumed that a mutation in a given exon yields an additive effect on the radiosensitivity. Since some cell lines expressed mutations in multiple exons, a mean value was calculated for those cell lines. To be able to perform a standard ordinary least square regression method, the zero hypothesis in testing the mean level of SF2 for the different exon groups was termed SF2=1. Thus, a mutation in exon 7 alone gives a significant mean SF2 value of 0.40 ($p=0.019$), while other exon mutations alone yielded non-significant mean radiosensitive values (the zero hypothesis thus cannot be rejected). The small number of observations in this study ($n=9$) is a limiting factor for the analysis, but the level of the association between mutations within exon 7 and increased radiosensitivity ($p=0.019$) is strong.

Concerning cytotoxic sensitivity/ and or resistance, cytotoxic compounds that expressed intermediate sensitivity were excluded from the analyses. No significant associations between cytotoxic sensitivity/ and or resistance and mutations within different exons could be found.

In **Article IV** the Kaplan-Meier product-limit for estimation of survival functions was used and the median survival time was estimated with linear interpolation in the survival function. Cox-regression analysis was applied to study if certain factors had an impact on survival. In cases where there was only one dichotomous explanatory factor, the results from the Cox-analysis are the same as from the log rank test. Throughout this study a 5 % significance level is used in the statistical tests.

RESULTS AND DISCUSSION

Lung cancer is a malignancy with poor prognosis, and treatment for patients with SCLC is based on chemotherapy, whereas for NSCLC patients different treatment options exist. For certain subgroups of patients, both SCLC and NSCLC, radiotherapy with curative intention is recommended (**TABLE I+II)**, since it increases local control and survival. Treatment with curative intention needs irradiation doses with toxic effects. Data on the radioresponsiveness from measurements in head and neck carcinomas and cervix carcinomas, as well as analyses from other clinical studies (Brodin, 1990), indicate that tumours with SF2 above 0.4 have a much lower probability to be cured by irradiation. The question of predicting radiosensitivity prior to radiotherapy is thus of vital importance since the radioresponsiveness varies between tumours with the same histopathological characteristics, as well as the side effects of treatment can be hazardous (Deacon et al., 1984; Fertil and Malaise, 1985; Peters, 1990). Fertil & Malaise established the concept by measuring the surviving fraction of cancer cells irradiated *in vitro*. An index of the intrinsic radiosensitivity was calculated and based on the evaluation of the survival of 59 different established human cancer cell lines in colony formation assays. The authors concluded that the surviving fraction after *in vitro* irradiation at 2 Gy could be used to predict the clinical response to radiotherapy (Fertil and Malaise, 1981).

The most common method to predict radiation sensitivity is the clonogenic assay. However, this method has several disadvantages. It depends on the establishment of a cell line from each individual patient. The establishment of cell lines from NSCLC patients is a very difficult task and the majority of attempts fail. Moreover, the period of time it generally takes to perform the clonogenic assay is two-four weeks and this fact, in combination with low plating efficacy and clumping artefacts, makes the concept implausible as a predictive assay in clinical praxis (Rockwell, 1985). However in a Swedish study, using a soft agar clonogenic assay, published in 1998, the authors had successfully established growth in 110 out of 156 specimens from head and neck cancers. SF2 values were obtained from 99/156 specimens. The authors concluded that tumour SF2 was a significant prognostic factor for local control but not for overall survival (Bjork-Eriksson et al., 1998). The results from *in vitro* studies of SF2 in lung cancer have revealed a wide range for each category (SCLC and NSCLC) of lung cancer (Brodin, 1990) but to our knowledge, no data exist concerning predictive assays measuring SF2 prior to radiation therapy for these patients.

In the present project, we have tried to find a correlation between the number of induced micronuclei in cell lines and data from the clonogenic assay (Bergqvist et al., 2001). There was an increase in the number of micronuclei after irradiation but the method was not sensitive enough for estimation of radiosensitivity in clinical doses. We also investigated if the FMCA-method could be used in predicting cellular radiosensitivity, but found that the FMCA assay was not useful as a quick screening method for the radioresponsiveness in vitro of human tumour cell lines (Xing et al., 2001).

Ionising radiation causes a spectrum of lesions such as DNA single-and-double strand breaks, damage to the DNA bases and sugars, as well as protein crosslinks (Olive and Banath, 1995). In response to irradiation, eukaryotic cells activate DNA repair pathways as well as slow down proliferation by halting the cell cycle at distinct cell cycle checkpoints. Radiation induces two different modes of cell death termed mitotic or clonogenic cell death, and apoptosis. (Ross, 1999). Studies by several authors have claimed that the single most lethal event

induced by radiation treatment is the induction of DNA DSB, and that cell killing correlates to the number of DNA DSB (Frankenberg-Schwager, 1989; Iliakis, 1991; Nunez et al., 1996). DNA DSB are generated when two complementary strands of the DNA double helix are broken simultaneously at sites that are so close to one another that base pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed. As a result, the broken DNA ends might migrate from each other, causing insufficient repair. Correlations have been found between unrepaired DNA DSB and residual chromosome breaks in cells (Badie et al., 1995; Cornforth and Bedford, 1987).

Five different methods are available by which estimations concerning the number of DNA DSB can be made. These methods can, in broad terms, be described as follows; Sucrose gradient sedimentation, in which DNA fragments are separated by centrifugation through a sucrose density gradient according to their molecular mass (Lehmann and Ormerod, 1970). Neutral filter elution, in which the elutions of DNA from paper filters are measured. To obtain the number of DNA DSBs, a calibration method is performed (Bradley and Kohn, 1979)., Pulsed field gel electrophoresis, in which DNA fragments are separated in agarose gels according to their size. The name of this method refers to the use of a pulse switch of the electric field (Schwartz and Cantor, 1984). The halo method uses the radiation-induced DNA unwinding, and after staining with propidium iodide, the resultant halo might be used as a measurement of induced DNA SSB (Roti Roti and Wright, 1987) and finally the comet assay (Hellman et al., 1995)

The comet assay can be used to measure low levels of SSB with high sensitivity (Singh et al., 1988). This method, called the alkaline version, is very sensitive and has been used in biomonitoring studies. However, confounding variables exist (age, gender, nourishment) and thus these should be considered when designing a study making use of this method (Moller et al., 2000). The comet method can also be performed under neutral conditions according to a protocol by Olive PL, thus detecting DNA DSB (Olive, 1989). The neutral method was established to detect subpopulations of cells with varying sensitivity to different drugs and/or radiation (Fairbairn et al., 1995).

In **Article I**, using the comet assay, a clear dose-response relationship for radiation-induced DNA SSB, DSB was found but there was no consistent pattern in the way the radiosensitive and radioresistant cell lines responded to the radiation-induced DNA damage. All cell lines showed a remarkably efficient repair of both the DNA single strand and double strand breaks one hour after irradiation, but again there was no consistent pattern in the way the various cell lines rejoined the different types of DNA strand breaks. When the rejoining rate of DNA double strand breaks was investigated in one radiosensitive U-1285 and one radioresistant cell line U-1810, the latter was found to have a more efficient repair during the first 15 min following the *in vitro* irradiation.

U-1810 and U-1285 have previously been investigated for the induction of DNA DSB and DNA DSB repair. Using pulsed field gel electrophoresis and supralethal doses of radiation (0-50 Gy), the authors concluded that the numbers of induced DNA DSB were the same for both cell lines, but they differed in the velocity of repair. The U-1810 cells exhibited a fast component of repair and about half of the DNA DSBs were rejoined during the first fifteen minutes while this was not the case for U-1285 (Cedervall et al., 1994).

As described by Frankenberg-Schwager, the repair curve of mammalian cells is often divided into two episodes. The first have a short repair half-life of 3-10 minutes whereas the repair rate of the second phase is slower, with a T ½ of 40-60 minutes (Frankenberg-Schwager, 1989). Cedervall et al. suggested that the DNA repair in U-

1285, that apparently lacks an early phase for DNA DSB repair, exhibits this pattern since either the fast repair is delayed or the cell line expresses mainly slow repair (Cedervall et al., 1994). In **Article I**, we also found that U-1810 has a fast component of repair in comparison with U-1285 and that the numbers of DNA DSB and SSB were not statistically associated with cellular radiosensitivity. However, the severity of the induced lesion as well as the compactness of the chromatin might also influence repair velocity, so the explanation of the described repair kinetics might thus be found in the regulation of the DNA repair enzymes.

Further, in a review published in 1997, a total of 26 studies had been performed investigating DNA DSB and intrinsic radiosensitivity in human tumour cells. Out of 110 cell lines, only 24 cell lines expressed a positive connection between DNA DSB induction and radiosensitivity. The same review reports that in 89 of 101 cell lines, the radiosensitive cell lines repaired the induced DNA DSB slower/ or incompletely in comparison with the radioresistant cell lines (Foray et al., 1997). A correlation has also been found for SF2 of human tumour cells and the repair of DNA DSB (Powell and McMillan, 1994) as well as a high rate of misrepair in hypersensitive cells (Powell et al., 1992).

However, the usefulness of the comet assay depends on the standardisation of the method and, as pointed out by Vaghef, interlaboratory standardisation, calibration and quantification of the assay must be performed to make it possible to compare different studies performed with this method (Vaghef, 1997).

When a DNA DSB is induced, the cell responds to this threat through several defence systems. The purpose of these processes is the restoration of DNA to prevent the development of malignant cell clones, and the core of these processes is the recruitment of DNA repairing proteins. In dividing cells, the cell cycle is slowed down and if the DNA damage is induced in G1, the cells are prevented from entering the S-phase, whereas if the damage is induced in the S-phase, the S-phase is slowed down. This also applies to cells in G2, that are prevented from entering into mitosis (Khanna and Jackson, 2001). ATM is crucial component in the DNA DSB signalling system and it is hypothesized that ATM is recruited to and activated at DNA DSB sites. ATM exerts control over several signalling pathways by phosphorylating key players resulting in a variety of effects on cell cycle progression and DNA repair (Andegeko et al., 2001). ATR is another DNA surveillance protein which especially signals DNA damage during the S-phase (Cliby et al., 2002) and both ATR and ATM share homologies in their kinase domains with the DNA DSB repair protein DNA- PKcs (Jackson, 2002).

There are two main pathways for repair of DNA DSB, non-homologous end joining (NHEJ) and homologous recombination (HR), as previously discussed. NHEJ predominates especially during G0 and G1 whereas HR predominates during the S and G2 phases (Johnson and Jasin, 2000).

p53 and radiobiology/ radiotherapy

These different repairing systems are controlled by a number of different genes but the p53 gene has evolved as one of the most important ones and gained the term “guardian of the genome”. P53 exerts these functions through interactions with several different genes, of which some have been described previously.

As described earlier, two different pathways, NHEJ and HR, perform repair of DNA DSB.

In broad terms, NHEJ is performed as follows: A central protein in NHEJ is the Ku protein. This is composed by a heterodimer of two subunits called Ku 70 and Ku 80 (Critchlow and Jackson, 1998). The Ku protein has been found to bind to DNA in a non-sequence-dependent manner and, in general, forms an open ring-type structure that can be threaded onto a DNA end (Jackson, 2002). NHEJ is initiated when Ku binds to both ends of a double-

strand break. After this procedure, DNA-PK_{cs} (an inactivated protein kinase) is recruited to the site and an active heterodimer protein kinase called DNA-PK is formed (Hammarsten et al., 2000).

DNA-PK aligns the ends of the double-strand break and DNA ligase IV, ligates the strands.

HR repair mechanism results in sequence information that has been lost due to damage in a double-stranded DNA molecule is accurately replaced, by physical exchange, of a segment from a homologous intact DNA molecule. Most of the genes involved in this process belong to the Rad52 epistasis group but the majority of proteins that initially recognize and recruit recombination proteins, as well as the particular polymerases, ligases and nucleases, have not yet been defined (Thompson and Schild, 2001).

Several functions of p53 have been related to DNA repair processes as shown in **TABLE IV**.

TABLE IV. The role of p53 in supervising DNA repair

Type of repair	**Function**	**Reference:**
Nucleotide excision repair	XPB/D-helicase is supervised by p53	(Ford and Hanawalt, 1995)
Nucleotide excision repair	PCNA, GADD45, RPA and polymerase σ	(Lieter et al., 1996)
Mismatch repair	P53 binds and/or tranactivates the hMSH2 promoter	(Scherer et al., 1996)
Homologous recombination (HR)	P53 suppresses spontaneous HR	(Mekeel et al., 1997)
Non-homologous recombination (NHEJ)	P53 promotes NHEJ after irradation	(Tang et al., 1999)

DNA sensing genes such as ATM, ATR and the DNA-PK kinase, react when activated by phosphorylating p53-Ser 15. This phosphorylation after DNA damage is thought to prevent interaction with mdm2, thus inhibiting the degradation of p53 by the ubiquitin degradation pathway (Smits and Medema, 2001). As mentioned earlier, cells irradiated in G1 stop at the G1/S boundary. The D-type cyclins (D1-D3) bind to CDK 4 or its homologue CDK 6 and mediates the progression through G 1 phase. This is performed by phosphorylation of the RB protein, which is coupled in a complex with E2F (belonging to a family of transcription factors). When RB is phosphorylated, it is removed from the complex, and E2F induces E2F-dependent gene transcription allowing progression into S-phase (Milas, 2001). This checkpoint is tightly regulated by p53 through its ability of activating $p21^{WAF1/CIP1}$ that, in turn, inhibits the complex cyclin E:cdk2 (Izumi and Maller, 1993; O'Connell et al., 1997). Cells irradiated in G2 undergo cell cycle arrest at the G2/M checkpoint. The role for p53-mediated mechanisms behind this checkpoint is believed to be due to induction of a cdk-inhibitor, p14-3-3σ, as well as interacting with GADD 45by suppressing cyclin B **(FIG 3)** (Hermeking et al., 1997; Honda et al., 1998).

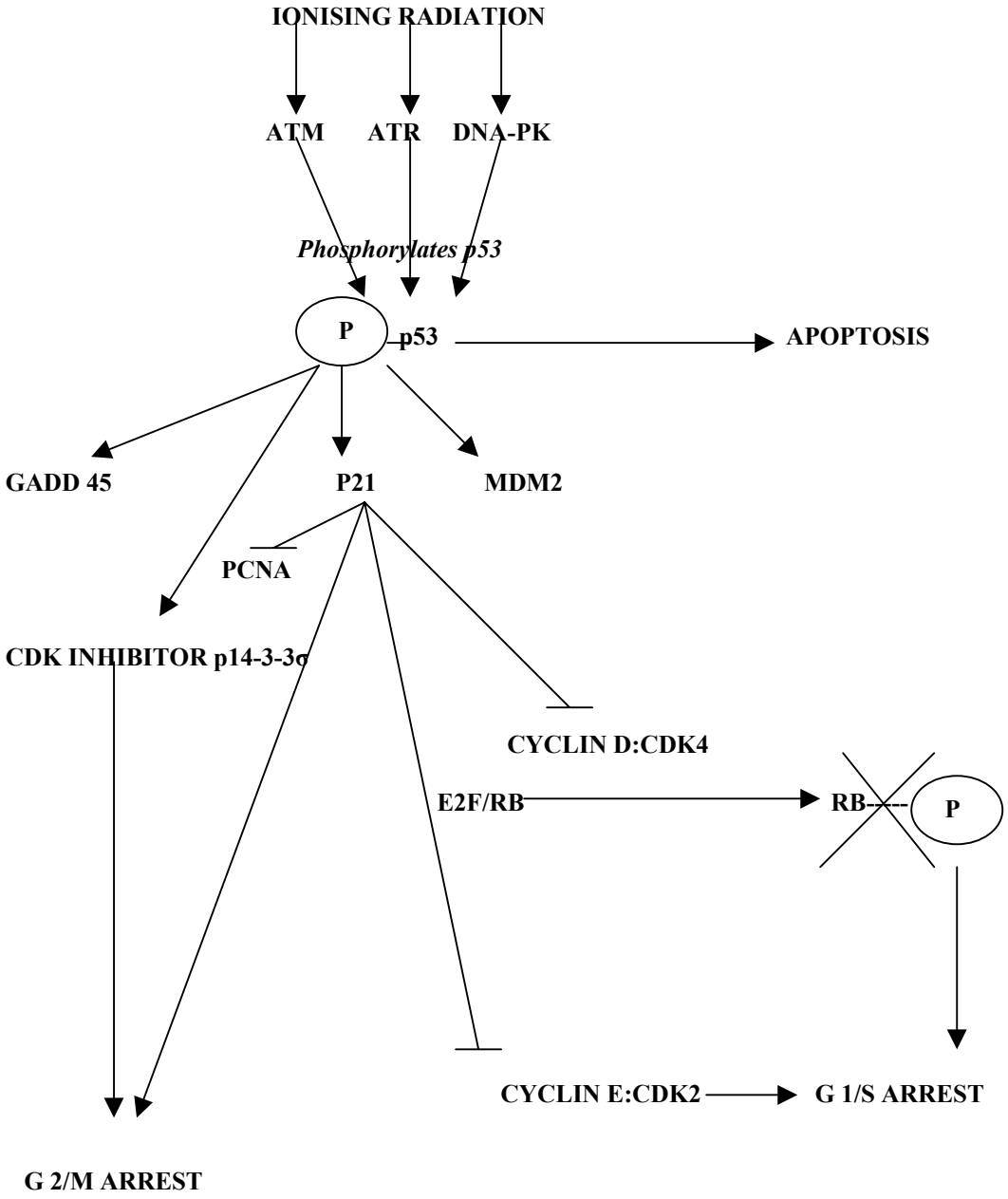

Fig 3

p53 and the cell cycle

Cells that are irradiated as lymphocytes and germ cells respond to DNA damage by inducing apoptosis rather than cell cycle arrest or repair. The threshold probably varies also between different cell-types and environment. Apoptosis is activated in G1 and is believed to results from genotoxic stresses by ionising radiation. p53 serves as a transcription factor for the bax promoter (Miyashita and Reed, 1995) but p53 also downregulates the apoptosis protector bcl-2 and thus induces apoptosis (Yarnold, 1997). PIGs (p53 inducible genes) can also be utilised by p53 to manoeuvre the cell into apoptosis.

In cells with haematopoietic origin, p53 dependent apoptosis is of great importance for cellular radiosensitivity (Clarke et al., 1993). However, in solid tumours there are data showing that solid tumours arise from apoptosis-resistant cell types; therefore p53 induced apoptosis might be of minor interest (MacCallum et al., 1996).

The issue concerning the role of p53 in cellular radiosensitivity is not fully elucidated. **TABLE V** shows a compilation of literature reporting the diverging data on p53 mutations and its impact on cellular response to radiation.

TABLE V. A compilation of literature regarding p53 mutations and cellular radiosensitivity in cell lines

Studies associated with no correlation between mutations within the p53 gene and cellular radiosensitivity			
Histology	**p-value**	**Number of cell lines**	**Author**
Head and neck cell lines	0.28	24	(Brachman et al., 1993)
Squamous cell carcinoma and osteogenic sarcomas	NS	4	(Huang et al., 1996)
Squamous cell carcinomas	NS	6	(Jung et al., 1992)
Human lymphoblast lines	NS	3	(Mallya and Sikpi, 1999)
Human ovarian cancers and cutaneous melanomas	NS	24	(Zaffaroni et al., 1995)
Studies associated with mutations within the p53 gene and increased cellular radioresistance			
Mixed	0.0046	8	(Siles et al., 1996)
Studies associated with mutations within the p53 gene and increased cellular radiosensitivity			
Head and neck cancer cell lines	<0.01	20	(Servomaa et al., 1996)
Oral cavitary carcinomas	0.023	16	(Pekkola-Heino et al., 1996)

Prognostic significance of p53 mutations in the clinical situation

Results found in the literature concerning the outcome of radiation treatment and different malignancies have shown diverging results. A study was performed on 316 breast cancer patients using complete coding of the p53 gene. This study concluded that patients with mutations within the evolutionary conserved regions II and V were

associated with worse prognosis (Bergh et al., 1995). The question concerning p53 mutational status and radiotherapy was further studied in a group of 206 primary breast cancer patients without axillary metastases. It was shown that, after receiving locoregional radiotherapy, patients expressing p53 mutations had a significantly improved relapse free survival (Jansson et al., 1995). Using polymerase chain reaction-single strand conformation polymorphism and direct sequencing, exons 5-8 of the p53 gene were investigated in a study consisting of 204 patients with NSCLC. It was found that mutations within exons 7 and 8 of p53 were poor prognostic factors for these patients (Huang et al., 1998). In **TABLE VI**, a compilation of literature associated with radiation therapy and with prognosis in patients with p53 mutations is shown.

TABLE VI. Prognosis in patients treated with radiation therapy and its association with p53 mutations

Histology	**Number of patients**	**Treatment**	**p-value**	**Results**	**Author**
Locally advanced prostate cancer	129	External-beam radiation therapy alone or total androgen blockade before and during the radiation therapy	1) 0.02 2) 0.03 3) 0.58	1) Abnormal p53 protein expression and decreased overall survival, 2) decreased progression-free survival 3) No association between abnormal p53 protein expression and time to pregression	(Grignon et al., 1997)
Non-small cell lung cancer	36	Radiation therapy	NS	A trend towards p53 overexpression and response to radiation therapy in NSCLC	(Hayakawa et al., 1998)
Head and neck squamous cell carcinoma	39	Radiotherapy and/or chemotherapy	1)0.003 2) 0.02	1) Unfavourable overall survival 2) Unfavourable disease-free survival	(Hegde et al., 1998)
Locally advanced breast cancer	42	Neoadjuvant chemotherapy followed by mastectomy and radiotherapy	NS	Nuclear accumulation of p53 was not an independent prognostic factor for disease-free survival or overall survival	(Honkoop et al., 1998)

Head and neck squamous cell carcinoma	110	Radiation therapy	0.02	Mutation of the p53 gene was associated with increased risk of locoregional failure	(Koch et al., 1996)
Malignant gliomas of childhood	29	Different treatment approaches	1) 0.019 2) 0.013	1) A significant association between p53 overexpression and shorter progression-free survival and 2) overall survival	(Pollack et al., 1997)
Squamous cell carcinomas of the head and neck	101	Radical radiotherapy	Relative risk of 3.78	Patients with p53 expressing tumours had an increased risk of not having their tumours controlled with radiation therapy in comparison with p53 negative patients	(Raybaud-Diogene et al., 1997)
Esophageal carcinoma	42	Preoperative chemotherapy/radiotherapy	1)0.0038 2) 0.0004	1) patients with p53 mutations had a shorter survival 2) and shorter disease free survival	(Ribeiro et al., 1998)
Primary epidermoid carcinoma of the anal canal	58	Radiation therapy (XRT), 5-fluorouracil (5-FU), and mitomycin C (MMC)	0.01	p53 protein expression is of prognostic value for disease-free survival	(Wong et al., 1999)
Muscle-invasive transitional cell carcinoma of the bladder	109	Preoperative radiotherapy			(Wu et al., 1996)
Squamous cell carcinoma of the uterine cervix	46	Radiation therapy	NS	No association between p53 protein levels	(Ebara et al., 1996)

Cervical cancer	64	Radiation therapy	<0.01	Patients with p53 protein expression had a shorter 5-year survival than p53 negative patients	(Nakano et al., 1998)
Nasopharyngeal carcinoma	30	Radiation therapy	NS	No association between p53 protein levels	(Roychowdhury et al., 1996)
Rectal carcinoma	123	Treated with surgery and postoperative radiotherapy	NS	No association between p53 protein levels	(Wiggenraad et al., 1998)
Squamous cell carcinoma of the glottic larynx	86	Radiation therapy	NS	p53 status was not predictive of treatment outcome parameters including local-regional failure rate and disease-free survival rate	(Pai et al., 1998)

p53 alterations and survival in NSCLC have been controversial. p53 overexpression has been variously correlated with worse outcome, better prognosis, or no influence on patient survival (Carbone et al., 1994; Lee et al., 1995; McLaren et al., 1992; Pappot et al., 1996; Quinlan et al., 1992). Results from DNA sequencing studies are shown in **TABLE VII.**

TABLE VII. *Result from p53 DNA sequencing studies in lung cancer*

Author	**Method**	**Number of pat**	**% mutated**	**Treatment**	**Survival**
(Mitsudomi et al., 1993)	Exon 5-8	120	43		↓ 0.01
(Horio et al., 1993)		71	49	Primary resected NSCLC	↓ 0.014
(Hashimoto et al., 1999)	SSCP	144	45	Surgically treated NSCLC	↓ (P = 0.03) in stage I

(Tomizawa et al., 1999)	SSCP	103	48	Surgically resected NSCLC	Missence mutations have poorer prognosis than patients with null mutations in stage 1 $p<0.001$
(Skaug et al., 2000)	SSCP	148	54		Patients with mutations in p53 had a significantly higher risk for lung cancer-related death and for death from all causes than those with wild-type p53
(Huang et al., 1998)	SSCP	204	37		Patients with mutations in exon 7 and 8 had worse survival when compared with patients with other mutations or no mutations
(Vega et al., 1997)	SSCP	5-9	81	Surgically resected NSCLC	Patients with p53 mutations in exon 5 had a worse survival

The role of p53 mutations and chemoresistance

Many patients with NSCLC are diagnosed at advanced stages and are thus candidates for systemic chemotherapy despite the fact that the majority of these tumours have at diagnosis, limited chemosensitivity both *in vivo* and *in vitro* (Nishio et al., 1999; Vogt et al., 1999). The selection of chemotherapy is, in general, founded on evidence-based medicine. The effect in the individual patient is determined after some cycles of chemotherapy (Fruehauf, 1993). The need for investigation of drug resistance in individual patients in predicting outcome of treatment can be performed with several methods (Fruehauf, 1993; Weisenthal et al., 1983). Using the FMCA method, a good correlation can be obtained between *in vitro* and *in vivo* (Csoka et al., 1994).

P53 mutations have been associated, *in vitro*, to sensitisation to Taxol (Wahl et al., 1996) whereas etoposide, nitrogen mustard and cisplatin failed to induce G1 arrest in cells containing mutant p53 genes (Fan et al., 1994). Regarding chemosensitivity data from the FMCA method used in **Article II**, no correlation between increased or decreased responsiveness of the investigated chemotherapeutics and mutational status of the p53 gene was found.

In breast cancer patients, mutations in the P53 gene were associated with primary resistance to doxorubicin therapy and early relapse in breast cancer patients (Aas et al., 1996). In **Article III**, mutations within the p53 gene were compared with data from the FMCA method. Our data indicated that tumours expressing p53 mutations seemed to be more resistant to cisplatin and cyclophosphamide. In a similar study, *in vivo* chemosensitivity testing using Adenosine Triphosphate cell viability assay (ATC) indicated that tumours with mutated p53 were significantly more resistant to the combination treatment cyclophosphamide/ etoposide/ epirubicin, than tumours with wild-type p53 (Vogt et al., 2002). Paclitaxel was investigated in conjunction with p53 mutational status in twenty-five patients with NSCLC. In this treatment setting, p53 mutations did not adversely affect the response of Paclitaxel (King et al., 2000).

The role of p53 antibodies in serum

In 1979, it was found that animals bearing SV40 tumours elicited an immune response specific for p53 (Melero et al., 1979; Rotter et al., 1980). These works were followed by studies in humans (Crawford et al., 1982).

However, p53 antibodies in sera are not present in all patients with mutations within the p53 gene and several hypotheses have been raised to elucidate this issue.

-The first hypothesis is that p53 antibody response is due to the site and type of mutation within the p53 gene. The majority of mutations within the p53 gene are located within the central core, and therefore a majority of p53 antibodies would thus be directed to this region. However, in a study in which 45 serum samples were correlated with p53 sequencing analysis, it appeared that mutations in exons 7 and 8 did not yield antibody response, whereas mutations within exons 5 and 6 resulted in the expression of p53 antibodies (Davidoff et al., 1992). Further, patients with stop, splice/stop, splice or frameshift mutations did not yield p53 antibodies whereas patients with missence mutations resulted in the appearance of p53 antibodies (Winter et al., 1992). The issue of comparing sequencing analysis with the expression of p53 antibodies in sera is perhaps not meaningful. When sequencing analyses are being performed, only a small sample of the tumour tissue is investigated, whereas analyses of serum correlate with the immune- response within the individual (Soussi, 2000). Thus, due to tumour heterogeneity, some mutations might be missed and therefore should conclusions concerning mutational status and p53 antibodies in sera should be interpreted with caution.

-The second hypothesis of p53 serum antibody response has been the accumulation of the protein within the cell (Davidoff et al., 1992; Winter et al., 1992). The accumulation of p53 within the cell is, as described previously, often the result of cellular stress and/or mutations stabilizing the protein. Therefore, it seems plausible that increased protein levels might elicit an immune response due to a self-immunization process. However, this hypothesis has been questioned since a mutation might cause a truncated polypeptide lacking the carboxyl terminus containing the signal for nuclear localisation. Further, a mutation might be silent and, additionally, p53 might be stabilized by binding to other proteins such as mdm2 (Wild et al., 1995).

-The third hypothesis of p53 antibody response is the individual host response. Anti-p53 sera recognize both wild type and mutant p53 conformational and denaturation resistant epitopes and no evidence has been found that the mutant p53 molecules contain dominant antigenic epitopes which are not present on the wild type p53 protein (Labrecque et al., 1993). p53 is heavily phosphorylated at the amino and carboxy termini and such phosphorylation has an influence on the reactivity of p53 antibodies towards the p53 protein (Soussi, 2000). In a series of 188 patients, 7 % of the p53 antibodies were directed towards the amino terminal regions whereas 14 % were directed towards the C-terminal epitopes (Mitsudomi et al., 1998). These data were contradicted by a study comprising 136 patients in which the antibodies were directed towards the N-terminal region in 15 of 16 p53 antibodies positive seras (Wild et al., 1995).

Further, in the previously mentioned study, the expression of p53 antibodies differed in two patients with identical mutations at codon 245 (GGC-TGC) and 273 (CGT-CTT). One was positive, the other negative. It has also been proposed that since tumours in general have a high degree of necrosis, and that parts of the tumour, due to treatment or inadequate blood supply, disintegrate through disease progression, this might lead to the release of p53 protein in sera, which could provoke an immune response. However, p53 proteins have not been found in sera of cancer patients and, therefore, p53 protein is either quickly degraded or other mechanisms for antigen presentation exist (Levesque et al., 1996). Isotyping of p53 antibodies has shown that they resemble IgG1 and IgG2 subclasses and, since no IgG3 or IgG4 was detected, it has been suggested that an active humoral response is needed to elicit p53 antibodies in sera (Lubin et al., 1995a). Perhaps complexes between p53 and a

70-kDa heat shock protein are needed for antigenic presentation of p53 since all antibody-eliciting tumours contained this 70-kDa heat shock protein in a study on breast cancer patients (Davidoff et al., 1992).

In conclusion, the reason why some patients express p53 antibodies in sera and others do not, is not fully elucidated. This makes it more interesting to investigate if patients who express p53 antibodies in sera, have increased survival, or if they express better response towards different treatments, such as, for example, radiation and/or chemotherapy.

Results from studies performed in lung cancer patients investigating p53 antibodies in serum and its correlation to p53 mutations and p53 accumulation are shown in **TABLE VIII.** The results from studies performed in non-small cell lung cancer patients investigating survival and its correlation to p53 antibodies are shown in **TABLE IX.**

TABLE VIII *Results from studies performed in lung cancer patients investigating p53 antibodies in serum and its correlation to p53 mutations and p53 accumulation*

Correlation with p53 accumulation and missence mutations	(Winter et al., 1992)
A correlation with the frequency of p53 gene alteration and seropositives for p53	(Lubin et al., 1993)
Serum p53 antibodies were detected only in a proportion of lung cancer cases. However, these were specifically associated with a detectable p53 mutation in the tumour	(Wild et al., 1995)
Missense mutations were identified in five of nine patients who had serum antibodies recognizing p53	(Guinee et al., 1995)
A statistically significant association was found between p53 protein overexpression, serum p53-antibodies and the presence of a p53 gene alteration	(Laudanski et al., 2001)
A statistically significant association was found between p53 protein overexpression, serum p53-antibodies	(Iizasa et al., 1998)

TABLE IX *Results from studies performed in non-small cell lung cancer patients investigating survival and its correlation to p53 antibodies*

Number of NSCLC patients	% expressing p53 ab	Survival	Ref
188	20	No correlation between survival and the presence of p53 ab	(Mitsudomi et al., 1998)
102	25	No correlation between survival and the presence of p53 ab	(Laudanski et al., 2001)
99	13	A significant correlation with shorter survival in NSCLC (p=0.01)	(Mack et al., 2000)
84	23	Patients with a positive result from the p53-Antibodies test had lower probability of overall and disease-free survival	(Laudanski et al., 1998)
140	12	No correlation between p53 antibodies and survival	(Komiya et al., 1997)

Lung cancer studies investigating the role of p53 antibodies in sera have generally been performed in patients in conjunction with pulmonary surgery (Iizasa et al., 1998; Laudanski et al., 1998; Mitsudomi et al., 1998). In **Article IV**, we investigated the relationship between p53 antibodies, prior to radiotherapy since the majority of NSCLC patients are diagnosed with advanced stage and since presumably, during the progression of the disease, they will become candidates for radiation therapy. Our study included 67 patients with NSCLC and serum samples were investigated using a commercially available kit (Dianova, Hamburg, Germany). Antibodies were detected in 27 % of these patients and these data are well in accordance with other studies (Laudanski et al., 1998; Lubin et al., 1995b; Mitsudomi et al., 1998). The majority of patients, in our study, were classified as stage IIIA and IIIB, and there were a trend indicating that the higher stage, the higher the expression of p53 antibodies. These data are supported in a study consisting of 188 NSCLC patients in which patients with stage (III-IV) had increased expression of p53 antibodies in sera (Mitsudomi et al., 1998). However, other studies have not shown a correlation between tumour stage and p53 antibodies (Iizasa et al., 1998; Laudanski et al., 1998; Segawa et al., 1998). Questions concerning histology and p53 antibodies expression have shown diverging results. In **Article IV**, 42 patients were classified as squamous cell carcinomas whereas 21 patients were classified as having adencarcinomas whereas four patients were classified as large cell carcinomas. The expression of p53 antibodies was 50% (2/4) in patients with large cell carcinoma whereas the corresponding values were 31% for patients with squamous cell carcinoma and 14 % for patients with adencarcinomas. In a prospective study investigating p53 antibodies in 58 patients, two patients were diagnosed as having large cell carcinomas and they both expressed high p53 antibody index levels (Segawa et al., 1998). In 62 cases of resected NSCLC, these data were also confirmed since four patients were diagnosed as large cell cancer and all of these patients expressed p53 antibodies in sera (Iizasa et al., 1998). Only few patients had large cell carcinoma in our study and thus no major conclusions should be drawn, but our data indicate that the immune system might be activated in a different way in these patients. A study consisting of 140 patients with squamous cell carcinoma reported that seronegative patients, whose primary tumours overexpressed p53, survived significantly longer (Komiya et al., 1997).

In **Article IV**, the presence of p53 antibodies prior to radiation treatment was significantly associated with increased survival ($p=0.025$). Are these data the result of mere chance? Perhaps they are, since the study was retrospective with fairly few patients. These results can also be explained by these patients with p53 antibodies in sera possibly being in a very good performance status, data that we have not been able to be investigate since the study was retrospective. Neither do we have information about the treatment these patients received when relapse occurred, which might also influence survival. However, the material has not been selected; we investigated the first serum sample of all patients that had a histopathologically diagnosed NSCLC. Additionally, the statistical analysis was performed in collaboration with an experienced statistician and we believe that these data are legitimate. So, how can we explain why p53 antibodies in sera prior to radiation treatment prognosticates increased survival? Further analysis in **Article IV** showed that patients with squamous cell carcinoma, expressing p53 antibodies in sera, had a better survival than those that did not express p53 antibodies in sera. It might, thus, be a matter of histology. However, as described previously, p53 antibodies in sera might indicate an activated immune system, more capable of interacting with the disease and thus the presence of p53 antibodies might indicate this. However, in colorectal carcinoma, mutations in sero-positive patients were mainly located in exons 5 and 7 (Forslund et al., 2001). Thus, in **Article II** we showed that cell lines expressing

mutations with exon 7 had a statistically significant increase in radiosensitivity. Therefore, hypothetically, our results could indicate that patients with NSCLC expressing p53 antibodies in sera prior to radiotherapy, might have a more radiosensitive tumour, thus indicating that these patients might respond better to radiation treatment than patients not expressing p53 antibodies in sera.

CONCLUSIONS

I. The comet assay is not feasible for a valid characterization of radiosensitivity in lung cancer, even if it seems to reflect different degrees of radiosensitivity. Using clinical irradiation doses it seems that the difference between the radiosensitive and the radioresistant cell line is that of early repair of DNA DSB in the radioresistant cell line.

II. p53 mutations within exon 7 were associated with increased radiosensitivity, in comparison with other mutations. No correlation to data on chemosensitivity could be found.

III. It was possible to perform cDNA analysis in tumour tissue and these data showed that 50 % of the patients were mutated in p53.

IV. The presence of p53 antibodies in sera prior to radiation therapy was associated with increased survival for those patients that expressed the antibodies.

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