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Radiogenomics in gynecological cancer patients

Radiogenomics bij gynaecologische kankerpatiënten

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2007

Thesis submitted in fulfillment of the requirements for the degree of
Doctor in Medical Sciences

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Woord vooraf

Zes jaar geleden kwam ik voor het eerst terecht op de Dienst Medische Fysica. Meteen was ik helemaal gewonnen voor het vooropgestelde project dat uiteindelijk tot dit proefschrift zou leiden. Nu, zoveel jaren later, is het proefschrift afgewerkt. En hoewel enkel mijn naam vermeld staat op de omslag, zou ik dit werk nooit tot een goed einde kunnen gebracht hebben zonder de hulp en steun van velen.

Vooreerst zou ik mijn promotor, Professor Dr. H. Thierens, hartelijk willen bedanken voor het vertrouwen, de steun en de vele constructieve bijdragen tijdens het uitvoeren van de studies gebundeld in dit werk. Bert, uw wetenschappelijke ondersteuning en uw enthousiasme voor mijn onderzoek hebben steeds inspirerend gewerkt en lieten mij toe om dit werk tot een goed einde te brengen.

Professor Dr. A. Vral, mijn co-promotor, zou ik willen bedanken voor de steun en de professionele inleiding tot de wereld van de cytogenetica. Dank ook aan Professor Dr. R. Cornelissen en Professor Dr. L. De Ridder voor de interesse in mijn onderzoek en de vele nuttige discussies tijdens de traditionele donderdagmiddagvergaderingen.

Dit proefschrift zou er nooit gekomen zijn zonder de uitstekende samenwerking met de dienst Radiotherapie van het UZ Gent. Ik zou dan ook alle stafleden en verpleegkundigen willen bedanken voor hun steun. Professor Dr. M. Van Eijkeren verdient een speciale vermelding. Marc, bedankt voor het verzamelen en opvolgen van de patiënten die de basis vormen van dit proefschrift, voor het beantwoorden van mijn vele vragen en voor uw bereidwillige medewerking aan andere initiatieven. Mevrouw Goethals zou ik willen bedanken voor de vele efficiënte telefoontjes en de leuke babbels wanneer ik mijn “cadeautjes”, de stalen, kwam ophalen.

Dankzij de vlotte samenwerking met de dienst Arbeidsgeneeskunde van het UZ Gent, waren wij in staat genetisch materiaal van gezonde controle-individuen te verzamelen. Dank daarvoor aan Dr. R. Morthier en zijn medewerksters. Jacqueline, Martine en Kristel, bedankt om dit

extra werk op jullie te nemen en mij steeds te verwittigen als de "bloedjes" er waren. Ook bij jullie was het steeds aangenaam om eens langs te komen!

Dank ook aan alle stafleden, doctoraatsstudenten en laboranten van de dienst Medische Genetica van het UZ Gent. Een dikke merci voor Dr. Ir. K. Claes voor de vakkundige ondersteuning. Ilse, Leen en Nathalie, bedankt om mij de moleculaire technieken die ik toepaste in dit proefschrift aan te leren. Annick, Katia en Elien, bedankt voor de assistentie bij het gebruik van de toestellen.

Een speciaal woordje van dank ook aan de medewerkers van het Westlakes Research Institute (UK) en in het bijzonder aan Dr. C. Wilding. Craig, thanks a lot for the great idea to combine our expertises. This had led to a super efficient collaboration and a very valuable publication!

Toffe collega's en een aangename werksfeer maken het zonder enige twijfel makkelijker om een doctoraat tot een goed einde te brengen. Dat dit in ons labo geen probleem zou zijn, had ik reeds begrepen vanaf de eerste dag. Daarom een dikke merci voor Isabelle en Virginie voor de organisatie van de vele etentjes. Myriam en Nancy zou ik willen bedanken voor de aangeboden hulp in drukke tijden. An, Evelien, Barbara en Nele, bedankt voor de toffe tussentijdse babbels. Bedankt Nick, Frederik en Tom voor jullie interesse. Joke, bedankt om een aantal van de nog lopende projecten van mij over te nemen. Virginie, je hulp bij het telwerk, de bloedscheidingen en de DNA isolaties werd zeer geapprecieerd. Jij was bovendien steeds bereid om nieuwe technieken aan te leren en in te springen wanneer het druk werd. Hartelijk dank daarvoor!

Aan al mijn familieleden en vrienden: jullie hebben er dikwijls naar gevraagd en lang op moeten wachten, maar eindelijk is het vele werk van de laatste jaren gebundeld in dit proefschrift en ben ik "afgestudeerd"! Bedankt voor jullie interesse, het luisterend oor en de aangename ontspanningsmomenten die me terug de nodige energie gaven. Liefste ouders, bedankt voor jullie steun en vertrouwen gedurende de voorbije jaren. Mede dankzij jullie heb ik dit proefschrift kunnen voltooien.

Eén "collega" ben ik daar daarnet vergeten vermelden. Mijn allerliefste Klaus, bedankt voor je interesse in mijn werk, je rust op gespannen momenten, je hulp bij grote en kleine problemen, je liefde. We hebben nu beiden ons doctoraat afgewerkt. Tijd voor andere grote projecten…

Kim, juni 2007

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List of abbreviations

^{192}Ir: Iridium-192

3DCRT: 3-Dimensional Conformal Radiotherapy

AOA1: Ataxia-oculomotor Apraxia 1

AP: Apurinic/Apyrimidinic

APE1: AP Endonuclease-1

APEX: Arrayed Primer Extension

AT: Ataxia Telangiectasia

ATM: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia Related

BAX: BCL2-Associated X protein

BCL2: B-cell CLL/lymphoma-2

BED: Biological Equivalent Dose

BER: Base Excision Repair

BRCA: Breast Cancer

CBP: Creb Binding Protein

CDC25: Cell Division Cycle-25

CHK: cell cycle Checkpoint Kinase

Col1a2: Collagen type 1, alpha-2

CTC: Common Toxicity Criteria

CTCAE: Common Terminology Criteria for Adverse Events

CTGF: Connective Tissue Growth Factor

CuZnSOD: Copper/Zink Superoxide Dismutase

CYP2D6: Cytochrome P450, family 2, subfamily 6

DGGE: Denaturing Gradient Gel Electrophoresis

DHPLC: Denaturing High-Performance Liquid Chromatography

DNA-PKcs: DNA-dependent Protein Kinases

ECM: Extracellular Matrix

ED-A FN: ED-A Fibronectin

EGF: Epidermal Growth Factor

EORTC: European Organization for Research and Treatment of Cancer

ERCC: Excision Repair Cross-Complementing

ESTRO: European Society for Therapeutic Radiology and Oncology

FEN1: Flap structure-specific Endonuclease-1

FGF: Fibroblast Growth Factor

Gene-PARE: Genetic Predictors of Adverse Radiotherapy Effects

GENEPI: Genetic pathways for the Prediction of the effects of Irradiation

hHR21: human Homologue of Rad21

HNPCC: Hereditary Non-Polyposis Colorectal Cancer

HPV: Human Papillomavirus

HR: Homologous Recombination

IARC: International Agency for Research on Cancer

IFNγ: Interferon-γ

IGF1: Insulin-like Growth Factor-1

IL: Interleukin

IMRT: Intensity Modulated Radiotherapy

IVS: Intervening Sequence

LAP: Latency Associated Peptide

LENT/SOMA: Late Effects Normal Tissue/Subjective Objective Management Analytical

MGMT: O^6-methylguanine-DNA methyltransferase

MLH1: MutL Homologue-1

MMP: Matrix Metalloproteinase

MMR: Mismatch Repair

MnSOD: Manganese Superoxide Dismutase

MRE11: Meiotic Recombination-11

NBS: Nijmegen Breakage Syndrome

NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End Joining

NTCP: Normal Tissue Complication Probability

OGG1: 8-oxoguanine DNA glycosylase

PAI1: Plasminogen Activator Inhibitor-1

PARP: Poly(ADP-ribose)polymerase

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

PDGF: Platelet-Derived Growth Factor

PFGE: Pulsed Field Gel Electrophoresis

PKC: Protein Kinase C

PNK: Polynucleotide Kinase

RACE: Radiation Complications and Epidemiology

RAPPER: Radiogenomics: Assessment of Polymorphisms for Predicting the Effects of Radiotherapy

RAS: Renin-Angiotensin System

RFLP: Restriction Fragment Length Polymorphism

RNS: Reactive Nitrogen Species

ROCK: Rho Kinase

ROS: Reactive Oxygen Species

RPA: Replication Protein A

RTOG: Radiation Therapy Oncology Group

SCGE: Single-Cell Gel Electrophoresis

SF: Surviving Fraction

Smurf: Smad ubiquitination regulatory factor

SNP: Single Nucleotide Polymorphism

SOD: Superoxide Dismutase

SSCP: Single-Strand Conformation Polymorphism

TCP: Tumor Control Probability

TD: Tolerance Dose

TGFβ: Transforming Growth Factor-β

TGFβR: TGFβ receptor

TIMP: Tissue Inhibitor of Metalloproteinase

TNFα: Tumor Necrosis Factor-α

UTR: Untranslated Region

XP(A-G): Xeroderma Pigmentosum complementation group A-G

XP: Xeroderma Pigmentosum

XRCC: X-ray Repair Cross-Complementing

Summary

Radiation therapy is a cornerstone in the management of cancer with approximately half of all newly diagnosed cancer patients receiving radiotherapy at some point in the treatment of their disease. Although the irradiation techniques have improved considerably over the years, normal tissue toxicity still occurs and varies widely from patient to patient. As the number of long-term cancer survivors increases, preventing or reducing late side effects has become a priority. The development of an *in vitro* assay capable to predict individual radiosensitivity prior to radiotherapy would enable the individualization of treatment schemes. As a consequence, radiation induced normal tissue toxicity could be minimized.

Gynecologic cancers account for an important percentage of cancers in women worldwide. Most gynecologic malignancies, particularly cervical and endometrial cancers, are treated surgically followed by radiotherapy. In the framework of the scientific work for this thesis, a patient group consisting of women with cervical or endometrial cancer who received radiotherapy was followed for late radiation toxicity.

During the past few decades, several *in vitro* assays have been developed in order to predict normal tissue reactions after radiotherapy. In the first part of this thesis, the value of the cytogenetic G_2 assay on peripheral blood lymphocytes in predicting late clinical radiosensitivity was assessed. With this test, the mean number of chromatid breaks per cell was found to be significantly higher in patients with late normal tissue reactions than in patients without normal tissue reactions. However, the G_2 assay lacked sensitivity to identify high-risk patients at the individual level. This leads to the conclusion that the G_2 assay has limited use in the clinical screening for individual radiosensitivity.

Currently, normal tissue radiosensitivity is considered as an inherited complex trait dependent on the interaction of multiple genes or gene products. As a result, it is assumed that normal tissue reactions could be predicted from individual genetic profiles. Because of the importance of DNA repair in cell and tissue response to radiation, the association

between eight single nucleotide polymorphisms (SNPs) in the DNA repair genes *XRCC1*, *XRCC3* and *OGG1* and the development of late radiotherapy reactions was investigated. The selection of these SNPs was based on literature data regarding a possible involvement in cancer predisposition and radiosensitivity. This study showed that the *XRCC3* IVS5-14 polymorphism is significantly associated with the risk of developing late radiation injury. The remaining individual SNPs could not be linked to an increased radiation toxicity, but it was shown that clinical radiosensitivity is significantly associated with a combination of different SNPs in *XRCC1* and *XRCC3* applying a risk allele analysis.

In the second part of this thesis, the involvement of three microsatellite polymorphisms in the DNA repair genes *XRCC1*, *XRCC3* and *XRCC5* in late clinical radiosensitivity was studied. This study was started because of promising results reported in the literature on the association between clinical radiosensitivity and rare microsatellites. Although no significant association was found between the repeat length at any of the microsatellites and the incidence of late radiotherapy complications, the possible involvement of small and large *XRCC1* repeats in clinical radiosensitivity could not be completely ruled out.

Over the years, it has become clear that normal tissue radiosensitivity is not exclusively caused by cell killing, but is the result of multicellular interactions between various cell types within a specific tissue or organ. The early activation of cytokine cascades after radiation exposure strongly affects the normal tissue response, and the profibrotic cytokine TGFβ1 plays a crucial role in the development of radiation induced late normal tissue reactions. Therefore, in the last part of this thesis, the association between six *TGFβ1* polymorphisms and the occurrence of late normal tissue reactions was investigated. This study showed that the -1.552delAGG, the -509C>T and the Leu10Pro polymorphisms are tightly linked, and that the risk of late radiation toxicity may be modulated by these polymorphisms.

In conclusion, the studies performed for this thesis contribute to the ongoing research aiming at the identification of genetic profiles associated with normal tissue radiosensitivity. Indications have been provided that polymorphisms in the *XRCC1* and *XRCC3* DNA repair genes and in the cytokine *TGFβ1* may be involved in the development of late radiation complications. The fact that similar associations for *TGFβ1* SNPs have also been reported by other independent studies, supports the possible involvement of these *TGFβ1* SNPs in late radiotherapy toxicity. However, to achieve a comprehensive understanding of the genetic basis underlying clinical normal tissue radiosensitivity and in order to use

genotype data for clinical radiosensitivity screening, large scale investigations making use of high-throughput technologies in a multicenter setting are required.

Samenvatting

Radiotherapie wordt toegepast bij ongeveer de helft van alle kankerpatiënten en vormt bijgevolg één van de belangrijkste behandelingsmethoden tegen kanker. Niettegenstaande de bestralingstechnieken de laatste jaren veel verbeterd zijn, treedt schade aan normale weefsels nog steeds op. Bovendien variëren deze stralingsgeïnduceerde complicaties sterk van patiënt tot patiënt. Door de stijgende overlevingsgraad van kankerpatiënten is de preventie of reductie van late neveneffecten ten gevolge van radiotherapie een prioriteit geworden. Met een *in vitro* test die de individuele radiosensitiviteit kan voorspellen vóór de radiotherapiebehandeling, zouden de behandelingsschema's verder kunnen aangepast worden zodat stralingsgeïnduceerde toxiciteit aan normale weesfels tot een absoluut minimum beperkt wordt.

Gynaecologische tumoren maken een groot deel uit van het aantal kankers in vrouwen over de hele wereld. De meeste van deze maligniteiten, in het bijzonder cervix- en endometriumkanker, worden operatief behandeld gevolgd door radiotherapie. Voor deze thesis werd een patiëntengroep bestaande uit vrouwen behandeld voor cervix- of endometriumkanker opgevolgd voor late stralingstoxiciteit.

De laatste 10-tallen jaren werden verschillende *in vitro* testen ontwikkeld teneinde schade aan normale weefsels ten gevolge van radiotherapie te voorspellen. In het eerste deel van deze thesis werd de toepasbaarheid nagegaan van de cytogenetische G_2 test op perifere bloedlymfocyten als biomerker voor late klinische radiosensitiviteit. Er kon aangetoond worden dat het gemiddeld aantal chromatidbreuken per cel significant hoger is in patiënten met late normale weefselreacties dan in patiënten zonder deze reacties. De G_2 test miste echter sensitiviteit om individuele patiënten met een verhoogd risico te identificeren. Bijgevolg heeft de G_2 test een beperkte bruikbaarheid bij de klinische screening voor individuele radiosensitiviteit.

Momenteel wordt radiosensitiviteit van normale weefsels beschouwd als een overerfbare complexe aandoening die bepaald wordt door de

interactie tussen verschillende genen of genproducten. Er wordt bijgevolg verondersteld dat klinische radiosensitiviteit voorspeld kan worden van individuele genetische profielen. DNA herstel is één van de belangrijkste mechanismen van cellen en weefsels als respons op straling. Daarom werd de associatie bestudeerd tussen acht single nucleotide polymorfismen (SNPs) in de DNA herstelgenen *XRCC1*, *XRCC3* en *OGG1* en de ontwikkeling van laattijdige radiotherapiereacties. De selectie van deze SNPs was gebaseerd op literatuurdata betreffende de mogelijke betrokkenheid van deze SNPs in kankerpredispositie en radiosensitiviteit. In dit werk werd aangetoond dat het *XRCC3* IVS5-14 polymorfisme significant geassocieerd is met het risico op de ontwikkeling van laattijdige radiotherapiereacties. De overige individuele SNPs konden echter niet geassocieerd worden met een verhoogde stralingstoxiciteit. Klinische radiosensitiviteit bleek wel significant geassocieerd met een combinatie van verschillende SNPs in *XRCC1* en *XRCC3* op basis van een risico-allel analyse.

In het tweede deel van deze thesis werd de betrokkenheid bestudeerd van drie microsatelliet polymorfismen in de DNA herstelgenen *XRCC1*, *XRCC3* en *XRCC5* bij late klinische radiosensitiviteit. Deze studie werd uitgevoerd uitgaande van veelbelovende literatuurdata die wijzen op de associatie tussen klinische radiosensitiviteit en zeldzame microsatellieten. Hoewel geen significante associatie gevonden werd tussen de lengte van één van de microsatellieten en het voorkomen van laattijdige radiotherapiereacties, kon de mogelijke betrokkenheid van korte en lange *XRCC1* herhalingen niet uitgesloten worden.

De laatste jaren is het duidelijk geworden dat radiosensitiviteit van normale weefsels niet enkel veroorzaakt wordt door celdood, maar het resultaat is van multicellulaire interacties tussen verscheidene celtypes in een specifiek weefsel of orgaan. De vroege activatie van cytokine cascades na stralingsblootstelling heeft een grote invloed op de respons van normale weefsels. Het profibrotische cytokine TGFβ1 speelt een cruciale rol in de ontwikkeling van stralingsgeïnduceerde late weefselreacties. Bijgevolg werd in het laatste deel van deze thesis de associatie nagegaan tussen zes polymorfismen in *TGFβ1* en het voorkomen van late toxiciteit van normale weefsels. Deze studie toonde aan dat het -1.552delAGG, het -509C>T en het Leu10Pro polymorfisme sterk met elkaar geassocieerd zijn, en dat het risico op late stralingstoxiciteit gemoduleerd kan worden door deze polymorfismen.

De studies uitgevoerd in deze thesis hebben bijgedragen tot het lopende onderzoek met als doel genetische profielen te identificeren die geassocieerd zijn met radiosensitiviteit van normale weefsels. Er werden

indicaties gegeven dat polymorfismen in de *XRCC1* en *XRCC3* DNA herstelgenen en in het cytokine *TGFβ1* betrokken kunnen zijn in de ontwikkeling van late radiotherapiereacties. Gelijkaardige associaties voor de *TGFβ1* SNPs werden ook reeds gerapporteerd in andere onafhankelijke studies. Dit ondersteunt de mogelijke betrokkenheid van deze *TGFβ1* SNPs in laattijdige radiotherapiereacties. Om tot een volledig begrip van de genetische basis van klinische radiosensitiviteit te komen en om genetische data aan te wenden in de screening naar klinische radiosensitiviteit, zijn echter grootschalige studies noodzakelijk die gebruik maken van high-troughput technologieën in een multicenter setting.

Résumé

Presque de la moitié des patients atteints de cancer sont traité par radiothérapie, montrant que la radiothérapie est un des plus importants traitements contre le cancer. Bien que les techniques d'irradiations soient améliorées considérablement les dernières années, du dommage aux tissus se présente encore. En outre les complications induites par l'irradiation varient de patient à patient. Comme le nombre de survivants au cancer augmente, la prévention ou la réduction d'effets secondaires tardifs à cause de la radiothérapie est devenue une priorité. La mise au point d'un test *in vitro* capable de prévoir la radiosensibilité préalablement à la radiothérapie permettrait d'individualiser le schèmes de traitement, de telle sorte que la toxicité induite par l'irradiation aux tissus normaux est réduite au maximum.

Les cancers gynécologiques représentent un pourcentage important des cancers chez les femmes dans le monde entier. La plupart de ces malignités, en particulier les cancers cervicaux et endométriaux, sont traités chirurgicalement suivi par la radiothérapie. Pour cette thèse un groupe de patients composé de femmes atteintes d'un cancer du cervix ou de l'endométre, traitées par radiothérapie a été suivi pour la toxicité tardive d'irradiation.

Au court des dernières décennies, plusieurs testes *in vitro* ont été mis au point à fin de prédire le dommage des tissus à la suite de la radiothérapie. Dans la première partie de cette thèse, la pertinence du test cytogénétique G2 sur des lymphocytes du sang périphérique, pour prévoir la radiosensibilité clinique tardive a été examiné. Ce test a démontré que le nombre moyen de cassures chromatides par cellule est significativement élevé chez des patients avec des réactions des tissus normaux par rapport à ceux sans réactions. Néanmoins, le teste G2 manque de sensibilité pour identifier les patients à haut risque au niveau individuel. Par conséquent le teste G2 n'a qu'une valeur restreinte en cas de screening clinique pour la radiosensibilité individuelle.

Actuellement, la radiosensibilité de tissus normaux est considérée comme une affection génétique héritée complexe, dépendant de l'interaction de gènes multiples ou de produits de gènes. Ainsi, il est supposé que les réactions de tissus normaux pourraient être prédit, se basant sur les profils individuels génétiques. Le mécanisme le plus important dans les cellules et dans les tissus, qui répond à l'irradiation est la réparation de l'ADN. Pour cette raison l'association entre huit single nucleotide polymorphismes (SNPs) dans des gènes de réparation de l'ADN *XRCC1*, *XRCC3* et *OGG1*, et l'apparition de réactions tardives de radiothérapie ont été examinées. Les SNPs ont été sélectés à base d'information déjà publiée concernant leur implication dans la prédisposition au cancer et à la radiosensibilité. Cette étude a démontré que le polymorphisme *XRCC3* IVS5-14 est significativement associé avec le risque de développer des réactions tardives à l'irradiation. Les autres SNPs individuels ne pouvaient pas être mis en rapport avec une radiosensibilité élevée, mais la radiosensibilité clinique s'est avérée associée avec une combinaison de différents SNPs dans *XRCC1* et *XRCC3* en appliquant l'analyse d'allèle de risque.

Dans la deuxième partie de cette thèse, l'implication de trois polymorphismes microsatellite dans les gènes de réparation d'ADN notamment *XRCC1*, *XRCC3* et *XRCC5* est examiné. Cette étude a été réalisée en raison de résultats prometteurs qui ont été publié concernant l'association entre la radiosensibilité clinique et des microsatellites peu fréquents. Bien qu'aucune association significative n'ait été trouvée entre la longueur de la répétition des microsatellites et l'incidence des complications tardives dues à la radiothérapie, l'implication possible de répétitions courtes et longues de *XRCC1* dans la radiosensibilité clinique ne peut pas être complètement exclue.

Au fil des années, il est apparu clairement que la radiosensibilité de tissus normaux n'est pas uniquement causée par la mort cellulaire, mais qu'elle est le résultat d'interactions multicellulaires entre différents types de cellules dans un tissu en particulier ou un organe. L'activation précoce des cascades de cytokines après l'exposition à l'irradiation, influence fortement la réaction des tissus normaux. La cytokine profibrotique TGFβ1 joue un rôle crucial dans le développement de réactions tardives de tissus normaux. Par conséquent, dans la dernière partie de cette thèse, l'association entre six polymorphismes de *TGFβ1* et la présence de réactions tardives de tissus normaux ont été examinées. Cette étude a démontré que les polymorphismes -1.552del AGG, -509C>T et Leu10Pro sont étroitement liés, et que le risque de toxicité tardive peut être modulée par ces polymorphismes.

En conclusion, les études réalisées pour cette thèse contribuent à la recherche en cours qui tache d'identifier des profiles génétiques liés à la radiosensibilité de tissus normaux. Des indications sont fournies que les polymorphismes dans les gènes réparateurs *XRCC1* et *XRCC3* et la cytokine *TGFβ1* puissent être impliqués dans le développement de complications tardive dues à la radiothérapie. Le fait que des associations semblables à propos des SNPs *TGFβ1* ont été rapportées par d'autres études indépendantes, soutient l'implication éventuelle de ces SNPs *TGFβ1* dans les réactions tardives de tissus normaux. Pour obtenir une compréhension complète de la base génétique sous la radiosensibilité clinique de tissus normaux et pour appliquer les données génétiques dans le dépistage de la radiosensibilité clinique, des études multicentres à grande échelle utilisant des technologies à haut débit sont nécessaires.

Chapter 1

Introduction

1.1 The biology of radiotherapy

Radiation therapy uses ionizing radiation in the treatment of patients with malignant diseases. While in the past radiotherapy used Cobalt-60 gamma rays, contemporary radiotherapy is based on high-energy X-rays and electrons generated by linear accelerators. The aim of radiation therapy is to deliver a precisely measured dose of radiation to a defined tumor volume with as minimal damage as possible to surrounding healthy tissues, resulting in eradication of the tumor, a high quality of life and prolongation of survival.

1.1.1 Mechanism of cell killing by ionizing radiation

Ionizing radiation results in at random excitations and ionizations within the material of interaction. Depending on the localization of these interactions in the cellular environment, the effect is classified as direct or indirect (figure 1.1). The direct effect occurs when radiation deposits its energy directly into critical biological molecules (e.g. DNA), resulting in ionization of this target. In the indirect effect, the damage to the target is caused by reactive species produced by ionizations elsewhere in the cell. These indirect effects result mainly from the ionization of water molecules, leading to the formation of highly reactive free radicals (reactive oxygen species or ROS) which cause most of the damage in the cell (1).

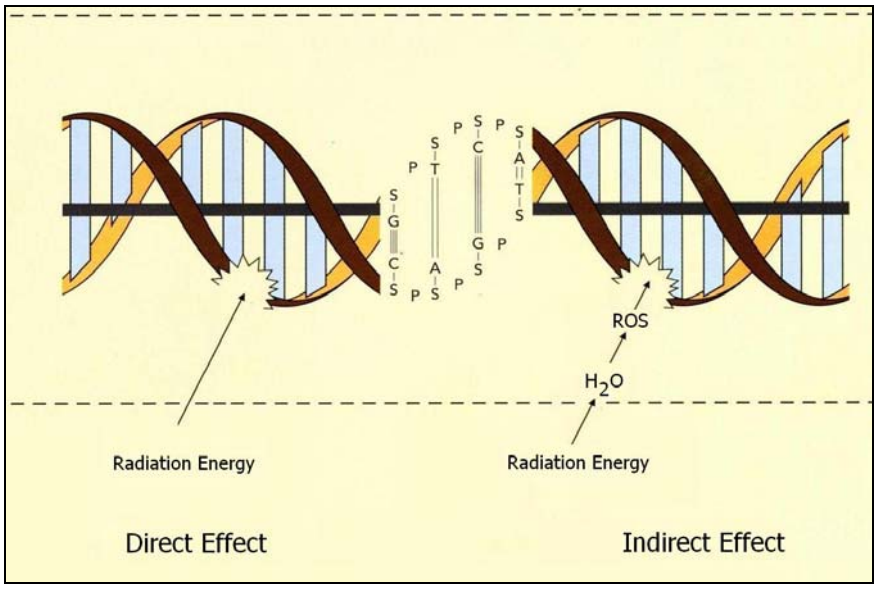

Figure 1.1. Direct and indirect effects of ionizing radiation on DNA. Modified from Vanmarcke *et al.* (2).

The main biological effect of radiation is a result of damage to DNA, which is the principal target of ionizing radiation within the cell. The produced lesions include base or sugar damage, DNA single-strand and double-strand breaks, DNA-protein crosslinks and DNA-DNA crosslinks. These different types of DNA damage can occur separately or together and result in complex damaged sites. Base damage and DNA single-strand breaks are of little consequence to cancer treatment by ionizing radiation because cells have evolved efficient error-free DNA repair mechanisms to repair this type of damage. However, if the density of ionizations (dose) is high enough, two single-strand breaks can be close together and result in a double-strand break. Induction of DNA double-strand breaks is the dominant mechanism of cell killing by ionizing radiation. Together with complex damaged sites they are often unrepaired or misrepaired, resulting in stable or unstable chromosomal aberrations. Unstable chromosome aberrations can result in mitotic cell death, whereas stable chromosome aberrations can lead to mutations and altered gene expression. Apart from mitotic cell death following DNA damage, irradiation can also lead to cell death via apoptosis and necrosis. Apoptosis is a process of active cell death resulting from DNA or cell membrane damage. The process consists of sequential stages of nuclear condensation, fragmentation, phagocytosis and degradation. Necrosis is a passive form of cell death and is characterized by an early loss of membrane permeability (1,3).

1.1.2 Cellular response to ionizing radiation damage

Radiation induced DNA damage initiates a complex series of cellular responses. As shown in figure 1.2, these responses occur after a cascade of reactions in which the DNA damage is detected by sensor proteins that trigger the activation of transducer proteins which amplify and diversify the signal by targeting specific downstream effectors. The outcome of this dynamic cascade includes cell cycle control, DNA damage repair, apoptosis, and cytokine transcription. Multiple proteins, including poly(ADP-ribose)polymerase (PARP), DNA-dependent protein kinases (DNA-PKcs) and Ku70/80, are involved in the sensor phase. Ataxia telangiectasia mutated gene (ATM), ataxia telangiectasia-related gene (ATR) and p53 play a central role in the transducer phase (4,5). Non-DNA lesions can also stimulate various signal transduction pathways, as the ceramide and the protein kinase C (PKC) pathway, leading to cellular responses including apoptosis and altered cytokine transcription (1).

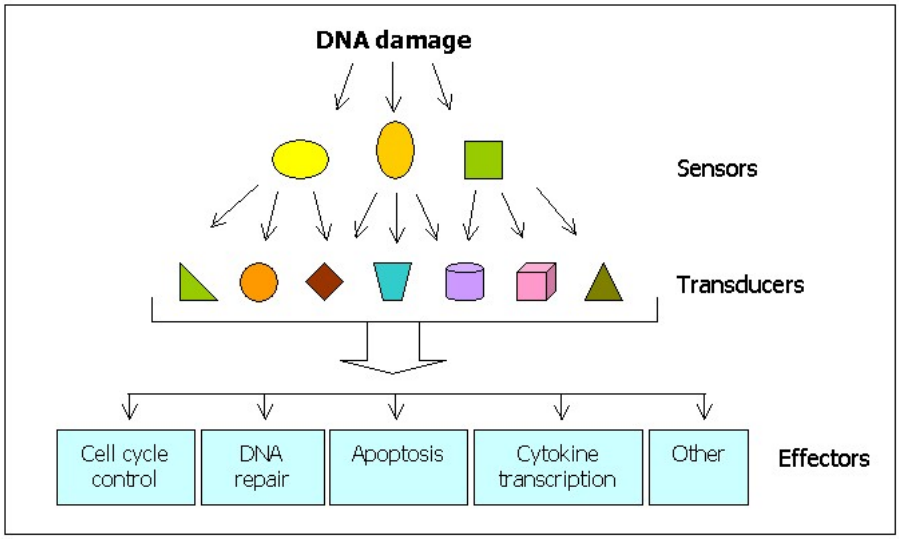

Figure 1.2. General cascade of cellular responses to radiation induced DNA damage. Modified from Jackson *et al.* (5).

1.1.2.1 Cell cycle control

Cell cycle progression is monitored by cell cycle checkpoints which represent intracellular signalling pathways leading to cell cycle delay. The delay allows cells to initiate an event only when all conditions for successful completion of this event are fulfilled. DNA damage can lead to activation of cell cycle checkpoints at G1, S and G2 allowing actively dividing cells to arrest and repair DNA damage before initiating or continuing replication of their genome, or before segregating the

replicated genome into daughter cells. Depending on the complexity of the DNA damage and on the efficiency of the DNA repair mechanisms, cells will either resume cycling or undergo apoptosis (1). Key players in DNA damage induced checkpoint responses are ATM, cell cycle checkpoint kinase-1/2 (CHK1/2), p53, and cell division cycle-25 (CDC25) family proteins (6).

1.1.2.2 Repair of DNA damage

Cells respond to DNA damage by activating DNA repair mechanisms which can be divided in error-free, where the DNA is repaired correctly, or error-prone mechanisms, which can result in misrepair. Depending on the type of DNA damage, specific pathways are recruited. The five major, partly overlapping DNA damage repair pathways are base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) (7).

BER repairs non-bulky lesions such as small chemical base alterations and DNA single-strand breaks caused by ionizing radiation or oxidative damage. The intact complementary strand is used as template which results in a mainly error-free repair. In the case of base damage, BER starts with the recruitment of specific DNA glycosylases (e.g. 8-oxoguanine DNA glycosylase or OGG1), which bind to and release the modified base resulting in an apurinic/apyrimidinic (AP) site. An AP site can also occur spontaneously by hydrolysis. Bi-functional glycosylases further cleave the sugar-phosphate backbone, whereas repair initiated by mono-functional glycosylases requires the activity of AP endonucleases (e.g. APE1) to incise the sugar-phosphate backbone. In the case of single-strand breaks, the reaction is initiated by the recruitment of PARP and polynucleotide kinase (PNK) to protect and prepare the ends for repair. The short-patch pathway is the dominant mode for the remainder of the reaction. DNA polymerase-β incorporates a nucleotide and removes the abasic residue, ligation is performed by the ligase3/x-ray repair cross-complementing group 1 (XRCC1) complex. The XRCC1 scaffold protein interacts with most of the above BER core components and is therefore crucial in protein exchange. A subpathway of BER, the long-patch repair, results in the replacement of several nucleotides and is used in the presence of modified AP sites which can not be excised by DNA polymerase-β. This pathway involves DNA polymerase-β, polymerase-δ/ε and proliferating cell nuclear antigen (PCNA) for repair synthesis, as well as the flap structure-specific endonuclease-1 (FEN1) to remove the displaced DNA flap and ligase1 for sealing (7,8,9). Both pathways are

illustrated in figure 1.3. No human diseases are currently known to be associated with a defect in BER, which may be due to embryonic lethality or functional redundancy (8). However, it has recently been suggested that ataxia-oculomotor apraxia 1 (AOA1), which is caused by aprataxin gene mutations, may be a novel DNA damage response-defective disease as aprataxin physically interacts with XRCC1 and as cells deficient in aprataxin are defective in DNA single-strand break repair (10). In contrast to the BER pathway, which requires a number of proteins, some small chemical base alterations such as O-alkylated bases can also be repaired by the action of a single protein which directly removes the damage. An example is O^6-methylguanine which is repaired by O^6-methylguanine-DNA methyltransferase (MGMT) (8).

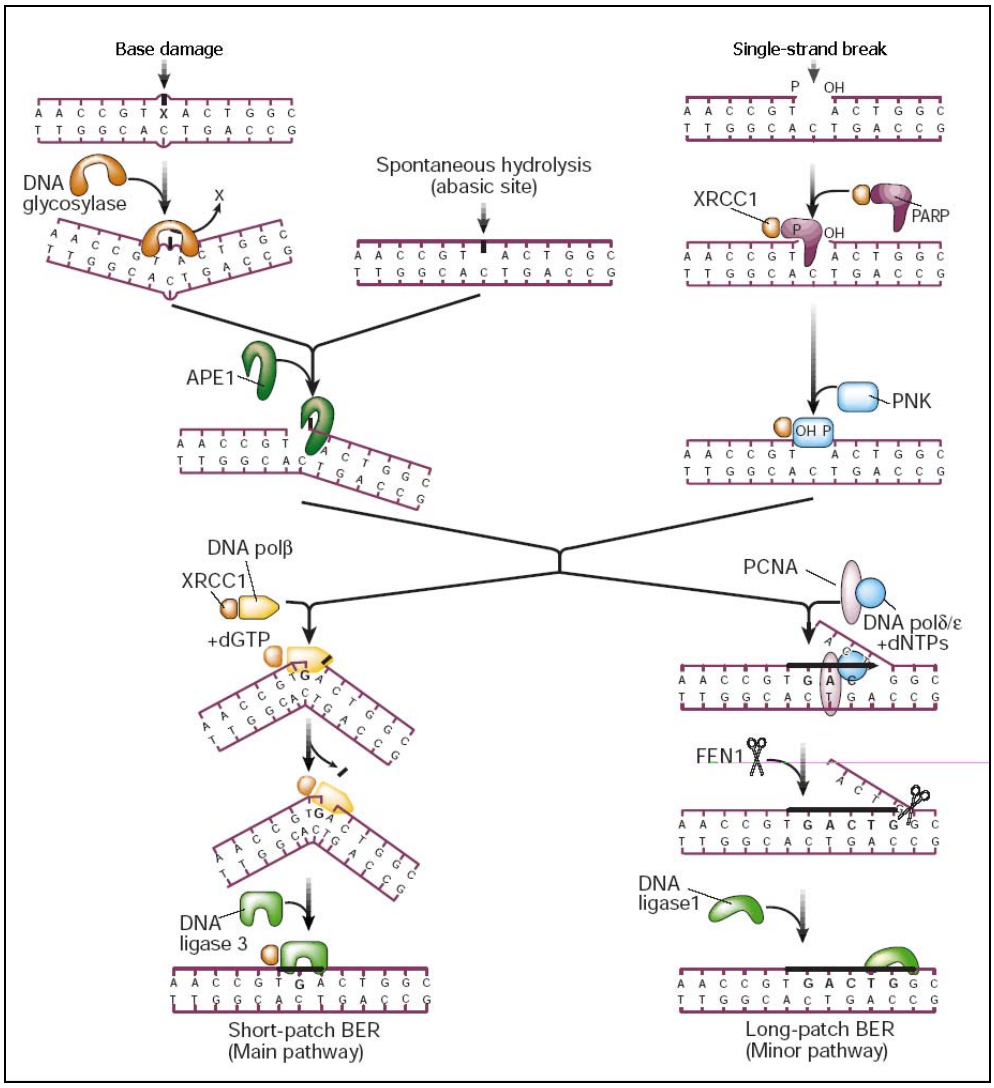

Figure 1.3. Mechanism for base excision repair. Modified from Hoeijmakers *et al.* (7).

NER and MMR are less important to repair DNA damage induced by ionizing radiation. NER is able to detect a variety of bulky DNA lesions including DNA adducts and cross-links induced by UV light. Two NER subpathways exist: global genome NER which surveys the entire genome for damage, and transcription-coupled NER which focuses on damage that blocks transcription. Both NER mechanisms involve damage recognition and assembly of the incision complex, dual DNA incision and damage excision, DNA repair synthesis and ligation. More than 25 proteins, including the XP(A-G) proteins, participate in the NER pathway. The human syndrome xeroderma pigmentosum (XP), which is characterized by extreme photosensitivity and an increased incidence of sunlight-induced skin cancer, is caused by genetic defects in this pathway (11). MMR removes nucleotides misrepaired by DNA polymerases and insertion/deletion loops that result from slippage during replication of repetitive sequences or during recombination. MMR proceeds via mismatch recognition, identification of the wrong strand, degradation past the mismatch, resynthesis of the excised fragment, and ligation. These processes involve multi-member families of the MSH2 and MLH1 proteins. Defects in the MMR system dramatically increase mutation rates and are associated with hereditary non-polyposis colorectal cancer (HNPCC) (7).

DNA double-strand breaks, the dominant type of DNA damage by ionizing radiation, can be repaired by HR or NHEJ. HR uses a homologous DNA template and is highly accurate, whereas NHEJ rejoins the broken ends without using a template, which often results in the loss of a few nucleotides. NHEJ is the main pathway to repair double-strand breaks in mammals and is more active in the G1 cell cycle phase. HR acts mainly during S and G2 phases using the undamaged sister chromatid or the homologous chromosome as template (8). The two pathways are well described in literature and are presented in figure 1.4. HR starts with nucleolytic resection of the double-strand break by the MRE11/Rad50/NBS1 complex, forming single-stranded DNA fragments which are bound by replication protein A (RPA). Next, a Rad51 nucleoprotein filament, including Rad51-related proteins XRCC2, XRCC3 and Rad51B-D, is assembled stimulated by Rad52. The Rad51 filament catalyzes, facilitated by Rad54, strand invasion into a homologous DNA duplex. Subsequently, the strand is extended by DNA polymerase, the ends are ligated by DNA ligase1, and DNA crossovers (Holiday junctions) are resolved by resolvases (5,7,8). The NHEJ pathway is initiated by binding of the Ku70/Ku80 complex to the free DNA ends, followed by recruitment of DNA-PKcs. Double-strand breaks that are not suitable for direct ligation may be processed by the MRE11/Rad50/NBS1

complex and other nucleases, such as FEN1. In addition, a DNA polymerase may be required. Finally, the DNA ends are rejoined by the ligase4/XRCC4 complex (5,7). Defective repair of DNA double-strand breaks can result in genomic instability or mutations. A number of human syndromes, such as ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS), as well as breast and ovarian cancer caused by BCRA1/2 mutations, are associated with defects in the regulation of double-strand break repair (8).

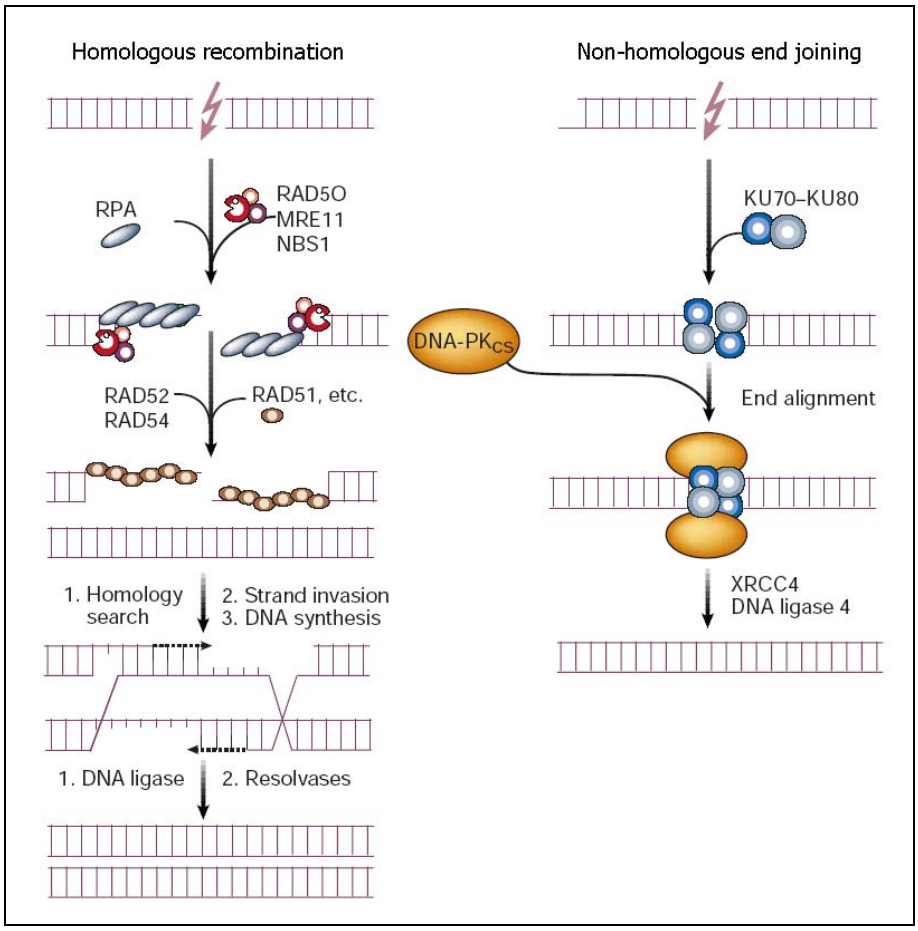

Figure 1.4. Mechanism of homologous recombination and non-homologous end joining. Modified from Hoeijmakers *et al.* (7).

1.1.2.3 Apoptosis

Apoptosis or programmed cell death is a precisely regulated, complex cascade of molecular events to eliminate individual cells. The process can be considered as a protective mechanism of the cell since it removes highly damaged cells. The occurrence of radiation induced apoptosis depends on the cell type, cell cycle phase, dose rate and dose fractionation. The triggers for apoptosis include signals in the nucleus, as

well as in the cell membrane, where the ceramide pathway is initiated. Apoptosis is regulated by a number of proteins including p53, ATM, c-myc, B-cell CLL/lymphoma (BCL2) and BCL2-associated X protein (BAX), and can be mediated by p53-dependent and p53-independent pathways (1,12).

1.1.2.4 Cytokine transcription

Radiation mediated gene expression also results in the induction and release of cytokines and growth factors by many cell types including macrophages, epithelial cells and fibroblasts (13). These proteins influence the communication between cells and allow a signalling cell to transfer and amplify the radiation damage response to other exposed and non-exposed cells (14). Examples of radiation induced cytokines are tumor necrosis factor-α (TNFα), interleukin-1 (IL1) and IL6. Radiation inducible growth factors include transforming growth factor-β (TGFβ) and platelet-derived growth factor (PDGF). Cytokines can provoke an inflammatory response and contribute, together with growth factors which stimulate the expression of extracellular matrix proteins, to the pathogenesis of tissue damage (15).

1.1.3 Tissue response to ionizing radiation – adverse radiation effects

The pathological processes of tissue response to radiation start immediately after exposure, but the clinical and histological features may not become visible for weeks, months or even years after treatment. Radiation responses are generally classified as acute, consequential or late effects according to the time of appearance of symptoms. Acute (or early) effects are observed during treatment or within a few weeks after treatment. Consequential effects, or consequential late effects, appear later and are caused by persistent acute damage. Late effects emerge typically after latency periods of months to years (16). The terms acute and late have been used for convenience in radiation therapy, but because the underlying molecular and cellular processes are complex and lead to a range of events, the definitions may be more operational than mechanistic (17).

1.1.3.1 Acute effects

Acute radiation damage is most prominent in tissues with rapidly proliferating cells, such as epithelial surfaces of the skin or gastro-intestinal tract and red bone marrow. Radiation effects occur when functional cells are lost as part of normal tissue turnover and are not replaced because of damage to the stem cell compartment. Compensatory proliferation within the stem cells, followed by replacement of functional cells may lead to rapid recovery of the injury (18). Although cell killing is a critical part of the etiology of tissue response after radiation, not all acute tissue responses can be explained by parenchymal cell death. Radiation induced inflammatory responses, vascular injury and wound-healing processes may be involved in the development of acute radiation effects such as skin edema and erythema (19).

1.1.3.2 Late effects

Late effects tend to occur in tissues with a slow turnover of cells, such as subcutaneous tissue, muscle, kidney, and liver, and in sites of slow turnover within tissues that contain rapidly proliferating cells, such as the wall of the intestine (18). For many years, cell depletion and the long cell cycle time of the target cells has been considered to be responsible for late radiation injury (20). However, is has become clear that late radiation effects are not exclusively caused by parenchymal or vascular cell killing, but develop through interactions between multiple cell types within a particular organ (21). The radiation induced acute inflammatory response is followed by an aberrant chronic inflammatory/wound-healing response that develops over months to years and leads, together with cell loss, to cellular infiltration of e.g. macrophages and fibroblasts, fibrin leakage, collagen deposition and dysregulated cellular interactions (figure 1.5) (19,22).

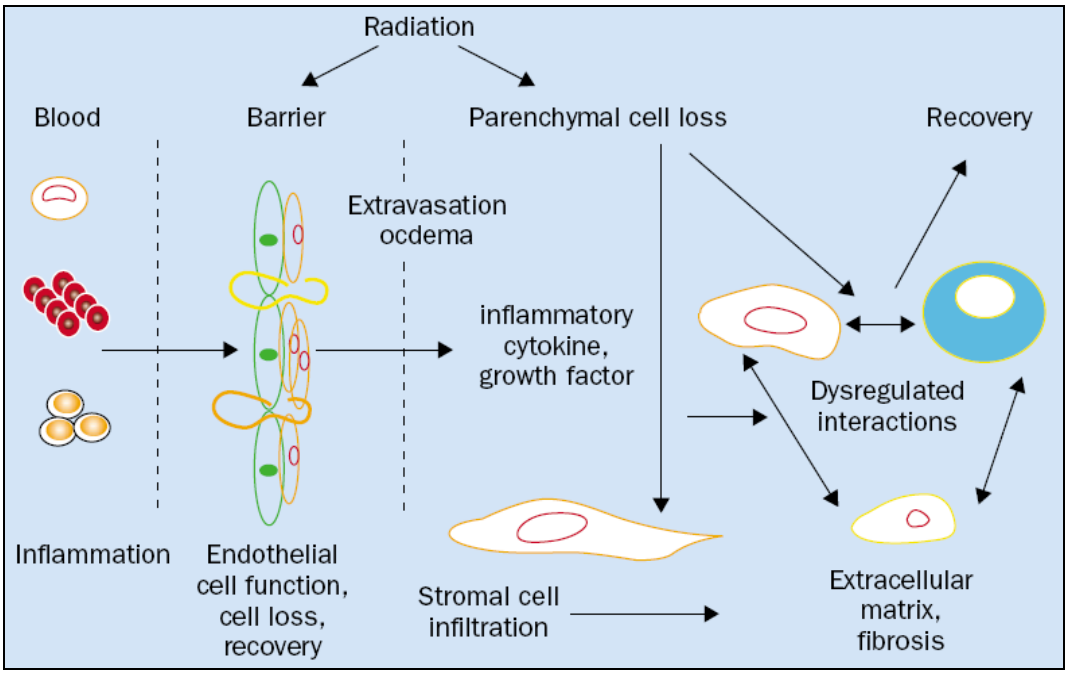

Figure 1.5. Scheme of the tissue response after radiation exposure. From Stone *et al.* (18).

Generally, vascular and parenchymal cell dysfunction and cell loss, associated with chronic overproduction of cytokines and growth factors, results in fibrosis, necrosis, atrophy and/or vascular damage, depending on the involved organ (23,24). The response may be perpetuated by post-radiation tissue hypoxia, since hypoxia can generate ROS, promote inflammation and collagen formation, increase vascular permeability and activate profibrotic cytokines (25). The multifunctional cytokine TGFβ plays a significant role in late tissue reactions, particularly in fibrosis, via the induction and deposition of extracellular matrix proteins (e.g. collagen), inhibition of proteases involved in the degradation of the extracellular matrix, growth stimulation of fibroblasts and inhibition of endothelial cell proliferation. TGFβ is secreted as an inactive latent complex and can be activated by radiation and ROS (24,26).

1.1.3.3 Consequential late effects

Acute reactions which fail to heal completely can persist into the late period and add to the overall damage. The resulting chronic lesions are termed consequential late effects. Because of the introduction of unconventional aggressive treatment regimens with combined modalities, consequential late effects are increasingly observed. These effects are mainly found in organ systems where the acute response (of the epithelial lining) is associated with an impairment of the barrier

against mechanical or chemical stress, which may cause additional trauma to the underlying tissues. Therefore urinary and intestinal systems, mucosa, and skin are most susceptible. One prominent example is acute, confluent oral mucositis in head and neck tumour patients, which may result in chronic, non-healing ulcers and necrosis (27,28).

1.1.3.4 Treatment related factors

The risk, severity and type of early, consequential and late tissue reactions depend on several factors. Radiation related treatment factors include total dose, dose per fraction and treatment schedule. Due to the observations that late effects are less severe and better local tumor control rates can be achieved with multiple small radiation fractions than with one or a few large fractions, fractionated radiotherapy treatments are currently being used. Late effects are generally more sensitive to changes in fraction size, and less sensitive to changes in overall treatment time than early responses (29,30). The use of chemotherapy can intensify the reactions (31). The volume of normal tissue receiving high radiation doses is also important, with larger volumes carrying higher risk of functional damage (32). Additionally, the functional reserve and structural organization of normal tissue may determine the tolerance of the tissue. For example, the lung is able to tolerate a high dose in a small volume, but is less able to tolerate a low dose to the whole lung. On the other hand, a high dose to a small volume of the spinal cord can be dangerous, whereas a low dose to a large area can be harmless (33).

1.1.3.5 Patient related factors

Patient related factors in normal tissue response after radiotherapy treatment include trauma or surgery in irradiated sites and coexisting morbidities such as diabetes or hypertension (34,35). Age and lifestyle related factors (e.g. smoking, nutrional status) may also be associated factors (36). Studies from intra- and inter-patient variability in radiation response indicate the existence of a genetic or physiological factor that alters the expression of a specific type of normal tissue reaction after radiotherapy, and the probability that this factor is differentially expressed in various cells or tissues (37-40). The hypothesis that genetic variations are involved in radiosensitivity has found support in the observed hyper-radiosensitivity associated with rare genetic syndromes such as AT or NBS, which both result from rare, highly penetrant germ-line mutations in DNA repair genes (41). Despite the fact that these disease causing mutations provide the proof of principle for the link

between genetic factors and radiosensitivity, they do not seem to be responsible for the patient-to-patient variability observed in individuals receiving radiotherapy (42-44). Further evidence for a genetic basis of radiosensitivity came from the observation that certain strains of mice have a greater genetic predisposition to develop radiation reactions (45).

1.1.3.6 The role of the tumor

Since tumors have the ability to change their surroundings, the presence of the tumor may affect normal tissue injury. Tumors physically modify normal tissue architecture resulting in defects that can add to damage produced by therapy (46,47). Moreover, tumors release proteolytic enzymes that facilitate cell invasion and metastasis (48). Tumor vessels leak fibrinogen, which is converted to fibrin, resulting in collagen deposition and fibrosis (49). Loss of heterozygosity in genes has been observed in tissues adjacent to breast and bladder tumors, and in a gene, possibly affecting bio-availability of proinflammatory and profibrotic cytokines, in tumor cells of lung cancer patients (50,51).

1.1.3.7 Assessing normal tissue responses

Multiple scoring systems have been developed for the grading of adverse effects after cancer treatment. The most widely used systems over the last 20 years are the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer (RTOG/EORTC) scoring system for assessing acute and late radiotherapy effects, the Late Effects Normal Tissue/Subjective Objective Management Analytical (LENT/SOMA) scoring system for evaluating late radiotherapy effects and the Common Toxicity Criteria v2.0 (CTCv2.0) scoring system for assessing acute radiotherapy and chemotherapy effects (52-54). Recently, the Common Terminology Criteria for Adverse Events v3.0 (CTCAEv3.0) scoring system was developed from the CTCv2.0 scale and the LENT/SOMA scale (55). The merged scoring system includes early and late responses, and is applicable to radiotherapy, chemotherapy, surgery, other treatment modalities, and combinations of therapies. The scale is adequate for all organs and displays grades 1 through 5 with unique clinical descriptions of severity for each adverse effect (56). For example, radiation associated skin dermatitis is described as faint erythema or dry desquamation (grade 1), as moderate erythema, moist desquamation mostly confined to skin folds and moderate edema (grade 2), as moist desquamation outside skin folds and bleeding induced by minor trauma

or abrasion (grade 3), as skin necrosis or ulceration and spontaneous bleeding (grade 4) and as death (grade 5).

1.1.4 Tumor response to ionizing radiation

Tumors are composed of proliferative cells, resting cells, differentiated cells and dead or dying cells. The tumor growth rate is determined by the cell cycle time, the growth fraction and the rate of cell loss, and can vary widely among tumors of the same histological type (57). Radiation therapy aims at killing the clonogenic cells leading to tumor cure. The response of a tumor to radiation is seen by regression and is determined by tumor hypoxia, proportion of clonogenic cells, inherent radiosensitivity of tumor cells, and repair of radiation damage. Accelerated repopulation and the tumor-bed effect are other important processes in tumor radiobiology (58). Cytokine alterations in immune, inflammatory and angiogenic regulatory routes within the tumor microenvironment may also play a critical role in tumor response to radiation therapy (59).

1.1.5 Tumor control probability and normal tissue complication probability

Whereas the probability of killing the last surviving clonogenic cell in a tumor is expressed as tumor control probability (TCP), the probability to produce damage to normal tissues is defined as normal tissue complication probability (NTCP). The relationship between both probabilities and dose is threshold-sigmoid (figure 1.6). Above a certain threshold, tumor control and complication rates increase steeply. Curves for normal tissue complications are less well-defined than tumor control curves. However, it is likely that they are steeper than tumor control curves, reflecting less heterogeneity in the biology of normal tissues than of tumors. Thus, in practice, the TCP curve is always likely to be shallower than that for complication and may actually cross it. Radiotherapy treatment aims at maximal TCP, which increases with increasing dose, but which is restricted by NTCP. The therapeutic ratio of the treatment increases with a greater shift of the NTCP to the right. This displacement can be achieved by other treatment modalities like surgery or chemotherapy, or dose fractionation or dose rate effects (60).

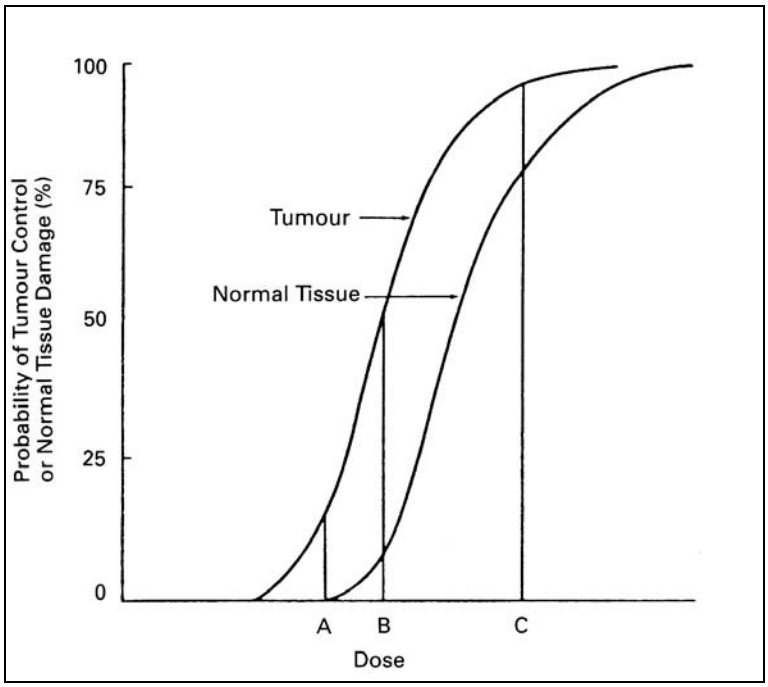

Figure 1.6. Dose response curves for tumor control probability and normal tissue complications. A, B and C illustrate doses yielding different ratios of tumor control to normal tissue complications. From Withers (60).

1.1.6 Dose fractionation

The total dose in radiotherapy is almost always given as a series of fractions, commonly once a day, five days a week. In curative therapy, doses of 1.8-2 Gy per fraction are most often used. Each equal dose-fraction kills the same proportion of cells, resulting in a logarithmic decrease in the number of surviving cells. Consequently, small differences in survival from each dose-fraction amplify into large differences after 25-35 fractions. The biologic basis of dose fractionation can be considered in terms of the 4Rs: repair of cellular damage, repopulation by surviving viable cells, redistribution within the mitotic cycle and reoxygenation in tumors (3,61).

1.1.6.1 Repair

Repair of sublethal cellular injury following irradiation is completed in a few hours and the degree and the rate varies among tissues. In general, slowly proliferating tissues are capable of greater repair than highly proliferating normal tissues and most malignant tumors. Thus, by using a large number of small dose fractions spaced by at least six hours, the

greater recovery in late responding normal tissues is exploited. This leads to relatively higher survival of cells in those tissues compared to tumor cells, and an exponential increase in therapeutic differential.

1.1.6.2 Repopulation

Following irradiation of normal and malignant tissues, dead cells are replaced by recruitment of resting cells into the cell cycle. This is a rapid process in highly proliferative normal tissues and in malignant tumors. As a result, such tissues can tolerate higher doses if treatment duration is extended. In both normal tissues and tumors, repopulation is preceded by a lag period of varying length during which there is no evidence of regeneration. In general, the lag period is shorter and repopulation is faster in highly proliferative normal tissues than in tumors. Consequently, the therapeutic differential between these tissues and tumors is usually enhanced by prolongation of the overall duration of radiation therapy. Nevertheless, the enhancement varies with the kinetic profile of the tumor and the possibility of accelerated repopulation in treated tumors should be taken into consideration. In those cases the radiation dose should be delivered in the shortest possible time within the limits of acute radiation tolerance. Cells in slowly proliferating normal tissues do not repopulate within the time course of radiotherapy and are not influenced by a prolonged course of radiotherapy.

1.1.6.3 Redistribution

The radiation sensitivity of cells varies according to the cell cycle phase they are in at the time of radiation exposure. In general, cells are most resistant in the S phase and most sensitive in the late G2 and M phases. When a proliferative tissue such as a tumor is exposed to small doses, the radiosensitive subpopulations are affected more than cells in the radioresistant phases of the cell cycle. Fractionation of the radiotherapy treatment allows the surviving, relatively radioresistant cells to progress towards more radiosensitive cell cycle phases. Highly proliferating normal tissues will also undergo the self-sensitizing effect of cell cycle redistribution between dose fractions. In contrast, for slowly proliferating normal tissues, redistribution has a neutral effect since cells in these tissues are static within the relatively radioresistant G_0 phase. Radiation induced cell cycle delay also influences the process of redistribution.

1.1.6.4 Reoxygenation

As solid tumors grow, their increased demand for nutrients can often not be met by their vascular supply. As a result, poorly vascularised tumors develop areas of hypoxia and necrosis. Hypoxic cells are known to be 2-3 times more radioresistant than well oxygenated cells. The oxygen effect occurs because oxygen expands the damage by producing free radicals. The existence of hypoxia in tumors is a potential cause of treatment failure in radiation therapy. If a large single dose is replaced by multiple small fractional doses, the influence of hypoxic cells can be reduced or eliminated by a process of reoxygenation during the interfraction intervals. Reoxygenation of hypoxic cells can occur by recirculation of blood through vessels that were temporarily closed, or by reduced metabolic activity of sterilized cells. Other possible mechanisms include removal of sterilized cells which permits better access to oxygen, and a reduction in intercapillary distances allowing oxygen to reach the hypoxic cells. Furthermore, reoxygenation of hypoxic tumor cells leads to DNA damage resulting in cell death.

1.1.7 Dose rate effect - brachytherapy

The dose rate at which irradiation is delivered influences the biologic response to a given dose. The dose rate effect is most apparent between 6 Gy/hr and 60 Gy/hr. External beam radiotherapy uses dose rates in the range of 60-300 Gy/hr (1-5 Gy/min). As the dose rate is lowered, the time to deliver a certain radiation dose increases and biologic processes that modify radiation response can take place during irradiation. The same processes (repair, repopulation, redistribution, reoxygenation) that apply to dose fractionation, are involved in low dose rate continuous irradiation. Continuous low dose rate irradiation is particularly applied in interstitial or intracavitary radiotherapy (brachytherapy), delivering doses at the rate of 0.4 to 2 Gy/h. An additional physical dose effect appears after irradiation from an implanted source within a tumor. The rapid decline of dose with distance results in a high dose rate and subsequently a high degree of cell killing close to the irradiation source, and a lower dose rate and less cell killing further away from the irradiation source ensuring sparing of surrounding normal tissues. At present, the brachytherapy dose is often delivered in a series of pulses (pulsed dose rate) where every pulse delivers approximately 0.5 Gy per 10-minute exposure every hour (62,63).

1.2 Gynecologic cancer

1.2.1 Overview

Cancers of the female genital tract account for an important proportion of cancers in women. The global cancer statistics from the International Agency for Research on Cancer (IARC) indicate that gynecologic cancers accounted for 19% of the 5.1 million estimated new cancer cases, 2.9 million cancer deaths and 13 million 5-year prevalent cancer cases among women in the world in 2002. Cervical cancer accounted for 493.000 new cases and 273.000 deaths; ovarian cancer[1] for 204.000 new cases and 125.000 deaths; endometrium cancer for 199.000 new cases and 50.000 deaths; cancers of the vulva, vagina and choriocarcinoma together constituted 45.900 cases. More than 80% of the cervical cancer cases occurred in developing countries and two-thirds of endometrium cancer cases occurred in the developed world (64).

1.2.1.1 Cancer of the cervix

Cancer of the cervix is the second most common life-threatening cancer in women worldwide (65). This cancer is more frequent in women who had intercourse at early age, have a history of sexual promiscuity, or had a large number of pregnancies. Epidemiological and clinical data indicate that human papillomaviruses (HPV), especially HPV-16 and HPV-18, play a major role in the etiology of cervical cancer. Other factors, such as herpex simplex virus type 2 infections, cigarette smoking, vaginal douching, nutrition, and use of oral contraceptives, have been proposed as contributing factors. Cervical cytology screening programs have led to a reduction in the incidence and mortality in the developed world. Prophylactic HPV vaccines protect against persistent infection in fully vaccinated women, but are unfortunately not available in the developing world (65-67). Different types of treatment are available for patients with cervical cancer. These comprise surgery, external beam radiotherapy, brachytherapy, chemotherapy or a combination of these treatments. After cervical cancer therapy, patients can be treated with replacement hormones (66).

[1] Ovarian cancers together with fallopian tube cancers and extraovarian primary peritoneal cancers

1.2.1.2 Cancer of the ovary

Ovarian cancer is the most lethal gynecologic malignancy. The high mortality can be attributed to the lack of symptoms related to the disease. As a result, most cases are diagnosed at an advanced stage. Ovarian cancer is a disease of older women, with a peak incidence in the 50- to 70-year age group. Endocrine, genetic and environmental factors have been identified in the carcinogenesis of ovarian cancer. Although the majority of ovarian cancer cases are sporadic, family history of the disease is the strongest risk factor. The risk is also increased in women with a history of breast cancer and in *BRCA1/2* mutation carriers. Furthermore, ovarian cancer is part of the phenotype of the HNPCC syndrome. The incidence is reduced by pregnancy, lactation, the oral contraceptive pill and tubal ligation (68-70). Fallopian tube cancer is a very rare female genital tract malignancy and is considered along with ovarian cancers because the two diseases often have analogous histological, biological and clinical characteristics (64). Treatment of ovarian cancer is dependent on clinical stage, but most patients will require pelvic clearance and adjuvant chemotherapy. For a minority of patients hormonal therapy is a therapeutic option (68).

1.2.1.3 Cancer of the endometrium

Endometrial cancer, cancer of the lining of the uterus, is the most common gynecologic malignancy in the developed world. The peak incidence occurs in postmenopausal women between 50 to 70 years old. Mortality rates for endometrial cancer are relatively low because early-stage localized disease usually leads to abnormal uterine bleeding resulting in early detection. Many of the risk factors for development of endometrial cancer have been identified, and most relate to estrogen levels. Obesity, hypertension and diabetes also predispose women to development of endometrial cancer. A small increase in risk is found in women with a family history of endometrial cancer and the malignancy can also be part of the HNPCC syndrome (71-73). The basic treatment for patients with endometrial cancer is radical surgery, postoperative external beam pelvic irradiation and vaginal brachytherapy. A number of patients will benefit from additional chemotherapy treatment. Many reports have documented that progestational agents are effective in selected patients with endometrial cancer (71).

1.2.1.4 Cancer of the vulva, vagina and choriocarcinoma

Cancer of the vulva and the vagina are relatively rare malignancies, accounting respectively for approximately 3% and 2% of all primary genital cancers. Both are mainly diseases of women beyond the menopause. Choriocarcinoma is the rarest genital tract carcinoma constituting 0.6% of all gynecologic cancers (64). Epidemiological studies have identified sexual factors, particularly HPV infection, as increasing risk for vulvar cancer. Smoking also increases the risk by interacting synergistically with HPV infection. No apparent cause or associated factors that predispose women to vaginal cancer have been found. The standard treatment for cancer of the vulva and vagina is radical surgery and radiotherapy. Choriocarcinomas are usually treated by surgical removal of the tumor and chemotherapy (74,75).

1.2.2 Radiotherapy for gynecologic cancer

Radiotherapy is essential in the management of gynecologic malignancies. It is the definitive treatment modality for locally advanced cervical carcinomas, for most cases of vaginal cancer, as an organ-sparing approach in locally advanced vulvar cancer, and for inoperable endometrial cancer. Radiotherapy is also beneficial as an adjuvant treatment after hysterectomy for cervical cancer with high-risk features (positive margins, involved parametrium[2], positive nodes, deep stromal invasion) or for high-risk endometrial cancer (high grade, deep myometrial[3] invasion). It also prevents relapse of excised vulvar cancer, and it improves survival in vulvar cancer patients with more than one positive inguinal node. Radiotherapy remains the primary method to cure endometrial or cervical cancer that recurs after surgery. It is also useful for palliation of local symptoms due to metastatic disease (76).

1.2.2.1 External beam radiotherapy

External beam radiation can be administered by conventional external beam radiotherapy, by three dimensional conformal radiotherapy (3DCRT) or by intensity modulated radiotherapy (IMRT). In the past, pelvic malignancies were most often treated with conventional external

[2] The connective tissue of the pelvic floor
[3] The muscular outer layer of the uterus

beam radiotherapy. Three dimensional conformal planning has contributed in reducing the radiation dose to normal tissues, but the treatment of patients with gynecologic malignancies also requires treating the regional nodes which still results in a large volume of small bowel and rectum being irradiated. In contrast to conventional external beam radiotherapy and 3DCRT, where the radiation intensity is generally uniform within the radiation field, IMRT can vary the dose intensity within a field. Pelvic IMRT can be used to deliver a specified dose to the pelvic lymph node, the vaginal vault, and the paracervical and upper paravaginal tissue, while reducing the dose and the dose per fraction to rectum, bladder, and small bowel. The radiation therapy is typically administered 5 days per week for 5-6 weeks up to a total dose of 50 Gy via parallel and opposed anterior and posterior portals and lateral fields (76,77).

1.2.2.2 Brachytherapy

Brachytherapy is an important component in the radiation treatment of gynecologic malignancies and uses small sealed γ-ray sources to irradiate tissues from inside. Intracavitary therapy applies sources which are arranged in a suitable applicator to irradiate the walls of a body cavity. The most commonly used device is the Fletcher-Suit application created by an intra-uterine tandem and vaginal ovoids. Given at low dose rates, this type of irradiation takes several days and can be used for many anatomic sites, the most common being the uterine cervix. Interstitial therapy uses sources which are implanted directly into the diseased tissues. The most widely used radionuclide in both techniques is ^{192}Ir. While intracavitary brachytherapy is the standard technique, interstitial brachytherapy is usually applied for more complicated clinical scenarios. Dose specification of intracavitary brachytherapy is based on the location of the ureter and the pelvic lymph nodes. The brachytherapy dose depends largely on the tumor stage but also on the prescription of the department, and is defined in accordance with the whole-pelvis external radiation dose and the parametrial dose. Doses ranging from 20 to 35 Gy at dose-maximum points are most often used (77,78).

1.2.3 Pelvic normal tissue adverse effects

The dose that can be administered by radiotherapy is limited by the tolerance dose (TD) of the surrounding normal tissues for late effects. Tolerance doses are commonly reported as $TD_{5/5}$, defined as the average

dose that causes 5% complication risk within 5 years. The radiation tolerance of pelvic organs is considerably variable. The uterus, cervix and upper vagina can tolerate very high doses of radiation, while the colon, rectum and especially the small intestine are much more susceptible to radiation injury. As a consequence, the latter are the dose-limiting organs during pelvic and abdominal radiation therapy (77,79). Acute reactions of pelvic radiotherapy result from inflammation of the small intestine (enteritis), the rectum (proctitis), the bladder (cystitis), the vagina (vaginitis) and the skin (dermatitis). The symptoms are abdominal pain, diarrhea, increased secretion of mucus, rectal bleeding, dysuria, pollakiuria, hematuria, erythema, edema or desquamation. These effects are usually reversible and manageable with medical support. The most common late rectal effects include increased stool frequency, spotting of blood, and partial incontinence. Less common are mucosal ulceration, severe bleeding, pain, stricture, severe incontinence, and fistula. Fibrosis and ischemia in the submucosa and muscularis are largely responsible for these effects, accompanied by telangiectasia and other vascular abnormalities, mucosal congestion, collagen deposition and abnormal fibroblasts. Late radiation damage to the urinary tract, the vagina and the bones include, respectively, hemorrhagic radiocystitis and ureteric stricture, vaginal atrophy with telangiectasia and adhesions, and edema, pelvic fibrosis and sacrum fracture (osteoradionecrosis). Late effects are generally irreversible and progressive (18,76,79-81). Because of large differences in applied treatment schedules, length of follow-up and used scoring system, late complication rates vary considerably among studies. As a consequence, late complication rates of 7-28% have been reported in patients treated with radiotherapy for cervix cancer (82). More homogeneous studies have indicated that approximately 2-9% of the patients develop severe late gastrointestinal toxicity after pelvic external beam radiotherapy using total doses of 45-55 Gy with conventional fractionations (83,84).

1.3 Radiosensitivity assays

For a long time, many research groups have tried to develop an *in vitro* radiosensitivity assay capable of predicting the extent of normal tissue damage in radiotherapy patients. Despite limited success, the effort to achieve this objective continues because an assay capable of predicting susceptibility for the development of adverse radiation effects would allow individualization of radiotherapy schemes. It has been estimated that personalized radiotherapy protocols could result in a significant improvement in the therapeutic index (85,86). Numerous assays have

been proposed to predict the outcome after irradiation, but none have become established in daily practice. The assays can be grouped in cellular radiosensitivity assays and molecular radiosensitivity assays.

1.3.1 Cellular radiosensitivity assays

Different cell functions, such as cell survival, formation of chromosome aberrations and/or micronuclei, DNA repair capacity, and apoptosis are affected by individual radiosensitivity. *In vitro* assays using these endpoints have been applied in different test systems to estimate individual radiosensitivity (87).

1.3.1.1 Clonogenic assays

The clonogenic assay has often been employed to measure cell survival after *in vitro* or *in vivo* irradiation of cells. In this assay, the capacity of single cells to multiply and form a colony or clone after irradiation is measured. The fraction of colony-forming cells as a function of radiation dose is graphically displayed by the cell survival curve and cellular radiosensitivity is estimated as the surviving fraction (SF) at a certain dose (e.g. at 2 Gy, SF_2). The formation of a clone from a single cell requires sustained cell division and is the ultimate proof of reproductive integrity. Consequently, the colony forming assay measures the end result of a combination of several cellular responses to irradiation including reproductive cell death, death by apoptosis, accelerated differentiation and senescence. Fibroblasts are the most common type of cells used in clonogenic assays (88). Early studies with a limited number of patients showed a moderate correlation between the *in vitro* radiosensitivity of normal human skin fibroblasts and late radiotherapy side effects using the clonogenic assay, but larger scaled studies did not find significant correlations between clonogenic *in vitro* radiosensitivity and clinical response (89-94). On the other hand, a relatively large study, using an alternative clonogenic assay on lymphocytes, showed a significant correlation between lymphocyte radiosensitivity and late normal tissue toxicity after radiotherapy (95).

1.3.1.2 Cytogenetic assays

During the last few years the suitability of cytogenetic assays, such as the G_2 assay, the dicentrics assay and the micronucleus assay, have been

investigated as candidate biomarkers for predicting radiation sensitivity. The assays measure the capacity of cells to repair radiation induced chromosomal damage. All assays can be applied for any dividing cell population, but are most often used to measure the frequency of chromatid aberrations, chromosome aberrations or micronuclei in stimulated peripheral blood lymphocytes (88). In the G_2 assay, lymphocytes are irradiated *in vitro* during the G_2 phase of the cell cycle to induce DNA damage (typical dose: 0.4 Gy or 0.5 Gy). Subsequently, cells are allowed to progress to metaphase where chromatid breaks can be observed and scored (96). Figure 1.7 shows an example of a metaphase cell comprising chromatid breaks.

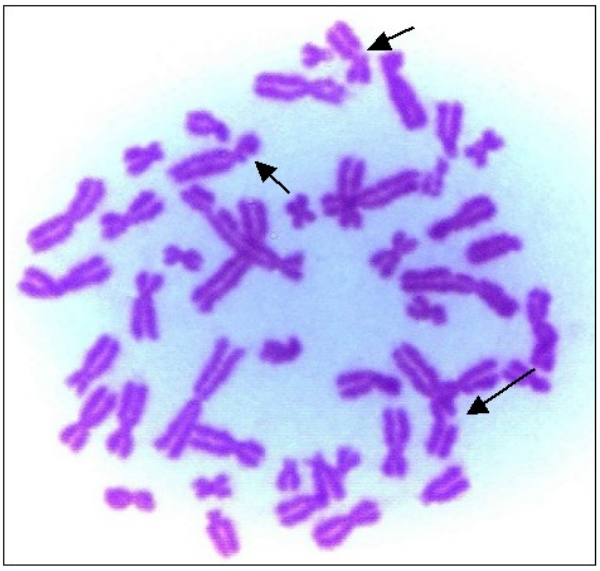

Figure 1.7. Pictures of a metaphase cell with chromatid breaks. Chromatid breaks are indicated by arrows.

The dicentrics assay and the micronucleus assay involve *in vitro* irradiation of lymphocytes during the G_0 phase of the cell cycle. In both assays, irradiation doses ranging from 2 to 5 Gy are most often used. In the dicentrics assay, cells are blocked in metaphase and unstable chromosome aberrations, such as dicentric chromosomes and acentric fragments, can be detected in metaphase cells. In the micronucleus assay, micronuclei, composed of acentric fragments, whole chromosomes and complex chromosomal fragments, can be scored in binucleated cells after cytokinesis blocking (97). Up to now, only a few studies investigated the predictive value of the G_2 assay (98,99), and in one of the studies the number of chromatid breaks was found to be highly correlative with the occurrence of acute skin reactions (98). The usefulness of the micronucleus assay in predicting clinical radiosensitivity seems to be limited since contradictory results have been obtained in different studies

(98,100-103). The dicentrics assay generally showed positive correlations between the number of dicentrics and acute and late normal tissue injury after radiotherapy treatment (104-106).

1.3.1.3 Assays evaluating the DNA repair capacity

The comet assay or single-cell gel electrophoresis (SCGE) assay is a rapid and sensitive fluorescent microscopic method to examine DNA damage and repair in individual cells. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleus when a potential is applied. Evaluation of the DNA 'comet' tail content and length allows for assessment of DNA damage. By performing the comet assay at several time points after *in vitro* irradiation of cells (typical dose: 2-5 Gy), the DNA repair kinetics can be analyzed. In the alkaline comet assay, cell lysis and electrophoresis is performed under alkaline conditions, whereas in the neutral comet assay these steps are done under neutral conditions. As a consequence, the alkaline comet assay measures the induction and repair of various types of DNA damage (e.g. single- and double-strand breaks, alkali labile sites) and can be seen as a test to evaluate the 'global repair phenotype'. On the other hand, the neutral comet assay allows to quantify induction and repair of double-strand breaks only. The assay can be applied on several cell types of which lymphocytes are most applicable (107). Initially, the alkaline comet seemed to be a valuable technique for predicting acute clinical radiosensitivity (108,109), but larger scaled studies could not support the predicting capacities of the assay (110).

Pulsed field gel electrophoresis (PFGE) is a technique for separating large DNA fragments and analyzing induction and repair of DNA double-strand breaks following irradiation. The test operates by alternating electric fields to run DNA through an agarose gel. Quantification of double-strand breaks is determined from DNA smears on the gel after electrophoresis. The DNA repair kinetics can be investigated by varying the post-irradiation time. Fibroblasts are the most common type of cells used in PFGE assays and the applied doses range from to 5 Gy up to 150 Gy (111). In the past, the assay has not been used frequently to predict clinical radiosensitivity and although a strong correlation has been found between residual DNA damage and late normal tissue response when fibroblast were used, this correlation was not found when keratinocytes were applied (112,113). Moreover, contradictory results between the initial number of DNA double-strand breaks and acute normal tissue

reactions have recently been reported in studies applying the PFGE assay on lymphocytes (114,115).

1.3.1.4 Assays evaluating apoptosis

The detection of apoptosis is frequently used as a parameter for radiation induced DNA damage. Various techniques are available to detect apoptotic cells, e.g. electron microscopy, fluorescence nuclear staining with propidium iodide, annexin V labeling, observation of oligonucleosomal DNA fragments by DNA laddering in agarose gels (87). Doses used to measure apoptosis range from 2 to 8 Gy. Recently, it has been shown that an assay of intrinsic radiosensitivity based on apoptosis in CD4 and CD8 lymphocytes is able to significantly predict radiation induced late toxicity (116).

1.3.2 Molecular radiosensitivity assays

Since today normal tissue radiosensitivity is considered as a complex trait dependent on the interaction of multiple genes or gene products, molecular techniques are now also being used to predict normal tissue radiosensitivity. The techniques allow the study of relevant target genes at the DNA, RNA or protein level.

1.3.2.1 Assays at the DNA level - Radiogenomics

Radiogenomics is the study of genetic variations in relation to inter-individual differences in radiotherapy response. Most of the research so far has concentrated on single nucleotide polymorphisms (SNPs) in candidate genes (24). SNPs represent the most abundant type of sequence variation in the human genome and are thought to be the genetic basis for most genetic diseases. Currently, more then 9 million SNPs are available in the public SNP databases (117,118). A SNP is defined as a single base alteration in genomic DNA found in at least 1% of the population. The mechanisms by which they affect phenotype and influence complex diseases vary according to their genomic location. Substitutions in coding regions may affect the amino acid sequence of predicted proteins, reducing or abolishing functions like DNA binding, catalytic activity and receptor-ligand contact. SNPs may interrupt the initiation or the termination codon, or introduce errors in the reading frameshift, all with consequences for insufficient or prematurely truncated peptides. SNPs

located in regulatory regions may influence gene expression, whereas SNPs in non-coding sequences may affect splicing or RNA cleavage, stability and export (119). Apart from SNPs, other genetic variations such as deletions, insertions and repeats (mini- or microsatellites) can also be studied to identify individual genetic profiles which may predict normal tissue radiosensitivity. Approaches for detecting DNA variability include DNA sequencing, single-strand conformation polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (DHPLC), restriction fragment length polymorphism (RFLP) analysis, single base extension technique (SNapShot®), arrayed primer extension (APEX) technique, and a number of high-throughput SNP genotyping platforms such as the TaqMan® SNP genotyping assay and the SNPlex™ genotyping system (120,121). Over the last few years, several studies have investigated possible associations between SNPs in selected candidate genes and the risk of developing various normal tissue complications. The results of these studies will be handled in the discussion section of this thesis.

1.3.2.2 Assays at the RNA and protein level

Variations in gene expression at the RNA and protein levels have also been evaluated as prognostic factors for complex diseases such as clinical radiosensitivity. Techniques to measure RNA expression are *in situ* hybridization, reverse transcription PCR methods and micro-arrays. Assays for detecting protein levels include immunohistochemistry, flow cytometry, Western blots and various proteomic methods, generally using mass spectrometry or antibody arrays (122,123). The transcriptional response of normal cells and tissues to radiation has been investigated in several studies using cDNA micro-arrays and many genes that could be relevant for predicting radiation induced morbidity have been identified from these studies (124-128). Studies investigating the utility of protein levels in tissues and blood serum as markers for the development of adverse radiation reactions also highlighted a number of possibly related proteins, of which TGFβ1 seemed to be the most promising (129-134).

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Chapter 2

Aim and outline of the thesis

2.1 Aim

During the past few decades, numerous *in vitro* radiosensitivity assays have been developed in order to predict normal tissue reactions after radiotherapy. One of the most promising assays is the cytogenetic G_2 assay (1,2). As it was shown previously that the number of chromatid breaks after *in vitro* irradiation, determined by this assay, is highly correlative with the occurrence of acute radiotherapy reactions (3), the first aim of this thesis was ***to assess the value of the G_2 assay in predicting late clinical radiosensitivity after radiotherapy***.

Today, normal tissue radiosensitivity is considered to be a complex trait dependent on the interaction of multiple genes or gene products. As a result, it is assumed that the development of normal tissue reactions could be predicted from individual genetic profiles (4,5). Because of the importance of DNA repair in cell and tissue response to radiation, genetic variants in genes responsible for DNA damage detection and repair are suitable candidates to study normal tissue radiosensitivity (6,7). Therefore, the second aim of this thesis was ***to investigate the association between single nucleotide polymorphisms (SNPs) in the DNA repair genes XRCC1, XRCC3 and OGG1 and the development of late radiotherapy reactions***. In addition, as suggested by literature data, ***the involvement of microsatellite polymorphisms in the DNA repair genes XRCC1, XRCC3 and XRCC5 in late clinical radiosensitivity was also examined***.

Over the years, it has become clear that normal tissue radiosensitivity is not exclusively caused by cell killing, but is the result of multicellular interactions between various cell types within a specific organ (8). The early activation of cytokine cascades after radiation exposure strongly affects the normal tissue response (9). Since the multifunctional

profibrotic cytokine TGFβ1 plays an important role in radiation induced normal tissue reactions (8,10,11), the last aim of this thesis was ***to study the association between TGFβ1 polymorphisms and late clinical radiosensitivity***.

2.2 Outline

Gynecologic cancers account for an important percentage of cancers in women worldwide (12). Most gynecologic malignancies, particularly cervical and endometrial cancers, are treated surgically followed by radiotherapy (13). Although the irradiation techniques have improved considerably, normal tissue reactions in the pelvic area still occur and vary widely from patient to patient (14). Therefore, the occurrence of late clinical radiotherapy reactions was studied within the framework of the present thesis using a patient group consisting of women with cervical or endometrial cancer.

In the first study described in chapter three (**part I**, p 45), the correlation between *in vitro* chromosomal radiosensitivity and clinical radiosensitivity was assessed using the G_2 assay. To this end, whole blood cultures were irradiated *in vitro* and the mean number of radiation induced chromatid breaks was compared between non-radiosensitive and radiosensitive patients.

Based on the hypothesis that normal tissue radiosensitivity is the result of multiple subtle genomic alterations, and based on the positive results of our initial cytogenetic study, the involvement of single nucleotide polymorphisms (SNPs) in the DNA repair genes *XRCC1*, *XRCC3*, and *OGG1* in clinical radiosensitivity was studied in **part I (**p 45). For this study, genomic DNA was extracted from isolated lymphocytes and the polymorphic sites were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays.

As polymorphic microsatellites within the *XRCC1* and *XRCC3* genes have previously been suggested to be associated with clinical radiosensitivity (15), the association of these microsatellite polymorphisms in the development of late pelvic radiotherapy reactions was studied in **part II** (p 67). A microsatellite in the *XRCC5* gene was included based on the involvement of this gene in DNA repair. The number of tandem repeat units at each locus was determined by PCR on lymphocyte DNA followed by automated fragment analysis.

In radiogenomics, genetic polymorphisms in DNA repair genes are not the only interesting candidates to study. In fact, as the cytokine TGFβ1 plays a significant role in the cell and tissue response to radiation, genetic variations in this gene can also be important to study in relation to clinical radiosensitivity (11). Therefore, in **part III** (p 83), the association between *TGFβ1* polymorphisms and the occurrence of late normal tissue reactions was investigated. The polymorphic sites were examined by PCR-RFLP, single base extension and genotyping assays on lymphocyte DNA. Furthermore, *TGFβ1* haplotypes were determined and linkage disequilibrium between the different polymorphisms was calculated.

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Chapter 3

Original research: results

3.1 Part I

Radiation-induced damage to normal tissues after radiotherapy in patients treated for gynecologic tumors: association with single nucleotide polymorphisms in *XRCC1*, *XRCC3*, and *OGG1* genes and *in vitro* chromosomal radiosensitivity in lymphocytes

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Reprint from IJROBP 2005; 62:1140-1149

Abstract

Purpose: To examine the association of polymorphisms in *XRCC1* (194Arg/Trp, 280Arg/His, 399Arg/Gln, 632Gln/Gln), *XRCC3* (5' UTR 4.541A>G, IVS5-14 17.893A>G, 241Thr/Met), and *OGG1* (326Ser/Cys) with the development of late radiotherapy (RT) reactions and to assess the correlation between *in vitro* chromosomal radiosensitivity and clinical radiosensitivity.

Methods and Materials: Sixty-two women with cervical or endometrial cancer treated with RT were included in the study. According to the Common Terminology Criteria for Adverse Events, version 3.0, scale, 22 patients showed late adverse RT reactions. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays were performed to

examine polymorphic sites, the G2 assay was used to measure chromosomal radiosensitivity, and patient groups were compared using actuarial methods.

Results: The *XRCC3* IVS5-14 polymorphic allele was significantly associated with the risk of developing late RT reactions (odds ratio 3.98, $p = 0.025$), and the *XRCC1* codon 194 variant showed a significant protective effect ($p = 0.028$). Patients with three or more risk alleles in *XRCC1* and *XRCC3* had a significantly increased risk of developing normal tissue reactions (odds ratio 10.10, $p = 0.001$). The mean number of chromatid breaks per cell was significantly greater in patients with normal tissue reactions than in patients with no reactions (1.16 and 1.34, respectively; $p = 0.002$). Patients with high chromosomal radiosensitivity showed a 9.2-fold greater annual risk of complications than patients with intermediate chromosomal radiosensitivity. Combining the G2 analysis with the risk allele model allowed us to identify 23% of the patients with late normal tissue reactions, without false-positive results.

Conclusion: The results of the present study showed that clinical radiosensitivity is associated with an enhanced G2 chromosomal radiosensitivity and is significantly associated with a combination of different polymorphisms in DNA repair genes.

Introduction

The ultimate goal of curative radiotherapy (RT) is to achieve tumor control without producing complications due to damage to surrounding normal tissues. With respect to normal tissue reactions, RT dose fractionation schemes are based on α/β values and other parameters such as the $TD_{5/5}$, the tolerance dose producing 5% of a specific, either acute or late, radiotoxic effect within 5 years after treatment in a patient population (1). Evidence is now emerging that normal tissue radiation effects are also due to a patient-related variability in response, which is determined mainly by genetic factors (2). The first indications for an inherited basis for clinical radiosensitivity came from patients with rare genetic syndromes such as ataxia telangiectasia, Nijmegen breakage syndrome, Fanconi anemia, and Bloom syndrome. These patients exhibit, not only an enhanced clinical, but also an enhanced *in vitro* cellular radiosensitivity. All these syndromes are associated with germline mutations in genes involved in the detection of DNA damage and DNA repair (3). Mutations in repair genes have also been detected in very radiosensitive cancer patients who do not have these syndromes (4). The importance of genetic factors in clinical radiosensitivity has been further supported by the results of controlled animal studies showing that some strains of mice have a greater genetic predisposition to develop typical late reactions such as fibrosis (5).

Except for patients with rare genetic syndromes, the data available today have indicated that clinical normal tissue radiosensitivity should be regarded as a complex phenotype dependent on the interplay of several gene products (6). This is supported by the observation that in overreacting patients no association has been found between the different

radiotoxic effects: the genetic determinants exhibit differential expression in different cell types and tissues (7, 8). At the interindividual level, single nucleotide polymorphisms (SNPs) represent approximately 90% of the naturally occurring sequence variation (9). Complex diseases, such as the considered radiopathologic effects, are determined most likely by genetic variants with a relatively high allelic frequency. This leads to the idea that common SNPs in a limited number of genes could be the inherited basis of clinical radiosensitivity.

In view of the importance of DNA repair in cell and tissue response to radiation, SNPs in the genes responsible for DNA damage signalling and repair pathways are suitable candidates in the search for the genetic basis of clinical normal tissue radiosensitivity. The *XRCC1* and the *OGG1* genes, involved in base excision repair, have a key role in the efficient repair of DNA damage caused by ionizing radiation and oxidative stress. Mutations in *XRCC1* result in an increased sensitivity and an increased number of chromosomal aberrations (10). The 194Arg/Trp and 399Arg/Gln polymorphisms in *XRCC1* were associated with an increased risk of developing an adverse response to RT in breast cancer patients (11). The *XRCC1* 280Arg/His polymorphism appeared to be correlated with *in vitro* bleomycin hypersensitivity (12). The 241Thr/Met SNP in the *XRCC3* gene, involved in the homologous recombination pathway, as well as the *XRCC1* 399Arg/Gln SNP, has been associated with increasing chromosome deletions after *in vitro* irradiation (13).

In the past decade, numerous *in vitro* radiosensitivity assays have been developed aimed at prediction of individual radiosensitivity and individualizing treatment schedules. Studies correlating *in vitro* radiosensitivity using different biologic endpoints and cells have led to contradictory results (14–16). Although initially encouraging results were obtained with the alkaline comet assay (15, 17), larger scale studies did not support the comet assay as a valuable prediction test for individual clinical radiosensitivity (18). Among the cytogenetic test systems on peripheral blood lymphocytes, the G2 chromatid break assay can be considered as the standard measure of chromosomal radiosensitivity (19). The study performed by Barber *et al.* (20) showed that highly radiosensitive patients with respect to acute effects had significantly greater G2 scores than patients with reactions considered to be normal. The value of the G2 assay has, up to now, never been confirmed for clinical radiosensitivity prediction.

In the present study we investigated whether eight SNPs selected in *XRCC1* (194Arg/Trp, 280Arg/His, 399Arg/Gln, 632Gln/Gln), *XRCC3* (5'UTR 4.541A>G, IVS5-14 17.893A>G, 241Thr/Met), and *OGG1*

(326Ser/Cys) genes are associated with an increased risk of adverse reactions to RT in patients treated for gynecologic tumors. Furthermore, the reliability of the G2 assay in the prediction of late reactions within the studied population was investigated.

Methods and Materials

Population

The study population consisted of 62 women with cancer of the cervix (n = 30) or endometrium (n = 32). All patients were treated at the Ghent University Hospital between December 1993 and May 2002. They received fractionated external beam RT to the pelvis (one anterior and two lateral fields, 25-MV photons), followed by a brachytherapy boost. Of the 62 patients, 15 received a tumor dose of 45 Gy in 25 fractions of 1.8 Gy within 5 weeks, 22 received a tumor dose of 46 Gy in 23 fractions of 2 Gy within 4.6 weeks, 22 received a tumor dose of 50 Gy in 25 fractions of 2 Gy within 5 weeks, 2 received 46 Gy supplemented with a 14-Gy (7 fractions of 2 Gy) parametrial boost within 6 weeks, and 1 received a total tumor dose of 66 Gy in 33 fractions of 2 Gy, with the final 20 Gy given by three-dimensional conformal therapy within 6.4 weeks. Except for 2 patients, all patients additionally underwent brachytherapy using vaginal ovoids (after hysterectomy), Fletcher-type applications, or perineal implants. All brachytherapy was performed using a pulsed-dose-rate technique with ^{192}I. The dose rate was between 0.5 and 0.65 Gy/h at 0.5 cm from the applicator surface for ovoids and perineal implants and to point A for the Fletcher-type application. The total doses from brachytherapy ranged from 15 to 35 Gy. Of the 30 patients with cervical cancer, 18 received combined radiochemotherapy and were treated with 40 mg/m^2 cisplatinum weekly during the external beam RT period. Forty-six patients underwent Wertheim Meigs hysterectomy. The mean patient age at treatment was 59 years (range, 24–80 years).

The reactions of all patients were scored with respect to several different normal tissue reactions according the Common Terminology Criteria for Adverse Events scale, version 3.0, of the National Cancer Institute (21). Of the 62 patients, 40 had no or very light adverse reactions to RT and were classified as CTC0-1, and 14 experienced side effects according to CTC2 (radiation enteritis with diarrhea and secondary hemorrhoidal bleeding, miction problems with pollakisuria and incontinence, radiocolitis and radiation rectitis, and vaginal atrophy with telangiectases and synechia). Six patients experienced side effects classified as CTC3 (dehydration as a

consequence of extreme diarrhea, anemia caused by rectal blood loss, vaginal atrophy, pelvis fibrosis, edema of the upper leg, sacrum fracture and osteoradionecrosis, and hemorrhagic radiocystitis). Finally, 1 patient experienced side effects classified as CTC4 (radiation enteritis with pelvic fibrosis, anal blood loss, and rectal ulcus leading to intestinal resection), and 1 patient died as a consequence of RT (perforation of the small intestine) and was classified as CTC5. All normal tissue reactions appeared between 6 months and 5.7 years after RT and could be considered as late normal tissue reactions. The mean time of follow-up was 4.8 years (range, 0.7–10.6 years).

From each patient in the study, a heparinized blood sample was taken at least 6 months after the last therapy session. Part of the blood sample was used on the sampling day to perform the G2 assay. Lymphocytes were isolated from the remaining blood and frozen for genotyping analysis. To determine the frequency of the considered SNPs in a Belgian control population, a blood sample was taken of 150 healthy individuals, working at the Ghent University Hospital, at their annual occupational medical examination. Lymphocytes were isolated and used for the SNP analysis. Patient samples were collected from January 2003 to July 2004 and control samples from October 2003 to July 2004. The local ethical committee approved the study, including the genotyping analysis on DNA of all individuals. All participants provided written informed consent.

G2 assay

The G2 assay procedure (Paterson Institute, Manchester, UK) was followed with some minor changes. In brief, heparinized blood was kept at room temperature before culturing within 4 h after blood sampling. To a tissue culture flask (25 cm^2), 0.5 mL of blood was added to 4.5 mL of complete RPMI-1640 culture medium supplemented with 10% fetal calf serum (Life Technologies), 1% L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin. The lymphocytes were stimulated to divide with 1% phytohemagglutinin (Life Technologies). Two cultures were started per donor, one served as the control, the other for irradiation. After 70-h incubation in a CO_2 incubator at 37°C, the cultures were irradiated with a dose of 0.4 Gy ^{60}Co rays at 37°C. At 30-min after irradiation, 75 µL of colcemid (final concentration 0.15 µg/mL, Sigma-Aldrich) was added to block the cells at metaphase; 90 min after irradiation, the cultures were arrested by putting them on ice for 5 min. The cells were harvested by centrifugation of the samples, and the cell pellets were resuspended in 5 mL of 0.075 M KCl for 15 min on ice. After the hypotonic shock, the cells were fixed three times in 5 mL of cold methanol acetic acid (3:1). Finally,

the cells were dropped on clean slides and stained with 4% Azur B SCN solution (Serva Polylab). Fifty well spread metaphases were analyzed by two independent scorers on coded slides for the appearance of chromatid breaks. All types of single chromatid breaks in which a clear discontinuity was present were scored (22).

Genotyping analysis

Polymorphic sites in *XRCC1* (194Arg/Trp, 280Arg/His, 399Arg/Gln, 632Gln/Gln), *XRCC3* (5'UTR 4.541A>G, IVS5-14 17.893A>G, 241Thr/Met), and *OGG1* (326Ser/Cys) were examined by polymerase chain reaction (PCR)-restriction length fragment polymorphism analysis. Genomic DNA was extracted from the isolated lymphocytes using a commercially available kit (QIAamp DNA Blood Mini Kit, Qiagen). PCR-restriction fragment length polymorphism (RFLP) assays were performed on each DNA sample using the primer sequences, annealing temperatures, and restriction enzymes detailed in Table 1. The PCR reactions contained 0.2 mM dNTPs (Amersham Biosciences), 1X PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 1 µM forward and reverse primer, 0.6 U Platinum taq DNA Polymerase (Invitrogen), and 200 ng DNA in a 25-µL reaction volume. The PCR amplification conditions for the SNPs in *XRCC3* and *OGG1* consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 1 min at 95°C, 1 min at the appropriate annealing temperature T_a (Table 1), 1 min at 72°C, and a final extension step at 72°C for 10 min. For the amplification of the DNA regions containing the polymorphic sites for *XRCC1,* the following touchdown program was used: an initial denaturation step at 95°C for 2 min, 12 cycles of 20 s at 95°C, 15 s at the initial annealing temperature T_a (Table 1), 1 min at 72°C, followed by 24 cycles of 40 s at 95°C, 40 s at T_a-12, 30 s at 72°C, and a final extension step of 10 min at 72°C. The amplified fragments were digested with the appropriate restriction endonucleases listed in Table 1. After 3 h of incubation (2 h for FokI and Fnu4HI) at 37°C, 20 µL of digested product was analyzed by gel electrophoresis on a 2% agarose gel and visualized under ultraviolet light after staining with ethidiumbromide. Table 1 lists the polymorphic variants found at each site and the fragment combinations identifying the different genotypes.

Table 1. Technical details of PCR-RFLP analysis

Gene	SNP	Primers	PCR product size (bp)	Restriction enzyme	Variant	Fragments identifying genotypes (bp)
XRCC1	194 Arg/Trp	F=GTTCCGTGTGAAGGAGGAG R=CTTGGAGGTGCTGCCTATG T_a=67°C	504	PvuII	C → T	CC=431 CT=368+431 TT=368
	280 Arg/His	F=CTGGACTGCTGGGTCTGAG R=CTCCAGATTCCTGGCATTGC T_a=69°C	849	RsaI	G → A	GG=153+597 GA=153+597+696 AA=153+696
	399 Arg/Gln	F=CTGGACTGCTGGGTCTGAG R=CTCCAGATTCCTGGCATTGC T_a=69°C	849	HpaII	G → A	GG=321+461 GA=321+461+528 AA=321+528
	632 Gln/Gln	F=AGTTACTTCCTCACCAGCTC R=AAGATACAGGTGTGGCTCAG T_a=67°C	333	HaeIII	G → A	GG=195 GA=195+234 AA=234
XRCC3	5′ UTR 4.541	F=TGAGGCGCCTAATCAGCTG R=TGGACTGTGTCAAGCAGCG T_a=58°C	293	FokI	A → G	AA=111+182 AG=111+182+293 GG=293
	IVS5-14 17.893	F=GACACCTCTACAGAGGACG R=TTCTCGATGGTTAGGCACAG T_a=58°C	650	PvuII	A → G	AA=283+367 AG=283+367+650 GG=650
	241 Thr/Met	F=GACACCTCTACAGAGGACG R=TTCTCGATGGTTAGGCACAG T_a=58°C	650	NlaIII	C → T	CC=281+298 CT=193+281+298 TT=193+281
OGG1	326 Ser/Cys	F=GTGGATTCTCATTGCCTTCG R=CTGTTGCTGTCGAGACTGC T_a=58°C	672	Fnu4HI	C → G	CC =553 CG=154+399+553 GG=154+399

Abbreviations: PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; F = forward; R = reverse; T_a = annealing temperature.

Statistical analysis

Statistical analysis was performed using MedCalc, version 4.0, and Statistical Package for Social Sciences, version 10.0, software (SPSS Inc., Chicago, IL). For the comparison of the G2 scores between different groups, the Mann-Whitney *U* test was applied. Differences in the proportions of sensitive patients and nonsensitive patients were compared using the chi-square test. Actuarial fibrosis rates were obtained from Kaplan-Meier analysis. The rate of complications in the different groups was analyzed with the log–rank test. The annual incidence of radiation-induced complications was estimated according to Jung *et al.* (23) using the actuarial Kaplan-Meier data. Genotype and allele frequencies were calculated by direct counting. Allele frequencies were examined by calculating the odds ratios (OR), with 95% confidence

intervals (95% CIs) using the Fisher exact test, with the wild-type alleles used as the reference group.

Results

G2 assay

Chromosomal radiosensitivity G2 data were collected for 58 patients. Stimulation of the cultures of blood samples of 4 patients failed. The average yield of chromatid breaks per cell for patients in the CTC0-1, CTC2, and CTC3-4-5 group was 1.16, 1.33 (p = 0.012), and 1.37 breaks/cell (p = 0.022), respectively. Of the 20 patients with reactions to RT (CTC2+), 9 could be judged as highly radiosensitive with respect to chromosomal radiosensitivity on the basis of the 90th percentile cutoff value of the patient group with no severe normal tissue radiation effects (1.39 breaks/cell, p = 0.001). An overview of the results is given in Table 2.

Table 2. Overview of G2 data of different CTC groups

	CTC 0-1	CTC 2	CTC 3-4-5	CTC 2-3-4-5
Population size*	38	14	6	20
Mean ± SD†	1.16 ± 0.18	1.33 ± 0.19	1.37 ± 0.19	1.34 ± 0.19
p value (Mann-Whitney U test)		0.012	0.022	0.002
Cut off value‡	1.39			
% > cut off value	5	43	50	45
p value (χ^2)		0.004	0.011	0.001

* Number of successful blood samples, † Number of chromatid breaks per cell, ‡ Ninetieth percentile of nonradiosensitive patients.

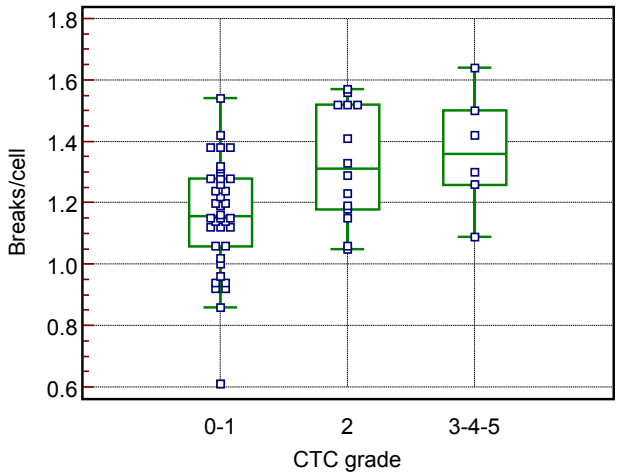

Fig. 1. Correlation of G2 data and clinical response according to Common Terminology Criteria for Adverse Events scale.

Figure 1 sketches the spread of the G2 data for the CTC groups, CTC0-1, CTC2, and CTC3-4-5.

The G2 data of all patients were normally distributed with a mean (μ) of 1.22 breaks/cell and standard deviation (SD) of 0.20 breaks/cell. In view of this distribution, three patient groups were defined: patients with low chromosomal radiosensitivity (≤μ-SD or ≤1.02 breaks/cell), patients with high chromosomal radiosensitivity (≥μ+SD or ≥1.42 breaks/cell), and patients with intermediate chromosomal radiosensitivity (>μ-SD but <μ+SD or >1.02 but <1.42 breaks/cell). The Kaplan-Meier plot analysis for the three groups showed that for patients with high chromosomal radiosensitivity, the probability of complication-free survival at 3 years was 0.20 versus 0.73 for patients with intermediate chromosomal radiosensitivity (p = 0.007; Fig. 2). Except for the group with low chromosomal radiosensitivity, the proportion of complication-free patients decreased exponentionally with the time after RT. The annual rate of complications was 9.2-fold greater in patients with high chromosomal radiosensitivity compared with patients with intermediate chromosomal radiosensitivity (55%/y vs. 6%/y). For all patients, the annual risk was 8%/y.

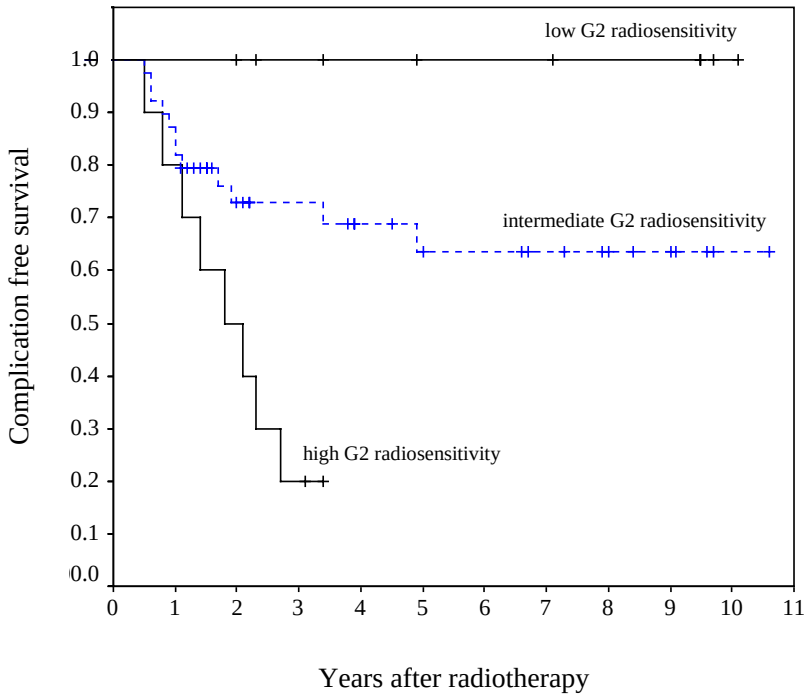

Fig. 2. Kaplan-Meier plot showing relationship between chromosomal radiosensitivity and occurrence of complications after radiotherapy. Actuarial rate of complication-free patients showing low, intermediate, or high chromosomal radiosensitivity plotted versus time after radiotherapy.

Genotyping analysis

The allele frequencies for the eight polymorphisms in the control group of healthy individuals were similar to those reported previously in white control subjects from Europe and America. Except for the *XRCC1* 632Gln/Gln polymorphism in the control group, all the genotype distributions in all groups considered were in Hardy-Weinberg equilibrium. Tables 3 and 4 show the genotype and allele frequencies obtained for each polymorphic site in the cancer patients with (CTC2+) and without (CTC0-1) reactions to RT and in the control population. The results of a comparative statistical analysis of these data are presented in Table 5.

In a first analysis, patients with and without normal tissue reactions were compared. A significant negative association (p = 0.028) was found between the *XRCC1* 194Arg/Trp polymorphism and the risk of normal tissue reactions after RT. Variant alleles of this polymorphism occurred only in patients without normal tissue reactions (allele frequency 14%). This observation indicates a radioprotective effect for the Trp allele. A greater number of patients with adverse reactions to RT possessed one variant *XRCC1* 280His allele, one variant *XRCC1* 399Gln allele, and two variant *XRCC1* 632Gln alleles. Variant alleles at these sites correlated positively with radiosensitivity (OR 2.65, 1.58, and 1.78, respectively). However, none of these results were statistically significant (Table 5).

The subgroup of patients with adverse reactions to RT had a greater percentage of individuals who were heterozygous for the polymorphic allele at IVS5-14 in *XRCC3* (54% vs. 30%) and a lower percentage who were homozygous for the normal alleles (32% vs. 65%). Individuals in the radiosensitive group had an OR of 3.98 (p = 0.025) for carrying this variant allele. No direct significant association was found between the polymorphic genotypes in *XRCC3* 5'UTR and the *XRCC3* 241 codon and normal tissue reaction risk. Variant alleles at these sites correlated negatively with an increased risk of normal tissue reactions (OR, 0.42 and 0.87, respectively). Furthermore, no major differences in *OGG1* 326Ser/Cys genotype distributions could be found between radiosensitive patients and patients with no adverse reactions. Slightly more homozygous mutants were found among radiosensitive patients, but, in contrast, radiosensitive patients included fewer heterozygous mutant allele carriers (Table 4).

Table 3. Genotype and allele frequencies of *XRCC1* polymorphisms in cancer patients with and without reactions to radiotherapy and healthy controls

	Position of the polymorphic site in the *XRCC1* gene (%)											
	194 Arg/Trp C>T			280 Arg/His G>A			399 Arg/Gln G>A			632Gln/Gln G>A		
	Cancer patients			Cancer patients			Cancer patients			Cancer patients		
	CTC 0-1	CTC 2+	Controls	CTC 0-1	CTC 2+	Controls	CTC 0-1	CTC 2+	Controls	CTC 0-1	CTC 2+	Controls
Genotype and allele frequencies	*n* = 40	*n* = 22	*n* = 150	*n* = 40	*n* = 22	*n* = 150	*n* = 40	*n* = 22	*n* = 150	*n* = 40	*n* = 22	*n* = 150
Genotype												
Homozygous normal alleles	75 (30)	100 (22)	83 (124)	90 (36)	77 (17)	90 (135)	42 (17)	32 (7)	39 (58)	40 (16)	27 (6)	31 (46)
Heterozygous	22 (9)	0 (0)	17 (25)	10 (4)	23 (5)	10 (15)	40 (16)	55 (12)	46 (69)	50 (20)	50 (11)	58 (88)
Homozygous polymorphic	3 (1)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	18 (7)	13 (3)	15 (23)	10 (4)	23 (5)	11 (16)
Allele frequency												
Normal allele	86	100	91	95	89	95	63	59	62	65	52	60
Polymorphic allele	14	0	9	5	11	5	37	41	38	35	48	40

Data in parentheses are numbers of individuals with each genotype.

Table 4. Genotype and allele frequencies of *XRCC3* and *OGG1* polymorphisms in cancer patients with and without reactions to radiotherapy and healthy controls

	Position of the polymorphic site in the *XRCC3* gene									Position of the polymorphic site in the *OGG1* gene		
	5′ UTR 4.541 A>G			IVS5-14 17.893 A>G			241 Thr/Met C>T			326 Ser/Cys C>G		
	Cancer patients			Cancer patients			Cancer patients			Cancer patients		
	CTC 0-1	CTC 2+	Controls	CTC 0-1	CTC 2+	Controls	CTC 0-1	CTC 2+	Controls	CTC 0-1	CTC 2+	Controls
Genotype and allele frequencies	*n* = 40	*n* = 22	*n* = 150	*n* = 40	*n* = 22	*n* = 150	*n* = 40	*n* = 22	*n* = 150	*n* = 40	*n* = 22	*n* = 150
Genotype												
Homozygous normal alleles	48 (19)	68 (15)	72 (108)	65 (26)	32 (7)	51 (76)	38 (15)	41 (9)	37 (56)	60 (24)	68 (15)	62 (93)
Heterozygous	42 (17)	27 (6)	27 (40)	30 (12)	54 (12)	39 (58)	50 (20)	50 (11)	42 (63)	38 (15)	23 (5)	34 (51)
Homozygous polymorphic alleles	10 (4)	5 (1)	1 (2)	5 (2)	14 (3)	10 (16)	12 (5)	9 (2)	21 (31)	2 (1)	9 (2)	4 (6)
Allele frequency												
Normal allele	69	82	85	80	59	70	63	66	58	79	80	79
Polymorphic allele	31	18	15	20	41	30	37	34	42	21	20	21

Data in parentheses are numbers of individuals with each genotype.

Table 5. Statistical comparison of data for cancer patients with and without reactions to radiotherapy and for cancer patients vs. healthy controls

			CTC 0-1 vs. CTC 2+			Controls vs. patients		
Gene	**SNP**	**Genotype**	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
XRCC1	194	Arg/Trp	0	/	0.039*	0.86	[0.38 – 1.96]	0.877
		Trp/Trp	0	/	0.862	2.38	[0.15 – 38.85]	0.882
		Arg/Trp + Trp/Trp	0	/	0.028*	0.92	[0.41 – 2.04]	0.991
	280	Arg/His	2.65	[0.63 – 11.13]	0.325	1.53	[0.63 – 3.70]	0.480
		His/His	0	/	/	/	/	/
		Arg/His + His/His	2.65	[0.63 – 11.13]	0.325	1.53	[0.63 – 3.70]	0.480
	399	Arg/Gln	1.82	[0.57 – 5.78]	0.463	0.98	[0.51 – 1.87]	0.915
		Gln/Gln	1.04	[0.21 – 5.23]	0.716	1.05	[0.44 – 2.54]	0.908
		Arg/Gln + Gln/Gln	1.58	[0.53 – 4.73]	0.580	1.00	[0.54 – 1.83]	0.883
	632	GA	1.47	[0.45 – 4.83]	0.740	0.74	[0.38 – 1.41]	0.452
		AA	3.33	[0.66 – 16.76]	0.280	1.18	[0.45 – 3.08]	0.934
		GA + AA	1.78	[0.57 – 5.51]	0.469	0.80	[0.43 – 1.50]	0.602
XRCC3	5′ UTR	AG	0.45	[0.14 – 1.41]	0.269	1.83	[0.96 – 3.47]	0.092
		GG	0.32	[0.03 – 3.14]	0.591	7.94*	[1.47 – 42.80]	0.019*
		AG + GG	0.42	[0.14 – 1.26]	0.194	2.12*	[1.15 – 3.91]	0.024*
	IVS5-14	AG	3.71*	[1.17 – 11.80]	0.046*	0.95	[0.51 – 1.78]	0.993
		GG	5.57	[0.77 – 40.12]	0.197	0.72	[0.24 – 2.13]	0.738
		AG + GG	3.98*	[1.31 – 12.05]	0.025*	0.90	[0.50 – 1.63]	0.851
	241	Thr/Met	0.92	[0.30 – 2.77]	0.898	1.15	[0.60 – 2.18]	0.797
		Met/Met	0.67	[0.11 – 4.18]	0.988	0.53	[0.20 – 1.36]	0.266
		Thr/Met + Met/Met	0.87	[0.30 – 2.51]	0.993	0.94	[0.51 – 1.73]	0.974
OGG1	326	Ser/Cys	0.53	[0.16 – 1.77]	0.457	0.94	[0.49 – 1.77]	0.965
		Cys/Cys	3.20	[0.27 – 38.43]	0.727	1.19	[0.28 – 5.01]	0.892
		Ser/Cys + Cys/Cys	0.70	[0.23 – 2.10]	0.716	0.96	[0.52 – 1.77]	0.974

Abbreviations: OR = odds ratio; calculated to homozygous normal genotype; 95% CI = 95% confidence interval.

* Statistically significant.

The data in Table 6 show the distribution of patients with and without adverse reactions to RT for the *XRCC1* (280Arg/Trp, 399Arg/His, 632Gln/Gln) genotypes, *XRCC3* (IVS5-14A>G) genotypes, and combined *XRCC1* (280Arg/Trp, 399Arg/His, 632Gln/Gln) and *XRCC3* (IVS5-14A>G) genotypes. For these polymorphisms, ORs >1 were found, which points to a positive correlation between their presence and the appearance of RT reactions. An analysis of the patient population according to these risk alleles showed that clinical radiosensitivity is significantly associated with the number of risk alleles in *XRCC1* (OR, 12.60; $p = 0.011$, for two or more risk alleles) and with the number of risk alleles in *XRCC1* combined with *XRCC3* (OR, 10.10; $p = 0.001$, for three or more risk alleles). Patients with one or no risk allele and with two or less

risk alleles served as a reference group, respectively, for *XRCC1* and combined *XRCC1* and *XRCC3* analysis.

Table 6. Association between *XRCC1* risk alleles and *XRCC1* and *XRCC3* risk alleles and clinical radiosensitivity

Gene(s)	Risk alleles* (*n*)	CTC 0-1	CTC 2+	OR	95% CI	*p*
XRCC1	≤1	37.5†	4.5‡	Ref		
	2	62.5	90.9	12.00	[1.46 – 98.78]	0.014
	≥2	62.5	95.4	12.60	[1.53 – 103.49]	0.011
XRCC1 & *XRCC3*	≤2	82.5§	31.8‖	ref		
	3	15.0	54.5	9.43	[2.63 – 33.74]	0.001
	≥3	17.5	68.1	10.10	[3.00– 33.96]	0.001

Abbreviations: OR = odds ratio; 95% CI = 95% confidence interval; Ref = reference. Patient percentages in the CTC0-1 group and the CTC2+ group for the different number of risk alleles given. * Risk allele: OR > 1 for CTC2+ group compared with CTC0-1 group, calculated to reference alleles. † Three patients with 0 risk alleles. ‡ No patients with no risk alleles. § Nine patients with 0 or 1 risk allele. ‖ No patients with 0 or 1 risk allele.

In a second analysis of the SNP data, all cancer patients were compared with the cohort of healthy individuals. Except for one polymorphism, no association was found between the presence of mutant alleles and the risk of cancer. Individuals with one or two mutant alleles of the *XRCC3* 5'UTR polymorphism had a twofold increased risk of developing a gynecologic tumor compared with individuals with only the wild-type allele (p = 0.024). Furthermore, homozygous carriers of the mutant 5'UTR G allele had an eightfold increased risk of developing a gynecologic tumor (p = 0.019).

Combined G2 and genotyping analysis

To investigate the correlation between the genotype and chromosomal data, a combined analysis between the G2 score and the number of risk alleles in *XRCC1* and *XRCC3* was performed. This combined G2/risk allele analysis is presented in Figure 3. The 90th percentile of the patient group without radiation reactions was used as the cutoff value for the number of chromatid breaks, and more than two risk alleles was adopted as the limit for the number of risk alleles pointing to enhanced radiosensitivity. Five patients, possessing three or more risk alleles in *XRCC1* and *XRCC3* and a G2 score greater than the cutoff value of 1.39 breaks/cell, all showed severe RT reactions. None of the patients without normal tissue reactions (CTC0-1) were found in this quartile. Of the 38 patients without reactions to RT and 20 patients with reactions to RT, 30

and 3, respectively, were located in the third quartile (two or less risk alleles and <1.39 chromatid breaks/cells). The latter indicates that a normal G2 score, together with a low number of *XRCC1* and *XRCC3* risk alleles, is predictive of no adverse reactions to RT.

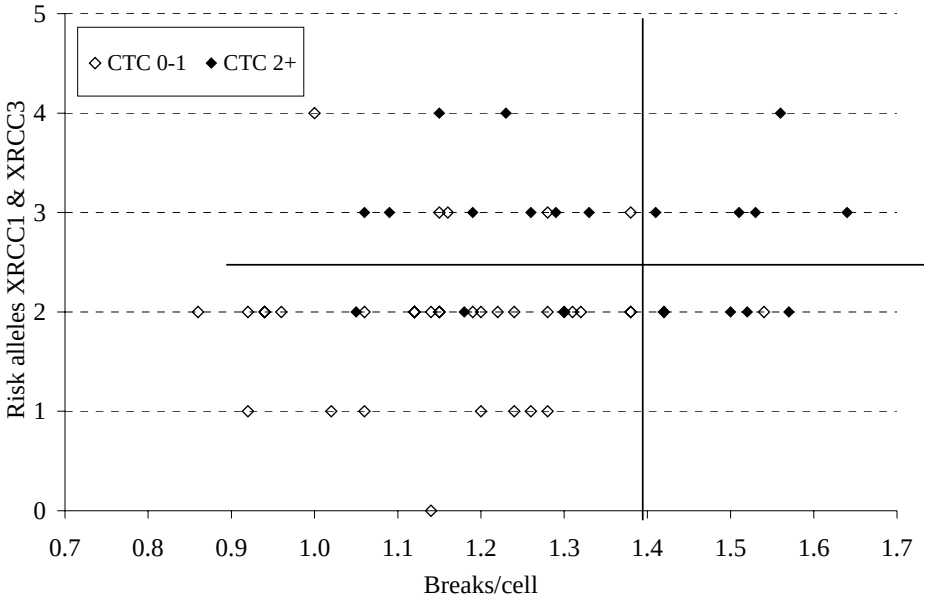

Fig. 3. Combined G2/risk alleles analysis. Vertical line represents 90th percentile cutoff value of number of chromatid breaks of patient group without radiation reactions (CTC0-1); horizontal line represents criterion for radiosensitivity with respect to number of risk alleles (>2).

Discussion

The aim of this study was to investigate the association between *in vitro* individual radiosensitivity using the G2 chromosomal assay and late normal tissue reactions. Furthermore, the involvement of four *XRCC1* (194Arg/Trp, 280Arg/His, 399Arg/Gln, 632Gln/Gln) polymorphisms, three *XRCC3* (5'UTR 4.541A>G, IVS5-14 17.893A>G, 241Thr/Met) polymorphisms, and one *OGG1* (326Ser/Cys) polymorphism in clinical radiosensitivity was studied.

Clinical endpoint and patient population

Late reactions in the pelvic area were used as the clinical endpoint. Because the scoring of these side effects is subjective, one staff oncologist was responsible for the follow-up of all patients in the study. The patients underwent a physical examination every 6 months for the first 2 years,

followed by an annual examination for up to 10 years after treatment. Normal tissue reactions were scored according to the Common Terminology Criteria for Adverse Events scale (21, 24). Various patient- and treatment-related factors may contribute to the development of adverse reactions to RT. For the patient group under study, age at treatment, follow-up period, total dose, and dose per fraction were largely uniform. The mean age at the end of the last RT session for the patients without adverse reactions (CTC0-1) was 57 years and for the patients with adverse reactions (CTC2+) was 62 years; the mean follow-up for both groups was, respectively, 5.0 and 4.5 years. Normal tissue reactions occurred 0.5–5.7 years (mean, 1.7 years) after the last treatment. For 8 patients without normal tissue reactions, the follow-up period was <1.7 years. This may constitute a shortcoming in the analysis when addressing normal tissue reactions that develop gradually after a latency period. However, excluding these patients from the data set did not result in a significant divergence for any of the conclusions. Different treatment schedules may also act as a confounding factor and affect the development of adverse normal tissue reactions. To investigate this, the patient population was sorted according to the treatment and CTC grading. The chi-square analysis showed no significant differences in CTC classification between patients with or without chemotherapy (p = 0.270) or between patients with or without surgery (p = 0.914).

Relationship between clinical and chromosomal radiosensitivity

To date, studies correlating cellular radiosensitivity (comet, micronucleus or colony-forming assays) and radiation toxicity have produced contradictory findings with some showing a relationship (15,26,27) and others not (18,25). In the present study, individual chromosomal radiosensitivity was determined by scoring chromatid breaks in lymphocytes irradiated *in vitro.* Our results confirm the findings of Barber *et al.* (20) that the number of *in vitro* radiation-induced G2 chromatid aberrations is greater for patients with complications after RT, but for a patient group treated for gynecologic tumors with late radiopathologic reactions. Of our patients with enhanced clinical radiosensitivity, 45% also showed a significantly increased chromosomal radiosensitivity based on the 90th percentile cutoff value. Actuarial analysis was used because of the time dependence of the late RT reactions and showed that patients with high chromosomal radiosensitivity (≥μ+SD) had a 9.2-fold greater annual risk of complications than patients with intermediate chromosomal radiosensitivity (<μ+SD). This is in agreement with the study of Hoeller *et al.* (28), in which patients were classified according to their cellular sensitivity. Although the sensitivity of the G2 assay (45%) is

not sufficient for individual tailoring of RT schedules, the technique can have a limited usefulness in patients in whom the clinician expects radiation hypersensitivity because of family history or associated medical conditions. The incomplete association between the chromosomal and clinical radiosensitivity can be explained by the existence of other susceptibility factors such as inflammatory reactions or cytokine production, in addition to a possible defect in DNA repair.

Relationship between clinical radiosensitivity and SNPs in DNA repair genes

Single nucleotide polymorphisms represent a very abundant type of genetic variation, and hundreds of genes can potentially participate in the response to RT (9). The selection of candidate genes and polymorphisms remains a crucial step in the search for an association with normal tissue radiosensitivity. For this study, we decided to screen for polymorphisms that have been shown to be involved in cancer predisposition and radiosensitivity according to the literature. In our first selection, we opted for functional polymorphisms in biologic processes. Later, this selection was expanded with (*1*) polymorphisms without amino acid change (*XRCC1 6*32Gln/Gln), (*2*) polymorphisms located in regulatory regions (*XRCC3* 5'UTR), and (*3*) polymorphisms in noncoding sequences (*XRCC3* IVS5-14). Furthermore, we decided to examine different polymorphisms within one gene to study intragenic associations.

Analysis of the individual polymorphism data showed that the strongest association with clinical radiosensitivity was observed for the *XRCC3* IVS5-14G variant (OR 3.98, $p = 0.025$). Because the *XRCC3* IVS5-14 17.893 polymorphism is located in a noncoding sequence region of the gene, the functional effect of this polymorphism is still incomprehensible. Apart from Kuschel *et al.* (29), who showed a protective effect of the variant G allele for developing breast cancer, no positive linkage studies have been reported with respect to this polymorphism (30, 31). The functional relevance could be associated with an alternative splicing variant or be involved in RNA instability. Another possibility is that the polymorphism is in linkage disequilibrium with other functional variants not tested here.

Recently, Moullan *et al.* (11) reported a positive association of the *XRCC1* 194Trp polymorphic allele, as well as the *XRCC1* 399Gln polymorphic allele, and the risk of developing normal tissue reactions after RT for the breast. In present study, we also established a positive association for the 399Gln polymorphic allele and clinical radiosensitivity, but a statistically

significant opposite association for the *XRCC1* 194Trp variant allele (p = 0.028) and clinical radiosensitivity was found. The latter could have been because individuals with the CT genotype were underrepresented in the patient group with reactions. Alternatively, it might be that different polymorphisms predispose different tissues to fibrosis-like reactions. Another explanation for the discrepancy may be that Moullan *et al.* (11) did not make a distinction between acute and late RT reactions.

Although the *XRCC3* IVS5-14G polymorphic allele was significantly associated with the occurrence of late normal tissue reactions, one-third of the radiosensitive patients were homozygous for the normal alleles. This reflects the possible role of additional genes and polymorphisms participating in the development of late normal tissue reactions. Combined analysis of positively correlated polymorphisms in *XRCC1* and *XRCC3* genes with late normal tissue reactions confirmed this hypothesis. In the present study, the His and Gln amino acids, the A and G alleles in *XRCC1* codon 280, codon 399, codon 632, and *XRCC3* IVS5-14 position, respectively, appeared to be risk factors for radiation-induced normal tissue reactions. These four alleles were defined as "risk alleles," and the patients were classified according to the total number of risk alleles they harbored. Patients with three and more risk alleles had a significantly increased risk of developing late normal tissue reactions (OR 10.10, p = 0.001). Of the 40 patients without reactions to RT, 17.5% possessed three or more risk alleles and could be considered as false positives. In an additional analysis, the risk allele model was combined with the G2 chromosomal data. Using the 90th percentile of the patient group without radiation reactions as the cutoff value for the number of chromatid breaks and more than two risk alleles as the criterion for radiosensitivity with respect to risk alleles, 23% of the patients with late normal tissue reactions could be identified without false-positive results. Likewise, 79% of the patients without normal tissue reactions could be identified, with 15% false-negative findings.

The positive association found in our study between the number of risk alleles and the clinical radiosensitivity supports the assumption that normal tissue radiosensitivity is determined by the combined effect of different genetic variations. Furthermore, Quarmby *et al.* (32) and Andreassen *et al.* (33) demonstrated in their studies of radiation-induced fibrosis that supplemental genes in the development of late normal tissue reactions could be located outside DNA repair genes.

Relationship between gynecologic cancer and SNPs in DNA repair genes

A lot of research has already been done on the association between polymorphisms in DNA repair genes and cancer risk (34). The primary goal of this study with respect to SNPs was the association with clinical radiosensitivity. Taking into account genotype data of a healthy control population, the obtained data also allowed us to study possible associations between polymorphisms and cervical and/or endometrial cancer risk. Except for the *XRCC3* 5'UTR 4.541 A>G polymorphism, no association of the selected SNPs with an increased risk of gynecologic tumors was found. Subdividing the patient group into cervical (n = 30) and endometrial (n = 32) cancer cases, it became clear that the *XRCC3* 5'UTR G variant allele is associated with the risk of cervical cancer exclusively (AA vs. GG, OR 15.43, p = 0.001; AA vs. AG + GG, OR 2.57, p = 0.038). Although our patient group was relatively small, our data support the results obtained in a recent study by Wu *et al.* (35) that did not find any correlation between polymorphisms in *XRCC1* (194, 280, 399) and the risk of cervical cancer. To date, only one study has examined the association between genetic variations in *XRCC3* (5'UTR 4.541, IVS5-14 17.893, 241) and endometrial cancer risk (31). That study could not provide evidence that women with polymorphisms at these sites had an altered risk of endometrial cancer, which is in agreement with our data set.

Conclusion

The data analysis of the present study showed that clinical radiosensitivity is related to an enhanced G2 chromosomal radiosensitivity and is significantly associated with a combination of different polymorphisms in DNA repair genes. Additional association studies of well-characterized large cohorts analyzing genes involved in the different mechanisms of the cellular response to radiation, not only DNA damage detection and repair, but also cytokines promoting fibrosis, could prove a promising approach to identify the genes associated with the adverse response to RT.

Acknowledgments

The work was supported by a grant from the Bijzonder Onderzoeksfonds (Gent University, No. 01109201) and the Federale Diensten voor

Wetenschappelijke, Technische en Culturele aangelegenheden (DWTC). We thank all the patients and control donors who participated in this study; special thanks to Virginie de Gelder for her help with the lymphocyte isolations, cell cultures, and scorings and to Valerie Breemersch. We also gratefully acknowledge the contribution of Jacqueline Cuypers, Martine De Vogelaere, and Kristel Van De Maele for the collection of the healthy control population, and Christiane Goethals for the prelevation of patient blood samples.

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3.2 Part II

Microsatellite polymorphisms in DNA repair genes *XRCC1*, *XRCC3* and *XRCC5* in patients with gynecological tumors: association with late clinical radiosensitivity and cancer incidence

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Reprint from Rad. Res. 2005;164, 237–244

Abstract

This study investigates the association of microsatellite polymorphisms in *XRCC1*, *XRCC3* and *XRCC5* with the development of late radiation-induced radiotherapy reactions and examines the correlation between these microsatellites and cancer incidence.

Sixty-two women with cervical or endometrial cancer treated with radiotherapy were included in the study. According to the CTCAEv3.0 scale, 22 patients showed late adverse radiotherapy reactions (grade 2 or more). PCR on lymphocyte DNA followed by automated fragment analysis was performed to examine the number of tandem repeat units at each locus.

No significant association was found between the repeat length at any of the microsatellites in *XRCC1*, *XRCC3* or *XRCC5* and the incidence of late radiotherapy complications. Since higher odds ratios (ORs) were found for the rare *XRCC1* $[AC]_{11}$ and $[AC]_{21}$ repeats (OR = 2.65, $p = 0.325$ and OR = 8.67, $p = 0.093$, respectively), the possible involvement of these small and large repeats in clinical radiosensitivity cannot be completely ruled out. When specific numbers of repeats were examined, no significant correlation was found between the microsatellite repeat length in *XRCC1* and *XRCC5* and cancer incidence. A weak correlation between *XRCC3* $[AC]_{16}$ homozygotes and cancer incidence was found (OR = 2.56, $p = 0.055$).

A large-scale multicenter study of cancer patients with a high number of radiosensitive individuals is needed to clarify the value of rare polymorphic microsatellite repeats in *XRCC1* and *XRCC3* as a biomarker of clinical radiosensitivity or increased cancer risk.

Introduction

In radiation oncology, radiation dose protocols are dependent on both the tolerance of healthy tissue and the tumor control probability. In a small percentage of patients, radiation doses that are usually well tolerated by the healthy tissues within the irradiation field result in unexpected acute and/or late radiotoxic effects. The development of predictive methods to determine the degree of radiosensitivity of both tumor and healthy tissue has become of major interest in radiobiological research (1).

Several observations indicate that normal tissue hypersensitivity may be related to genetic factors (2, 3). Several studies have reported a possible correlation between genetic polymorphisms and adverse radiotherapy reactions in patients (4–12). Although most studies have not detected a conclusive correlation between genotype and clinical radiosensitivity, Price *et al.* have reported a highly significant association between clinical radiosensitivity and rare microsatellites (unusually large or unusually small alleles) in the DNA repair genes *XRCC1* and *XRCC3* (13).

Microsatellites are tandemly repeated highly polymorphic sequences and are common throughout the human genome. Repeat units are gained and lost by DNA replication slippage, a mutation mechanism that results from the transient dissociation of the replicating DNA strands followed by misaligned reassociation (14, 15). Expansions of triplet repeats are the underlying cause of several genetic diseases such as myotonic dystrophy, Huntington's disease and fragile X syndrome (16–18). Furthermore, microsatellites are the molecular targets for malfunctioning repair and replication proteins in diseases such as hereditary non-polyposis colorectal carcinoma (HNPCC), where there is a defect in mismatch repair, and Bloom's syndrome, where a DNA helicase homologue is defective (19, 20).

XRCC1 plays an important role in the base excision repair pathway (BER) and participates as a scaffolding intermediate by interacting with ligase III, DNA polymerase β and poly(ADP-ribose) polymerase (21). XRCC3 functions in the homologous recombination repair pathway (HHR) by repairing double-strand breaks. *XRCC5* or *Ku80* is involved in the non-homologous end-joining repair pathway (NHEJ), and encodes, together with the *G22P1* (*KU70*) and *PRKDC* genes, components of a DNA-dependent protein kinase (DNA-PK) (22).

In the present study we investigated whether polymorphic microsatellites in three DNA repair genes, *XRCC1, XRCC3* and *XRCC5,* are associated with clinical radiosensitivity and cancer incidence. To this end we have

screened for these microsatellites in patients with cervical or endometrial cancer who received radiotherapy and in a control population of healthy individuals.

Methods and Materials

Participants

The patient group has been described previously (12). Sixty-two women with cancer of the cervix (n = 30) or endometrium (n = 32) were treated with fractionated external-beam radiotherapy to the pelvis (one anterior and two lateral fields, 25 MV photons) followed by a brachytherapy boost at the Ghent University Hospital. Fifteen patients received a tumor dose of 45 Gy (25 x 1.8 Gy), 22 patients received a tumor dose of 46 Gy (23 x 2 Gy), and 22 patients received a tumor dose of 50 Gy (25 x 2 Gy). Three patients received 46 Gy supplemented with a parametrial boost up to 60 Gy. Except for two patients, all patients were additionally treated by brachytherapy using either vaginal ovoids, Fletcher-type applications, or perineal implants. All brachytherapy was performed using a pulsed dose-rate technique with iridium-192. Total doses from brachytherapy ranged from 15 to 35 Gy (dose rate 0.5 to 0.65 Gy/h). Eighteen patients with cancer of the cervix received combined radiochemotherapy and were treated with 40 mg/m^2 cisplatinum per week during the period of the external radiotherapy. Forty-six patients were operatively treated with a Wertheim Meigs hysterectomy. Nine premenopausal patients received hormone replacement therapy (estrogen). The mean age of the patients at the time of treatment was 59 years (range 24–80 years).

All patients have been scored with respect to several different normal tissue reactions by the same oncologist according to the Common Terminology Criteria for Adverse Events (CTCAE) scale version3.0 of the National Cancer Institute (23). Forty patients showed no or very light reactions to radiotherapy (CTC0–1), 14 patients experienced intermediate but distinct radiotherapy reactions (CTC2), six patients showed severe radiotherapy reactions (CTC3), one patient experienced life-threatening radiotherapy reactions (CTC4), and one patient died as a consequence of the radiotherapy (CTC5). Complication specifications are described in more detail by De Ruyck *et al.* (12). All normal tissue reactions appeared 6 months to 5.7 years after radiotherapy and can be considered as late reactions. The mean time of follow-up was 4.8 years (range 0.7–10.6 years). Patients classified in CTC0–1 are indicated as nonradiosensitive patients, while patients classified in CTC2, CTC3, CTC4 and CTC5

(CTC2+) are indicated as radiosensitive patients. For these two patient groups under study, age at time of treatment and follow-up period were very similar. Mean age at the end of the last radiation treatment for the patients without adverse reactions (CTC0–1) was 57 years and for the patients with adverse reactions (CTC2+) 62 years, while the mean follow-up time for the two groups was 5.0 and 4.5 years, respectively.

A Caucasian control population of 118 cancer-free individuals was used to determine the overall population microsatellite frequency and allows association analysis of microsatellite genotype with cancer incidence. The control individuals were employees of the Ghent University Hospital and were recruited during the annual occupational medical examination. The mean age of controls was 38 years (range 22–62 years). The patient and the control populations were ethnically matched. All individuals were Belgian. The mean age of the patients was higher in comparison with the healthy controls, 59 years and 38 years, respectively. However, there are no indications that microsatellite frequencies at the loci considered vary with age. The healthy control population consisted of 53 men and 65 women, while the patients are all women. This lack of sex matching should not cause a problem since the loci studied are located on autosomes. Moreover, χ^2 tests on the control population verified that there are no differences between microsatellite frequencies in men and women ($0.16 < p < 0.96$ for all repeats tested separately in the three *XRCC* genes).

A heparinized blood sample was taken from each individual in the study, and lymphocytes were isolated and frozen for genotyping analysis. The study was approved by the Ghent University Hospital Ethical Committee. All participants received oral and written information concerning the study and signed the informed consent form.

Genotyping Analysis

Genomic DNA was extracted from isolated lymphocytes, and DNA analysis was successful on all samples. A $[AC]_n$ microsatellite repeat region in the 3′ untranslated region (3′ UTR) of *XRCC1* (accession number L34079), a $[AC]_n$ microsatellite repeat located in intron 3 of *XRCC3* (accession number AF000735), and a $[GAPyA]_n$ repeat located 120kb 5′ of *XRCC5* (accession number AF000736) were analyzed. The repeat regions were amplified by PCR, and sizes were analyzed using an ABI Prism 310 Genetic Analyser (PE Applied Biosystems). The *XRCC1* 3′ UTR microsatellite tandem $[AC]_n$ repeat region was amplified using the following primers (MWG Biotech): XRCC1F 5′-CCC GAT GGA TCT ACA GTT GC-3′ and XRCC1R 5′-CCC AGG GAG CCT CTT AGA GT-3′. The

forward primer was labeled with the fluorophore FAM-6. The intron tandem $[AC]_n$ repeat region in *XRCC3* was amplified with XRCC3F 5′-GAC AAT ATG CAT GTA TTA CTT TG-3′ and XRCC3R 5′-GTG TGC AGT TTA TAT AAG GCA GG-3′. The *XRCC5* $[GAPyA]_n$ repeat region was amplified using XRCC5F 5′-TGT TGC TAT TGT TGT CTA GC-3′ and XRCC5R 5′-AAG TCA CTC ACA TGT AAT CC-3′. Both XRCC3R and XRCC5R were labeled with the fluorophore TET. Multiplex PCR was undertaken in 12.5-µl volumes on an ABI9700 thermal cycler with conditions of 95°C for 15 min followed by 25 cycles of 94°C for 30 s, 57°C for 90 s and 72°C for 60 s, with a final 60°C hold for 30 min. PCR was undertaken using a multiplex PCR mix (Qiagen). Each reaction contained 1x Qiagen Multiplex PCR mix, 0.2 µM of each primer, and 0.5 µl template DNA. After PCR, fragment analysis was undertaken on an ABI Prism 310 Genetic Analyser. One microliter of PCR sample was mixed with 12 µl of deionized formamide and 0.5 µl of Genescan-500 TAMRA size standard (Applied Biosystems) and denatured for 3 min at 94°C. Capillary electrophoresis used POP-4 polymer with a 5-s injection time and 27 min electrophoresis at 60°C. Microsatellite allele sizes were converted to repeat lengths based on allele size as described by Price *et al.* (13). All genotyping was performed in duplicate.

Statistical analysis

Statistical analysis was performed by MedCalc 4.0. Allele frequencies of the different patient groups and the control population were determined and displayed graphically. Heterozygosities were calculated by dividing the number of heterozygotes by the total number of individuals. Odds ratios (ORs) with 95% confidence intervals (95% CI) were calculated for each microsatellite repeat length to evaluate the association of *XRCC1*, *XRCC3* and *XRCC5* microsatellite genotypes with both clinical radiosensitivity and cancer incidence. Corresponding *p* values were obtained using the χ^2 test. The reference genotype was a pooled sample of individuals with all repeat numbers, except the one examined. For clinical radiosensitivity, genotypes were compared between radiosensitive patients (CTC2+) and nonradiosensitive patients (CTC0–1). For cancer incidence, genotypes were compared between patients (total population) and control individuals. Impact of the different external radiotherapy doses, brachytherapy doses and total doses was evaluated with the Mann-Whitney test. Influence of chemotherapy, surgery and hormone therapy in the patient population and influence of gender in the control population was tested using the χ^2 test.

Table 1. Overview of cancer type, treatment protocols and clinical radiosensitivity according to the CTCAE Scale

Patient no.	Cancer type	Hormone therapy	External radiotherapy dose (Gy)	Brachytherapy dose (Gy)	Clinical radiosensitivity CTCAE
1	Cervix	yes	45	15	0
2	Cervix	yes	45	30	0
3	Cervix	no	45	20	0
4	Cervix	yes	45	19	0
5	Cervix	no	45	20	0
6	Cervix	no	45	30	0
7	Endometrium	no	45	30	0
8	Cervix	no	45	20	0
9	Cervix	no	45	20	0
10	Endometrium	no	45	27	0
11	Endometrium	no	45	22	0
12	Cervix	no	45	15	2
13	Cervix	no	45	25	0
14	Endometrium	no	45	20	0
15	Cervix	no	45	35	2
16	Cervix	no	46	30	3
17	Cervix	no	46	19	0
18	Cervix	no	46	15	3
19	Cervix	no	46	34	3
20	Endometrium	no	46	19	2
21	Cervix	no	46	24	0
22	Endometrium	no	46	34	3
23	Cervix	yes	46	30	0
24	Cervix	yes	46	20	0
25	Endometrium	no	46	19	0
26	Endometrium	no	46	19	1
27	Cervix	no	46	34	0
28	Endometrium	no	46	19	0
29	Endometrium	no	46	19	0
30	Cervix	no	46	19	3
31	Endometrium	yes	46	19	0
32	Cervix	no	46	35	3
33	Cervix	yes	46	19	0
34	Endometrium	no	46	19	0
35	Endometrium	no	46	19	0
36	Endometrium	no	46	24	2
37	Endometrium	no	46	19	2
38	Endometrium	no	50	/	5
39	Endometrium	no	50	15	2
40	Cervix	yes	50	19	4
41	Endometrium	no	50	15	2
42	Endometrium	no	50	15	1
43	Endometrium	no	50	15	0
44	Endometrium	no	50	15	0

Table 1. Overview of cancer type, treatment protocols and clinical radiosensitivity according to the CTCAE Scale (continued)

Patient no.	Cancer type	Hormone therapy	External radiotherapy dose (Gy)	Brachytherapy dose (Gy)	Clinical radiosensitivity CTCAE
45	Cervix	no	50	19	0
46	Cervix	no	50	15	0
47	Endometrium	no	50	15	2
48	Endometrium	no	50	15	2
49	Cervix	no	50	15	2
50	Endometrium	no	50	/	0
51	Endometrium	no	50	30	1
52	Endometrium	no	50	15	2
53	Cervix	yes	50	14	0
54	Endometrium	no	50	19	0
55	Endometrium	no	50	15	0
56	Endometrium	no	50	15	0
57	Endometrium	no	50	15	2
58	Endometrium	no	50	20	2
59	Endometrium	no	50	15	0
60	Cervix	no	60	19	0
61	Cervix	no	60	20	2
62	Cervix	no	60	15	0

Results

An overview of the radiotherapy treatment protocols and the radiotherapy reactions is given in Table 1. To investigate a possible bias of the treatment protocols, the delivered radiation doses of both the CTC2+ and the CTC3+ groups were compared with the CTC0–1 patient group using a two-tailed Mann-Whitney test. For the CTC0–1 and CTC2+ groups, no significant difference could be shown with respect to the external radiotherapy dose (p = 0.13), the dose delivered by brachytherapy (p = 0.74), and the summation of both doses (p = 0.72). The comparison of the CTC0–1 and CTC3+ groups resulted in nonsignificant p values of 0.58, 0.10 and 0.28, respectively. The impact of the chemotherapy treatment, surgery and hormone therapy on the clinical radiosensitivity in the population was evaluated by χ^2 analysis. Therefore, the patient population was sorted according to the treatment and the CTC grading. This analysis showed no significant differences in CTC classification between patients with or without chemotherapy (p = 0.27), with or without surgery (p = 0.91), and with or without hormone therapy (p = 0.20).

Figures 1, 2 and 3 show the allele frequencies of the different microsatellite repeats in *XRCC1*, *XRCC3* and *XRCC5*, respectively, for healthy controls, all cancer patients, nonradiosensitive cancer patients (CTC0–1), and radiosensitive cancer patients (CTC2+). The data on the association between the number of microsatellite repeats in *XRCC1*, *XRCC3* and *XRCC5* and clinical radiosensitivity are given in Table 2, while the data on the association between the microsatellites and cancer incidence are given in Table 3.

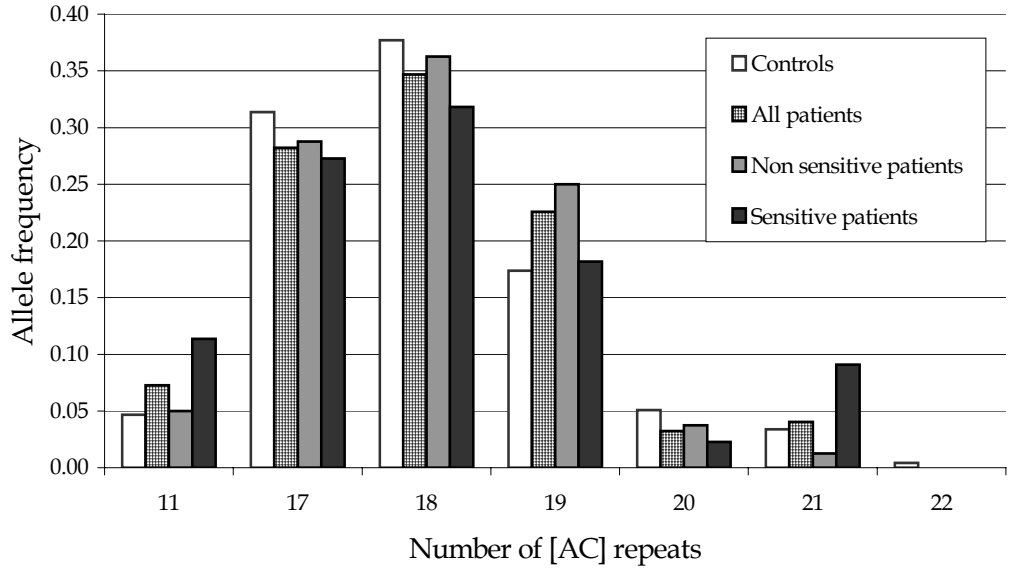

Fig. 1. Allele frequencies of the $[AC]_n$ microsatellite in the 3′ untranslated region of *XRCC1*. Number of healthy controls = 118; number of nonradiosensitive cancer patients = 40; number of clinically radiosensitive cancer patients = 22.

The *XRCC1* $[AC]_n$ microsatellite was highly polymorphic, with between 11 and 22 repeat units and an observed heterozygosity of 0.74. The most frequently occurring alleles were in the range $[AC]_{17}$ to $[AC]_{19}$. Allele frequencies for these repeats were similar in all groups considered. The smallest observed allele size ($[AC]_{11}$) had a frequency of 0.047 in healthy controls, 0.073 in all patients, 0.050 in nonradiosensitive patients, and 0.114 in radiosensitive patients. Patients with one $[AC]_{11}$ repeat had a 2.65 times higher risk of developing adverse radiotherapy reactions. This result, however, is not statistically significant ($p = 0.325$). With this obtained OR of 2.65, a sample size of 160 individuals is needed to reach statistical significance. Allele frequencies for $[AC]_{21}$ repeats were 0.034, 0.040, 0.013 and 0.091 for healthy controls, all cancer patients, nonradiosensitive cancer patients, and radiosensitive cancer patients, respectively. Four patients with one $[AC]_{21}$ repeat were found among the 22 clinically radiosensitive patients, while only one $[AC]_{21}$ heterozygote

was found in the 40 nonradiosensitive patients (OR = 8.67, p = 0.093). With this very high OR of 8.67, an increase of the sample size to 80 individuals would be needed to reach statistical significance. None of the patients were homozygous for $[AC]_{11}$ and $[AC]_{21}$ microsatellite copy numbers; thus we were unable to determine whether these alleles act in a recessive fashion. Comparison of cancer incidence with the presence of any number of microsatellite repeats in *XRCC1* did not show any distinct association (Table 3).

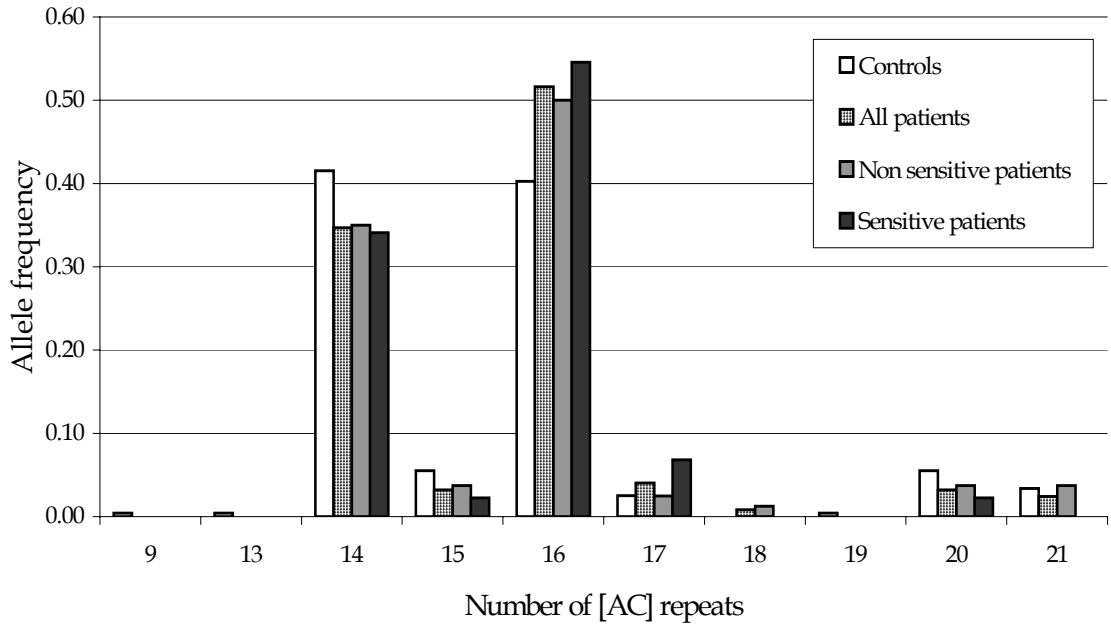

Fig. 2. Allele frequencies of the $[AC]_n$ microsatellite in intron 3 of the *XRCC3* gene. Number of healthy controls = 118; number of nonradiosensitive cancer patients = 40; number of clinically radiosensitive cancer patients = 22.

The *XRCC3* $[AC]_n$ microsatellite exhibited between 9 and 21 repeat units. The observed heterozygosity for this repeat was 0.63. The most frequently occurring alleles were $[AC]_{14}$ and $[AC]_{16}$. The largest allele, $[AC]_{21}$, was found at a frequency of 0.034 in controls, 0.024 in all cancer patients, 0.038 in nonradiosensitive cancer patients, and 0 in clinically radiosensitive cancer patients. Accordingly, no measure of association with radiosensitivity could be undertaken. Patients carrying one $[AC]_{17}$ repeat had a three times higher risk of developing normal tissue reactions after radiotherapy in comparison with patients having any other number of repeats, although this is not statistically significant (p = 0.479). None of the patients were homozygous for the $[AC]_{17}$ repeat (Table 2). A borderline significant positive association was found between the presence of two $[AC]_{16}$ repeats and cancer incidence (OR = 2.56, p = 0.055). Other [AC] repeats did not show any significant association with cancer incidence.

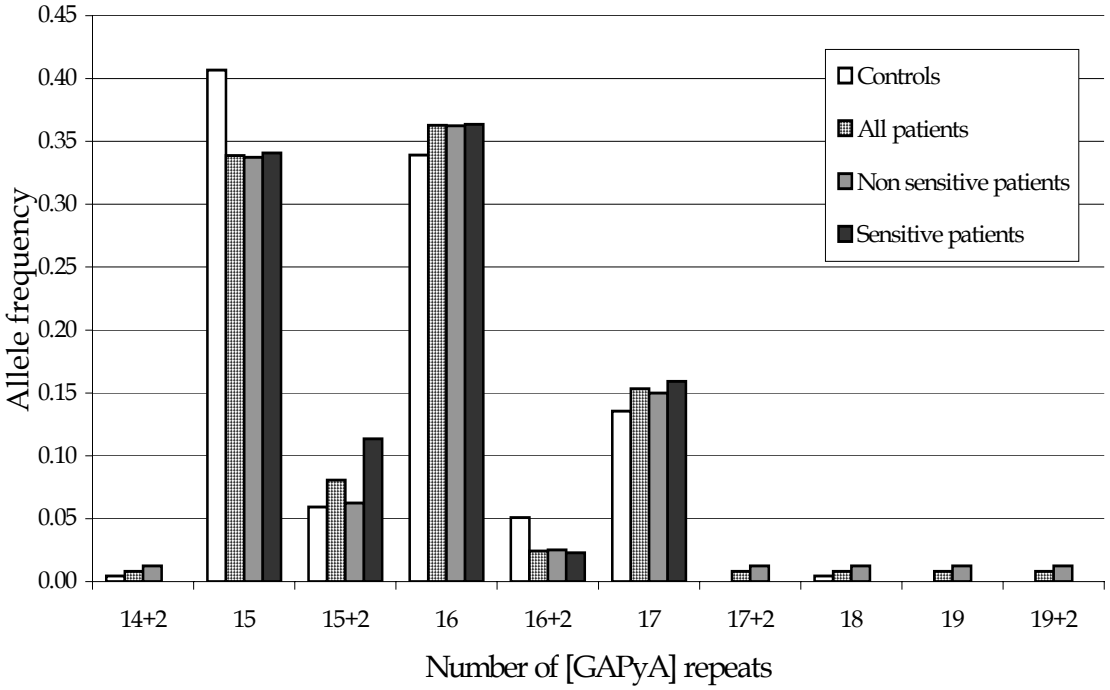

Fig. 3. Allele frequencies of the [GAPyA]$_n$ microsatellite 120 kb from the *XRCC5* gene. A small proportion of the alleles were extended by two nucleotides, resulting in [GAPyA]$_{14+2}$, [GAPyA]$_{15+2}$, [GAPyA]$_{16+2}$, [GAPyA]$_{17+2}$ and [GAPyA]$_{19+2}$ repeats. Number of healthy controls = 118; number of nonradiosensitive cancer patients = 40; number of clinically radiosensitive cancer patients = 22.

The *XRCC5* [GAPyA]$_n$ tetranucleotide microsatellite was polymorphic, with repeat units from 14 to 19. The observed heterozygosity for this repeat was 0.63. A small proportion of the alleles were extended by two nucleotides, resulting in [GAPyA]$_{14+2}$, [GAPyA]$_{15+2}$, [GAPyA]$_{16+2}$, [GAPyA]$_{17+2}$ and [GAPyA]$_{19+2}$ repeats. The most frequent alleles were [GAPyA]$_{15}$ and [GAPyA]$_{16}$. The rarest alleles were the smallest and the largest ([GAPyA]$_{14+2}$, [GAPyA]$_{17+2}$, [GAPyA]$_{18}$, [GAPyA]$_{19}$, [GAPyA]$_{19+2}$), representing together 0.8, 4, 6.3 and 0% in healthy controls, all cancer patients, nonradiosensitive cancer patients, and radiosensitive cancer patients, respectively (Fig. 3). Statistical analysis of clinical radiosensitivity or cancer incidence with the presence of any number of microsatellite repeats in *XRCC5* did not show any significant association.

Table 2. Association between microsatellite repeat number in *XRCC1*, *XRCC3* and *XRCC5* and clinical radiosensitivity

	Number of repeats					
***XRCC1*[a]**	**11**	**17**	**18**	**19**	**20**	**21**
NRS patients	4 (4/0)	18 (13/5)	20 (11/9)	17 (14/3)	3 (3/0)	1 (1/0)
RS patients	5 (5/0)	11 (10/1)	12 (10/2)	8 (8/0)	1 (1/0)	4 (4/0)
Odds ratio	2.65	1.22	1.20	0.77	0.59	8.67
95% CI	0.63 - 11.13	0.43 - 3.47	0.42 - 3.41	0.26 - 2.26	0.06 - 6.01	0.90 - 83.17
***XRCC3*[b]**	**14**	**15**	**16**	**17**	**20**	**21**
NRS patients	24 (20/4)	3 (3/0)	29 (18/11)	2 (2/0)	3 (3/0)	3 (3/0)
RS patients	13 (11/2)	1 (1/0)	17 (10/7)	3 (3/0)	1 (1/0)	0
Odds ratio	0.96	0.59	1.29	3.00	0.59	/
95% CI	0.33 - 2.78	0.06 - 6.01	0.38 - 4.35	0.46 - 19.51	0.06 - 6.01	/
***XRCC5*[c]**	**14+2**	**15**	**15+2**	**16**	**16+2**	**17**
NRS patients	1 (1/0)	22 (17/5)	4 (3/1)	24 (19/5)	1 (0/1)	12 (12/0)
RS patients	0	12 (9/3)	4 (3/1)	12 (8/4)	1 (1/0)	6 (5/1)
Odds ratio	/	0.98	2.00	0.80	1.86	0.88
95% CI	/	0.35 - 2.79	0.45 - 8.94	0.28 - 2.29	0.11 - 31.22	0.28 - 2.78

Notes. The number of individuals carrying at least one allele is given (with number of heterozygous/homozygous individuals indicated in parentheses). Odds ratios, with 95% CI, for risk of radiosensitivity are shown for nonradiosensitive (NRS) patients (n = 40) compared to radiosensitive (RS) patients (n = 22).

a Does not include $[AC]_{22}$ repeats for which only one individual was seen.

b Does not include $[AC]_{9}$, $[AC]_{13}$, $[AC]_{18}$, $[AC]_{19}$ repeats for which only one individual each was seen.

c Does not include $[GAPyA]_{17+2}$, $[GAPyA]_{19}$, $[GAPyA]_{19+2}$ for which only one individual each was seen and $[GAPyA]_{18}$ for which only two individuals were seen.

Discussion

This study investigated the association between polymorphic microsatellites in the DNA repair genes *XRCC1*, *XRCC3* and *XRCC5* and the risk of developing normal tissue reactions after radiotherapy treatment. Therefore, we have screened for these microsatellites in patients with cancer of the cervix or endometrium who received radiotherapy. To determine the overall microsatellite frequency in a Belgian population and to assess the correlation of these microsatellites with gynecological tumors, a control population consisting of healthy individuals was also screened. The microsatellites examined are located in different genomic contexts: the *XRCC1* microsatellite occurs in the 3'UTR of the gene, the *XRCC3* microsatellite is intronic, and the *XRCC5* microsatellite is located 120 kb from the gene. The microsatellites within *XRCC1* and *XRCC3* have previously been suggested to be associated with radiosensitivity and cancer incidence (13). The microsatellite in *XRCC5*

was included based on the involvement of the *XRCC5* gene in the NHEJ repair pathway.

Table 3. Association between microsatellite repeat number in *XRCC1*, *XRCC3* and *XRCC5* and cancer incidence

	Number of repeats					
***XRCC1*[a]**	**11**	**17**	**18**	**19**	**20**	**21**
Controls	11 (11/0)	67 (60/7)	74 (59/15)	36 (31/5)	12 (12/0)	8 (8/0)
Patients	9 (9/0)	29 (23/6)	32 (21/11)	25 (22/3)	4 (4/0)	5 (5/0)
Odds ratio	1.65	0.67	0.63	1.54	0.61	1.21
95% CI	0.64 - 4.23	0.36 - 1.24	0.34 - 1.18	0.81 - 2.92	0.19 - 1.97	0.38 - 3.86
***XRCC3*[b]**	**14**	**15**	**16**	**17**	**20**	**21**
Controls	76 (54/22)	11 (9/2)	77 (59/18)	5 (4/1)	13 (13/0)	8 (8/0)
Patients	37 (31/6)	4 (4/0)	46 (28/18)	5 (5/0)	4 (4/0)	3 (3/0)
Odds ratio	0.82	0.67	1.53	1.98	0.56	0.70
95% CI	0.43 - 1.54	0.20 - 2.20	0.77 - 3.03	0.55 - 7.13	0.17 - 1.79	0.18 - 2.74
***XRCC5*[c]**	**14+2**	**15**	**15+2**	**16**	**16+2**	**17**
Controls	1 (1/0)	80 (64/16)	10 (6/4)	72 (64/8)	12 (12/0)	31 (30/1)
Patients	1 (1/0)	34 (26/8)	8 (6/2)	36 (27/9)	2 (1/1)	18 (17/1)
Odds ratio	1.92	0.58	1.60	0.88	0.29	1.17
95% CI	0.12 - 31.20	0.31 - 1.09	0.60 - 4.29	0.47 - 1.65	0.06 - 1.36	0.59 - 2.33

Notes. The number of individuals carrying at least one allele is given (with number of heterozygous/homozygous individuals indicated in parentheses). Odds ratios, with 95% CI, for risk of cancer are shown for healthy controls (n = 118) compared to cancer patients (n = 62).

a Does not include $[AC]_{22}$ repeats for which only one individual was seen.

b Does not include $[AC]_{9}$, $[AC]_{13}$, $[AC]_{18}$, $[AC]_{19}$ repeats for which only one individual each was seen.

c Does not include $[GAPyA]_{17+2}$, $[GAPyA]_{19}$, $[GAPyA]_{19+2}$ for which only one individual each was seen and $[GAPyA]_{18}$ for which only two individuals were seen.

The overall microsatellite frequencies and distribution of repeat lengths in a healthy Belgian population were similar to those reported previously in studies of UK newborns and retired UK radiation workers (25, 26) and in an Australian twin study for *XRCC3* and *XRCC5* (27).

In 1997, Price *et al.* (13) reported a highly significant association between clinical radiosensitivity and rare microsatellites in *XRCC1* and *XRCC3* in a population of 19 cancer patients. In their study, rare microsatellites were alleles with less than 12 or more than 23 repeats for *XRCC1*, alleles with more than 20 repeats for *XRCC3*, and alleles with less than 14 or more than 18 repeats for *XRCC5.* All other microsatellite allele sizes were classified as common repeats. In this study we found a positive correlation between patients with $[AC]_{11}$ repeats and patients with $[AC]_{21}$ repeats in *XRCC1* and the risk of developing adverse radiotherapy

reactions, but these results were not statistically significant for both repeat numbers (p = 0.325 and p = 0.093, respectively). Alleles with more than 23 [AC] repeats were not present in the patient population or in the control population. Large *XRCC3* alleles ($[AC]_{20}$ and $[AC]_{21}$ repeats) did not correlate with clinical radiosensitivity, while *XRCC3* $[AC]_{17}$ repeats were slightly more common in patients with adverse radiotherapy reactions (p = 0.479). For *XRCC5*, no examples of rare (≤14 or ≥18) repeat lengths were identified in the clinically radiosensitive patient group. Accordingly, no measure of association with radiosensitivity could be undertaken. For the other more common repeat lengths, we found no association with clinical radiosensitivity.

Furthermore, we could not demonstrate an association between the rare microsatellite alleles considered and cancer incidence. However, a weak correlation was found between *XRCC3* $[AC]_{16}$ homozygotes and cancer incidence (p = 0.055). Subdividing the patient group into cervical and endometrial cancer cases, the positive association with the *XRCC3* $[AC]_{16}$ repeat is retrieved only for endometrial cancer (p = 0.057).

Although we found no significant association between the rare microsatellite alleles considered and clinical radiosensitivity or cancer incidence, the microsatellites could affect radiosensitivity or cancer in a recessive manner. Because of the small numbers of individuals homozygous for the rare microsatellite repeats, we are unable to test this hypothesis. For other microsatellite repeat lengths where sufficient variant homozygotes were detected, we found (*XRCC3* $[AC]_{16}$ excluded) no evidence for a recessive action of these polymorphisms. We have consequently assumed dominance and treated the data accordingly.

For this study, radiosensitivity classification of the patients is based on grading of the normal tissue reactions to the radiotherapy according to the CTCAE scale (23). Patients with intermediate but distinct radiotherapy reactions (CTC2) were pooled with patients with severe (CTC3) to life-threatening (CTC4/5) radiotherapy reactions. To assess possible differences in genotypes between the more severe radiosensitive individuals (CTC3/4/5), the intermediate radiosensitive patients (CTC2) and the nonradiosensitive patients (CTC0–1), we performed analyses of CTC3/4/5 (n = 8) compared to CTC0–1 (n = 40) and CTC2 (n = 14) compared to CTC0–1 (n = 40) analysis. The outcome of these analyses showed similar associations with the initial analysis (results not shown). Furthermore, the supplemental analysis showed that the *XRCC1* $[AC]_{11}$ repeat occurred 2.3 times more frequently in the CTC2 patient group than in the CTC3/4/5 patient group. On the other hand, the *XRCC1* $[AC]_{21}$ repeat was present more often in the CTC3/4/5 patient group, resulting

in an odds ratio of 13 compared the nonradiosensitive patient group (CTC0–1). Due to the low frequency of this microsatellite repeat and the small number of patients with very severe radiotherapy reactions, no statistical significance for this effect was reached ($p = 0.110$).

Although not statistically significant, higher odds ratios were obtained for *XRCC1* $[AC]_{11}$ and *XRCC1* $[AC]_{21}$ alleles in radiosensitive patients. Therefore, we are not able to completely reject the hypothesis that these small and large rare alleles may be associated with adverse radiotherapy outcome. The strongly positive associations found by Price *et al.* between *XRCC1* $[AC]_{24}$ and *XRCC3* $[AC]_{20}$ repeats and clinical radiosensitivity were not found in this study, nor could we show significant associations between rare microsatellite repeats and cancer incidence. These discrepancies could be due to the fact that the two studies are based on different cancer populations and studied different radiosensitivity end points. The fact that both studies could not demonstrate an association between the *XRCC5* microsatellite, clinical radiosensitivity and cancer incidence could be explained by the distant location of the microsatellite considered from the gene.

Due to the highly polymorphic nature of the loci considered, in the future, larger studies with larger numbers radiosensitive cases in a multicenter setting are needed to clarify the involvement of rare polymorphic microsatellites in *XRCC1* and *XRCC3* DNA repair genes in either clinical radiosensitivity or cancer incidence.

Acknowledgments

The work was supported by a grant of the Bijzonder Onderzoeksfonds (Gent University, No 01109201) and the Federale Diensten voor Wetenschappelijke, Technische en Culturele aangelegenheden (DWTC). We wish to thank all the patients and control donors who participated in this study. Special thanks to Virginie de Gelder and Valerie Breemersch for their help with the lymphocyte and DNA isolations. We also gratefully acknowledge the contribution of Jacqueline Cuypers, Martine De Vogelaere and Kristel Van De Maele for the collection of the healthy control population, and Christiane Goethals for the patient recruitment.

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3.3 Part III

TGFβ1 polymorphisms and late clinical radiosensitivity in patients treated for gynecologic tumors

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Reprint of IJROBP 2006; 65:1240-1248

Abstract

Purpose: To investigate the association between six transforming growth factor-β1 gene (*TGFβ1*) polymorphisms (-1.552delAGG, -800G>A, -509C>T, Leu10Pro, Arg25Pro, Thr263Ile) and the occurrence of late normal tissue reactions after gynecologic radiotherapy (RT).

Methods and Materials: Seventy-eight women with cervical or endometrial cancer and 140 control individuals were included in the study. According to the Common Terminology Criteria for Adverse Events version 3.0 (CTCAEv3.0) scale, 25 patients showed late adverse RT reactions (CTC2+), of whom 11 had severe complications (CTC3+). Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), single base extension and genotyping assays were performed to examine the polymorphic sites in *TGFβ1*.

Results: Homozygous variant -1.552delAGG, -509TT, and 10Pro genotypes were associated with the risk of developing late severe RT reactions. Triple (variant) homozygous patients had a 3.6 times increased risk to develop severe RT reactions ($p = 0.26$). Neither the -800A allele, nor the 25Pro allele or the 263Ile allele were associated with clinical radiosensitivity. There was perfect linkage disequilibrium (LD) between the -1.552delAGG and the -509C>T polymorphisms, and tight LD between the -1.552/-509 and the Leu10Pro polymorphisms. Haplotype analysis revealed two major haplotypes but could not distinguish radiosensitive from nonradiosensitive patients.

Conclusions: The present study shows that homozygous variant *TGFβ1* -1.552delAGG, -509TT, and 10Pro genotypes may be associated with severe clinical radiosensitivity after gynecologic RT.

Introduction

Late normal tissue complications in cancer radiotherapy (RT) are becoming of increasing concern as the number of long-term cancer survivors increases. For the radiation oncologist, a better understanding of the molecular events underlying normal tissue injury will allow a more rational approach to the prevention and treatment of normal tissue injury (1).

Late RT effects develop months or years after treatment through complex interacting processes and result in diverse pathologic lesions, including fibrosis, necrosis, atrophy, and vascular damage. Important in this response is the production of cytokines (2). Among them, the multifunctional, autoinducible transforming growth factor-β1 (TGFβ1) is considered as a master switch for fibrosis, contributing to both the influx and activation of inflammatory cells, as well as to the activation of fibroblasts to produce extracellular matrix. TGFβ1 is implicated in the development and continuation of postirradiation injury in various tissues (3). The cytokine is known to be upregulated in normal tissues damaged by radiation as well as to be overproduced by tumors (4–6). Circulating TGFβ1 levels are also linked to the development of fibrosis in irradiated normal tissues. Associations between radiation pneumonitis after RT for lung cancer and TGFβ1 plasma levels have been reported (7–9). For RT of breast cancer and head-and-neck tumors, similar associations have been observed with respect to late radiation-induced fibrosis (10, 11). Out of the three known isoforms, TGFβ1 is predominant in human plasma, whereas TGFβ2 and TGFβ3 account for less than 5% of its total plasma concentration (12).

Transforming growth factor-beta-1, located on chromosome 19q13.1, is synthesized as a large immature precursor molecule composed of a signal peptide, a latency-associated peptide, and active TGFβ1. The mature peptide contains two latency-associated peptide dimers with two disulfide cysteine bonds, bound to an active TGFβ1 dimer. In humans, mutations in the latency-associated peptide encoding sequence of the gene lead to the Camurati-Engelman syndrome (13).

Several polymorphisms in the *TGFβ1* gene have been detected (14–19). Grainger *et al.* have demonstrated that the *TGFβ1* promoter genotype is responsible for 54% of the variation in circulating levels of TGFβ1. They studied the -800G>A and the -509C>T polymorphisms and found that the -509TT genotype was significantly associated with increased serum levels of TGFβ1 (17). The Leu10Pro and the Arg25Pro polymorphisms in exon 1 of the *TGFβ1* gene are located in the signal peptide sequence that is

cleaved from the TGFβ1 precursor at codon 29. Whereas both leucine and proline are apolar, the Arg25Pro polymorphism leads to a change of the big polar amino acid arginine for the small apolar proline closely to the 3' end of the hydrophobic core of the signal sequence (20). Dunning *et al.* showed in *in vitro* experiments that the allele encoding 10Pro is associated with increased rates of TGFβ1 secretion (21). Yokota *et al.* reported that the amount of TGFβ1 in serum is higher for 10Pro homozygotes than for 10Leu homozygotes (22). In addition, three independent studies showed a significant association of -509TT and 10Pro homozygotes and the occurrence of late fibrosis in breast cancer patients after RT treatment (23–25). For the Arg25Pro polymorphism, however, homozygosity for the normal 25Arg allele is associated with higher TGFβ1 production *in vitro* and with fibrosis in lung allografts (26, 27). The Thr263Ile polymorphism is located in exon 5 and codes for that part of the TGFβ1 proprotein that is cleaved from the active part at codon 278 (20). Substitution of the polar amino acid threonine by the apolar isoleucine could affect the stability and the activation process of TGFβ1, leading to reduced concentrations of latent and subsequently active TGFβ1 (19).

These observations indicate that TGFβ1 could be involved in the pathogenesis of radiation-induced normal tissue damage and that polymorphisms in *TGFβ1* may predispose individuals to severe normal tissue morbidity after RT. To investigate this association, we performed genotyping for the -1.552delAGG, -800G>A, -509C>T, Leu10Pro (T>C), Arg25Pro (G>C), and Thr263Ile (C>T) polymorphisms in patients with cervix or endometrial cancer treated previously by RT.

Methods and Materials

Participants

The study population consisted of 78 women, of whom 62 were also included in previous studies (28, 29). The patients with cancer of the cervix (n = 35) or endometrium (n = 43) were treated at the Ghent University Hospital with fractionated external beam RT to the pelvis (1 anterior and 2 lateral fields, 25-MV photons) followed by a brachytherapy boost. Eighteen patients received a tumor dose of 45 Gy (25 x 1.8 Gy), 33 patients received a tumor dose of 46 Gy (23 x 2 Gy), and 23 patients received a tumor dose of 50 Gy (25 x 2 Gy). Four patients received 46 Gy supplemented with a parametrial boost up to 60 Gy. Except for 2 patients, all patients were additionally treated by brachytherapy using either vaginal ovoids, Fletcher type applications, or perineal implants. All

brachytherapy was performed using a pulsed dose rate technique with iridium-192. Total doses from brachytherapy ranged from 15 to 35 Gy (dose rate, 0.5 to 0.65 Gy/h) at 0.5 cm from the applicator surface for ovoids and perineal implants and to point A for the Fletcher type application. Twenty-one patients with cancer of the cervix received combined radio-chemotherapy and were treated with 40 mg/m2 cisplatinum per week during the period of the external RT. Sixty-one patients were operatively treated with a Wertheim-Meigs hysterectomy. Nine premenopausal patients received hormone replacement therapy (estrogen). The mean age of the patients at time of treatment was 59 years (range, 24–80 years).

All patients have been scored with respect to several different normal tissue reactions by the same oncologist according to the Common Terminology Criteria for Adverse Events (CTCAE) scale version 3.0 of the National Cancer Institute (30). Fifty-three patients showed no or very light reactions to RT (CTC0–1), 14 patients experienced intermediate but distinct RT reactions (CTC2), 7 patients showed severe RT reactions (CTC3), 3 patients experienced life-threatening RT reactions (CTC4), and 1 patient died as a consequence of the RT (CTC5). All normal tissue reactions appeared 6 months to 5.7 years after RT treatment and can be considered as late reactions. Of the 14 patients classified as CTC2, 13 patients suffered from rectal problems including radiation enteritis with diarrhea, rectal blood loss, and ulcus formation. Three patients had additional urinary problems, 1 patient additional urinary and vaginal side effects, and 1 patient had additional bone problems (osteoradionecrosis). One CTC2 patient had only bone problems (pubis fracture). Of the 7 patients classified as CTC3, 6 patients had rectal injury comprising radiation enteritis with extreme diarrhea, rectal blood loss, ulceration, and intestinal obstruction. One CTC3 patient had exclusively bone problems (pelvis fibrosis, sacrum fracture, and osteoradionecrosis). All the patients classified as CTC4 suffered from severe rectal problems including radiation enteritis with anal blood loss and rectal ulceration leading to intestinal resection. The CTC5 patient suffered from a perforation of the small intestine. This overview shows that, except for 2 patients, all adverse reactions scored comprise rectal injury.

To investigate a possible bias of the treatment protocols on the clinical radiosensitivity, the delivered radiation doses of the CTC2+ group were compared with the CTC0–1 group using a two-tailed Mann-Whitney test. No significant differences could be shown with respect to the external RT dose ($p = 0.224$) and the dose delivered by brachytherapy ($p = 0.549$). The impact of the chemotherapy treatment, surgery, and hormone therapy on

the clinical radiosensitivity was evaluated by chi-square analysis. Therefore, the patient population was sorted according to the treatment and the CTC grading. This analysis showed no significant differences in CTC classification between patients with or without chemotherapy (p = 0.900), with or without surgery (p = 0.976), and with or without hormone therapy (p = 0.293). An overview of the RT protocols according to cancer type and CTC classification is given in Table 1.

Table 1. Overview of the treatment protocol data according to cancer type and CTC classification

	n	Mean external RT dose (Gy)*	Mean brachytherapy dose (Gy)*	% Chemotherapy treatment	% Surgery	% Hormone therapy
Cervical cancer	35	47.4 (4.2)	21.8 (6.4)	51.4	71.4	22.9
CTC 0-1	23	47.3 (4.3)	21.3 (5.4)	47.8	73.9	30.4
CTC 2+	12	47.5 (4.3)	22.7 (8.3)	58.3	66.7	8.3
Endometrial cancer	43	47.9 (2.8)	19.4 (4.7)	7.0	83.7	2.3
CTC 0-1	30	47.6 (3.1)	19.6 (4.4)	10.0	83.3	3.3
CTC 2+	13	48.5 (2.0)	18.8 (5.6)	0	84.6	0

Abbreviations: CTC = Common Terminology Criteria; RT = radiotherapy.

* Standard deviation is in parenthesis.

The patients were subjected to a physical examination every 6 months for the first 2 years followed by an annual examination up to 11 years after treatment. The mean time of follow-up was 5.2 years (range, 0.9 –11.6 years). Patients classified in CTC0–1 are considered as nonradiosensitive patients. Of this category, 1 patient was lost from the follow-up as she died 1 year after RT treatment. In addition, 11% of the CTC0–1 patients left the follow-up prematurely. Patients classified in the CTC2, CTC3, CTC4, and CTC5 (CTC2+) groups are indicated as radiosensitive patients, whereas patients with the most severe RT reactions are classified in the CTC3+ group. For the three patient groups under study, age at time of treatment and follow-up period were very similar. Mean age at the end of the last radiation treatment for the patients without adverse reactions (CTC0–1) was 58 years, for the patients with adverse reactions (CTC2+) 62 years, and for the patients with the most severe adverse reactions (CTC3+) 64 years. The mean follow-up time was respectively 5.3, 5.1, and 4.4 years. To study the association of polymorphisms in *TGFβ1* with global clinical radiosensitivity, the CTC2+ patient group was compared with the CTC0–1 group. To evaluate the association with severe RT reactions, the CTC3+ patient group was compared with the CTC0–1 group.

A Caucasian control population of 140 cancer-free individuals was used to determine the frequency of the *TGFβ1* polymorphisms. The control population consisted of 65 men and 75 women, and the mean age was 42 years (range, 23–83 years). The majority of the control individuals were employees of Ghent University Hospital and were recruited on the occasion of the annual occupational medical examination. Samples of elderly control individuals were collected during a meeting of a local senior club. All patients and control individuals had Belgian nationality.

From each individual in the study, a heparinized blood sample was taken and lymphocytes were isolated and frozen for genotyping analysis. The study was approved by the Ghent University Hospital Ethical Committee. All participants received oral and written information concerning the study and signed the informed consent form.

Nomenclature of polymorphisms

Publications reporting linkage or association studies often use an equivocal description of polymorphisms. According to the nomenclature of the Human Genome Variation Society (31), the correct nucleotide number of polymorphisms in the 5' region of a gene is determined by counting back from the translation start. However, numerous studies use a nucleotide numbering beginning from the transcription start. Polymorphisms in the coding region of the gene are most often named by codon number. Table 2 gives an overview of the different *TGFβ1* polymorphisms, analyzed in this study, with their synonyms used in literature. Because most polymorphisms are already widely known by a certain name, this name was maintained throughout this study (bold in Table 2).

Table 2 . Characteristics of the *TGFβ1* polymorphisms analyzed in this study

Synonyms used in literature					
nt *	**nt†**	**Codon**	**Location**	**Substitution**	**Reference**
- 2.391	**- 1.552**		5' region	AGG deletion	14
- 1.639	**- 800**		5' region	G>A	15, 16, 17
- 1.348	**- 509**		5' region	C>T	15, 16, 17
29	+ 869	**Leu10Pro**	Exon 1 (SP)	T>C	15, 16, 18
74	+ 915	**Arg25Pro**	Exon 1 (SP)	G>C	15, 16, 18
788	+ 1.629	**Thr263Ile**	Exon 5 (LAP)	C>T	15, 16, 19

GenBank accession number = NT_011109 (reverse-complement); bold: nomenclature used in this study.

* Nucleotide number, counting from translation start (in agreement with the nomenclature according to the Human Genome Variation Society) (31).

† Nucleotide number, counting from transcription start.

Genotyping analysis

Polymorphic sites in *TGFβ1* at position -800 (G>A), -509 (C>T), codon 25 (Arg/Pro G>C), and codon 263 (Thr/Ile C>T) were examined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis; the *TGFβ1* Leu10Pro (T>C) polymorphism was determined by SNaPshot analysis and the *TGFβ1* -1.552delAGG polymorphic site was genotyped by direct sequencing. Genomic deoxyribonucleic acid (DNA) was extracted from the isolated lymphocytes using a commercially available kit (QIAamp DNA Blood Mini kit; Westburg, Leusden, The Netherlands). PCR was performed on each DNA sample using the primer sequences detailed in Tables 3 and 4.

Table 3. Technical details of PCR-RFLP analysis

Polymorphism	PCR primers	PCR product size (bp)	Restriction enzyme	Fragments identifying genotypes (bp)
-800 G>A	F = GCAGTTGGCGAGAACAGTTG R = TGGGTCACCAGAGAAAGAGG	681	HpyCh4IV	GG = 195 + 486 GA = 195 + 486 + 681 AA = 681
-509 C>T	F = GCAGTTGGCGAGAACAGTTG R = TGGGTCACCAGAGAAAGAGG	681	Bsu36I	CC = 193 + 488 CT = 193 + 488 + 681 TT = 681
Arg25Pro G>C	F = TGTTCGCGCTCTCGGCAG R = GACCTCCTTGGCGTAGTAG	365	BglI	GG = 252 GC = 252 + 312 CC = 312
Thr263Ile C>T	F = CTGCTCCTGTGACAGCAGG R = AGGCCTCCATCCAGGCTAC	361	FokI	CC = 361 CT = 116 + 245 + 361 TT = 116 + 245

Abbreviation: PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism.

The PCR reactions contained 0.2 mM deoxyribonucleoside triphosphates (Amersham Biosciences, Diegem, Belgium), 1x PCR buffer, 1.5 mM $MgCl_2$, 1 µM forward and reverse primer, 0.6 units Platinum Taq DNA Polymerase (all Invitrogen, Merelbeke, Belgium), and 200 ng DNA in a 25-µL reaction volume. The PCR amplification conditions for the polymorphisms at location -1552, -800, -509, and codon 263 consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and a final extension step at 72°C for 10 min. For the amplification of the DNA fragment containing the codon 10 and codon 25 polymorphic sites, the following touchdown program was used: an initial denaturation step at 95°C for 2 min, 12 cycles of 20 s at 95°C, 15 s at the initial annealing temperature (58°C), 1 min at 72°C, followed by 24 cycles of 40 s at 95°C, 40 s at 46°C, 30 s at 72°C, and a final extension step of 10 min at 72°C. For polymorphisms at -

800, -509, codon 25, and codon 263, the amplified fragments were digested with appropriate restriction endonucleases listed in Table 3. After incubation at 37°C, 20 µL of digested product was analyzed by gel electrophoresis on a 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide (Sigma-Aldrich, Bornem, Belgium). The -1.552delAGG polymorphism was genotyped by direct sequencing (ABI 3730XL DNA Analyzer, Applied Biosystems) using dye terminator chemistry (Big Dye version 3.1, Applied Biosystems). The codon 10 polymorphism was analyzed by single base extension using the ABI Prism SNaPshot Kit (Applied Biosystems, Lennik, Belgium). The DNA samples, containing extension products, and GeneScan-120 LIZ Size Standard (Applied Biosystems, Lennik, Belgium) were added to Amresco Capillary Electrophoresis (ACE) running buffer (Lucron Bioproducts, De Pinte, Belgium) and loaded onto the ABI Prism 3100 Genetic Analyzer. The results were analyzed using GeneMapper Software version 3.7 (Applied Biosystems).

Table 4. Technical details of PCR-single base extension and PCR-genotyping analysis

Polymorphism	PCR primers	PCR product size (bp)	Sequencing / SNaPshot primer
-1552delAGG	F = CCAGGTGGAAGGTGGATTAG R = CTCCCTGATACTCACTGGAG	429	CCAGGTGGAAGGTGGATTAG
Leu10Pro T>C	F = TGTTCGCGCTCTCGGCAG R = GACCTCCTTGGCGTAGTAG	365	TAGCCACAGCAGCGGTAGCAGCAGC

Abbreviation: PCR = polymerase chain reaction.

Statistical analysis

Statistical analysis was performed by MedCalc 4.0 software (MedCalc, Mariakerke, Belgium) and SPSS 10.0 software (SPSS Inc., Chicago, IL). Genotype and allele frequencies were calculated by direct counting. Allele frequencies were examined by calculating the odds ratios (OR), with 95% confidence intervals using Fisher exact test, with the wild-type alleles used as the reference group. Haplotypes were estimated by an expectation-maximization algorithm using the Haploview version 3.2 software (Haploview, Cambridge, MA) (32). This software packet was also used to calculate the linkage disequilibrium (LD) coefficient r^2. The impact of the external RT and brachytherapy doses on the clinical radiosensitivity in the population was evaluated with the Mann-Whitney test. The influence of the chemotherapy treatment, surgery, and hormone therapy was tested using the chi-square test.

Results

Linkage analysis and haplotype determination

The allele frequencies of the *TGFβ1* -1.552, -800, -509, codon 10, codon 25, and codon 263 polymorphisms were respectively 28%, 11%, 28%, 34%, 6%, and 1% in the control population, and 36%, 6%, 36%, 39%, 4%, and 4% in the total patient group. All genotype distributions in both groups considered were in Hardy-Weinberg equilibrium. The mutual position of the *TGFβ1* polymorphisms and the results of the linkage analysis for the healthy individuals are shown in Fig. 1. The first five polymorphisms are located closely together in a region spanning 2.5 kb, whereas the codon 263 polymorphism is approximately 11 kb downstream from the codon 25 polymorphism. There was perfect LD between the -1.552 and the -509 polymorphisms (r^2 = 1 for the control and patient population) and tight LD between the -509/-1.552 and the codon 10 polymorphism (r^2 = 0.71 for the control population, r^2 = 0.87 for the patient group). There was no LD between the other polymorphisms.

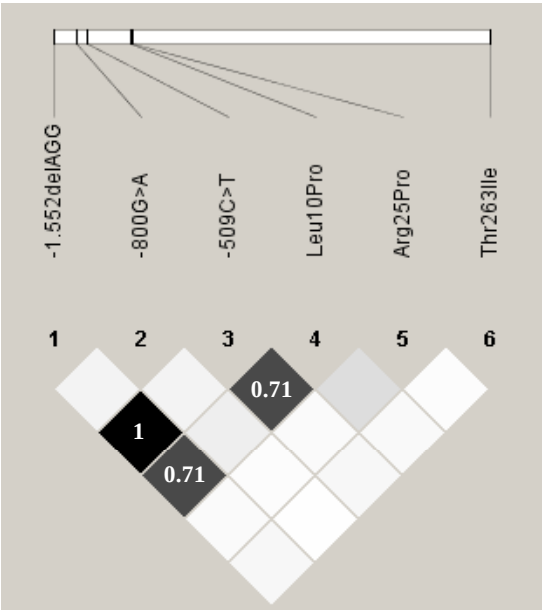

Fig. 1. Map and linkage disequilibrium coefficient (r^2) of *TGFβ1* polymorphisms in 140 healthy individuals.

The six polymorphisms have been used to reconstruct *TGFβ1* haplotypes (Fig. 2). Eight haplotypes (H1–H8) were found in the patient population, four in the control population (H1–H3, H5). The H1 haplotype, composed of all wild-type alleles, and the H2 haplotype, composed of the -1.552, -509, and codon 10 variant alleles, represented >85% of the population in both groups. To correlate haplotypes with clinical radiosensitivity,

haplotype frequencies were calculated for the CTC0–1, CTC2+, and CTC3+ patient groups. This analysis showed that the frequency of the H1 and the H2 haplotypes was similar for the CTC0–1 and the CTC2+ patient groups as for the control group. On the other hand, the H2 haplotype was more frequent in the CTC3+ patient group. Taking CTC0–1 patients and the H1 haplotype as reference, patients with the H2 haplotype had a 2.28 (p = 0.428) times higher risk to develop severe (CTC3+) late RT reactions. Three rare haplotypes (H6, H7, H8) occurred only within the radiosensitive patient group. Because of the low frequency of these haplotypes, they were restrained when calculating haplotype frequencies of the total patient group.

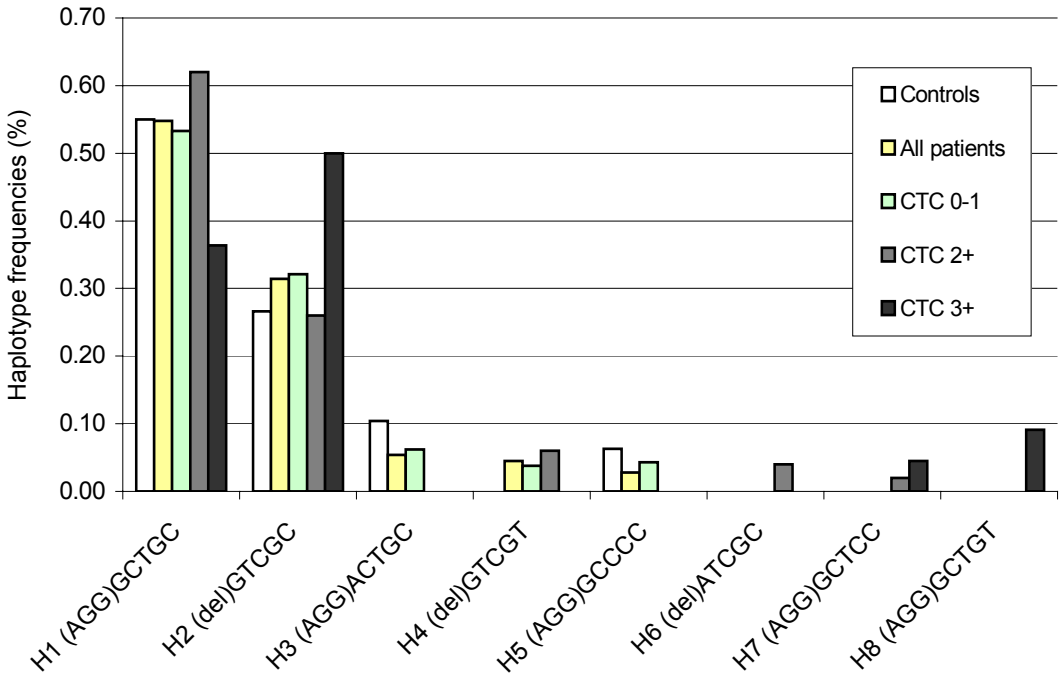

Fig. 2. Haplotype distribution of the control population, the total patient population, and the different Common Terminology Criteria (CTC) patient groups.

Genotyping analysis

The genotype numbers, frequencies, and comparative statistics of the -509 and the codon 10 polymorphisms for the different patient groups are summarized in Table 5. Because there was a perfect linkage between the -1.552 and the –509 polymorphisms, only the results for the -509 polymorphism are displayed. The patients with adverse reactions to RT (CTC2+) had, compared with the nonradiosensitive patients (CTC0–1), a lower percentage of individuals who were heterozygous for the polymorphic alleles at -509 and codon 10 (32% vs. 53% and 32% vs. 58%

respectively), and a greater percentage who were homozygous for the variant alleles at these sites (20% vs. 9% and 20% vs. 11%, respectively). The percentage of -509 and codon 10 homozygous variant individuals increased up to 27% for the subgroup of patients with the most severe RT reactions (CTC3+). Consequently, patients in this CTC3+ radiosensitive group had an OR of 4.00 (p = 0.323) for carrying two variant alleles at -509 and an OR of 2.67 (p = 0.573) for carrying two variant alleles at codon 10. Assuming a recessive action of both polymorphisms (the wild-type genotype together with the heterozygous genotype is the reference genotype), the association of the homozygous variant genotype with severe adverse RT reactions was more pronounced. Patients homozygous for the -509 polymorphism had a risk of severe RT reactions increased by 3.60 times (p = 0.260), and patients homozygous for the codon 10 polymorphism had a risk increased by 2.94 times (p = 0.364). Accordingly, triple (-1.552, -509, codon 10) variant homozygous patients had a 3.6 times increased risk to develop severe RT reactions (p = 0.260). Approximately 50% of the patients with the -509 and the codon 10 homozygous variant genotype are clinically radiosensitive (CTC2+), compared with 29% of the patients with the homozygous normal and the heterozygous genotype (p = 0.337). With respect to severe RT reactions (CTC3+), the percentages are 38% vs. 14% (p = 0.241).

Table 5. Genotype numbers, genotype/allele frequencies (%) and statistics of the *TGFβ1* -509 C>T and the codon 10 T>C polymorphisms in cancer patients with and without reactions to radiotherapy

	CTC0-1 (n = 53)	**CTC2+ (n = 25)**	**OR**	p	**CTC3+ (n = 11)**	**OR**	p
- 509 C>T							
CC	20 (38%)	12 (48%)	1		3 (27%)	1	
CT	28 (53%)	8 (32%)	0.48	0.266	5 (46%)	1.19	0.868
TT	5 (9%)	5 (20%)	1.67	0.738	3 (27%)	4.00	0.323
CT + TT	33 (62%)	13 (52%)	0.66	0.540	8 (73%)	1.62	0.754
CC + CT	48 (91%)	20 (80%)	1		8 (73%)	1	
TT	5 (9%)	5 (20%)	2.40	0.347	3 (27%)	3.60	0.260
Freq T allele	36%	36%			50%		
10 T>C							
TT	16 (30%)	12 (48%)	1		3 (27%)	1	
TC	31 (58%)	8 (32%)	0.34	0.089	5 (46%)	0.86	0.832
CC	6 (11%)	5 (20%)	1.11	0.832	3 (27%)	2.67	0.573
TC + CC	37 (70%)	13 (52%)	0.47	0.201	8 (73%)	1.15	0.865
TT + TC	47 (89%)	20 (80%)	1		8 (73%)	1	
CC	6 (11%)	5 (20%)	1.96	0.497	3 (27%)	2.94	0.364
Freq C allele	41%	36%			50%		

Abbreviations: CTC = Common Toxicity Criteria; OR = odds ratio.

For the polymorphisms at location -800, codon 25, and codon 263, the genotype numbers, frequencies, and statistics for the different patient groups are summarized in Table 6. No major differences in genotype distributions could be found between radiosensitive patients and patients without adverse RT reactions. The number of patients heterozygous for the codon 263 polymorphism increased from 8% for the CTC0–1 patient group to 18% for the CTC3+ patient group, resulting in an OR of 2.72 for the patients with severe RT reactions. On the other hand, the number of -800 variant allele carriers decreased slightly in the CTC2+ patient group and dropped to zero in the CTC3+ patient group. None of these results were, however, statistically significant.

Table 6. Genotype numbers, genotype/allele frequencies (%) and statistics of the *TGFβ1* -800 G>A, the codon 25 G>C and the codon 263 C>T polymorphisms in cancer patients with and without reactions to radiotherapy

	CTC0-1 (*n* = 53)	**CTC2+ (*n* = 25)**	**OR**	***p***	**CTC3+ (*n* = 11)**	**OR**	***p***
- 800 G>A							
GG	47 (89%)	23 (92%)	1		11 (100%)	1	
GA	5 (9%)	2 (8%)	0.82	0.847	0 (0%)		
AA	1 (2%)	0 (0%)			0 (0%)		
GA + AA	6 (11%)	2 (8%)	0.68	0.959	0 (0%)		
Freq A allele	7%	4%			0%		
25 G>C							
GG	48 (91%)	24 (96%)	1		10 (91%)	1	
GC	5 (9%)	1 (4%)	0.40	0.700	1 (9%)	0.96	0.594
CC	0 (0%)	0 (0%)			0 (0%)		
GG + GC	5 (9%)	1 (4%)	0.40	0.700	1 (9%)	0.96	0.594
Freq C allele	5%	2%			5%		
263 C>T							
CC	49 (92%)	22 (88%)	1		9 (82%)	1	
CT	4 (8%)	3 (12%)	1.67	0.828	2 (18%)	2.72	0.594
TT	0 (0%)	0 (0%)			0 (0%)		
CT + TT	4 (8%)	3 (12%)	1.67	0.828	2 (18%)	2.72	0.594
Freq T allele	4%	6%			9%		

Abbreviations: CTC = Common Toxicity Criteria; OR = odds ratio.

Discussion

The reaction of normal tissues to radiation is very similar to tissue wound healing. In case of irradiation, however, the tissue enters into a cycle involving hypoxia, angiogenesis, cell death, proliferation, and macrophage infiltration. Ultimately, this spiral leads to total replacement of the tissue by collagen, leaving few cellular elements (33). In

gynecologic RT, the rectum is the area most often affected by pelvic irradiation. The most common late effects include increased stool frequency, spotting of blood, and partial incontinence. Less common are ulceration, severe bleeding, pain, stricture, severe incontinence, and fistula. Fibrosis and ischemia in the submucosa and muscularis are largely responsible for these effects, accompanied by telangiectasia and other vascular abnormalities, mucosal congestion, collagen deposition, and abnormal fibroblasts (2, 34). The cytokine TGFβ has been demonstrated to be a key mediator of fibrogenesis in a number of pathologic conditions, including tissue reactions postirradiation (4, 35). Moreover, rectum radiation injury is associated with significant downregulation of the endothelial cell surface protein, thrombomodulin. Reduced thrombomodulin levels may contribute to hypercoagulation with increased fibrin formation, platelet aggregation, and subsequent upregulation or release of inflammatory and fibrogenic cytokines, such as TGFβ (36).

Numerous studies reporting the association between high TGFβ1 plasma levels, both before and after RT, in cancer patients and the development of severe radiation-induced fibrosis demonstrate the involvement of TGFβ1 and its functional polymorphisms in clinical radiosensitivity (7–11). To analyze the involvement of TGFβ1 in the pathobiology of radiation-induced damage to normal tissues, we studied six *TGFβ1* polymorphisms in a patient group treated with RT for cervix or endometrium cancer. Therefore, we decided to screen for variations that possibly contribute to the genetic control of TGFβ1 plasma levels. The -1.552delAGG, the -800G>A, and the -509C>T polymorphisms are located in the 5′ and promoter region of the gene and could affect the production of the cytokine. Both Leu10Pro and Arg25Pro polymorphisms are located in the signal sequence, which is responsible for the export of the newly synthesized protein across the membranes of the endoplasmic reticulum. The Thr263Ile polymorphism is situated in the latency associated protein and thus is possibly involved in the stability and activity of the protein. Whereas proven functional information for the -1.552delAGG, the -800G>A, and the Thr263Ile polymorphisms is lacking, both -509C>T and Leu10Pro homozygous variant genotypes have been shown to be associated with increased serum levels of TGFβ1 (17, 22, 37). Previously published studies addressing the influence of *TGFβ1* polymorphisms on clinical radiosensitivity established significant associations between the Leu10Pro as well as the -509C>T polymorphisms and increased risk of radiation-induced late complications after RT for breast cancer (23–25). This association was most pronounced (23) or retrieved only (24) when the mutant homozygous genotype was compared with the wild-type

genotype. In this study we could also only demonstrate an increased normal tissue complication risk for -509TT and 10Pro homozygotes when recessivity was assumed. Double (variant) homozygous patients had a 2.4 (p = 0.347) times increased risk for developing moderate or severe RT reactions and a 3.6 (p = 0.260) times increased risk to develop severe RT reactions.

For the remaining polymorphisms, the study did not provide any valuable associations with the risk of RT complications. Variant alleles at codon 263 correlated slightly positively with radiosensitivity, whereas variant alleles at -800 and codon 25 had a minor protective effect. This protective effect of the codon 25 Pro allele was expected to be higher because several investigations have previously reported a reduced production of TGFβ1 *in vitro* and a decreased risk of different fibro-proliferative conditions for this variant Pro allele (26, 27, 38, 39).

Linkage analysis of the six considered polymorphisms showed a strong linkage between the -509C>T and the Leu10Pro polymorphisms and a perfect linkage between the -1.552delAGG and the -509C>T polymorphisms. The effect on radiosensitivity and TGFβ1 serum levels reported in the literature could thus be caused as well by the Leu10Pro polymorphism by its influence on the intracellular trafficking or export efficiency of the propeptide, as by the -1.552delAGG or the -509C>T polymorphism, through their effect on the production of the proprotein. Kim *et al.* characterized the 5' region of the *TGFβ1* gene down to 1.362 bp from the transcription start and demonstrated that this promoter region consists of several transcriptional binding sites (40). As the -1.552delAGG polymorphism is located outside this screened region, it is unknown whether this deletion polymorphism is situated in or near a transcriptional binding site. Because the last regulatory element was found at position -1.232, the possibility exists that another upstream regulatory region is present. The -509C>T polymorphism lies several bases from nuclear hormone receptor binding sites (41). In this way, the polymorphism could influence transcription, because it has been shown that ligands for nuclear hormone receptors regulate TGFβ1 production *in vitro* and *in vivo* (42, 43).

Haplotypes are becoming important tools in genetic studies because they are theoretically more informative than single polymorphisms. In our patient group, eight haplotypes were identified by six polymorphisms with two major haplotypes that contained 86% of the haplotype distribution. The H2 haplotype, consisting of the -1.552, the -509, and the codon 10 variant alleles, was overrepresented in the CTC3+ patient subgroup (Fig. 2). Because analysis of the individual polymorphisms

showed that homozygous variant genotypes in the -1.552, -509, or codon 10 polymorphisms are required for an association with radiosensitivity, haplotype analysis using these polymorphisms is not ideal for interpretation of the results. On the other hand, the haplotype with the -1.552delAGG, the -800G>A, the -509C>T, and the Leu10Pro variant alleles (H6), the haplotype with the Arg25Pro variant allele (H7), and the haplotype with the Thr263Ile variant allele (H8) occurred only within the radiosensitive patient group.

For this study we evaluated the association between global clinical radiosensitivity and polymorphisms in *TGFβ1* by performing a CTC2+ vs. CTC0–1 analysis. To assess possible differences in genotypes between the more severe radiosensitive individuals, a CTC3+ vs. CTC0–1 analysis was performed. The outcome of both analyses showed that the strongest associations for the -1.592, the -509, and the codon 10 polymorphisms were found for the CTC3+ patient group. Moreover, supplemental CTC2 vs. CTC0–1 analysis revealed a further loss of the associations for the CTC2 radiosensitivity group (results not shown). This indicates that the CTC3+ patients were responsible for the positive odds ratios when comparing the CTC2+ group with the CTC0–1 group. In conclusion, homozygous variant -1.552, -509, and codon 10 individuals are at a higher risk to develop severe or life-threatening reactions after RT treatment.

Although the -1.552delAGG, the -509C>T, and the Leu10Pro homozygous variant genotypes were at higher risk to develop late RT reactions, this higher risk was not statistically significant ($p < 0.05$) in our study using late reactions in the pelvis region as endpoint. Possible explanations for the lack of statistical significance are the relatively small sample size, especially with respect to the CTC3+ patient group, and the uncompleted follow-up for 11% of the nonradiosensitive patients. Forty-eight percent of the patients with moderate and severe radiosensitivity (CTC2+) and 27% of the patients with severe radiosensitivity (CTC3+) were homozygous for the normal alleles. This is in accordance with the study of Quarmby *et al.* where over one-third of the patients with fibrosis in the breast after RT treatment were homozygous for the normal alleles (23). This finding indicates that other factors influencing TGFβ1 levels contribute to the differences in response. Examples are given by Roberts *et al.* and Flanders *et al.* demonstrating that disruption of the signal transduction pathway for TGFβ using transgenic mice deficient in Smad3, results in resistance to the development of radiation-induced fibrosis (44, 45). In addition, gene therapy with an adenoviral vector in rats, which results in increased expression of the type 2 TGFβ receptor (TGFβRII), reduces tissue levels of TGFβ and protects against radiation-induced

injury in the lung (46). Kang *et al.* showed that overexpression of extracellular superoxide dismutase in transgenic mice appears to be protective against radiation-induced lung injury. TGFβ is here also involved because latent TGFβ is activated in part by reactive oxygen species (47).

Conclusion

The present study shows that late severe clinical radiosensitivity may be associated with *TGFβ1* polymorphisms. The association holds only for patients with homozygous genotypes for the polymorphic alleles. The results encourage further investigation of the involvement of *TGFβ1* polymorphisms and TGFβ1 plasma levels in late fibrosis–associated RT complications.

Acknowledgments

The work was supported by a grant of the Bijzonder Onderzoeksfonds (Gent University, No. 01109201). We wish to thank all the patients and control donors who participated in this study. Special thanks to Virginie de Gelder for the help with the lymphocyte and DNA isolations. We also gratefully acknowledge the contribution of Jacqueline Cuypers, Martine De Vogelaere, and Kristel Van De Maele for the collection of the healthy control population, and Christiane Goethals for the patient recruitment.

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Chapter 4

General discussion and conclusions

4.1 Normal tissue toxicity

Radiation therapy remains a cornerstone of cancer management, with approximately half of all newly diagnosed cancer patients receiving radiotherapy at some point in the treatment of their disease (1). Compared with surgery, radiation therapy has the advantage of being non-invasive and potentially organ preserving. Unfortunately, early and late normal tissue toxicity limit the radiation dose, and may affect the long-term health-related quality of life of the patients. As the number of long-term cancer survivors increases, preventing or reducing late side effects has increasingly become a priority. The development of an *in vitro* assay capable of predicting individual radiosensitivity prior to radiotherapy would enable the identification of radiosensitive patients, modification of their therapy and consequently minimalisation of radiation induced normal tissue toxicity. Furthermore, individualized radiotherapy protocols would also allow intensification of therapy in relatively radioresistant patients.

The classical framework for discussing early and late radiation induced side effects is the "target-cell hypothesis". Following this theory, the main effect of ionizing radiation on tissues and organs is thought to be a direct consequence of cell killing, resulting in the depopulation of crucial cell populations and subsequent functional deficiency. Direct loss of parenchymal cells and loss of vascular endothelial cells were considered to be responsible for the clinical expression of radiation damage. The long latency period of late side effects was thought to be a silent interval, during which the irreversible cellular damage was expressed at the time when cells attempting mitosis died following mitotic cell death (2). Recent research in radiobiology and molecular pathology has caused a change of paradigm, particularly in the understanding of late effects.

According to the "orchestrated response hypothesis", radiation induced side effects are the result of a biological response at the cell and tissue level effected by the early activation of cytokine cascades which remain active until clinical expression of late normal tissue injury (3,4). In general, the "target cell hypothesis" remains a useful frame for discussing early radiotherapy effects. On the other hand, for late side effects, radiation induced cell killing is considered to be a triggering event followed by an orchestrated biological response induced by the early release of cytokines.

4.2 Cellular radiosensitivity assays

In the past, several cellular radiosensitivity assays have been developed in order to predict normal tissue response after radiotherapy treatment. These assays have mainly focused on colony forming assays, cytogenetic assays, and assays of residual DNA damage after *in vitro* irradiation. Although some authors found associations between clinical radiosensitivity and *in vitro* radiosensitivity, the observed correlations have been difficult to reproduce between studies (5-26). In this thesis, individual cellular radiosensitivity was determined by the G_2 assay (27). The mean number of chromatid breaks per cell was found to be significantly higher in patients with late normal tissue reactions than in patients without normal tissue reactions. Two other studies could, however, not find a correlation between the G_2 *in vitro* radiosensitivity and late normal tissue effects after breast or larynx irradiation (11,12). Nonetheless, although a positive association between *in vitro* and clinical radiosensitivity was found in our study, the G_2 assay lacked sensitivity to identify high-risk patients at the individual level. Moreover, based on the low reproducibility of the G_2 assay which has been shown by Vral *et al*., care has to be taken when results obtained from one single measurement are used to assess the individual *in vitro* radiosensitivity (28). These shortcomings, which also occur in other studies using different cellular radiosensitivity assays, lead to the conclusion that target cell assays have limited use as a clinical screen for individual increased radiosensitivity. As a consequence, much of the research interest has moved toward genotypic assays.

4.3 Radiogenomics

Radiogenomics is the study of genetic variations in relation to individual differences in radiotherapy response. Based on theoretical considerations and observations from the fields of molecular biology, population genetics and clinical radiobiology, a putative model for the allelic architecture underlying clinical radiosensitivity has been proposed (29). This model is based on the assumption that normal tissue radiosensitivity is dependent of the combined effect of a number common or rare genetic variations in several genes, and on the hypothesis that some genes (and their genetic alterations) are expressed selectively through certain types of normal tissue reactions, whereas others have a general impact on radiosensitivity. The model is illustrated in figure 4.1.

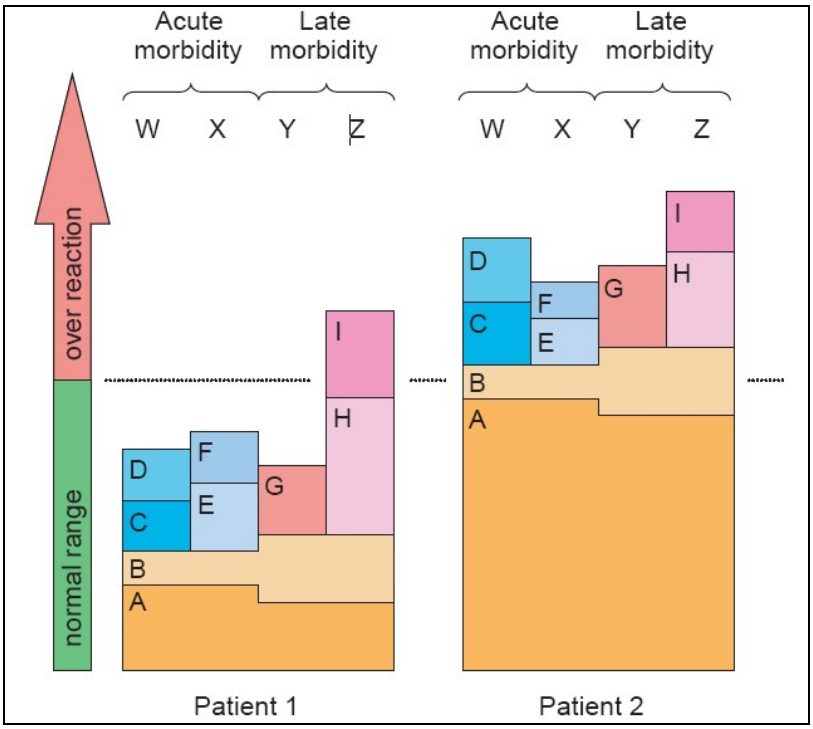

Figure 4.1. Hypothetical model illustrating the influence of different genetic alterations on clinical radiosensitivity. Boxes A-I represent a number of sequence variants in genes of which the gene products are involved in the response to ionizing radiation. Boxes C-I represent sequence variants that are expressed selectively through certain types of normal tissue reactions. Boxes A and B represent sequence variants that affect radiosensitivity in a generalized way. Patient 1 exhibits an increased susceptibility to normal tissue reaction Z due to the variants H and I. Patient 2 exhibits a severe global radiosensitivity due to the highly penetrant sequence alteration A. From Andreassen *et al.* (29).

Over the years, substantial work has been performed in order to identify radiosensitivity candidate genes as well as specific single nucleotide polymorphisms (SNPs) and other genetic variants associated with the

risk of developing normal tissue complications after radiotherapy. To this end, patient groups consisting of breast cancer patients, head- and neck cancer patients, prostate cancer patients and gynecological cancer patients were used. Studied candidate genes included genes coding for DNA damage detection and repair proteins, for endogenous anti-oxidant enzymes and for cytokines related to fibrogenesis and tissue remodeling.

4.3.1 DNA damage detection and repair genes

4.3.1.1 ATM

The first gene that received significant attention was the ataxia telangiectasia mutated (*ATM*) gene. This gene encodes a kinase that amplifies the DNA damage signal induced by DNA double-strand breaks and initiates processes that regulate DNA repair, cell cycle checkpoints and apoptosis (30). Homozygosity for truncating mutations in the *ATM* gene, which results in early termination of the *ATM* gene product, is the typical genetic lesion underlying the rare syndrome ataxia telangiectasia (AT) (31). The *in vitro* radiosensitivity of cells from AT patients is three times higher than that of normal human cells, and these patients exhibit extreme normal tissue reactions to radiotherapy (32). The frequency of AT heterozygotes in the population has been estimated to be approximately 1% (33). As cells from AT heterozygous individuals exhibit an intermediate *in vitro* radiosensitivity compared to cells of AT patients and healthy controls (34), it has been speculated that AT heterozygotes may represent a significant proportion of clinically radiosensitive patients. However, a large number of studies did not support the idea that individuals with heterozygous truncating *ATM* mutations are at a higher risk for developing normal tissue complications after irradiation (18,35-41). Nonetheless, this still leaves the possibility that minor genetic variations in the *ATM* gene, which result in protein alterations other than protein truncation, influence the radiation response. A number of studies investigated this assumption by screening either the entire *ATM* gene for genetic alterations or by analyzing specific genetic variations (42-49). Iannuzzi *et al*. screened 46 breast cancer patients for *ATM* sequence variants and showed that all patients with severe late subcutaneous radiation injury carried two *ATM* alterations (42). A study including 37 prostate cancer patients treated with brachytherapy reported that most of the patients experiencing adverse radiation responses carried an *ATM* sequence variation (43). The preliminary study of Andreassen *et al*. comprising 41 breast cancer patients detected 26 *ATM* variations and showed a significantly increased risk for subcutaneous fibrosis for the Asp1853Asn polymorphism (44). In

a relatively large study of Angele *et al*., 16 *ATM* variants were genotyped in 254 breast cancer patients and the codon 1853 SNP was also found to be significantly associated with increased acute and/or late clinical radiosensitivity. In addition, this study indicated a significant association between two intronic polymorphisms and the risk of normal tissue complications (45). In spite of the strong association found between the Asp1853Asn SNP and normal tissue radiosensitivity, two other relatively small breast and prostate cancer studies and the expanded breast cancer study of Andreassen *et al*. could only find a moderate correlation between the codon 1853 polymorphism and late normal tissue toxicity (46-48). Moreover, a study including 83 prostate cancer patients treated with 3DCRT could also not find a significant association between this *ATM* SNP and late rectal or bladder toxicity observed in 28 patients (49).

4.3.1.2 BRCA1/2

Among the downstream targets of ATM are the tumor suppressor genes *BRCA1* and *BRCA2* (breast cancer 1/2). The encoded proteins are involved in genome stabilization and repair of DNA double-strand breaks. Heterozygous germline mutations in the *BRCA1/2* genes account for a large proportion of hereditary breast cancer (50,51). As these pathogenic and mostly protein truncation mutations appear to be associated with an increased *in vitro* radiosensitivity (52), it has been thought that patients with *BRCA1/2* mutations may constitute a radiosensitive subpopulation. However, two small scale studies (53,54) and a large case-control study, which matched 71 breast cancer patients with a *BRCA1/2* mutation with 213 sporadic breast cancer patients (55), did not find an increased risk for early or late radiation toxicity in patients carrying highly penetrant *BRCA1/2* mutations. The involvement of minor *BRCA1/2* variations in relation to radiation response has up to now only been investigated in one study addressing late toxicity after prostate irradiation. In this study, no association with radiation toxicity was found for 9 SNPs in *BRCA1* and 7 SNPs in *BRCA2* (49).

4.3.1.3 Other DNA repair genes

The DNA double-strand break repair genes *NBS*, *MRE11*, *RAD50*, *DNA ligase4* and *hHR21* have also been screened for genomic alterations in groups of patients showing acute and/or late normal tissue reactions. A *DNA ligase4* single nucleotide deletion was detected in one of five chronic radiosensitive patients (18), and in *hHR21*, a T>C and a G>A substitution was found in, respectively, six and one of 19 radiosensitive patients (56).

Damaraju *et al*. found a significant association between the *DNA ligase4* Asp568Asp polymorphism and late toxicity after prostate irradiation. No association was found for the *NBS1* Glu185Gln polymorphism (49).

The XRCC3 protein participates in the repair of DNA double-strand breaks through the homologous recombination pathway. Up to now, four studies have evaluated the influence of the *XRCC3* Thr241Met polymorphism on the risk of developing late normal tissue damage. In the study based on 41 breast cancer patients from Andreassen *et al*., significant associations were found between the codon 241 Met allele and a decreased risk of subcutaneous fibrosis and telangiectasia (57). However, in two other studies including the one conducted by the same research group in order to confirm the initial results, no significant association between this SNP and risk for late toxicity was found (47,48). Moreover, our first study performed in this thesis in which 62 women with cervical or endometrial cancer were screened for three *XRCC3* polymorphisms, could also not demonstrate an association between the codon 241 SNP and the risk of developing late toxicity after pelvic irradiation. Yet, our study showed that heterozygosity for the intronic *XRCC3* IVS5-14 A>G polymorphism significantly increases the risk of late radiation injury (27). Because this polymorphism is located in a non-coding sequence region of the gene, the functional effect of the polymorphism is still incomprehensive. The functional relevance could be associated with an alternative splicing variant or be involved in RNA stability. Another possibility is that the polymorphism is in linkage disequilibrium with other functional variants not tested in this study. Nonetheless, the positive association between this intronic polymorphism and late clinical radiosensitivity could not be confirmed in the prostate cancer study of Damaraju *et al*. Moreover, in contrast to our study, the *XRCC3* 5'UTR 4.541 SNP was shown to be significantly associated with late radiation toxicity. In the same study, no associations with radiation toxicity were found for one *XRCC2*, one *Rad52* and two *Rad51* SNPs (49).

The XRCC1 protein plays a critical role in base excision repair. Several SNPs which have been associated with an altered cellular response to ionizing radiation have been documented in the *XRCC1* gene. A total of six studies have investigated the influence of the relatively common *XRCC1* Arg399Gln polymorphism on normal tissue radiosensitivity. In the previously mentioned study with 41 breast cancer patients, the codon 399 Gln allele was significantly associated with a decreased risk of subcutaneous fibrosis (57). Accordingly, the study of Chang-Claude *et al*. including 446 patients, also found a significantly decreased risk for acute skin toxicity after breast irradiation for codon 399 Gln carriers (58). In the

first study performed in this thesis, the impact of the *XRCC1* Arg399Gln polymorphism on radiation induced toxicity was also investigated. In this study, no significant association was found between the codon 399 polymorphism and the occurrence of late radiation toxicity (27). A study conducted in breast cancer patients, did also not demonstrate significant associations between the *XRCC1* Arg399Gln SNP and risk of altered late breast toxicity (47). Neither did the expanded breast cancer study of Andreassen *et al*. provide a significant association between this polymorphism and the risk of radiation induced subcutaneous fibrosis (48). Finally, in a study addressing various acute and/or late adverse reactions in 254 breast cancer patients, the codon 399 Gln allele was also not associated with an altered risk for normal tissue complications (59). Most of the aforementioned studies also investigated the association between other *XRCC1* polymorphisms and clinical radiosensitivity. In our study, the variant Trp allele of the Arg194Trp polymorphism was found to exhibit a significant protective effect for the development of late radiation injury (27). As a similar association has not been reported in any of the other studies (48,49,58,59), and as this polymorphism has a low allele frequency, it was thought that our result could be caused by an underrepresentation of heterozygous individuals in the patient group with radiotherapy reactions. Indeed, none of the 22 patients with adverse radiotherapy reactions possessed the variant 194Trp allele. In our study, no significant association with late clinical radiosensitivity was found for the *XRCC1* Arg280His and Gln632Gln polymorphisms and for the *OGG1* Ser326Cys polymorphism (27). For the codon 280 SNP, this is in accordance with the results of the other published studies (48,49,58,59). No additional data concerning clinical radiosensitivity are available for the *XRCC1* codon 632 and the *OGG1* codon 326 polymorphisms. On the other hand, Chang-Claude *et al*. and Brem *et al*. studied the association of, respectively, the *XRCC1* -77 polymorphism and the *APE1* Asp148Glu polymorphism. Only for the latter SNP, a significant association with radiation toxicity was found (58,60).

In 1997, Price *et al*. reported highly significant associations between the risk of various normal tissue reactions and rare microsatellite polymorphisms in *XRCC1* and *XRCC3* in a population of 19 cancer patients (61). In their study, rare microsatellites were alleles with less than 12 or more than 23 [AC] repeats for *XRCC1* and alleles with more than 20 [AC] repeats for *XRCC3*. In the second study performed in this thesis, the association of microsatellite polymorphisms in *XRCC1* and *XRCC3* with the development of late radiotherapy reactions was investigated in 62 women treated for gynecological tumors. In this study, a positive association with late radiotherapy reactions was found for patients with

11 and 21 [AC] repeats in *XRCC1* (62). Although these results were not statistically significant, patients with *XRCC1* $[AC]_{11}$ and $[AC]_{21}$ repeats were clearly overrepresented in the radiosensitive patient group. Alleles with more than 23 [AC] repeats in *XRCC1* were not present in the patient population and large *XRCC3* alleles ($[AC]_{20}$ and $[AC]_{21}$ repeats) did not correlate with clinical radiosensitivity. Both studies also investigated the influence of an *XRCC5* (*Ku80*) microsatellite polymorphism but could not demonstrate an association between any of the repeats and clinical radiosensitivity (61,62).

XPD (ERCC2) and XPF (ERCC4) are two proteins involved in nucleotide excision repair. In two studies, no associations between *XPD* SNPs and radiation toxicity after breast or prostate irradiation were found (49,58). One study investigating the influence of two *XPF* SNPs on normal tissue toxicity in 130 head- and neck cancer patients, found a significant association between the Ser835Ser polymorphism and radiation toxicity (63). Damaraju *et al*. could, however, not link this polymorphism to an enhanced rectal or bladder toxicity (49). Apart from the above mentioned genes and SNPs, this study also examined one polymorphism in the *BCL2* apoptosis gene, three polymorphisms in two mismatch repair genes, 10 polymorphisms in 9 steroid metabolism genes and two polymorphisms in two genes coding for cell signalling proteins. Variant alleles of the mismatch repair gene polymorphism *MLH1* Val219Ile were found to exhibit a protective effect, and variant alleles of the *CYP2D6*4* G>A polymorphism were positively associated with increased late rectal and bladder toxicity (49).

4.3.2 Endogenous anti-oxidant genes

The generation of reactive oxygen species (ROS) is the major mechanism by which the cytotoxic effect of ionizing radiation is induced. Moreover, sustained oxidative stress may be responsible for the development of late radiation reactions (64). Manganese superoxide dismutase (MnSOD) encoded by *SOD2* is an important scavenger of ROS and plays thereby a major role in the defense against oxidative damage (65). Four studies have investigated possible associations between clinical radiosensitivity and SNPs in *SOD2*. The study of Green *et al*. including 80 breast cancer patients could not find a significant association between the Ala16Val polymorphism and late skin reactions after radiotherapy. Moreover, no novel sequence variations were detected in any of the patients (66). The other studies considering the involvement of genetic variations in *SOD2*

were all performed by the research group of Andreassen *et al*. In their study considering 41 breast cancer patients, it was shown that the *SOD2* Val16Ala polymorphism was significantly associated with radiation induced subcutaneous fibrosis (57). However, this positive association could not be confirmed in their study with 120 breast cancer patients, nor in their study considering 52 breast cancer patients (47,48).

4.3.3 Fibrogenesis and tissue remodeling cytokine genes

A number of different cytokines and growth factors are involved in the acute and late response of normal tissues to ionizing radiation (67). Especially the multifunctional cytokine TGFβ1 plays a crucial role in the development of radiation induced fibrosis (68). A number of SNPs have been described in *TGFβ1*. Three of these SNPs (-509C>T, Leu10Pro, Arg25Pro) have gathered particular interest as they are located in the promoter or starting sequence of the gene, and as they have been demonstrated to affect TGFβ1 secretion and the risk of various pathologic conditions (69-72). Six studies have been performed to investigate the influence of *TGFβ1* SNPs on the risk of developing late normal tissue complications after radiotherapy. Quarmby *et al*. were the first to demonstrate significant associations between the -509C>T and the Leu10Pro polymorphisms and the risk of severe fibrosis after radiotherapy in 103 breast cancer patients (73). Two other studies in which the involvement of *TGFβ1* SNPs in, respectively, 41 and 52 breast cancer patients was investigated in relation to radiation induced subcutaneous fibrosis, also reported significant associations between these SNPs and the occurrence of skin fibrosis (47,57). These associations were most pronounced (73) or retrieved only (57) for variant homozygous genotypes. In the third study in this thesis, the association between six *TGFβ1* polymorphisms and the risk of late radiotherapy reactions was investigated in a group of 78 women treated for gynecological cancers. In this study, we could also demonstrate an increased, however not statistically significant, normal tissue complication risk for -509TT and 10Pro homozygotes (74). Moreover, as we showed the existence of a tight linkage disequilibrium between the -509C>T, the Leu10Pro and the -1.552delAGG polymorphisms, the effect on radiosensitivity and on TGFβ1 secretion could be caused by any of the three polymorphisms. In contrast to the above findings, the larger study of Andreassen *et al*. could not confirm the significant associations between these SNPs and the risk of radiation induced subcutaneous fibrosis (48). Given the very consistent results obtained in all four other conducted investigations, this finding

was very unexpected and the authors could not find any explanation for the observed inconsistency. Another very recent study including 83 prostate cancer patients, could not find an association between the *TGFβ1* -509C>T polymorphism and the occurrence of late rectal damage. The Leu10Pro polymorphism was not studied (49). None of the six studies could indicate a significant association between the Arg25Pro polymorphism and late normal tissue reactions. In our study, variant alleles at codon 25 had a minor protective effect which was expected to be higher since several investigations reported a reduced production of TGFβ1 for this variant Pro allele (74). Furthermore, our study could not provide valuable associations for the -800G>A and the Thr263Ile polymorphisms. For the -800 SNP, this is in accordance with the study of Quarmby *et al*. (73,74). No other data are available for the codon 263 polymorphism.

4.3.4 Methodological issues

From the overview of previous section it is clear that the associations between the studied genetic polymorphisms and normal tissue complications are not always entirely straightforward. The inconsistencies can be explained largely by a number of methodological issues since the performed studies differ from each other in several aspects. This makes a direct comparison of the results difficult.

First of all, a variety of different normal tissue damage endpoints were used to evaluate radiosensitivity. These ranged from basic endpoints such as telangiectasia, subcutaneous fibrosis and skin erythema to more complex endpoints as erectile dysfunction and rectal injury (27,43,49). In a number of studies, different types of normal tissue reactions were studied separately whereas in other studies, various acute and late effects were considered together (45,59,60). The latter approach may represent a problem as a number of genes (and their genetic alterations) are expressed only through separate types of normal tissue reactions.

In most of the studies, individual SNPs were analyzed with respect to clinical radiosensitivity. A few studies examined the influence of multiple SNPs based on the concept of risk alleles, which were defined as those being individually associated with increased radiosensitivity (27,44,57). Given the hypothesis that normal tissue radiosensitivity is determined by the combined effect of multiple genetic alterations, this approach seems to be very useful. In the first study in this thesis, two risk allele models based on SNPs in DNA repair genes have been proposed. According to

these models, patients with two or more risk alleles in *XRCC1*, and patients with three or more risk alleles in *XRCC1* and *XRCC3* have a significantly increased risk for developing late normal tissue reactions (27). Andreassen *et al*. also reported two risk allele models which could, however, not be confirmed in their larger scaled study (44,48,57). Although the risk allele approach allows to study the combined effect of different SNPs, this methodology does not consider possible interactions between SNPs. Up to now, the interaction between SNPs, which could be studied by multivariate analysis, has not been analyzed in any of the investigations. A number of studies considered haplotypes to investigate normal tissue radiosensitivity, thereby taking into account the existence of genetic linkage (45,58-60,74). As the haplotype is the basic unit that is inherited, it is interesting to investigate haplotypes rather than individual SNPs (75). The importance of evaluating haplotypes has been described in the study of Brem *et al*. that showed that the same allele of a specific polymorphism can be found on two haplotypes which are differently associated with radiation toxicity (60). On the other hand, in the case of a recessive action of a certain polymorphism, haplotype analysis is less suitable as was illustrated in the last study in this thesis considering *TGFβ1* haplotypes (74).

The most important factors influencing the risk, severity and type of normal tissue reactions are those related to radiation exposure, including dose, dose fractionation, volume and time. The use of concomitant chemotherapy and patient related factors may also affect the development of radiation injury (76). As a consequence, it is crucial to start from a homogeneous study population in which these confounding factors are already controlled for. In the three studies in this thesis, most of the patients received comparable external radiation doses. Although the range of the brachytherapy dose was broader, there was no significant difference with respect to the delivered brachytherapy dose between the radiosensitive and the non-radiosensitive patient groups. The external radiation dose and the total delivered dose did also not differ significantly between groups (27,62,74). However, due to the steepness of the dose response curves for most normal tissue reactions, small differences in absorbed dose can significantly influence the radiation response. In a group of patients apparently treated identically, differences in treatment characteristics may be present due to patient specific anatomical differences which necessitate adjusted field size and treated volume. If the target dose is specified at a variable depth, the dose and dose per fraction at the normal tissue at risk can vary considerably between patients. In addition, factors affecting dose build-up such as radiation type, varying organ anatomy, patient immobilizing and dose

modifying equipment can also influence the dose absorbed at the normal tissue at risk (29,77). In only one of the performed studies the patients were treated with 3DCRT, and mean doses and doses to partial volumes of the organs at risk were available from dose-volume histograms. In this study, significant associations with late rectal or bladder toxicity were found for mean rectal and mean bladder dose, and for dose to 30% of the rectum or bladder. However, no adjustments were made for these confounding factors when addressing the involvement of individual SNPs to bladder and rectal injury (49). As all patients in the other studies were treated by conventional external beam radiotherapy, similar dosimetric parameters were not available. Using the biological equivalent dose (BED) at a certain reference point, Chang-Claude *et al*. accounted for differences in fractionation size and treatment time (58). Andreassen *et al.* accounted for dose distributions between fields, radiation type and other relevant treatment parameters by calculating the BED (44,47,48,57).

As late reactions tend to increase in frequency and severity over time, the length of follow-up is another important factor that needs to be taken into account when studying late normal tissue reactions (78). Considering our gynecological study population, two patients developed severe radiotherapy reactions after a latency period of five years (27,62,74). Consequently, prolonged follow-up periods are necessary in order to exclude false negative samples.

The majority of the aforementioned studies are relatively small, with sample sizes between 5 and 446 patients. Consequently, the statistical power to detect associations with genetic alterations which have a minor impact on radiosensitivity is limited. As demonstrated in our microsatellite study, this is particularly pronounced for less frequent sequence alterations. It was calculated that in order to reach statistical significance for the association between late clinical radiosensitivity and *XRCC1* $[AC]_{11}$ and $[AC]_{21}$ repeats, the patient group had to increase to, respectively, 160 and 80 individuals (62).

4.4 Future prospects

The next logical step in the search for genetic variations affecting normal tissue reactions would be to confirm the already reported associations. Additionally, a broad-based candidate gene approach or genome-wide approach may be essential in order to unravel the genetics of clinical normal tissue radiosensitivity. Both approaches require large patient cohorts with detailed information about treatment characteristics and

normal tissue outcome. Moreover, the patients should be subjected to a long follow-up in which all relevant normal tissue endpoints are assessed. Large scale studies of this kind demand for multicenter programs and international cooperation. Currently, several initiatives have been taken in order to enroll several thousand well-characterized radiotherapy patients. The European GENEPI project coordinated through the ESTRO is the most comprehensive in this regard (79). Others are the British RACE and RAPPER studies, the Japanese RadGenomics project and the Gene-PARE studies (80,81).

4.4.1 Genome-wide approach

All of the association studies conducted so far have been based on the candidate gene approach in which the investigated genes are known to be involved in radiation induced cellular responses. Alternatively, genome-wide association studies could be performed in order to unravel the genetic background of normal tissue radiosensitivity. However, this approach still has a number of theoretical and practical restraints (82,83). It has been estimated that the human genome contains a total of 11 million SNPs of which approximately 9 million are already available in public databases (84,85). Recent research, such as the HapMap project, has revealed that the genome contains regions with pronounced linkage disequilibrium in which the sequence variants are strongly associated with each other (75). It has been estimated that only 200.000 to 500.000 well chosen representative SNPs, called tag SNPs, will be needed to cover most of the sequence variation in the genome (86). Despite recent advances in low-cost high-throughput genotyping, a genome-wide association study will still be extremely demanding, especially since sample sizes of at least 6.000 cases and 6.000 controls might be needed to ensure sufficient statistical power and stringency. Moreover, this approach has the disadvantage that it works less efficient in genomic regions with lower levels of linkage disequilibrium (82). Restricting the focus of genome-wide association studies to missense SNPs only would enable to reduce the number of SNPs needed to be genotyped to approximately 30.000-60.000 (83). However, this does not consider the possibility that non-coding regulatory variants, as one of the two *TGFβ1* SNPs which are quite consistently associated with fibrosis, might be involved in the response. As a consequence, the genome-wide approach seems less suited to study clinical radiosensitivity since, even in an international context, the high number of samples is unlikely to be collected.

4.4.2 Candidate gene approach

A broad-based candidate gene approach may be the best way to proceed in order to identify genetic profiles associated with the risk of developing normal tissue complications after radiotherapy. One possible way to achieve this goal could consist of the analysis of for example a few hundred carefully selected candidate genes. Compared to the already performed investigations, this study design would result in a more extensive examination. The relatively limited number of genes would facilitate a meaningful statistical analysis within a realistic sample size. The selected genes could be screened for common SNPs, thereby considering the available knowledge about linkage, or the selected genes could be resequenced. As interactions between genetic alterations in different genes may complicate the attempts to unravel the genetics of normal tissue radiosensitivity, the analysis of interactions could be limited to genes that participate in the same pathway or to genes of proteins that are known to form complexes and interact physically with each other (87,88). Candidate genes could be selected according to the studied normal tissue endpoint. For instance, in the case of fibrosis, proteins that take part in the fibrogenesis processes could be valuable candidates. As fibrosis is the most important late normal tissue reaction after radiotherapy and as fibrosis is the underlying cause of the normal tissue reactions of the patients considered in the studies for this thesis, we will explore the fibrogenesis processes to identify possible new candidate genes.

4.4.2.1 Radiation fibrogenesis

The biologic effects of ionizing radiation begin with cell damage mainly due to the generated reactive oxygen and nitrogen species (ROS/RNS). These immediate biochemical events rapidly trigger a series of cellular processes including the production of a number of proinflammatory and profibrotic cytokines. The most important cytokine in this context is TGFβ which is directly activated by ionizing radiation through the dissociation of the latency associated peptide (LAP) from the active mature form of TGFβ. The perturbation of the homeostatic control of ROS and RNS also leads to the activation of TGFβ and directly interferes with the Smad signalling pathway. These extracellular events activate the TGFβ Smad signalling pathway, which in turn produces various transcriptional responses, all of which lead to increased extracellular matrix (ECM) and collagen deposition (4). The radiation induced vascular damage and uncontrolled tissue remodeling can lead to tissue hypoxia, which could

be one of the mechanisms perpetuating the fibrogenic response (figure 4.2) (89).

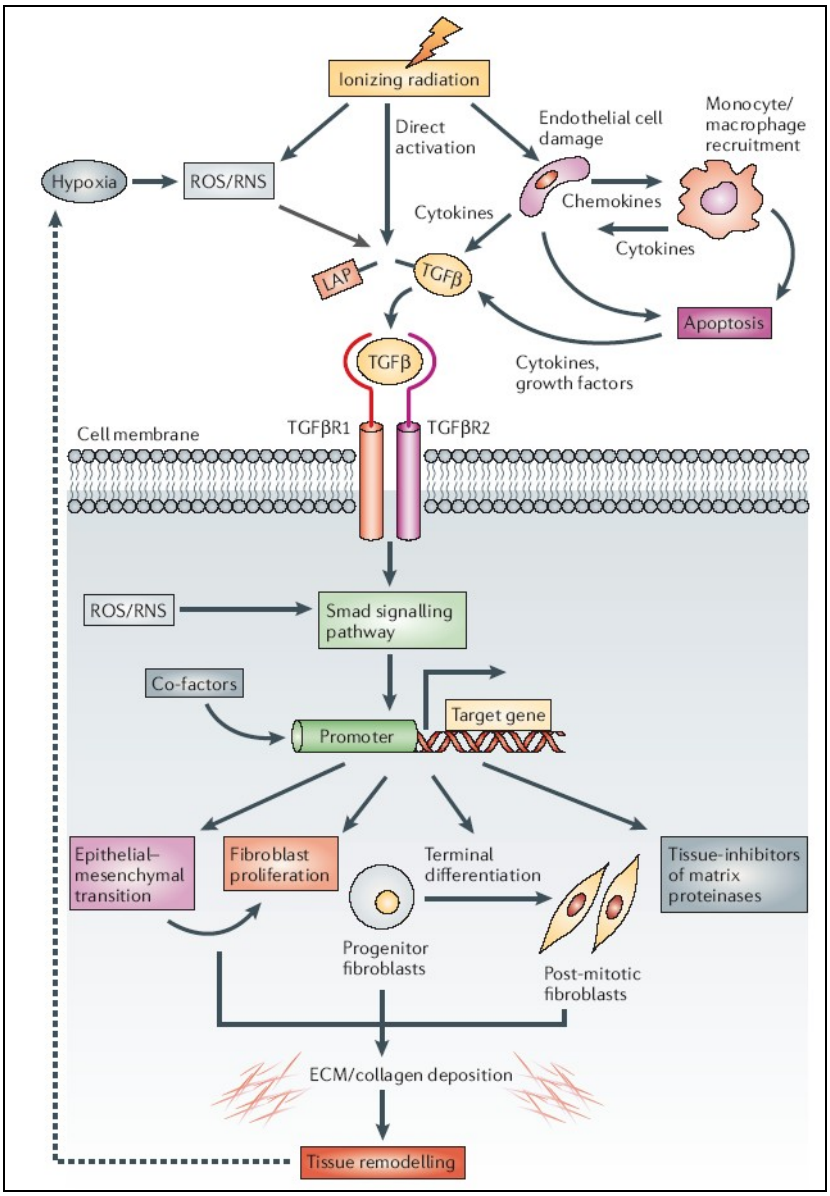

Figure 4.2. Key processes in radiation fibrogenesis. ROS/RNS: reactive oxygen and nitrogen species, LAP: latency associated protein, TGFβ: transforming growth factor β, TGFβR: transforming growth factor β receptor, ECM: extracellular matrix. From Bentzen 2006.

TGFβ exists in three isoforms (TGFβ1-3) of which TGFβ1 is predominant in human plasma. Active TGFβ binds, promoted by TGFβ receptor-3 (TGFβR3), to pairs of two distinct transmembrane receptors TGFβR1 and TGFβR2, and induces transphosphorylation of TGFβR1 by TGFβR2. The consequently activated TGFβR1 phosphorylates receptor-activated Smads (R-Smads: Smad2 and Smad3) which form complexes with Smad4 proteins. These complexes are translocated into the nucleus where they

regulate transcription of target genes through physical interaction and functional cooperation with transcription factors and co-activators or repressors. Two inhibitory Smads (Smad6 and Smad7) antagonize TGFβ signalling by preventing the activation of R-Smads. The ubiquitin ligases Smurf1 (Smad ubiquitination regulatory factor-1) and Smurf2 counteract TGFβ signalling by interacting with R-Smads and targeting them for degradation. Smurf1/2 can also degrade TGFβR1 through interacting with Smad6/7 (90). The signalling pathway is depicted in figure 4.3.

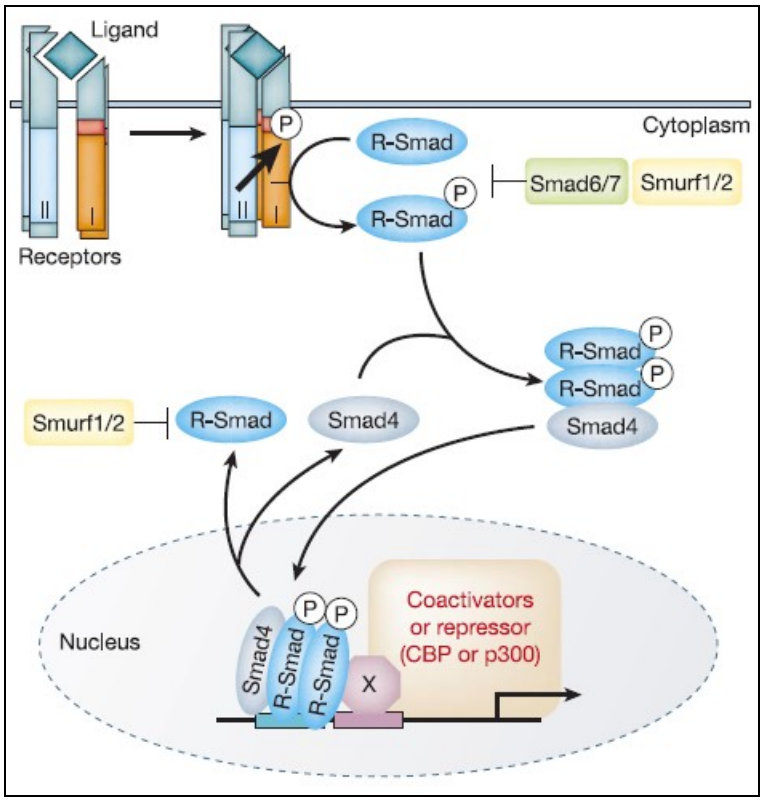

Figure 4.3. Transforming growth factor β (TGFβ) Smad signalling pathway. R-Smad: receptor-activated Smad, Smurf: Smad ubiquitination regulatory factor, X: transcription factor, CBP: CREB binding protein. From Derynck *et al.* 2003.

Although the Smad pathway is not the only TGFβ signalling pathway, it mediates most profibrotic activities (91). The involvement of TGFβ and Smad signalling proteins in radiation fibrosis has been showed by a number of studies. First of all, overexpression of TGFβ was described in diverse experimental models of late radiation injury (68), and increased TGFβ1 plasma levels have been associated with radiation induced fibrosis in radiotherapy patients (92-96). In various rodent studies, it has been possible to reduce the development of fibrosis by TGFβ1 neutralizing antibodies (97), *TGFβ1* antisense oligodeoxynucleotides (98) or recombinant TGFβ1 LAP (99). Furthermore, it was shown that disruption of the TGFβ signal transduction pathway, using transgenic mice deficient

in Smad3, prevents radiation induced fibrosis (100-102). Other rodent studies have established that the delivery of soluble TGFβR2 reduces the risk of radiation- or bleomycin induced lung injury (103,104). Finally, small molecule inhibitors which disrupt the TGFβ signalling pathway have been shown to diminish radiation induced fibrosis in mice (105). Apart from the observed effects of TGFβ and Smad signalling proteins on radiation fibrosis, it has recently been shown that loss of TGFβ signalling also disturbs the DNA damage response by reducing the ATM kinase activity (106,107). This indicates an additional role for TGFβ in regulating responses to genotoxic stress. Taken into account all previous indications, the genes coding for proteins involved in this signalling pathway could represent excellent candidate genes to study clinical radiosensitivity.

Despite the fact that TGFβ is a key cytokine, the fibrotic process involves a complex network of interacting cytokines and growth factors including platelet-derived growth factor (PDGF), interleukin-1 (IL1), insulin-like growth factor-1 (IGF1), and tumor necrosis factor-alpha (TNFα) (68). One of the downstream effects of active TGFβ is that it upregulates a number of cytokines and growth factors, including IL1, epidermal growth factor (EGF), fibroblast growth factor (FGF), connective tissue growth factor (CTGF) and TGFβ itself (108). This mechanism of auto-induction contributes to the maintained level of TGFβ and to the continuous production of cytokines and growth factors. In many aspects, radiation fibrosis resembles normal wound healing which is regulated by a complex balance between profibrotic proteins such as TGFβ and CTGF on the one hand, and antifibrotic proteins such as TNFα and interferon-γ (IFNγ) on the other. TNFα and IFNγ are expressed during normal wound healing and both cytokines have been shown to inhibit the TGFβ signalling pathway by inducing expression of Smad7 (109). In addition, IFNγ was shown to decrease bleomycin induced lung fibrosis in mice, and TNFα was shown to be downregulated and CTGF to be upregulated in fibrotic bowel tissue from patients with late radiation enteritis (110-112). Accordingly, the apparent dysfunctional homeostatic feedback control in radiation fibrogenesis also contributes to radiation fibrosis, and the genes encoding the involved cytokines and receptors could be appropriate candidate genes in a broad-based SNP approach.

Radiation induced fibrosis is characterized by a progressive and excessive accumulation of ECM. Factors that play a role in remodeling of the ECM include structural ECM proteins, ECM degrading proteases (matrix metalloproteinases or MMPs) and inhibitors of ECM breakdown (tissue inhibitors of metalloproteinases or TIMPs) (113). Apart from TIMPs, degradation of the ECM is also inhibited by plasminogen activator

inhibitor-1 (PAI1) which prevents proteolytic activation of latent MMPs, as well as fibrin breakdown, by inhibiting the plasminogen-plasmin activating cascade (114-115). Many different cytokines have been identified that regulate the transcription of genes implicated in the regulation of the ECM. Of these, TGFβ has been shown to be a potent transcriptional activator of ECM regulators such as PAI1 and TIMP1, and ECM components such as collagen-1a2 (Col1a2) and ED-A fibronectin (ED-A FN) (116,117). Several studies provided direct evidence that PAI1 plays a pivotal role in the pathogenesis of fibrosis: *PAI1* knockout mice were protected from the development of bleomycin induced lung fibrosis and showed a rapid removal of fibrin-rich matrix compared to wild-type mice (118-120). Furthermore, upregulation of several collagen types, MMPs, TIMPs and PAI1 has been found in strictured ileum from patients with late radiation enteritis (112,121). Based on the above findings, it would be interesting to investigate if polymorphisms in ECM modulating genes alter the response to either radiation or TGFβ.

Another key element in fibrogenesis is the homeostatic control of ROS/RNS in the cell which is altered by ionizing radiation and by hypoxia following tissue remodeling (64). Disturbed ROS/RNS levels initiate a number of cellular responses and activate TGFβ. MnSOD, CuZnSOD, gluthathione peroxidase and catalase are free radical scavenging enzymes that protect cells from oxidative stress. There is direct evidence that the SOD enzymes have an important role in fibrosis (122). Transfection with *SOD2* has been shown to protect against late radiation induced lung damage in mice (123,124), and injection of MnSOD in pigs has been shown to reverse established radiation induced fibrosis (125). In addition, it has been demonstrated that bleomycin induced lung fibrosis in mice could be reduced by inhibiting inducible nitric oxide synthase (126). Consequently, analysis of genes coding for anti-oxidant enzymes or nitric oxide synthases could also contribute to the identification of the genetic profile associated with fibrosis.

The renin-angiotensin system (RAS) and the Rho/ROCK pathway have both been shown to be involved in radiation induced fibrosis. A number of animal studies reported that RAS blocking reduces the level of damage after irradiation (127-131). Moreover, angiotensin II, which is the key protein in the RAS, is a potent proinflammatory agent, generates ROS, and has the ability to upregulate TGFβ and matrix protein synthesis, and to alter the regulation of ECM degradation through upregulation of PAI1 and MMP2 (132-135). The involvement of the Rho/ROCK pathway in radiation induced fibrosis is based on the observation that expression of genes coding for proteins of this pathway is enhanced in tissues derived

from radiation enteritis patients, and on the finding that inhibition of the pathway results in decreased CTGF, TGFβ1 and Col1a2 expression levels in cells derived from fibrotic bowel tissue (112,136,137).

The above overview indicates a high number of genes and pathways which can be studied in order to unravel the genetic background of radiation induced fibrosis. Although much of the emphasis has been placed on tissue remodeling as a crucial element in radiation fibrosis, it should be mentioned that the initial cellular processing of damage remains relevant as reduced initial cell killing will also reduce the cellular and tissue damage response.

4.5 Conclusions

In this thesis, the value of the cytogenetic G_2 assay in predicting late clinical radiosensitivity after pelvic radiotherapy was assessed. Although a positive association between mean *in vitro* and clinical radiosensitivity was found, the G_2 assay lacked sensitivity to identify high-risk patients at the individual level. This shortcoming leads to the conclusion that the G_2 assay has limited use in the clinical screening of patients for increased radiosensitivity.

The main aim of this thesis was to investigate the association between low penetrant genetic variations and late clinical radiosensitivity. The studied genes included the *XRCC1*, *XRCC3*, *XRCC5* and *OGG1* DNA repair genes, and the cytokine *TGFβ1*. Present work showed that the *XRCC3* IVS5-14 polymorphism is significantly associated with the risk of developing late radiation injury after pelvic radiotherapy. Although individual *XRCC1* SNPs could not be associated with the risk of developing radiation complications, clinical radiosensitivity seems to be significantly associated with a combination of different SNPs in *XRCC1* and *XRCC3*. Moreover, despite the fact that no significant association was found between the repeat length at any of the microsatellites in *XRCC1*, *XRCC3* or *XRCC5* and the incidence of late radiotherapy complications, the possible involvement of small and large *XRCC1* repeats could not be completely excluded. Finally, it was shown that the risk of fibrosis related late toxicity may be modulated by the *TGFβ1* -509C>T and Leu10Pro SNPs. The fact that these associations have also been reported by other independent studies, supports the possible involvement of these SNPs in late radiotherapy toxicity.

To get to a comprehensive understanding of the genetic basis underlying clinical normal tissue radiosensitivity and in order to use genotype data for clinical radiosensitivity screening, large scale investigations using high-throughput technologies are needed. Therefore, patients should be subjected to a long follow-up period in which all normal tissue endpoints are assessed. Moreover, all relevant dosimetric parameters should be taken into account. In addition, *in vitro* studies illustrating genotype-phenotype correlations are necessary to support the scientific background of the associations between genetic variations and clinical radiosensitivity. Currently, several initiatives are taken to establish bio-banks and databases needed for this purpose.

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Curriculum Vitae

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- Geagregeerde voor het secundair onderwijs (UGent, 2002)
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Awards

- Poster Award (Belgian Society of Nuclear Medicine, 2002)
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Membership

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Publications

A_1 Publications

[1] **De Ruyck K**, Lambert B, Bacher K, Gemmel F, De Vos F, Vral A, De Ridder L, Dierckx RA, Thierens H. Biologic dosimetry of Re-188-HDD/lipiodol versus I-131-lipiodol therapy in patients with hepatocellular carcinoma. J Nucl Med 2004; 45: 612-618.

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A_4 Publications

[1] Thierens H, Vral A, Baeyens A, **De Ruyck K**, De Ridder L, Barbe M, Meijlaers M, Claes K, Messiaen L. Application of cytogenetic radiosensitivity assays in breast cancer patients and temporary workers. Annalen van de Belgische Vereniging voor Stralingsbescherming 2003; 28: 93-103.

International communications

[1] 32nd Meeting of the European Environmental Mutagen Society. Warsaw, 3-7 IX, 2002.
De Ruyck K, Vral A, Lambert B, Dierckx R, Thierens H. The mutagenic potential of 131I-lipiodol therapy and combined 131I-lipiodol-cisplatinum therapy in HCC patients.

[2] 35th Meeting of the European Environmental Mutagen Society. Kos, 3-7 XII, 2005.
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National communications

[1] Annual Meeting of the Belgian Environmental Mutagen Society. Brussels, 3 X, 2002.
De Ruyck K, Lambert B, Thierens H. Radiosensitizers for hepatocellular carcinoma: toxicity and therapeutic efficacy.

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De Ruyck K, Thierens H. Radiation induced damage to normal tissues following radiotherapy in patients treated for gynecological tumors: association with single nucleotide polymorphisms in *XRCC1*, *XRCC3* and *OGG1* genes and *in vitro* chromosomal radiosensitivity in lymphocytes.

Invited lectures

[1] Annual Meeting of the Association for Radiation Research, Belfast, 3-5 IV, 2007.
Clinical radiosensitivity in patients treated for gynecologic tumors: association with genetic variants in DNA repair and fibrosis genes, and in vitro chromosomal radiosensitivity.

[2] Zorgprogramma Oncologie Gent (ZOG), Gent, 20 IV, 2007.
Polymorphisms in DNA repair genes in relation to lung cancer risk.