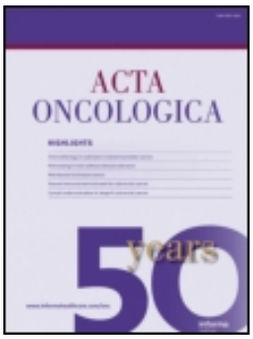

Acta Oncologica

ISSN: 0284-186X (Print) 1651-226X (Online) Journal homepage: informahealthcare.com/journals/ionc20

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To cite this article: Christian Nicolaj Andreassen (2005) Can risk of radiotherapy-induced normal tissue complications be predicted from genetic profiles?, Acta Oncologica, 44:8, 801-815, DOI: 10.1080/02841860500374513

To link to this article: https://doi.org/10.1080/02841860500374513

Acta Oncologica, 2005; 44: 801–815

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REVIEW ARTICLE

Can risk of radiotherapy-induced normal tissue complications be predicted from genetic profiles?

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Abstract
Over the last decade, increasing efforts have been taken to establish associations between various genetic germline alterations and risk of normal tissue complications after radiotherapy. Though the studies have been relatively small and methodologically heterogeneous, preliminary indications have been provided that single nucleotide polymorphisms in the genes *TGFB1* and *ATM* may modulate risk of particularly late toxicity. In addition, rare *ATM* alterations may enhance complication susceptibility. Nevertheless, we are still far from having an exhaustive understanding of the genetics that may underlie differences in clinical normal tissue radiosensitivity. Recent technical advances and emerging insights to the structure of inter-individual genetic variation open up unprecedented opportunities to dissect the molecular and genetic basis of normal tissue radiosensitivity. However, to fully exploit these new possibilities well-planed large-scale clinical studies are mandatory. Currently, international initiatives are taken to establish the bio banks and databases needed for this task.

Radiotherapy is a commonly used treatment modality for various solid tumours. The radiation dose prescribed, and thereby the probability of achieving tumour control, is usually limited by the tolerance of the surrounding normal tissues. It is a well-known clinical experience that cancer patients vary considerably with respect to their normal tissue response to radiotherapy [1]. If this variability could be taken into account in the treatment planning phase, the therapeutic strategy could be further individualised [2]. Thus, the ability to predict the individual risk of radiation induced normal tissue complications prior to cancer therapy has been a long sought goal in clinical radiobiology. Previously, the efforts in this regard have primarily focused on assays based on cellular radiosensitivity or sub-cellular damage endpoints [3]. However, within the last decade an increasing interest has been taken in the concept that normal tissue radiosensitivity could be predicted from individual genetic profiles [4,5]. This assumption is, among other observations, supported by the fact that certain inherited syndromes such as ataxia telangiectasia, Nijmegen breakage syndrome and Fanconi's anaemia have been reported to confer severe and devastating responses to radiotherapy [6–9]. Nonetheless, these syndromes are extremely rare and probably of limited relevance when addressing the average 'non-syndromic' cancer patient [10].

The purpose of the present article is to summarise the current understanding of the inherited basis that may underlie differences in clinical normal tissue radiosensitivity. Studies addressing possible associations between genetic germline variants and risk of normal tissue complications after radiotherapy will be reviewed and critically analysed. Furthermore, the article will discuss how recent technical advances and emerging insights to the 'allelic structure' of human genetic variation can be exploited in the future efforts to unravel the genetic and molecular basis of radiotherapy-associated normal tissue complications. By means of a MEDLINE search (August 2005), 26 studies were found that investigated the influence of genetic alterations upon clinical normal tissue radiosensitivity (Tables I and II). In addition, a study presented at a recent scientific meeting [11] and unpublished data provided by the author of the present article were included in this review. Twelve studies had a specific focus on SNPs (Table I) whereas the remaining investigations

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(Received 12 September 2005; accepted 14 September 2005)

ISSN 0284-186X print/ISSN 1651-226X online
DOI: 10.1080/02841860500374513

Tables I and Table II. Overview of studies addressing associations between genetic alterations and clinical normal tissue radiosensitivity. Table I provides a list of studies with a particular focus on single nucleotide polymorphisms. The studies listed in Table II investigated other types of genetic variants or screened the genes for various sequence alterations.

Table I.

Author, year [ref. #]	Gene(s) investigated	N	Conclusion
Angele S, et al. 2003 [13]	*ATM*	254	Significant association between the codon 1853 Asn/Asn genotype and risk of various acute and late adverse normal tissue reactions, intronic IVS22-77 CC genotype associated with reduced risk
Andreassen CN, et al. (in press) [32]	*ATM*	41	Risk of subcutaneous fibrosis significantly associated with the codon 1853 Asp/Asn and Asn/Asn genotypes
Moullan N, et al. 2003 [43]	*XRCC1*	254	Codon 399 Gln allele in combination with the codon 194 Trp allele associated with increased risk of various acute and late adverse normal tissue reactions
De Ruyck K, et al. 2005 [42]	*XRCC1*, *XRCC3*, *OGG1*	62	*XRCC3* IVS5-14 G allele significantly associated with increased risk of late gastro-intestinal damage, *XRCC1* 194 Trp allele with reduced risk
Chang-Claude J, et al. 2005 [41]	*XRCC1*, *APEX*, *XPD*	446	*XRCC1* codon 399 Gln allele in combination with *APEX* codon 148 Glu allele significantly associated with reduced risk of acute skin reactions in a sub group of 104 patients (8 cases and 96 controls)
Quarmby S, et al. 2003 [58]	*TGFB1*	103	Risk of subcutaneous fibrosis significantly associated with the position −509 T and codon 10 Pro allele in 15 breast cancer patients with severe subcutaneous fibrosis compared to 88 controls
Andreassen CN, et al. 2003 [40]	*TGFB1*, *SOD2*, *XRCC1*, *XRCC3*, *APEX*	41	Risk of subcutaneous fibrosis significantly associated with the *TGFB1* position −509 T and codon 10 Pro alleles, *SOD2* codon 16 Ala and *XRCC1* codon 399 Arg alleles. *XRCC3* codon 241 Thr allele associated with risk of subcutaneous fibrosis and telangiectasia
Andreassen CN, et al. 2004 [33]	*TGFB1*, *SOD2*, *XRCC1*, *XRCC3*, *APEX*, *ATM*	52*	Risk of altered breast appearance significantly associated with the position −509 T and codon 10 Pro alleles in 26 matched case-control pairs
Andreassen CN, et al. (unpublished data)	*TGFB1*, *SOD2*, *XRCC1*, *XRCC3*, *APEX*, *ATM*	120	No significant associations between the investigated SNPs and risk of radiation-induced subcutaneous fibrosis
De Ruyck K, et al. (abstract) [11]	*TGFB1*	62	Non-significant association between possession of the −509 TT and codon 10 Pro/Pro genotypes and risk of late gastro-intestinal damage
Green H, et al. 2002 [50]	*SOD2*	80*	No association between the *SOD2* codon 16 Val/Ala SNP and radiation-induced alteration of breast appearance in 41 cases and 39 matched controls
Kornguth DG, et al. 2005 [44]	*ERCC4*	130	Risk of need for long-term feeding tube after radiotherapy significantly reduced in patients with the T2505C (codon 835 Ser/Ser (silent)) SNP

Note: *The studies were based on patient cohorts that were partially identical, and the results for the *SOD2* codon 16 SNP can therefore not be considered as independent.

Table II.

Author, year [ref. #]	Gene(s) investigated	N	Conclusion
Appleby JM, et al. 1997 [15]	*ATM*	23	No *ATM* mutations detected in 23 patients with severe acute or late toxicity
Clarke RA, et al. 1998 [16]	*ATM*	9	No *ATM* truncations detected in neither five patients with severe acute toxicity nor in four controls
Ramsay J, et al. 1998 [14]	*ATM*	15	No *ATM* truncations detected in 15 patients with severe late toxicity
Weissberg JB, et al. 1998 [17]	*ATM*	13	No evidence of excessive acute or late toxicity in 13 ataxia-telangiectasia heterozygotes
Shayeghi M, et al. 1998 [18]	*ATM*	80	One *ATM* truncation in 41 patients with radiation induced breast shrinkage, none in 39 matched controls, difference not statistically significant
Hall EJ, et al. 1998 [19]	*ATM*	17	Three patients with 'significant' *ATM* mutations in 17 severe late reactors, no mutations in four controls, difference not statistically significant
Oppitz U, et al. 1999 [20]	*ATM*	20	No 'unequivocal' *ATM* mutations in 20 patients with severe acute or late toxicity
Iannuzzi CM, et al. 2002 [23]	*ATM*	46	Significant association between single base mutations and severe subcutaneous late damage.
Cesaretti JA, et al. 2005 [24]	*ATM*	37	Possession of missense mutations significantly associated with radiation-induced rectal bleeding and erectile dysfunction.
Bremer M, et al. 2003 [21]	*ATM*	10	No indications of increased acute or late radio-sensitivity in 10 patients being heterozygous for pathogenic *ATM* mutations
Borgmann K, et al. 2002 [22]	*ATM*, *NBS*, *MRE11*, *RAD50, DNA ligase IV*	5	No mutations detected in five patients with severe late toxicity, possible *DNA ligase IV* polymorphism detected in one patient
Gaffney DK, et al. 1998 [34]	*BRCA1*, *BRCA2*	21	No evidence of excessive acute toxicity in 21 patients with *BRCA1* or *BRCA2* mutations
Pierce LJ, et al. 2000 [35]	*BRCA1*, *BRCA2*	284	No exacerbation of acute or late toxicity in 71 patients with *BRCA1* or *BRCA2* mutations compared to 213 matched controls
Leong T, et al. 2000 [36]	*BRCA1*, *BRCA2*	22	No *BRCA1* or *BRCA2* mutations detected in 22 patients with severe acute or late toxicity
Severin DM 2001 [37]	*hHR21*	19	Non-conservative G1441A transition found in 1/19 and a T1440C substitution found in 6/19 radiosensitive patients. No control group to compare with
Price EA, et al. 1997 [38]	*XRCC1*, *XRCC3*, *XRCC5*	18	Significant associations between microsatellite polymorphisms in *XRCC3*/*XRCC5* and severe acute or late toxicity in 8 radiosensitive patients and 11 'normal reactors'

addressed various other classes of sequence alterations (Table II). The MEDLINE search was based on combinations of the key words: radiotherapy, predictive assays, polymorphisms, DNA repair genes, *BRCA* and *ATM*. Background literature was provided using the keywords: complex trait, quantitative trait, and association studies. Additional publications were located using the citations within the identified papers.

ATM

ATM encodes a protein kinase that plays a decisive role in the detection of DNA double strand breaks and initiation of processes that lead to cell cycle arrest, DNA repair or apoptosis [12]. Homozygosity for truncating mutations in *ATM* is the typical genetic lesion underlying the rare cancer prone syndrome Ataxia Telangiectasia (AT) [13]. As mentioned earlier, AT patients have been reported to experience severely enhanced normal tissue reactions to radiotherapy. Cells from AT heterozygotes exhibit intermediate radiosensitivity compared to cells from AT patients and healthy controls [14]. The frequency of AT heterozygotes in general population is estimated to be approximately 1% [4]. Overall, a relatively large number of studies have not provided indications that AT heterozygotes (carriers of one *ATM* copy with a truncating mutation) constitute a radiosensitive sub population [14–22]. Nonetheless, in three recently published studies the *ATM* gene was systematically screened for genetic changes using DHPLC, a technique capable of detecting minor sequence alterations such as single base substitutions. In 46 patients given adjuvant radiotherapy for early stage breast cancer, a total of nine single base changes were detected in six patients (three patients with two alterations each and three patients with only one alteration). All three patients manifesting severe late subcutaneous damage harboured *ATM* alterations (they all had two alterations each) whereas only three of 43 patients without this type of toxicity had a sequence alteration ($p = 0.001$) [23]. No significant associations were found for acute skin toxicity. Apparently, common base substitutions (i.e. SNPs) were excluded from the analysis. Furthermore, it should be noted that five of nine sequence alterations detected were either conservative or synonymous. In a study including 37 early stage prostate cancer patients given brachytherapy, a total of 21 *ATM* sequence alterations (SNPs as well as rare substitutions) were found in 16 subjects. The patients were evaluated for rectal bleeding, erectile dysfunction and reduced 'urinary quality of life'. Ten of 16 patients with an *ATM* sequence variation experienced at least one of the assessed adverse effects whereas this was only the case for three of 21 patients without any *ATM* alterations ($p = 0.005$). An even more pronounced skewing (7/9 versus 6/28) was observed when the analysis was restricted to missense alterations ($p = 0.004$) [24]. A similar screening of *ATM* has been applied to a cohort of 41 breast cancer patients given adjuvant radiotherapy after mastectomy. These patients represent a fraction of the so-called 'Danish post-mastectomy study cohort' [25], that has previously been subjected to various studies addressing quantitative clinical radiobiology [26–29]. For several reasons, it represents an interesting study object. Firstly, the frequency of late normal tissue damage among the patients was high due to the utilisation of a hypofractionated treatment schedule [30]. Furthermore, the patients had been scored for different types of normal tissue reactions in three different treatment fields from which exact dosimetric recordings were available [31]. Consequently, the data obtained were ideal for dose-response assessments (Figure 1). In this study, the influence of *ATM* alterations upon dose response-relationships for radiation induced subcutaneous fibrosis was investigated. A total of 26 *ATM* variants were found in 22 patients. No difference in risk of radiation induced subcutaneous fibrosis was found between patients with and without any *ATM* sequence variants. However, a significant difference in fibrosis risk was found when seven patients being either heterozygous or homozygous for the *ATM* codon 1853 Asp/Asn (G5557A) SNP were compared to 34 patients without this alteration ($p = 0.03$). The difference in

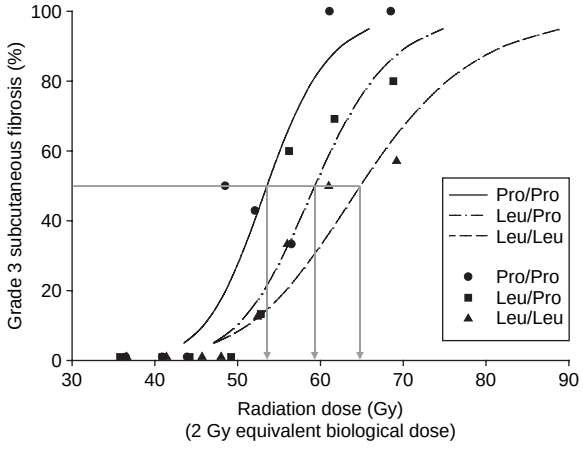

Figure 1. Curves illustrating the concept of estimating differences in radiosensitivity by analysis of dose-response relationships in patients scored for normal tissue morbidity in multiple treatment fields. Based on ED_{50} values (indicated by grey arrows), enhancement ratios can be calculated. Data from the study investigating the influence of the *TGFB1* codon 10 SNP on risk of radiation-induced fibrosis in 41 patients. Modified from ref. [40].

radiosensitivity corresponded to an enhancement ratio (ratio of the ED_{50} values for the two curves) of 1.13 (95% CI 1.05–1.22) [32]. This observation is consistent with the findings of a relatively large study in which 16 SNPs (or single base substitutions with 'sub-polymorphic' allele frequencies) were assessed in 254 breast cancer patients, of whom 70 experienced various acute and/or late normal tissue reactions after radiotherapy. Homozygote carriers of the codon 1853 Asn allele were significantly over-represented among radiosensitive subjects (OR 6.76, 95% CI 1.19–38.43). In addition, indications were provided that heterozygosity for the intronic IVS22-77 T>C SNP may inflict a reduced risk of normal tissue complications (OR 0.45, 95% CI 0.24–0.85) [13]. Two relatively small studies have investigated the *ATM* codon 1853 SNP in patients given radiotherapy for prostate and breast cancer respectively. Although the findings of these studies did not reach statistical significance a moderate accumulation of the codon 1853 Asn allele was found in subjects experiencing late normal tissue toxicity [19,33]. Recently, an investigation was conducted with the intention to seek a confirmation of the associations found in the previously described 41 Danish breast cancer patients. This study was based on 120 breast cancer patients also accrued from the 'Danish post-mastectomy study cohort' and utilised a methodology very similar to that used in the original study. Though the findings of this investigation did not reach statistical significance, the 35 patients being heterozygous for the *ATM* codon 1853 Asn allele were slightly more radiosensitive than the remaining 85 subjects with the Asp/Asp genotype (enhancement ratio 1.04, 95% CI 0.99–1.10) (unpublished data, Andreassen CN).

DNA repair genes

Since DNA damage (particularly DNA double strand breaks) is assumed to be the typical molecular event underlying radiation induced cell killing [12], it seems likely that alterations in DNA repair genes may interfere with risk of normal tissue complications after radiotherapy. *BRCA1* and *BRCA2* were among the first such genes to be investigated in this regard. According to three published studies, the possession of mutations in *BRCA1* and *BRCA2* do not seem to be associated with enhanced clinical radiosensitivity [34–36]. The genes *NBS*, *MRE11*, *RAD50*, *DNA ligase IV* and *hHR21* have been screened for sequence alterations in relatively small series of selected radiosensitive patients. A possible DNA ligase IV SNP was detected in one of four subjects [22]. In *hHR21* a nonconservative G1441A transition in was found in one of 19 subjects and a T1440C substitution was found in six of 19 patients [37]. The studies did not include a control group to compare with. In 1997, highly significant associations were reported between risk of various normal tissue reactions and microsatellite polymorphisms in *XRCC3* and *XRCC5* [38]. The study was rather small and it seems unclear whether these polymorphisms are functionally significant or should be regarded as markers of other alterations. To our knowledge, the results have not been confirmed by other groups.

Recently, several studies have addressed the influence of *XRCC1* and *XRCC3* SNPs upon risk of adverse reactions to radiotherapy. The reason for selecting these variants for investigation is probably that *XRCC1* and *XRCC3* participate in base excision repair and homologous recombination, processes involved in repair of DNA single- and double strand breaks respectively [39]. Furthermore, a number of SNPs in these genes have been reported to affect the risk of different malignancies and to be associated with various biological markers of impaired DNA repair [10,40].

A total of six studies have investigated the influence on clinical normal tissue radiosensitivity of the relatively common *XRCC1* codon 399 Arg/Gln SNP, either alone or in conjunction with other SNPs. In the 41 patient subset of the 'Danish post-mastectomy study cohort', the codon 399 Arg allele was significantly associated with an enhanced risk of radiation-induced subcutaneous fibrosis. The enhancement ratio between the codon 399 Gln/Gln and Arg/Arg genotypes was 1.15 (95% CI 1.02–1.29) [40]. In a relatively large study addressing acute skin reactions in 446 women given radiotherapy after breast conserving surgery, no significant impact on complication risk was found for the *XRCC1* codon 399 SNP in the cohort as a whole. However, when the analysis was restricted to patients with normal body mass index, carriers of the *XRCC1* codon 399 Gln allele in combination with the *APEX* codon 148 Glu allele had a significantly lower risk of acute skin toxicity (hazard ratio 0.19, 95% CI 0.06–0.56). In the same investigation, no association was found for two *XPD* SNPs [41]. A recent study investigated the impact of eight SNPs in three DNA repair genes (*XRCC1*, *XRCC3* and *OGG1*) upon late gastro-intestinal toxicity after radiotherapy for gynaecological tumours. No significant association was found for the *XRCC1* codon 399 SNP, but the codon 194 Trp allele in the same gene was found to exhibit a significant protective effect ($p = 0.03$) [42]. Conversely, in a study based on the previously described 254 breast cancer patients of whom 70 exhibited various acute and/or late adverse reactions, the codon 399 Gln allele in

combination with the codon 194 Trp allele was significantly overrepresented among radiosensitive patients (OR 4.33, 95% CI 1.24–15.12) [43]. Finally, a study conducted in breast cancer patients given post-lumpectomy radiotherapy, did not demonstrate significant associations between the *XRCC1* codon 399 SNP and risk of altered breast appearance [33]. Nor did the investigation based on 120 breast cancer patients from the 'Danish post-mastectomy study cohort' provide a significant association between this SNP and risk of radiation induced subcutaneous fibrosis (unpublished data, Andreassen CN). As mentioned earlier, this investigation utilised a study design very similar to that earlier applied to the 41 subjects from the 'Danish post-mastectomy study cohort' [40].

Four studies have addressed the influence of the *XRCC3* codon 241 Thr/Met SNP upon risk of late normal tissue damage. In the study based on the 41 patients from the 'Danish post-mastectomy study cohort', significant associations were found between the codon 241 Thr allele and increased risk of subcutaneous fibrosis (enhancement ratio for Thr/Met versus Thr/Thr 1.17, 95% CI 1.09–1.26) as well as telangiectasia (enhancement ratio for Met/Met versus Thr/Thr 1.25, 95% CI 1.04–1.51) [40]. However, in three other studies [33,42], including the one (unpublished data, Andreassen CN) conducted with the specific aim to seek a confirmation of the results from the 41 breast cancer patients, support was not provided for an association between this SNP and risk of late toxicity. Yet, in the study addressing gastrointestinal toxicity in patients treated for gynaecologic cancers, the *XRCC3* IVS5-14 G allele was reported to increase the complication risk (OR 3.98, $p = 0.025$) [42].

A single study has investigated the influence of two *ERCC4* SNPs on risk of need for long term gastrostomy feeding tube in 130 patients given radiotherapy for head and neck cancer. The *ERCC4* 2505 C allele was demonstrated to inflict a reduced risk of conditions requiring tube feeding [44]. This finding was somewhat unexpected as the SNP did not result in any amino acid change.

Scavengers of reactive oxygen species

Induction of reactive oxygen species seems to be a major mechanism by which the acute cytotoxic effect of ionizing radiation is mediated. Furthermore, it has recently been suggested that sustained oxidative stress may precipitate the development of late radiation reactions, in particular fibrosis [45]. Manganese superoxide dismutase (MnSOD) is an important scavenger of reactive oxygen species [10]. Increased expression of *SOD2*, the gene of manganese super oxide dismutase, has been found in lung tissue after *in vivo* radiation of mice [46] and transfection with *SOD2* has been demonstrated to protect against late radiation-induced lung damage [46,47]. Injection of MnSOD in rats before irradiation has provided partial protection of the parotid glands [48]. In addition, injection of MnSOD in pigs has been shown to reverse established radiation induced subcutaneous fibrosis [49]. A non-conservative SNP in codon 16 of *SOD2* has been described. This SNP is suspected to alter the protein configuration and has in some instances been associated with enhanced cancer risk [10].

A total of three independent studies have investigated possible associations between clinical radiosensitivity and SNPs in *SOD2*. In the 41 subjects from the 'Danish post-mastectomy study cohort', indications were provided that patients with the *SOD2* codon 16 Val/Ala genotype had a higher risk of radiation induced subcutaneous fibrosis than those homozygous for the codon 16 Val allele (enhancement ratio 1.15, 95% CI 1.01–1.30) [40]. Due to a low number of subjects with the codon 16 Ala/Ala genotype, a meaningful statistical analysis could not be made for this group. In the study with 120 breast cancer patients from the 'Danish post-mastectomy study cohort', based on a very similar study design, no significant association were detected between this SNP and risk of subcutaneous fibrosis (unpublished data, Andreassen CN). Furthermore, no association with risk of altered breast appearance after post-lumpectomy radiotherapy were found for *SOD2* codon 16 SNP [33,50].

Cytokines related to fibrogenesis and tissue remodelling

Several different cytokines are assumed to take part in the acute and late response of normal tissues to ionizing radiation [10]. In particular, the versatile cytokine TGF-β1 seems to play a crucial role in the development of radiation induced fibrosis [51]. In animal models, the *TGFB1* transcription increases after irradiation [52–54]. Administration of TGF-β1 *in vivo* results in fibrotic lesions and transgenic mice that over express *TGFB1*, the gene of TGF-β1, in specific organs develop fibrosis [51]. In various settings, it has been possible to reduce the development of fibrosis by TGF-β1 neutralising antibodies, antisense nucleotides or the TGF-β1 binding protein Latency Associated Protein (LAP) [51]. Furthermore, indications exist that interference with downstream mediators of TGF-β1, such as 'smads' and 'connective tissue growth factor', may prevent radiation induced fibrosis [55–57]. A number of SNPs have been described in *TGFB1*. Three of these

SNPs (position −509, codons 10 and 25) have gathered particular interest as they are located in regulatory regions (promoter region and starting sequence) and have been demonstrated to affect the TGF-β1 secretion rate and risk of various fibrotic disorders [10].

Four studies have been conducted to investigate the impact of *TGFB1* SNPs upon risk of late normal tissue complications after radiotherapy. In a cohort of 15 breast cancer patients with severe fibrosis and 88 without this sequel after post-lumpectomy radiotherapy, significant associations were found between fibrosis risk and the *TGFB1* position −509 C/T as well as the codon 10 Leu/Pro SNPs ($p = 0.004$ and 0.04 respectively). Subjects being homozygous for the −509 T allele or the codon 10 Pro allele were reported to be between seven and 15 times more likely to develop severe fibrosis [58]. Another study investigated *TGFB1* SNPs in 26 breast cancer patients with marked changes in breast appearance after post-lumpectomy radiotherapy compared to 26 matched controls. Also in this study the codon 10 Pro and position −509 T alleles were significantly associated with late radiotherapy complications. In 11 pairs, the index case had a higher number of codon 10 Pro alleles than the matched control whereas only one pair was characterised by the opposite finding ($p = 0.005$). For the position −509 SNP, 11 pairs were identified in which the case had a higher number of T alleles than the matched control whereas in only three pairs, the control had a higher number of T alleles than the case ($p = 0.018$) [33]. An unpublished study, recently presented at an international scientific meeting, reported a non-significant correlation between the *TGFB1* position −509 as well as the codon 10 SNP and risk of late gastro-intestinal damage in patients treated for gynaecological cancers. The frequencies of patients being double homozygous carriers of the position −509 T and codon 10 pro alleles were 9%, 21% and 33% in patients with CTC gastro-intestinal toxicity grade 0–1, grade 2 and grade 3+ respectively. Thus, the double homozygous subjects had a 4.9 times increased risk of severe normal tissue reactions ($p = 0.16$) [11]. In the study including the previously described 41 patients from the 'Danish post-mastectomy study cohort', the impact of *TGB1* SNPs on dose response relationships for subcutaneous fibrosis was investigated (Figure 1). Consistent with the above mentioned findings, this study demonstrated that the codon 10 Pro and position −509 T alleles were significantly associated with enhanced fibrosis risk (Enhancement ratio for codon 10 Leu/Leu versus Pro/Pro 1.21, 95% CI 1.06–1.39, for position −509 C/C versus T/T 1.14, 95% CI 1.01–1.27) [40]. Nonetheless, in the larger study conducted to seek a confirmation of this finding, no significant associations were found between these two SNPs and risk of radiation induced subcutaneous fibrosis (unpublished data, Andreassen CN). Given the very consistent and quite convincing results obtained in the previously conducted investigations, this finding was somewhat surprising. The study was fairly well powered and utilised a study design similar to that applied to the original investigation. A detailed analysis did not provide any obvious biological explanation for the observed inconsistency. In the study conducted on the 120 subjects, formalin fixed tissue samples were used for the genotyping procedure. DNA extracted from this source is often challenging to deal with. However, the utilised assays were carefully validated [59] and high error rates in the genotyping results therefore seem unlikely.

Studies addressing multiple genetic markers

In the study based on the 41 patients from the 'Danish post-mastectomy study cohort', a model was established that demonstrated a correlation between the total number of 'risk alleles' possessed at the 6 polymorphic sites *TGFB1* position −509, codon 10 and codon 25, *XRCC1* codon 399, *XRCC3* codon 241 and *SOD2* codon 16 [40]. The risk alleles were defined as those being individually associated with enhanced radiosensitivity. Later on the model was revised and the *ATM* codon 1853 SNP was incorporated [32]. Another author has also analysed multiple SNP data based on the concept of risk alleles [42]. Given the hypothesis that normal tissue radiosensitivity is determined by the combined influence of multiple genetic alterations (see below), such approach seems very appealing. Nonetheless, the initial results found in the 41 Danish breast cancer patients were not confirmed in a larger study using a similar methodology (unpublished data, Andreassen CN). In addition some of the SNPs included in the other 'multiple SNP model' [42] have not been consistently associated with radiosensitivity. Therefore, these models should certainly be interpreted very cautiously at the current stage.

Methodological issues

Methodologically, the reviewed studies differed from each other in several respects, which may hinder a direct comparison of the results. 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 induration and skin erythema to more complex endpoints like erectile dysfunction and

'urinary quality of life' [24]. In a number of studies, different types of reactions were addressed separately whereas in other instances, various acute and late effects were considered together [13,43]. The latter approach may represent a problem under the assumption that some genetic alterations are only expressed through separate types of normal tissue reactions [1,28] (see below). The subjects included in the investigations varied from groups of selected over-reactors [37] to cohorts of consecutive patients [40]. In between, several studies compared 'reactors' and 'non-reactors' in a case-control like manner (Tables I and II). These differences in patient allocation, may potentially affect the results. It cannot be excluded that subjects selected from different parts of the 'radiosensitivity spectrum' may have different types of genetic alterations as determinants of their radiosensitivity. Thus, a finding obtained in a group of highly selected 'over reactors' may not necessarily be reproducible in cohorts selected from less strict criteria. In most of the studies addressing polymorphisms, the data were analysed with regard to individual SNPs opposed to a few that examined the influence of haplotypes [13,43], thereby taking the existence of genetic linkage into account. From a biological point of view, it seems logical to investigate haplotypes rather than individual SNPs as the haplotype is in fact the basic unit that is inherited [60]. Furthermore, the subsequent statistical analysis may be facilitated when studies address haplotypes rather than a larger number of SNPs in the same gene.

Some of the investigations were based on groups of patients that were very heterogeneous as to treatment characteristics [15,37,42] and occasionally little attention was paid to potential confounders such as radiation dose, fractionation, treated volume and use of concomitant chemotherapy. Due to the steepness of the dose-response curves for most normal tissue reactions, even small differences in absorbed dose are likely to be significant. In addition, the risk of particularly late normal tissue damage is known to be very sensitive to differences in fractionation [30] and it is well-documented that the risk of acute reactions is highly dependent on the treated volume [61] and overall treatment time [62]. Even in a group of patients apparently treated identically, differences in treatment characteristics may be often be present. Field size and treated volume may vary due to anatomical differences. If target dose is specified at a variable depth (for instance according to tumour location), the dose (and dose per fraction) at skin- or subcutaneous level is likely to vary considerably between patients. In addition, factors affecting dose build-up such as radiation type, juxta posed skin surfaces, immobilising and dose modifying equipment are also likely to influence the dose absorbed at skin-level [31]. Furthermore, late reactions tend to increase in frequency and severity over time [27,63]. Therefore, length of follow-up is another factor that often needs to be taken into account in studies addressing late normal tissue endpoints. The variables described above can be encompassed in the analysis of radiobiological data in different ways. A relatively straight forward approach is to carefully match the investigated patients with regard to treatment characteristics and length of follow-up [33]. Alternatively, mathematical models can be utilised to correct for these external factors. Such models will often be based on dose-response formalisms in which the influence of various other parameters can be incorporated [31]. This methodology, however, requires access to very detailed dosimetric recordings [10].

The majority of the reviewed investigations were relatively small (Tables I and II), with sample sizes between 5 and 446 subjects (median 41). Consequently, many of these provided limited statistical power to detect associations for genetic alterations with only modest influence on radiosensitivity. This problem was particularly pronounced for sequence alterations characterised by low population frequencies. Furthermore, it should be noticed that none of the studies took any measures to counteract a potential 'multiple testing problem'.

What has been accomplished so far?

To sum up the present knowledge about the genetics that may underlie differences in clinical normal tissue radiosensitivity, support has not been provided that heterozygous carriers of truncating *ATM* mutations constitute a radiosensitive subpopulation. Nor does *BRCA* mutations seem to confer a major enhancement of radiation-induced normal tissue reactions. Nonetheless, recently published investigations have demonstrated significant associations between *ATM* single base alterations and increased risk of late adverse reactions. Over the last three years, several studies have investigated possible associations between SNPs in selected candidate genes and risk of various normal tissue complications. A total of 34 SNP in the genes *ATM*, *TGFB1, SOD2, XRCC1*, *XRCC3*, *OGG1*, *APEX*, *ERCC4*, and *XPD*, and have been subjected as part of this research (Table I). Even though the results have not been entirely consistent, increasing evidence indicates that the risk of fibrosis-related late toxicity is modulated by the *TGFB1* position −509 and codon 10 SNPs. In addition, the results suggest that the *ATM* codon 1853 SNP may also influence normal tissue radiosensitivity, particularly with regard to

late reactions. Opposed to this, the studies addressing *XRCC1* SNPs have yielded more conflicting results.

A putative model for the 'allelic architecture' underlying clinical radiosensitivity

Overall, it must be concluded that we are still far from having a comprehensive understanding of the sequence alterations that may underlie variability in clinical normal tissue radiosensitivity. On the other hand, the available data seem to provide a reasonable proof of principle that the risk of radiation induced normal tissue complications among unselected cancer patients is influenced by genetic factors. Based on theoretical considerations and observations from the fields of molecular biology, population genetics and clinical radiobiology, the following three hypotheses can be established with respect to the genetic basis that may underlie differences in normal tissue complication risk [10]:

1. *Clinical normal tissue radiosensitivity should be regarded as a so-called quantitative or complex trait dependent on the combined influence of variation in several genes*. The plain observation that normal tissue radiosensitivity shows a continuous variation rather than falling into distinct categories is per se indicative of a polygenic background. Furthermore, numerous genes take part in the biological response to ionising radiation [12]. Given the widespread occurrence of genetic variation, several loci with potential impact on the clinical response to radiotherapy are therefore likely to exist [10].
2. *Single nucleotide polymorphisms make up a proportion of the genetic determinants affecting normal tissue radiosensitivity*. The completion of the human genome project has provided remarkable insights to the genetic variation that exist within human populations. One of the noteworthy findings is that single nucleotide polymorphisms (SNPs) exist in large numbers and account for approximately 90% of inter-individual sequence variation. SNPs in coding regions that inflict an amino acid substitution may alter protein function whereas SNPs in regulatory regions may influence gene expression/protein secretion rates. Thus, SNPs have the potential to affect various phenotypes including clinical normal tissue radiosensitivity. In addition, rare sequence alterations may also impact the risk of radiation induced normal tissue complications. Thus, it seems plausible that the genetic determinants of clinical radiosensitivity will be made up by a spectrum of common and rare sequence alterations that are likely to differ with regard to their penetrance [10].
3. *Some genetic alterations are expressed selectively through certain types of normal tissue reactions whereas others exhibit a general impact on radiosensitivity*. Clinical studies addressing quantitative radiobiology have demonstrated that the risks of certain different types of normal tissue reactions are not associated with each other when corrections are made for differences in treatment characteristics and length of follow-up [26,28]. Under the assumption that genetic factors are major determinants of normal tissue complication risk, some genetic alterations have to exhibit their influence differentially through various types of normal tissue reactions. On the other hand, patients suffering from some of the previously mentioned rare radiosensitive syndromes seem to exhibit a generalised enhancement of radiosensitivity, indicating that some genetic alterations affect radiosensitivity in a broader perspective. Also from a mechanistic point of view, these assumptions seem reasonable, as the biological processes underlying different normal tissue reactions are hardly completely identical. Consequently, some genes are probably specifically involved in certain types of reactions, thereby giving way for a differential impact of certain sequence alterations

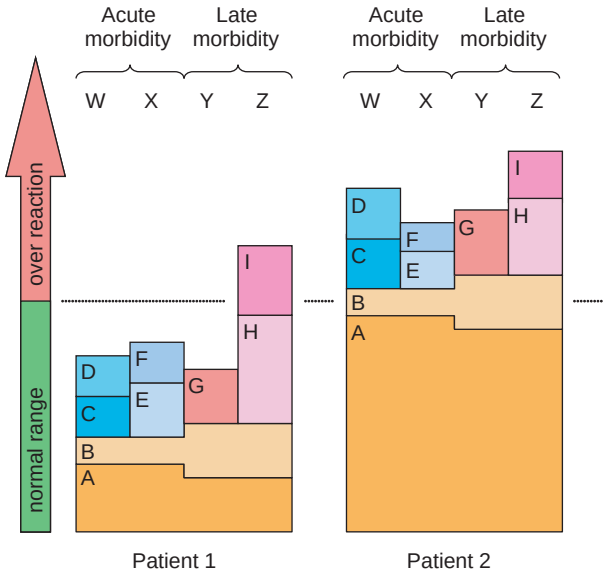

Figure 2. A hypothetical and presumably very simplified model illustrating how different genetic alterations may influence clinical normal tissue radiosensitivity. The underlying assumption is that normal tissue complication risk is determined by the cumulative effect of a number of sequence variants (boxes A-I) that inflict a suboptimal function of gene products involved in the biological response to ionising radiation. Some of these alterations (boxes C-I) are expressed selectively through certain types of normal tissue reactions, whereas others (boxes A and B) affect radiosensitivity in a generalised way. Patient 1 exhibits a selectively increased susceptibility to normal tissue reaction Z due to the variants H and I. In contrast patient 2 exhibit a severe 'global' enhancement of radiosensitivity due to a highly penetrant sequence alteration (box A). Modified from ref. [10].

[10]. These assumptions are graphically illustrated in Figure 2.

Though sparse, the evidence provided by the reviewed studies seem to be consistent with the working hypothesis that individual radiosensitivity is determined by the combined influence of several genetic variants in different genes. Furthermore, preliminary indications suggest that common sequence variations (i.e. SNP) as well as rare alterations (such as *ATM* missense mutations) may affect the risk of radiotherapy induced adverse events. As yet, sufficient data does not exist to bring the 'hypothesis of differential expression' to a final test. However, the *TGFB1* SNPs have so far primarily been associated with normal tissue endpoints that are likely to have the development of fibrosis as an important underlying mechanism. Given the known pro-fibrotic properties of TGF-β1, the *TGFB1* SNPs are obvious candidates for sequence alterations with a differential impact on radiosensitivity.

Nevertheless, the main conclusion that that should be drawn from the reviewed investigations at this point is probably that small studies each addressing a few genetic alterations are unlikely to provide a comprehensive understanding of the genetics that may underlie normal tissue radiosensitivity. Thus, a much more systematic large-scale approach is presumably needed if our knowledge in this area should be dramatically improved.

Theoretical considerations and lessons learned in other scientific fields

Over the last decade, an increasing number of studies have been conducted with the intention to unravel the genetic basis of various human biomedical traits that are assumed to depend on polygenic inheritance (i.e. those referred to as complex or quantitative traits) [64]. The typical study design used for this purpose has been the so-called association study, basically a case-control study in which the frequency of genetic markers (usually SNPs in selected candidate genes) is compared between affected and unaffected subjects [65]. Despite great efforts, these attempts have generally been faced with severe difficulties. Non-replication of previous results has been the rule rather than the exception and only for a very limited number of potential complex trait loci irrefutable associations have been established [64,66]. These difficulties can to some extent be attributed to false positive findings due to type I errors. It should be kept in mind that an extremely high number of variants exist within the human genome. Therefore, the prior probability that a given genetic alteration will be 'truly' associated with the investigated phenotype may occasionally be very low. Under this assumption, the majority of associations reported are likely to be 'false positives' when a typical 5% or 1% significance level is utilised. Publication bias may further exacerbate this problem. At the same time, underpowered studies presumably contribute to the lack reproducibility due to 'false negative' findings (type II errors). In this context, it should be emphasised that most variants that have been demonstrated to affect the risk of various common diseases have been found only to increase disease risk by two-fold or less [65,66].

Apart from these statistical considerations, the limited success in the attempts to unravel the genetics of complex traits has raised some broader methodological questions. Most of the association studies conducted until now have relied on the hypothesis that complex traits are primarily influenced by common genetic alterations such as SNPs. However, very little is still known about the 'allelic architecture' of human complex traits [66]. Even though convincing evidence has been provided that associations exist between common alterations and various complex traits [65], rare alterations might be of equal importance. This assumption is supported by the fact that most cancer susceptibility alleles identified so far have had population frequencies below 1% [67]. If this possibility should be taken fully into account, a complete re-sequencing of the investigated genes would be required. Compared to the assessment of a few selected markers in each gene, such approach is very laborious and expensive. In addition, the subsequent statistical analysis may represent a challenge due to the low frequency and heterogeneity of the detected genetic alterations [67].

Virtually all association studies conducted so far have been based on some sort of a 'candidate gene approach' in which the investigated genes have been selected from various criteria, typically derived from functional knowledge about the gene products. It has been claimed that such approach might be fundamentally insufficient, particularly when the physiology underlying a given phenotype is very complex or poorly understood [64]. The 'genome-wide association study' represents a radical solution to this problem [64,66]. It is estimated that the human genome contains a total of 11 million SNPs of which approximately 9 million are already available in public databases (e.g. www.ncbi.nlm.nih.gov/projects/SNP). Recent research, such as the 'HapMap project' has revealed that most of the genome falls into regions with pronounced linkage disequilibrium in which the sequence variants (mainly SNPs) are strongly associated with each other [60]. This means

that one SNP can serve as a proxy for many others. It is estimated that only 200 000 to 500 000 well chosen 'tag SNPs' will be needed to cover most of the sequence variation in the genome. Nonetheless, the comprehensive high-density SNP maps required for this purpose are not yet available. Despite recent advances in low-cost high-throughput genotyping, a genome-wide association study will still be extremely demanding. This is underscored by the fact that sample sizes of at least 6 000 cases and 6 000 controls might be needed to ensure sufficient statistical power and stringency in a genome wide association study [64]. In this perspective, it should be emphasised that some of the new high throughput technologies only convert about 50% of SNPs into robust assays [66]. Furthermore, this approach has the disadvantage that it works less efficient in genomic regions with lower levels of linkage disequilibrium and that it is unlikely to capture the impact of rare sequence alterations [64]. It has been suggested to restrict the focus of genome-wide association studies to missense SNP (SNPs that result in an amino acid substitution). Thereby, the number of SNPs needed to be genotyped could be reduced to somewhere between 30 000 and 60 000 (on average 1–2 SNPs per gene) [64]. However, such 'missense approach' does not take into account the possibility that non-coding regulatory variants might be of importance for complex traits. In this context it should be mentioned that none of the two *TGFB1* SNPs quite consistently associated with fibrosis risk are expressed in the mature TGF-β1 protein.

Interactions between genetic alterations in different genes may also complicate the attempts to unravel the genetics of phenotypes assumed to depend on polygenic inheritance [68]. Even for a few independent bi-allelic variants, the total number of possible multi-loci genotypes will be overwhelmingly high (note that 10 independent SNPs can be combined into more than 50 000 genotypes) [67]. An unconstrained search for interaction is therefore unlikely to be possible [64]. However, if detailed functional knowledge about the investigated genes is available, the analysis of interactions can be limited to genes that participate in the same pathway or even to genes of proteins that are known to form complexes and interact physically with each other [67]. Alternatively, some of the advanced computational analysis tools (such as cluster analysis) developed for gene expression arrays could be applied to genotype data with the intention to get around a potential 'interaction problem' [64].

Radiogenomics – a potential rewarding task?

The problems generally encountered in the efforts to unravel the genetics of various complex traits may not leave much optimism for the attempts to provide a detailed and cohesive understanding of the genetics probably underlying normal tissue radiosensitivity. However, compared to many other phenotypes, the clinical response to radiotherapy has some distinctive features. Radiation-induced normal tissue damage is the results of a very well defined external exposure. In that respect clinical radiosensitivity is likely to be 'less complex than other complex traits' that usually depend on multiple environmental factors. Furthermore, a detailed understanding exists for at least some of the biological responses to ionising radiation. For instance, studies of yeast and other primitive organisms have provided important insights to some of the key processes related to DNA damage detection, cell cycle control, signal transduction, DNA repair and apoptosis [12]. Finally, radiosensitivity is a phenomenon that can be meaningfully studied in various test systems such as cells, tissue culture or animal models. Therefore, the attempt to unravel the genetics of clinical normal tissue radiosensitivity may constitute a relatively feasible project and a candidate approach might be particularly suitable for this phenotype. Many processes underlying the development of normal tissue complications probably still need to be elucidated however. For instance, the mechanisms involved in radiation induced long-term tissue remodelling and cell-cell interactions in a complex microenvironment are as yet quite poorly understood [12]. Nevertheless, recent technological advances can presumably be used to further 'dissect' the molecular basis of normal tissue complications, and thereby hold the promise to provide a more comprehensive list of 'radiosensitivity candidate genes'.

Micro-array based gene expression profiling represents a promising technology that is likely to shed new light on molecular radiobiology. By determining expression patterns before and after irradiation of cells or tissues, new radiation induced genes could be identified. In addition, experiments comparing the gene expression between radioresistant and radiosensitive cell lines (or between cells/tissues from clinically resistant and sensitive patients) may uncover mechanisms responsible for differences in radiosensitivity [69]. It has recently been suggested that RNA interference could also be used to screen the genome for potential radiation susceptibility genes [70].

In a few instances, family based linkage studies have been successfully used to locate potential complex trait loci to certain chromosomal regions

[64,67]. However, such approach requires access to families in which all (or most) of the members are in fact at risk to develop the phenotype of interest. This condition is rarely fulfilled with regard to clinical radiosensitivity, as families in which all members have received radiotherapy are (fortunately) unusual. Nevertheless, it has recently been demonstrated that variation in the radiation-induced expression level of certain genes seems to have a heritable component. This implies that the radiation induced gene expression pattern obtained *in vitro* could serve as a 'surrogate phenotype' that may allow family based linkage studies to be conducted [71]. Similarly, it has been suggested that congenic strains of radiosensitive animals could be exploited in the efforts to map radiation sensitivity susceptibility genes [72]. Investigations addressing genetic predisposition to cancer and the molecular mechanisms underlying carcinogenesis are also likely to provide insights that could become useful in the field of normal tissue radiobiology [73]. Finally, various test systems (such as computational algorithms, biochemical assays and animal models) exist that can be utilised to determine whether a given sequence alteration in a candidate gene is likely to be functionally significant [74].

How to proceed?

The next logical step in the exploration of the genetic alterations that may affect normal tissue complication risk would be to seek a consolidation of the associations already reported (e.g. for SNPs in *TGFB1* and *ATM* and for rare sequence alterations in *ATM*). However, as indicated above, there are reasons to believe that a more 'broad-based' candidate gene approach may represent a fruitful strategy in the upcoming efforts to unravel the genetics of clinical normal tissue radiosensitivity. One possible way to realise this project, would be to develop a high throughput platform customised for the assessment of variation in, for instance, a few hundred carefully selected 'radiosensitivity candidate genes'. Compared to the investigations carried until now, such approach would offer a much more comprehensive survey for genotype-phenotype associations. At the same time, the relatively limited number of genes would facilitate a meaningful statistical analysis within a manageable sample size. The genes selected could either be assessed with regard to common alterations (SNPs) taking into account the available knowledge about haplotype formation or certain genes could be subjected to a 're-sequencing approach' in which the entire sequence is systematically screened for alterations, including the rare ones.

Nevertheless, a successful utilisation of the new molecular technologies presumably requires large patient cohorts (several hundred or even thousands of subjects) with detailed information about treatment characteristics and normal tissue outcome [10]. In order to fully encompass the temporal occurrence of most normal tissue reactions and the possibility that some genetic alterations are only expressed through certain types of reactions, the patients should ideally be subjected to a longitudinal follow-up including a systematic assessment of all relevant normal tissue endpoints. Furthermore, high quality biological specimens (such as lymphocytes or cultured fibroblasts) should be accessible to allow various *in vitro* functional studies to be conducted in conjunction to the genotyping procedures. Large-scale investigations of this kind are challenging and call for multi centre programmes and international cooperation. Currently, initiatives are taken to establish a large European tissue bank linked to a detailed outcome database. This so-called ESTRO GENEPI project has enrolled several thousand well-characterised radiotherapy patients with the specific aim of facilitating a further investigation of the genetic aspects underlying normal tissue and tumour responses to therapeutic radiation [40]. Similar projects are underway in other parts of the world [75].

Conclusion

So far, only a limited number of relatively small and methodologically heterogeneous studies have addressed possible associations between genetic germline variation and clinical normal tissue radiosensitivity. Preliminary results suggest that certain SNPs as well as rare sequence alterations may influence normal tissue complication risk. However, to get a comprehensive understanding of the genetic basis that may underlie clinical normal tissue radiosensitivity, large-scale investigations making use of novel high throughput technologies are probably needed. Currently, initiatives are taken to establish the bio banks and databases needed for this purpose and the coming years are likely to shed new light on genetic and molecular aspects related to clinical radiobiology.

Acknowledgements

This work was supported by grants from the Danish Cancer Society and was conducted as part of the ESTRO GENEPI project.

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