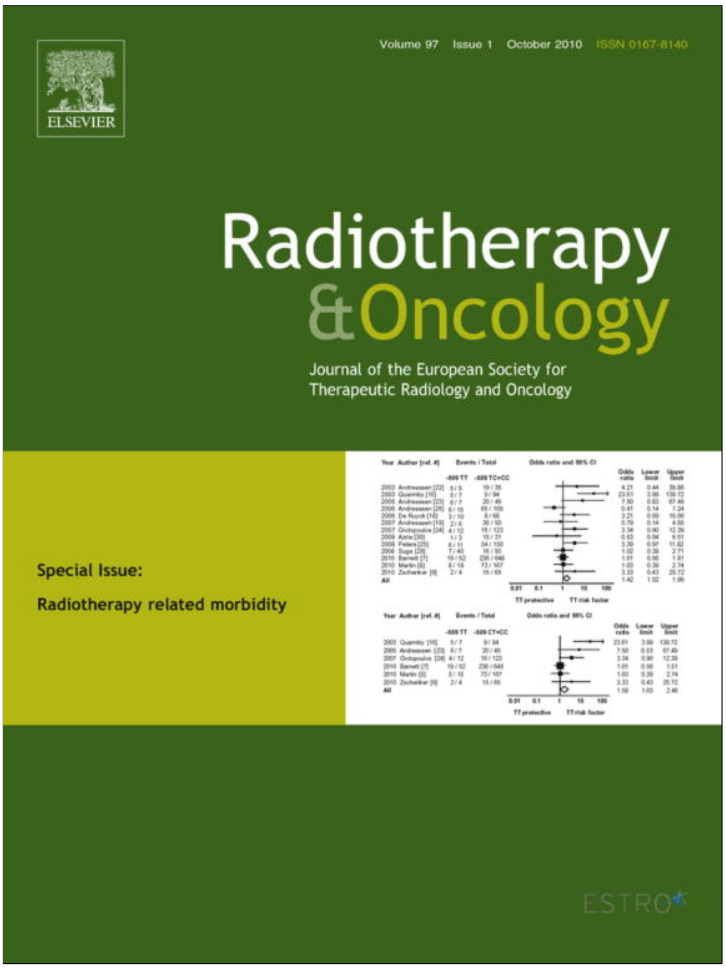

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Molecular genetics of RT side-effects

Test of association between variant tgβ1 alleles and late adverse effects of breast radiotherapy

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ABSTRACT

Purpose: To test for association between single nucleotide polymorphisms at the TGFβ1 locus and the risk of late normal tissue injury following whole breast radiotherapy.
Methods: A retrospective study compared the number of variant alleles at −509 and codons 10 and 25 of the TGFβ1 locus in women followed up in two prospective clinical trials who developed either marked radiotherapy adverse effects or no adverse effects after matching on fractionation schedule, breast size, surgical deficit, chemotherapy and length of follow up.
Results: Median follow up in the two trials was 7.4 (maximum 15) years and 5.3 (maximum 5.3) years. 1237/1716 (72%) women with photographic assessments of radiotherapy adverse effects were alive and well, and 147/1237 (12%) potential cases with the most marked change in photographic change in breast appearance were matched to potential controls recording no change. In an unmatched analysis of 82 cases and 108 controls, no significant difference in the number of genetic variants was observed.
Conclusions: No association was detected between sequence variations at the TGFβ1 locus and the risk of late adverse effects of breast radiotherapy.

Background

The available evidence strongly suggests that inter-patient variation in normal tissue response to radiotherapy is predominantly deterministic rather than to the random nature of radiation cell killing [9,16]. As extrinsic factors, such as radiotherapy dosimetry, become better controlled, factors intrinsic to the patient emerge as potentially more important in determining tissue tolerance [17]. This recognition has spurred investigation of genetic factors associated with late adverse effects of radiotherapy, and several high quality reviews describe the current state of research [1,3,14]. A large number of exploratory studies and a small number of confirmatory studies have claimed associations between the probability of developing late adverse effects of radiotherapy and the presence of specific SNP and mutations. Positive associations have been recently reviewed in APEX, ATM, CYP2D6, DNA ligase IV, eNOS, ERCC2, ERCC4, GSTP1, SOD2, TGFB1, XPD, XRCC1, XRCC3 and XRCC5 genes [1–2]. However, there is considerable residual uncertainty for a number of reasons, including variation in the clinical phenotype reported, difficulty in controlling for extrinsic factors, the small size of most studies and publication bias. The largest number of reports address polymorphisms at the TGFβ1 locus, although not all have been positive [6]. The current investigation of three alleles at the TGFβ1 locus was based on two randomised trials evaluating radiotherapy in 1716 women with early breast cancer, in whom the development of late adverse effects was prospectively recorded for a minimum of 5 years [11,18].

Patients and methods

Patients

A total of 1716 patients randomised into the Royal Marsden Hospital/Gloucestershire Oncology Centre (RMH/GOC) Breast Fractionation trial (1986–1998) or the RMH Breast Radiotherapy Dosimetry trial (1997–2000) were alive and well at the time of patient selection for the current study. The breast fractionation trial randomised 1410 patients after complete local excision of early breast cancer between a standard radiotherapy regimen (50 Gy in 25 fractions) and two dose levels of a test schedule delivering 39.0 Gy and 42.9 Gy, respectively, in 13 fractions over 5 weeks [18]. Eligibility criteria included: early stage breast cancer, invasive carcinoma, breast-preserving surgery and patient consent. Post-surgical photographs of both breasts were taken under standard conditions before radiotherapy, annually to 5 years, and change in breast appearance from baseline scored on a three-point scale.

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Annual physician assessments of breast induration were scored on four-point graded scales. The RMH Breast Radiotherapy Dosimetry trial randomised 306 patients between 2D versus 3D radiation dosimetry following breast-preserving surgery for early breast cancer [11]. Eligibility included: early breast cancer, invasive carcinoma, breast-preserving surgery, higher than average risk of radiation normal tissue changes (typically, large breast size) and written informed consent. Radiotherapy delivered 50 Gy in 25 fractions to the whole breast followed by an electron boost of 10 Gy in five fractions. Post-surgical photographs and clinical assessments were performed as in the fractionation trial.

Endpoint evaluation, case-control selection and sample collection

In each trial, three independent observers graded change in breast appearance in post-treatment photographs, using the post-surgical appearance as a comparator. Intra-observer variation and inter-observer variation confirmed the reproducibility of this approach based on a simple graded scale (no change, mild change, marked change). The risk of scoring any change (any change vs. no change) in breast appearance by 5 years on serial photographs after randomisation to 13 fractions or 3.0 or 3.3 Gy was 30.3% and 45.7%, respectively, confirming the sensitivity of this endpoint to small dose differences [18]. Annual clinical assessments of palpable induration also confirmed a dose response, with moderate or marked induration by 5 years reported after 13 fractions of 3.0 or 3.3 Gy in 16% and 35.6% of women, respectively. 1237/1716 (72%) women with photographic assessments of change in breast appearance were alive and well at a median follow up of 7.4 (maximum 15) years and 5.3 (maximum 5.3) years in the two trials, of whom 424 (35%) subjects scored some degree of change in breast appearance. The present study planned the identification and recruitment of cases with the most marked changes in photographic appearance and controls with no evidence of radiation-induced change in breast appearance matched for non-genetic co-variants, including fractionation schedule, breast size, surgical deficit, chemotherapy and length of follow up. DNA was isolated from 20 ml whole blood collected from each research volunteer.

DNA sequencing

Polymorphisms in the vicinity of the TGFβ1 locus (−509 C/T, codon 10 Leu/Pro & codon 25 Arg/Pro), previously reported as showing an association with late adverse effects, were analysed by uni-directional sequencing of the SNP region. We used primers CACATGGGAGGTGCTCAGTA and CAGGCGGAGAAGGCTTAAT to generate a PCR amplicon encompassing the −509 C/T variant. We used primers CCACACCAGCCCTGTTC and CTGTTGTACAGGGCGAGCAC to generate a PCR amplicon that encompassed both the codon 10 leu/pro variant and the codon 25 arg/pro variant. We used a 60–58 Touchdown protocol for both PCRs. Amplicons were then sequenced using the BigDyeTerminator Cycle Sequencing Kit and an ABI 3730 automated sequencer (Applied Biosystems). Sequence traces were analysed using Mutation Surveyor software v3.20 (SoftGenetics) and by visual inspection.

Analysis and statistical considerations

Restraining genotypic risks to only account for 30% of the severe radiation phenotype, an analysis of 100 cases (with normal tissue damage) and 200 matched controls (without damage) was estimated to provide 80% power ($\alpha = 0.05$) to demonstrate a three-fold increase in risk associated with an allele, assuming a carrier frequency >0.06. Matched analysis was not undertaken because the number of matched pairs available for analysis was reduced due to the failure in many instances to obtain samples from both the case and her two assigned controls.

Ethics committee approval

Local approval was obtained from the Research Ethics Committees at the Royal Marsden NHS Foundation Trust and the Gloucestershire Hospitals NHS Trust.

Results

One hundred and forty-seven patients were identified with the most marked radiotherapy phenotype based on the degree of change in breast appearance, representing approximately 12% (147/1237) of the total sample. As a comparator, 378 controls matched on randomised RT regimen, boost, breast size, chemotherapy, tamoxifen and axillary surgery were identified from 813 women recording no change in breast appearance, representing approximately 30% of the total. Demographic data and treatment characteristics are shown in Table 1. The planned matched analysis was not possible, since not all cases had matched controls by the time of the sequencing deadline. Unmatched analysis was carried

Table 1
Demographic and treatment details of cases and controls from breast dosimetry trial (BDT) and breast fractionation trial (BFT) submitted for genetic analysis.

	BDT				BFT			
	Cases		Controls		Cases		Controls	
Dose								
50 Gy	31		32		20		26	
42.9 Gy	0		0		21		32	
39 Gy	0		0		10		18	
Breast size								
Small	2		3		3		5	
Medium	26		27		34		58	
Large	3		2		15		13	
Median age years (interquartile range)	55 (50–62)		55 (50–60)		55 (47–61)		55 (46–60)	
Total patients	31		32		51		76	
	63				127			
	Yes	No	Yes	No	Yes	No	Yes	No
Chemotherapy	11	20	10	22	18	33	24	52
Axillary surgery	28	3	27	5	37	14	54	22
Axillary RT	1	30	16	16	7	44	10	66
Tamoxifen	[a]	[a]	[a]	[a]	43	8	66	10

[a] Data not collected at baseline.

Table 2
Distribution in clinical cases and controls of variant alleles at candidate loci in the TGFβ1 locus previously associated with late radiotherapy adverse effects.

#SNP rs#	Role	Amino acid change	Number cases/control	Genotype	Cases	Controls	Dominant Odds Ratio (95%CI) p value
rs1800469	[a]Promoter, 3′ UTR	–, –	[b]81/104	C/C (WT)	44	50	0.78 (0.44–1.39) $p = 0.46$
				C/T (het)	29	44	
				T/T (hom)	8	10	
rs1800470	Coding exon	L/P	82/108	P/P (WT)	14	19	1.04 (0.49–2.22) $p = 1.00$
				P/L (het)	36	40	
				L/L (hom)	32	49	
rs1800471	Coding exon	R/P	82/108	R/R (WT)	66	91	1.3 (0.61–1.40) $p = 0.56$
				R/P (het)	16	13	
				P/P (hom)	0	4	

[a] UTR = untranslated region.
[b] DNA sequencing failed in 1 case and 4 controls.

out on 82 cases and 108 controls, excluding samples that had been included in an earlier study of SNP [4]. The distribution of SNP around candidate TGFβ1 loci failed to confirm an association with late adverse effects of radiotherapy, see Table 2.

Discussion

No evidence of association between three previously examined polymorphic TGFβ1 alleles and clinical phenotype was detected. Several reasons for missing an effect of SNP at the TGFβ1 locus can be considered, including the clinical endpoint of radiotherapy effect, the matching of case-control pairs and statistical power. Where the clinical endpoint is concerned, change in breast appearance has been shown to be sensitive to radiotherapy dose intensity. In 1487 patients randomised to 13 fractions of 3.0 versus 3.2 Gy whole breast radiotherapy in the UK START-A trial, the hazard ratio for the photographic endpoint after 3.0 Gy compared to 3.2 Gy fractions was 0.65 (95% CI 0.49–0.85), a difference equivalent to less than 4 Gy in 2.0 Gy fractions [8]. Telangiectasia was excluded from the overall score, since it was typically seen in areas vulnerable to moist desquamation, especially inframammary fold, where it signifies loss of dose sparing. The sensitivity of the photographic endpoint to dose is a strength, but scores of breast shrinkage and distortion correlate to underlying atrophy and fibrosis, respectively, each of which may have distinct genetic determinants. No subgroup analysis was undertaken to attribute the relative contributions of breast shrinkage and distortion to clinical phenotype.

The selection of cases and controls took account of patient and treatment related factors that were systematically and prospectively recorded in trial registration, treatment and follow up proformas. These included breast size and surgical deficit scored from the surgical photographs (scored on three-point graded scales), whole breast radiotherapy dose, tumour bed boost dose, adjuvant endocrine therapy, adjuvant cytotoxic therapy and duration of follow up. Factors that were not prospectively recorded, and could not be taken into account, included histories of acute skin reaction, hypertension, diabetes, smoking, alcohol intake, non-cancer medication and collagen vascular disease. It is difficult to estimate the significance of these factors for the late outcome of breast radiotherapy, but several are risk factors for radiotherapy at other anatomical sites [9]. In breast skin, age, menopausal status, smoking habits, hypothyroidism, diabetes, hypertension, blood pressure, cardiovascular and autoimmune disease appear to have no influence on telangiectasia risk [17]. Overall, it seems unlikely that inability to control for these factors undermined the study, since genotypic risks were restrained in our sample size calculation to account for only 30% of the radiation phenotype.

Previous reports have suggested an association between variant TGFβ1 alleles and risk of late adverse effects after radiotherapy [4–5,10,15]. Our negative study is consistent with a recent investigation of 120 patients from the Aarhus post-mastectomy trial. The Aarhus cohort was scored for subcutaneous fibrosis after hypofractionated radiotherapy without chemotherapy, including patients who had not been previously analysed and for whom DNA could be extracted from paraffin tumour blocks [6]. Based on success in identifying breast cancer predisposition genes, there were reasons to believe at the time our study was conceived that a limited number of high penetrance alleles might contribute to late radiation effects and be detectable with a case-control design of 100–200 patients [12]. A polygenic model of late radiation effects is now considered likely, involving 100s or 1000s of low penetrance alleles impacting on gene regulation rather than structural alterations in proteins, as postulated for diabetes, hypertension and other complex traits [2,13]. Collaborative studies based on well-characterised patients entered into national and international radiotherapy trials are needed for this. Assay technologies and costs are now able to support such studies, opening the way for analysis of DNA banked as part of ESTRO-GENEPI initiatives [7].

Conflict of interest

We declare no conflict of interest.

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