A replicated association between polymorphisms near TNFα and risk for adverse reactions to radiotherapy

CJ Talbot*[,1], GA Tanteles[1,2], GC Barnett[3,4], NG Burnet[3], J Chang-Claude[5], CE Coles[3], S Davidson[6], AM Dunning[4], J Mills[2], RJS Murray[1], O Popanda[7], P Seibold[5], CML West[8], JR Yarnold[9] and RP Symonds[2]

[1]*Department of Genetics, University of Leicester, Adrian Building, University Road, Leicester LE1 7RH, UK;* [2]*Department of Cancer Studies and Molecular Medicine, University Hospitals of Leicester, Level 2, Osborne Building, Leicester Royal Infirmary, Leicester LE1 5WW, UK;* [3]*Department of Oncology, University of Cambridge, Oncology Centre, Cambridge University Hospital NHS Foundation Trust, Hills Road, Cambridge CB2 0QQ, UK;* [4]*Department of Oncology, Strangeways Research Laboratories, Cambridge CB1 8RN, UK;* [5]*Unit of Genetic Epidemiology, Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 581, 69120 Heidelberg, Germany;* [6]*Clinical Oncology, The Christie NHS Foundation Trust, Manchester M20 4BX, UK;* [7]*Division of Epigenomics and Cancer Risk Factors, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany;* [8]*School of Cancer and Enabling Sciences, University of Manchester, Christie Hospital, Manchester M20 4BX. UK;* [9]*Division of Radiotherapy and Imaging, The Royal Marsden NHS Foundation Trust and The Institute of Cancer Research, Sutton SM2 5PT, UK*

BACKGROUND: Response to radiotherapy varies between individuals both in terms of efficacy and adverse reactions. Finding genetic determinants of radiation response would allow the tailoring of the treatment, either by altering the radiation dose or by surgery. Despite a growing number of studies in radiogenomics, there are no well-replicated genetic association results.

METHODS: We carried out a candidate gene association study and replicated the result using three additional large cohorts, a total of 2036 women scored for adverse reactions to radiotherapy for breast cancer.

RESULTS: Genetic variation near the *tumour necrosis factor alpha* gene is shown to affect several clinical endpoints including breast induration, telangiectasia and overall toxicity. In the combined analysis homozygosity for the rare allele increases overall toxicity ($P = 0.001$) and chance of being in the upper quartile of risk with odds ratio of 2.46 (95% confidence interval 1.52–3.98).

CONCLUSION: We have identified that alleles of the class III major histocompatibility complex region associate with overall radiotherapy toxicity in breast cancer patients by using internal replication through a staged design. This is the first well-replicated report of a genetic predictor for radiotherapy reactions.

Radiotherapy is an important treatment modality for local tumour control and has contributed to declining mortality rates, for example, in breast cancer. Most patients show either no reaction or only transient skin reactions to radiation treatment, but a minority show life-long adverse reactions including vascular damage and fibrosis (Bentzen *et al*, 2003). Depending upon the tissue irradiated these reactions can lead to severe and sometimes life-threatening complications such as pulmonary fibrosis or oesophagitis (O'Sullivan and Levin, 2003). In low survival rate cancers there would be a benefit to identifying patients resistant to radiotherapy reactions to target for higher radiation doses, whereas in high survival rate tumour types such as breast cancer, the drive is to identify patients sensitive to adverse reactions, with the aim of offering alternative treatment. In breast cancer patients could be offered mastectomy instead of wide local excision followed by radiotherapy.

The variation in clinical response to radiotherapy is partly explained by treatment factors such as radiation dose, medical history or body type, and with an unknown contribution from genetic factors. Direct estimation of the heritability of clinical radiosensitivity is difficult to achieve, but is likely to be somewhat lower than for chromosomal and cellular radiosensitivity, which have been calculated as 58–78% (Finnon *et al*, 2008; Curwen *et al*, 2010).

Acute reactions to radiotherapy are either inflammation-related or through target cell depletion, and predict late reactions especially fibrosis (Barnett *et al*, 2009a). Broadly, late adverse reactions fall into two different pathophysiological categories namely vascular damage and fibroblast proliferation. Genes that affect processes early in DNA repair or inflammation pathways may be expected to lead to a wide range of late reactions, with other gene effects being more specific for damage to particular tissues or manifestations.

Identified predictors of radiosensitivity are aimed to ultimately contribute to an algorithm for guiding oncology treatment decisions, but there is no unanimity in the field about the most useful output measure of clinical radiosensitivity. Possibilities would include estimating the risk that an individual would develop late reactions with a particular score on one of the commonly used clinical scales (e.g., Common Terminology Criteria for Adverse Events or Late Effects of Normal Tissue-Subjective Objective Management Analytical (LENT-SOMA)), a score of combined

toxicity (e.g., Standardized Total Average Toxicity (STAT) score (Barnett *et al*, 2011b)) or for being in a high risk group (e.g., being in the upper quartile of overall toxic reactions).

Previous studies in radiogenomics have focussed on a wide range of candidate genes, but no genetic association results have yet proved replicable (Andreassen and Alsner, 2009, Barnett *et al*, 2009a, 2011a). More recently researchers have moved towards genome-wide association studies (GWAS) to overcome the well-documented problems with the candidate gene approach, and these are coming to fruition (Kerns *et al*, 2010). GWAS are very effective at detecting association with common causative alleles, with their power determined mainly by cohort size. Whether association results derive from candidate or genome-wide approaches, there is a pressing need for within-experiment replication through either staged design or combined analyses.

In this paper we describe a genetic association study used a three-staged design to ensure internal replication.

MATERIALS AND METHODS

Description of cohorts

LeND cohort This study had been undertaken with the written consent of patients attending the oncology departments or breast units from three hospitals in the East Midlands region of England; the University Hospitals of Leicester–Glenfield Hospital ($n = 566$; 89.4%), the Nottingham University Hospitals–City Hospital Ca mpus ($n = 35$; 5.5%) and the Derby Hospitals–Royal Derby Hospital ($n = 32$; 5.0%). In total, 633 women with a unilateral or bilateral, histologically confirmed early breast cancer (T1-3, N0-1, M0 at presentation) or ductal carcinoma *in situ* (DCIS) receiving adjuvant breast or chest wall irradiation after complete macroscopic tumour excision by breast-conserving surgery ($n = 493$) or mastectomy (no reconstruction; $n = 140$) were recruited from the follow-up clinics. Radiotherapy was given with 6–10 MV photons using tangential opposed fields at a variety of dose-fractionation schedules, but for most patients 50 Gy in 25 fractions over 5 weeks prescribed to the ICRU reference point. Boost irradiation with 9–15 Gy in 3–5 fractions was given to women with poor prognostic features. The sample collection and study were carried out with local and national ethics approval. Adverse effects of radiotherapy were scored on the LENT-SOMA scale with a median follow-up time of 62 months. Further details of the LeND cohort are as described previously (Giotopoulos *et al*, 2007, 2008; Tanteles *et al*, 2009; Murray *et al*, 2011).

German cohorts: ISE and MARIE$_{RAD}$ For both the ISE and the MARIE$_{RAD}$ study populations, histologically confirmed early breast cancer or *in situ* patients were recruited from the Rhine-Neckar-Karlsruhe region in Germany. Date of breast cancer diagnosis was between 1998 and 2001 for the ISE study (Lilla *et al*, 2007), and between 2002 and 2005 for the MARIE study, from which patients for the MARIE$_{RAD}$ study were drawn (Flesch-Janys *et al*, 2008). Briefly, breast cancer patients were eligible if they were treated unilaterally with radiotherapy(but not with chemotherapy) after breast-conserving surgery. For the ISE study at three sites the radiotherapy was given to the whole breast, either 50 Gy in 25 fractions or 50.4 Gy in 28 fractions, followed by a photon or electron boost with doses ranging from 5 to 20 Gy. At the fourth site, patients received 56 Gy in 28 fractions without boost. In the MARIE$_{RAD}$ study, radiotherapy was applied as whole-breast irradiation, followed by additional boost irradiation of the tumour bed for three quarters of patients. Whole-breast irradiation was either applied in daily doses of 1.8 Gy (28 fractions) or 2.0 Gy (25 fractions), summing up to a total whole-breast irradiation dose predominantly between 50.0 and 56.0 Gy. Median total irradiation dose applied including boost irradiation was 60.4 Gy. Two endpoints were assessed: skin alterations/telangiectasia and fibrosis at the irradiated breast. Late adverse effects were documented by a study physician according to a standard protocol using the RTOG/EORTC scoring, ranging from 0 = no late adverse effects to 4 = severe adverse effects. Late adverse effects were defined as ⩾ grade 2. Median follow-up time was 51 months for the ISE study and 68 months for the MARIE$_{RAD}$ study. For the ISE study, genotype data were available for 390 of the 418 patients with follow-up data who were treated with conventional radiotherapy (three patients treated with interstitial boost were omitted from the analysis). After exclusion of 27 patients who received intraoperative or interstitial boost irradiation in the MARIE$_{RAD}$ study, 363 of 387 patients treated with conventional radiotherapy were included in the genotype analysis. Written informed consent was obtained and approval from the Local Ethics Committee.

RAPPER The RAPPER study (UKCRN1471) is a large UK sample collection study, opened in 2005, which recruits patients from clinical trials and other well-designed studies. All patients in the Cambridge Intensity Modulated Radiotherapy (IMRT) and Manchester prospective trials were offered recruitment to RAPPER when they enroled in the component study; blood was taken for RAPPER before radiotherapy. Toxicity data for all patients were collected prospectively within the component clinical trial. RAPPER is approved by the Cambridgeshire 2 Research Ethics Committee (05/Q0108/365). All patients gave written informed consent that their samples could be used for genetic research.

Samples were obtained from 942 of the 1145 women recruited into the Cambridge Breast IMRT Trial (ISRCTN21474421) who underwent conservative surgery followed by adjuvant radiotherapy (Barnett *et al*, 2009b, 2011). All patients were treated to a dose of 40 grey (Gy) in 15 fractions, 5 days a week over 3 weeks with 6 MV photons prescribed to the ICRU 50 reference point. In this study, patients with significant dose inhomogeneities, defined by a volume of 2 cm³ or more exceeding 107% of the prescribed dose, were randomised to either standard breast RT (control arm) or to a simple method of forward-planned IMRT (interventional arm). A total of 34 samples were from patients enroled in a prospective study of breast toxicity in women who received conservative surgery and adjuvant radiotherapy at the Christie Hospital, Manchester. Samples were obtained from 63 patients recruited to the intensity modulated and partial organ radiotherapy low trial of partial breast radiotherapy (Coles *et al*, 2006). Samples were obtained from 179 patients from the Radiation Complications and Epidemiology (RACE) study (Martin *et al*, 2010). The RACE study recruited 82 cases from the Royal Marsden Hospital/Gloucestershire Oncology Centre (RMH/GOC) Breast Fractionation trial (Yarnold *et al*, 2005) and the RMH Breast Radiotherapy Dosimetry trial (Donovan *et al*, 2007) with marked changes in photographic appearance and 108 controls with no evidence of radiation-induced change in breast appearance.

The Cambridge Breast IMRT trial had as primary end point photographic assessment of late cosmetic effects. Breast shrinkage changes were recorded on a three-point scale (none/minimal = 1, mild = 2 and marked = 3) by three observers comparing baseline and 2-year photographs and generating a consensus score. Clinical assessment was made by a trained specialist radiographer with a 2-year follow-up time after radiotherapy. The breast was examined after treatment for telangiectasia, oedema, change in pigmentation and palpable induration. Induration of the breast was defined as hardening of the tissue and was used to assess fibrosis. Each of the secondary end points was scored 0–3 (none, a little, quite a bit and very much) on the scale used in the START trials. Pigmentation change was scored from 0 to 2 according to the LENT-SOMA scale.

Genotyping

The LeND cohort was genotyped by using SNPlex technology (Applied Biosystems, Foster City, CA, USA). In all, 5 SNPs failed genotyping (*TGF-β1* rs2241718, *RAD9A* rs2286620, exp *ACVR2A* rs286385, *CDC25A* rs3731487 and *RAD9A* rs91757), and 43 SNPs were successfully genotyped (Table 1). Tests for deviation from Hardy–Weinberg equilibrium showed that none of the 43 assays had a *P*-value <0.01, and only 5 had a *P*-value <0.1 (close to the average expected number of 4.3).

Table I Genotyped SNPs and associated genes

Gene	Chr	Position	Pathway	SNP
APEX	14	19 994 994	DNA repair	rs1130409
ATM	11	107 730 871	Cell cycle	rs664143
ATR	3	143 764 302	Cell cycle	rs2227928
AXIN2	17	60 979 143	Wnt signalling	rs11079571
CCND1	11	69 170 912	Cell cycle	rs647451
CDKN2A (p16)	9	21 963 412	Cell cycle	rs7036656
CHK1	11	125 025 731	Cell cycle	rs567889
CHK2	22	29 132 990	Cell cycle	rs5762764
CTGF	6	132 273 257	Growth factor	rs6918698
ERCC2 (XPD)	19	45 854 919	DNA repair (NER)	rs13181
ERCC4	16	13 922 582	DNA repair (NER)	rs744154
ERCC5	13	102 326 003	DNA repair (NER)	rs17655
exp *ACVR2A (AXIN1)*	16	316 781	TGF-β	rs7195617
exp *E2F5*	12	364 160	TGF-β	rs1860360
exp *ID2*	7	108 617 064	TGF-β	rs10953597
exp *ID3*	2	168 930 735	TGF-β	rs6717927
exp *SMAD1*	18	62 060 696	TGF-β	rs1873481
FGFR2	10	123 352 317	Growth factor	rs2981582
GSTP1	11	67 109 265	Detoxification	rs1695
IER2	19	12 836 792	Growth factor	rs1042164
IL12RB2	1	67 836 060	Inflammation	rs3790568
KLF4	9	109 289 326	Cell cycle	rs2236599
LIG3	17	30 355 688	DNA repair (BER)	rs1052536
LIG3	17	30 313 159	DNA repair (BER)	rs3744355
LIG3	17	30 358 225	DNA repair (BER)	rs3744357
NEK11	3	130 947 435	Cell cycle	rs3738000
PTTG1	5	159 775 043	Cell cycle	rs2910190
PTTG1	5	159 786 856	Cell cycle	rs2961951
PTTG1	5	159 779 450	Cell cycle	rs3811999
RAD21	8	117 938 641	DNA repair	rs16888927
RAD21	8	117 945 589	DNA repair	rs16888997
SOD2	6	160 033 862	Oxidative stress	rs4880
TGFB1	19	41 865 643	TGF-β	rs11083616
TGFB1	19	41 845 801	TGF-β	rs11466338
TGFB1	19	41 851 509	TGF-β	rs4803455
TGFBR2	3	30 637 332	TGF-β	rs1036095
TGFBR2	3	30 643 688	TGF-β	rs4522809
TNFα	6	31 651 010	Cytokine	rs1800629
VEGF	6	43 846 328	Growth factor	rs2010963
XRCC1	19	48 747 566	DNA repair (BER)	rs25487
XRCC3	14	103 235 680	DNA repair (HR)	rs1799796
XRCC4	5	82 684 699	DNA repair (NHEJ)	rs1805377
XRCC5 (KU80)	2	216 774 882	DNA repair (NHEJ)	rs3835

Abbreviations: SNP = single nucleotide polymorphism; TGF = transforming growth factor. Genes preceded by 'exp' indicate that the SNP was found to associate with expression of the gene in Smirnov *et al*, 2009, but is not physically in the gene.

For both German study populations, the tumour necrosis factor alpha (*TNFα*) SNP rs1800629 was genotyped by using iPlex application (Sequenom, SanDiego, CA, USA). There was no deviation from Hardy–Weinberg in both study populations.

In the RAPPER cohort the rs2857595 SNP was genotyped on an Illumina CytoSNP 12 array (Illumina Inc., San Diego, CA, USA). Genotype calling was performed with the GenCall software application to automatically cluster, call genotypes and assign confidence scores. The GenCall application incorporates a clustering algorithm (GenTrain) and a calling algorithm (GenCall software, Illumina Inc.).

Statistical analysis

Phenotypes used in this study were the residuals from linear regression incorporating known predictors on a study-specific basis into the model. For the LeND cohort the telangiectasia regression covariates were type of surgery, radiation boost and bra cup size. The STAT score incorporated induration, telangiectasia, oedema and atrophy. Standardized Total Average Toxicity regression covariates were surgery and bra cup size. For the German cohorts the telangiectasia regression covariates were as follows: BMI, radiation dose at the skin and clinic. The fibrosis regression covariates were as follows: BMI, radiation dose at the skin and bra cup size. The STAT score incorporated telangiectasia and fibrosis. The STAT regression covariates wereas follows: bra cup, smoking pack years, BMI and dose at the skin. For the RAPPER cohorts STAT incorporates telangiectasia, oedema, shrinkage, pigmentation and pain. The STAT regression covariates were as follows: breast volume, smoking, diabetes, post-op infection and acute score + boost.

Clinical endpoints were combined within and between cohorts by conversion to Z scores, i.e., number of s.d.s from the cohort mean and then averaging the Z scores to generate the STAT score (Barnett *et al*, 2011b). To enable bivariate analyses an arbitrary breakpoint was chosen by defining 'cases' as being in the upper quartile of risk (i.e., the 25% of patients with the highest unexplained adverse reactions).

Statistical analysis used SPSS v16.0 (IBM software, Armonk, NY, USA). Genetic analysis was carried out using the Plink v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) and SimHap v1.02 (http://www.genepi.meddent.uwa.edu.au/software/simhap). For calculation of empirical *P*-values 20 000 permutations were performed.

RESULTS

LeND cohort genotyping

In all, 43 candidate SNPs were genotyped in 35 genes, most of them as putatively affecting gene expression or protein function directly, or by *trans* effects on expression of TGFβ pathway genes. The latter SNPs were derived from unpublished data in Smirnov *et al*, 2009 provided by the authors. The direct candidate genes were drawn mainly from DNA repair, cell cycle and TGFβ pathways (Table 1).

Polymorphisms were assessed for association with two specific endpoints, fibrosis and telangiectasia, and for overall toxicity as measured by STAT score. All three phenotypes were the residuals from linear regression with known predictors included in the model (see Methods). Multiple testing was corrected for by

Table 2 s1800629 in LeND cohort

Genotype	GG	AG	AA
Count	197	128	15
Telangiectasia score Mean (25%, 75%)	− 0.07 (− 0.43, − 0.11)	+ 0.07 (− 0.27, + 0.05)	+ 0.29 (− 0.19, + 0.57)
STAT score Mean (25%, 75%)	0.00 (− 0.43, + 0.27)	− 0.02 (− 0.44, + 0.26)	+ 0.26 (− 0.03, + 0.65)

Abbreviation: STAT = Standardized Total Average Toxicity. Telangiectasia and STAT scores are residuals calculated from regression of clinical endpoints on known predictive factors. Standardized Total Average Toxicity score is a measure of the overall toxicity calculated by combining clinical endpoints.

permutation of the data. The most significant pointwise *P*-value was for telangiectasia with the minor A allele of rs1800629 in the *TNFα* gene ($P = 0.0028$), but which was not significant experiment wide as calculated by permutation ($P = 0.13$). The mode of inheritance observed from these data is intermediate between additive and recessive, as evidenced by the heterozygote AG telangiectasia score being between the GG homozygote score and the mid-point between the homozygotes (mid-point = +0.27; Table 2). The only other SNP with pointwise significant association with overall toxicity was rs3738000, a coding polymorphism in *NEK11* (p.E488V; pointwise $P = 0.04$, empirical $P = 0.83$).

The rare allele of the rs1800629 *TNFα* SNP is only marginally associated with fibrosis in the LeND cohort, but is significantly associated with overall toxicity (Mann–Whitney $P = 0.02$). For risk of being in the top quartile of adverse reactions, homozygosity for the rare allele shows marginal association (odds ratio 2.6, 95% confidence interval (CI) 0.9–7.1; Figure 1).

Replication in German cohorts

To follow-up the suggestive association of rs1800629, we genotyped two German cohorts for the same SNP. Residual telangiectasia was associated with the rs1800629 genotype under a recessive model in the two cohorts combined ($P = 0.01$; Table 3).
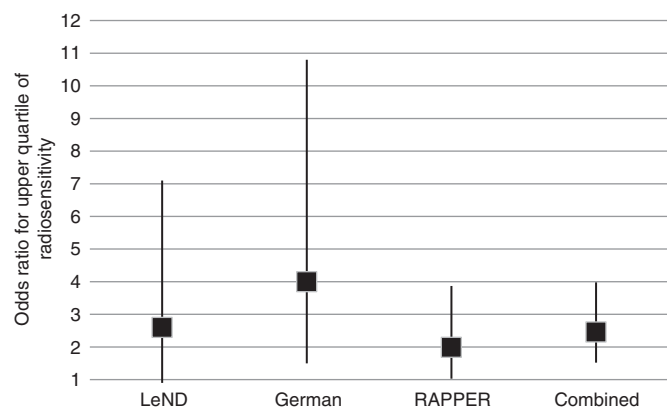
Figure 1 Plot showing odds ratios under a recessive model (genotype AA *vs* AG, GG) for being in upper quartile of adverse reactions to radiotherapy by cohort. Square shows central estimate of odds ratio, whiskers shows 95% confidence intervals.

Furthermore fibrosis was also significantly raised in AA homozygotes ($P = 0.02$), and therefore overall toxicity, as measured by STAT score, was also increased ($P = 0.02$). Regression analysis shows that rs1800629 genotype accounts for 0.6% of the phenotypic variance of STAT score in the German cohorts, with all known predictive factors accounting for 9.4% of phenotypic variance. If we define the patients with serious adverse reactions as being the top quartile, then being homozygote for rs1800629 gives an increased risk for being in this group with an odds ratio of 4.0 (95% CI 1.5–10.8; Figure 1).

Replication in RAPPER cohort

For a second replication we used data from the RAPPER consortium. Data on rs1800629 were lacking and we therefore selected a SNP with the strongest linkage disequilibrium to rs1800629 as calculated from the HapMap data, which is rs2857595 ($D' = 0.95$, $R^2 = 0.86$). rs2857595 is an intergenic SNP that is 25.7 kb from rs1800629, lying between the *NCR3* and *AIF1* genes.

Under a recessive model rs2857595 is significantly associated with STAT score in the breast cancer RAPPER cohort ($P = 0.01$; Table 4). For risk of being in upper STAT quartile for rs2857595 homozygotes odds ratio is 1.99 (95% CI 1.03–3.87; Figure 1).

Combined analysis A combined analysis of association between STAT score and homozygosity for the rare allele of the typed SNP in 2036 women from all four cohorts combined gives a Mann–Whitney *P*-value of 0.001. For risk of being in the top quartile of radiotherapy toxicity, the *P*-value is 1.5×10^{-4} with an odds ratio of 2.46 (95% CI 1.52–3.98; Figure 1).

DISCUSSION

The purpose of finding genetic loci in studies of complex diseases is normally to shed light on the underlying pathophysiology of the condition, with a long-term aim of developing therapies that target the causative genes. In therapeutic genetics, however, (e.g., pharmacogenetics and radiogenetics), the primary aim is to identify predictors to guide treatment, with any biological insights as secondary benefits. To be realistic components of a predictive algorithm for adverse reactions to radiotherapy, genetic effects will need to be robust in terms of replication and the central question will be their effect on an individual not a population, i.e., positive predictive value is more important than population attributable risk.

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Table 3 rs1800629 in German cohorts

Genotype	GG	AG	AA
Count	522	210	16
Telangiectasia score Mean (25%, 75%)	−0.024 (−0.728, +0.764)	+0.030 (−0.729, +0.910)	+0.490 (−0.244, +1.185)
Fibrosis score Mean (25%, 75%)	−0.054 (−1.006, +0.455)	+0.108 (−0.958, +0.486)	+0.421 (−0.370, +1.235)
STAT score Mean (25%, 75%)	−0.038 (−0.694, +0.580)	+0.061 (−0.640, +0.823)	+0.517 (+0.092, +1.214)

Abbreviation: STAT = Standardized Total Average Toxicity. Telangiectasia, fibrosis and STAT scores are residuals calculated from regression of clinical endpoints on known predictive factors. Standardized Total Average Toxicity score is a measure of overall toxicity calculated by combining clinical endpoints.

Table 4 rs2857595 in RAPPER breast cohort

Genotype	GG	AG	AA
Count	625	284	39
STAT score Mean (25%, 75%)	−0.041 (−0.710, +0.410)	+0.038 (−0.640, +0.555)	+0.157 (−0.635, +0.795)

Abbreviation: STAT = Standardized Total Average Toxicity.

The candidate genes and SNPs tested in this study are drawn from a variety of sources, including SNPs that control radiation-dependent expression of genes in the TGFβ pathway, which is known to be central to fibrogenesis. Tumour necrosis factor alpha is a multi-functional cytokine produced by a variety of cells after injury or infection, which also increases TGFβ expression in fibroblasts (Sullivan et al, 2009). The TNFα SNP typed in the LeND and German cohorts, rs1800629, is in the promoter lying 308-bp upstream of the transcript start site, and > 1 kb downstream of the lymphotoxin alpha (LTA) gene. A recent meta-analysis concluded that the G and A alleles do not significantly alter TNFα expression, although it cannot be discounted that they affect LTA expression (Mekinian et al, 2011). The rs2857595 SNP tested in the RAPPER cohort, lies in the same linkage disequilibrium block as rs1800629, being in the major histocompatibility complex (MHC) class III region.

Given that we find association with both SNPs, we are unable to determine which is the causative variation affecting radiosensitivity, or that neither are. In this study, the important question is whether we have found genetic variation that predicts radiosensitivity, and whether another polymorphism might provide higher predictive value. The MHC region is known to harbour long distance haplotypes, so it is conceivable that SNPs some considerable distance away may yield higher odds ratios. Major histocompatibility complex associations have been found for many diseases, but it remains problematic to identify the causative variation. Current radiogenomics GWAS will help in this as will developing methods for data analysis (Raychaudhuri et al, 2012).

In conclusion, we have identified that alleles of the class III MHC region associate with overall radiotherapy toxicity in breast cancer patients by using internal replication through a staged design. This is the first well-replicated report of a genetic predictor for radiotherapy reactions.

Conflict of interest

The authors declare no conflict of interest.

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