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

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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.

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Keywords: breast cancer; normal tissue radiation injury; genetics; tumour necrosis factor alpha (TNFα).
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 (Andreasen and Alsnor, 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 (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 4–15 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; Tantale et al, 2009; Murray et al, 2011).

German cohorts: ISE and MARIERAD For both the ISE and the MARIERAD 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 MARIERAD 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 MARIERAD 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 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 MARIERAD 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 MARIERAD 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 enrolled in a prospective study of breast toxicity in women with 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.

British Breast Unit (BBU) 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.

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Genetics and Genomics
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 3 had a P-value <0.05 (close to the average expected number of 4.3).

Table 1 Genotyped SNPs and associated genes

| Gene        | Chr | Position | Pathway | SNP       |
|-------------|-----|----------|---------|-----------|
| APEX        | 14  | 19994994 | DNA repair | rs1130409 |
| ATM         | 11  | 10730871 | Cell cycle | rs64143  |
| ATR         | 3   | 43764302 | Cell cycle | rs227928  |
| AXIN2       | 17  | 6097943  | Wnt signalling | rs1079571 |
| CND1        | 11  | 69170912 | Cell cycle | rs647451  |
| Cicators (P16) | 9   | 21963412 | Cell cycle | rs7036565 |
| CHK1        | 11  | 12505731 | Cell cycle | rs567889 |
| CHK2        | 22  | 29132990 | Cell cycle | rs5762764 |
| CTGF        | 6   | 13227257 | Growth factor | rs918698 |
| ERCC2 (XPD) | 19  | 45854919 | DNA repair (NER) | rs13818 |
| ERCC4       | 16  | 13922582 | DNA repair (NER) | rs744154 |
| ERCC5       | 13  | 10326302 | DNA repair (NER) | rs76556 |
| exp ACVR2A (AXIN1) | 16  | 316781 | TGF-β1 | rs7195617 |
| exp E2FS    | 12  | 364160 | TGF-β1 | rs860360 |
| exp ID2     | 7   | 10617064 | TGF-β1 | rs1053597 |
| exp ID3     | 2   | 16893735 | TGF-β1 | rs6717927 |
| exp SMAD7   | 18  | 6204696 | TGF-β1 | rs1873481 |
| FGR2        | 10  | 12335217 | Growth factor | rs2981582 |
| GSTP1       | 11  | 67109265 | Detoxification | rs169 |
| IER2        | 19  | 12836792 | Growth factor | rs1042164 |
| ILI2RB2     | 6   | 18736060 | Inflammation | rs3790568 |
| KLF4        | 9   | 10938926 | Cell cycle | rs2263599 |
| LIG3        | 17  | 30355688 | DNA repair (BER) | rs1052536 |
| LIG3        | 17  | 30313159 | DNA repair (BER) | rs3744357 |
| LIG3        | 17  | 30352825 | DNA repair (BER) | rs3744357 |
| NEK1I       | 3   | 13094743 | Cell cycle | rs7383000 |
| PITG1       | 5   | 15877004 | Cell cycle | rs2910190 |
| PITG1       | 5   | 15878656 | Cell cycle | rs2961951 |
| PTG1        | 5   | 15977945 | Cell cycle | rs5819999 |
| RAD21       | 8   | 11793864 | DNA repair | rs1688927 |
| RAD21       | 8   | 11794559 | DNA repair | rs1688997 |
| SOD2        | 6   | 16003382 | Oxidative stress | rs4880 |
| TGBF1       | 19  | 41865643 | TGF-β1 | rs1083616 |
| TGBF1       | 19  | 41865801 | TGF-β1 | rs1466338 |
| TGBF1       | 19  | 41851509 | TGF-β1 | rs4803455 |
| TGBF2       | 3   | 30637332 | TGF-β1 | rs1060956 |
| TGBF2       | 3   | 30643687 | TGF-β1 | rs4522809 |
| TNFα        | 3   | 616510 | Cytokine | rs1800629 |
| VEGF        | 4   | 43846328 | Growth factor | rs201963 |
| XRC2C       | 18  | 48747566 | DNA repair (BER) | rs52487 |
| XRC2C       | 14  | 10323560 | DNA repair (HR) | rs799796 |
| XRC2C       | 5   | 82684699 | DNA repair (NHEI) | rs1805377 |
| XRC2CC (KUBO) | 2  | 21677482 | DNA repair (NHEI) | 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.

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 predictors. Standardized Total Average Toxicity score is a measure of the overall toxicity calculated by combining clinical endpoints.

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 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 TGF/β 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 TGF/β 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 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.).
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 \((\text{mid-point} = +0.27; \text{Table 2})\). The only other SNP with pointwise significant association with overall toxicity was rs3738000, a coding polymorphism in NEK11 \((P = 0.04\); pointwise \(P = 0.83\)).

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 \((P = 0.02)\). For risk of being in the top quartile of adverse reactions, homozygosity for the rare allele shows marginal association \((\text{odds ratio} 2.6, 95\% \text{ confidence interval} (CI) 0.9–7.1; \text{Figure 1})\).

**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 \((P = 0.01; \text{Table 3})\).

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 \((95\% \text{ CI} 1.5–10.8; \text{Figure 1})\).

**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 \((P = 0.01; \text{Table 4})\). For risk of being in upper STAT quartile for rs2857595 homozygotes odds ratio is 1.99 \((95\% \text{ CI} 1.03–3.87; \text{Figure 1})\).

**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 \(\times 10^{-2}\) with an odds ratio of 2.46 \((95\% \text{ CI} 1.52–3.98; \text{Figure 1})\).

**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.

### 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.
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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.

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Conflict of interest

The authors declare no conflict of interest.
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