Independent validation of genes and polymorphisms reported to be associated with radiation toxicity: a prospective analysis study

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Summary

Background Several studies have reported associations between radiation toxicity and single nucleotide polymorphisms (SNPs) in candidate genes. Few associations have been tested in independent validation studies. This prospective study aimed to validate reported associations between genotype and radiation toxicity in a large independent dataset.

Methods 92 (of 98 attempted) SNPs in 46 genes were successfully genotyped in 1613 patients: 976 received adjuvant breast radiotherapy in the Cambridge breast IMRT trial (ISRCTN21474421, n=942) or in a prospective study of breast toxicity at the Christie Hospital, Manchester, UK (n=34). A further 637 received radical prostate radiotherapy in the MRC RT01 multicentre trial (ISRCTN47772397, n=224) or in the Conventional or Hypofractionated High Dose Intensity Modulated Radiotherapy for Prostate Cancer (CHHiP) trial (ISRCTN97182923, n=413). Late toxicity was assessed 2 years after radiotherapy with a validated photographic technique (patients with breast cancer only), clinical assessment, and patient questionnaires. Association tests of genotype with overall radiation toxicity score and individual endpoints were undertaken in univariate and multivariable analyses. At a type I error rate adjusted for multiple testing, this study had 99% power to detect a SNP, with minor allele frequency of 0·35, associated with a per allele odds ratio of 2·2.

Findings None of the previously reported associations were confirmed by this study, after adjustment for multiple comparisons. The p value distribution of the SNPs tested against overall toxicity score was not different from that expected by chance.

Interpretation We did not replicate previously reported late toxicity associations, suggesting that we can essentially exclude the hypothesis that published SNPs individually exert a clinically relevant effect. Continued recruitment of patients into studies within the Radiogenomics Consortium is essential so that sufficiently powered studies can be done and methodological challenges addressed.

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Introduction

Late side-effects from radiotherapy are often irreversible, can decrease health-related quality of life, and limit treatment intensity in radical radiotherapy regimens. Quantification of late toxicity is therefore crucial in assessment of the therapeutic benefit of radiotherapy, and recommendations for its reporting have been made.[1,2] If the hypothesis that there is a sizeable subpopulation of patients who have a significantly increased risk of developing toxicity proves correct, it is likely that this subset of cases currently limits our ability to maximise toxicity-free local control through higher doses of radiation to the remaining cases. The RAPPER (Radiogenomics: Assessment of Polymorphisms for Predicting the Effects of Radiotherapy) study was designed to identify common genetic variation associated with the development of late radiation toxicity[3] as a first step in the process of identifying such a subset of toxicity-prone patients.

Apart from one small genome-wide study,[4] single nucleotide polymorphism (SNP) association studies of radiotherapy toxicity published to date have used a candidate gene approach. Radiation-induced cell killing, for which DNA damage is a major mechanism, is thought to be a triggering event in the development of radiotherapy toxicity. Additionally, the release of cytokines[5] is thought to initiate biological responses in multiple cell types leading to the development of late toxicity. The focus of candidate gene studies has thus been on genes involved in DNA damage recognition and repair (eg, *ATM*, *BRCA1*, *BRCA2*, and *TP53*), free radical scavenging (eg, *SOD2*), and anti-inflammatory response (eg, *TGFB1*). Studies to date, reviewed recently,[6–8] have been underpowered, including fewer than 500 samples; they have tested many

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SNPs without adjusting for multiple comparisons and findings have proved difficult to replicate. No individual SNP or combined risk SNP signature[9–14] has yet been validated as a risk factor for radiation toxicity.

We aimed to confirm reported associations between candidate SNPs or SNP signatures and radiation toxicity in a large, well powered prospective study of patients with breast and prostate cancer.

Methods

Patients

1613 patients from four studies were included in this analysis. Treatment was given according to the component study protocols and details were recorded for all patients.

Samples were obtained from 942 of 1145 women recruited into the Cambridge breast IMRT trial (ISRCTN21474421) who underwent conservative surgery followed by adjuvant radiotherapy.[15] In this study, patients with significant dose inhomogeneities, defined by a volume of 2 cm^3 or more exceeding 107% of the prescribed dose, were randomly assigned to receive either standard breast radiotherapy (control) or a straightforward method of forward-planned intensity modulated radiotherapy (IMRT; intervention). 34 samples were from patients enrolled in a prospective study of breast toxicity in women who received conservative surgery and adjuvant radiotherapy at the Christie Hospital (Manchester, UK). Blood samples were also obtained for 224 of the 843 patients with localised prostate cancer randomly assigned in the MRC RT01 multi-centre trial (ISRCTN47772397) to standard-dose or escalated-dose conformal radiotherapy after neoadjuvant androgen suppression.[16–18] At the time of initial analysis in June, 2009, for the study we report here, 647 patients had been recruited to both the Conventional or Hypofractionated High Dose Intensity Modulated Radiotherapy for Prostate Cancer (CHHiP) trial (ISRCTN97182923) and the RAPPER study, and 2-year toxicity data were available for analysis for 413 patients. CHHiP patients with localised prostate cancer underwent IMRT after neoadjuvant androgen suppression and were randomly assigned to one of three dose schedules.[19] Recruitment to both the CHHiP and RAPPER studies was ongoing at the date of data release.

The RAPPER study (UKCRN1471) reported here 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 IMRT and Manchester prospective trials were offered recruitment to RAPPER when they enrolled in the component study; blood samples were taken for RAPPER before radiotherapy. Patients recruited to the MRC RT01 trial and under continuing follow-up in 2005 were offered participation in the RAPPER study at a follow-up appointment; blood samples were therefore taken at least 2 years after the start of their radiotherapy. The distribution of toxicity in patients recruited to RT01 but not RAPPER was broadly similar to that seen in patients recruited to both RT01 and RAPPER (data not shown). CHHiP patients could be recruited to the RAPPER study either concurrently or after consenting to participate in the CHHiP trial. Toxicity data for all patients were obtained prospectively within the component clinical trial. RAPPER is approved by the Cambridgeshire

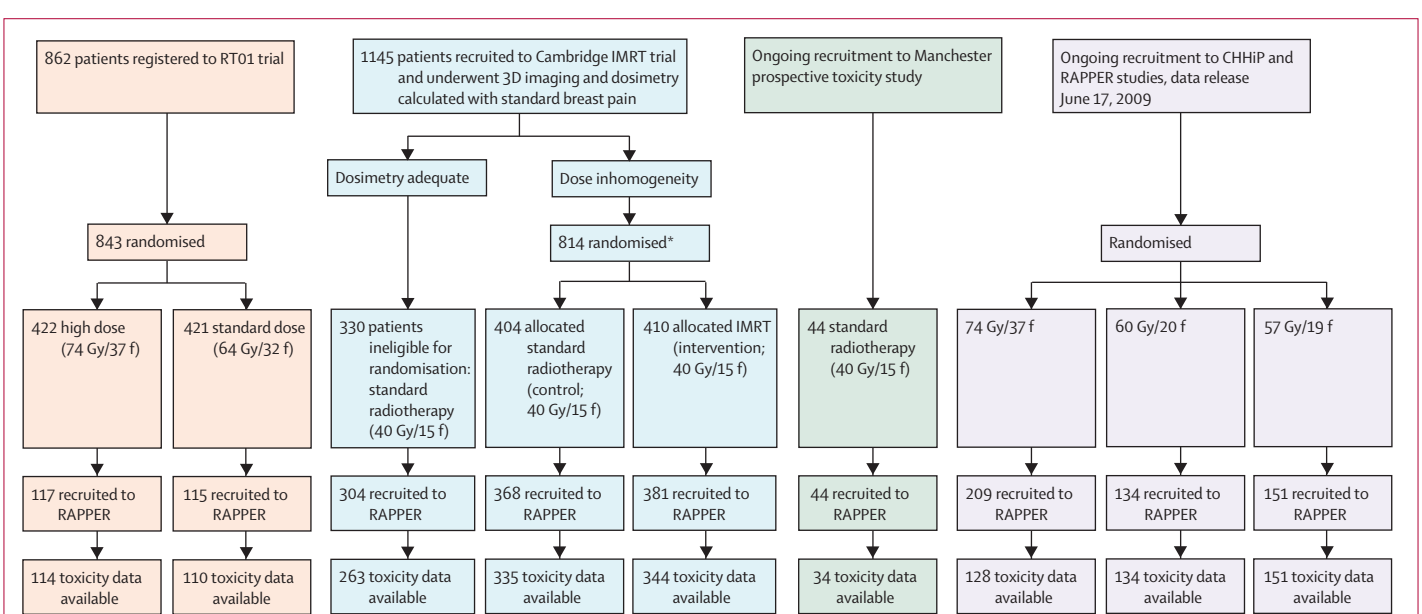

Figure 1: **Trial profile**
IMRT=intensity modulated radiotherapy. *One patient was not randomised because the standard plan was unacceptable, owing to a significant region of low dose, so IMRT was prescribed; patient was withdrawn from the late toxicity analysis.

2 Research Ethics Committee (05/Q0108/365). All patients gave written informed consent for use of their samples in genetic research. Figure 1 shows the trial profile for the RAPPER study.

Procedures

Data were obtained for age, smoking history, diabetes mellitus, and acute radiotherapy toxicity. In patients with breast cancer, body-mass index, use of tamoxifen and chemotherapy, breast boost, breast volume, ethnic origin, cardiovascular disease, and cosmesis after surgery were also recorded. In patients with prostate cancer, data for hypertension, previous surgery, clinical stage, risk of seminal vesicle involvement, prescribed dose, and baseline symptoms were also documented.

Toxicity was recorded with standardised scoring systems; the scales and incidence of each toxicity endpoint used (acute and late toxicity) are shown in appendix 1. An effect of poor surgical cosmesis on clinical assessment of breast shrinkage or induration at 2 years has been reported for patients in the IMRT trial;[15] thus, endpoints that accurately reflect late toxicity, due to radiotherapy rather than surgery, were used (appendix 1). Details of the methods used are available elsewhere.[15]

For patients with prostate cancer, five rectal (bleeding, proctitis, sphincter control, stool frequency, tenesmus) and four urinary endpoints (frequency, nocturia, incontinence, and decreased stream) were chosen to represent the range of rectal and bladder toxicity reported by patients 2 years after radiotherapy to the prostate. The rectal endpoints have previously shown a dose-volume response for at least one dose level with the volume of rectum receiving between 30 Gy and 70 Gy expressed as a percentage of the total rectal volume.[20] Sexual dysfunction was not analysed because few men had adequate, self-reported, erectile function at baseline before hormone therapy was commenced.[21] The symptoms of late toxicity after radiotherapy to the pelvis are non-specific and might be present before treatment. We took the effect of baseline function into account by calculating changes in scores from baseline (pre-hormone treatment) to those recorded at 2 years for each endpoint[22] (appendix 1). If pre-hormone symptom scores were unavailable, pre-radiotherapy scores were used. If symptom scores improved (ie, there were fewer symptoms after than before radiotherapy), these were recorded as a zero score. If the final symptom score was zero, then change in toxicity was also scored as zero if the pre-hormone or pre-radiotherapy scores were not recorded. By definition, there was no baseline measurement of proctitis on the Radiation Therapy Oncology Group (RTOG) scale. Further information is available elsewhere.[22]

Standardised total average toxicity (STAT) scores were derived to assess late overall toxicity with the individual endpoints listed on appendix 1. The rationale and validation of the STAT score in patients with breast cancer has previously been reported.[23] We also obtained STAT scores to measure overall acute toxicity in patients with breast cancer and, by combining acute bladder and bowel toxicity, in those with prostate cancer. STAT scores were derived with the following method. For an individual patient (k), a standardised Z score, $Z_{k,i}$, was derived for each toxicity endpoint (i) for which that patient had a valid (non-missing) score, $s_{k,i}$: $Z_{k,i}=(s_{k,i}-\text{mean}_i)/SD_i$, where mean_i and standard deviation (SD_i) were taken over all cases in the study population for which toxicity data were available. Conversion of individual toxicity scores to Z scores eliminated the problem of grades for one toxicity item not being directly comparable with grades for another item. Z scores defined, for a particular endpoint, whether a patient's score was high or low relative to the distribution of the scores of other patients in the population. The STAT score for patient k, $STAT_k$, was the average of all non-missing Z scores for that patient: $STAT_k=\text{mean}\ Z_{k,i}$.

See Online for appendix 1

To select SNPs, we undertook a comprehensive literature search of Medline and PubMed databases using the keywords "radiotherapy", "radiation", "toxicity", "adverse effects", "genetic variation", and "polymorphism". This search identified 69 in-vitro or in-vivo studies published up to Dec 31, 2009. 66 SNPs reported to be associated with late radiotherapy toxicity were identified from these studies. Additionally, we examined the most frequently studied genes, *ATM*, *TGFB1*, *XRCC1*, *XRCC3*, and *HIF1A*, in more detail by choosing a set of tag SNPs to capture all common genetic variation in these five genes with r^2 greater than 0·8.[24] Six new candidate SNPs were also selected: two in *ATM* (rs1800054, rs4986761) reported to be associated with breast cancer susceptibility; two in *MRE11* (rs569143, rs2155209) reported to be associated with breast and bladder cancer susceptibility; and rs1800734 in *MLH1* and rs2303428 in *MSH2*, reported to be associated with acute myeloid leukaemia after chemotherapy. Many of the SNPs included in the SNP signatures described in previous reports[9–14] were genotyped in this study. Where possible, risk alleles in each of the signatures were summed and related to each toxicity endpoint with regression analysis.

DNA was extracted from blood by Gen-Probe Life Sciences (Manchester, UK) and all samples were normalised to 40 ng/μL before arraying in 384-well

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	OR	P_0 (%)	P_C (%)	P (%)	ΔD_{esc} (Gy)
MAF 50%	2·1	20%	34·4%	27·2%	2·1
MAF 35%	2·2	20%	35·0%	25·3%	1·5
MAF 20%	2·5	20%	38·3%	23·7%	1·1
MAF 10%	3·3	20%	45·0%	22·5%	0·7
MAF 5%	4·9	20%	55·2%	21·8%	0·5

OR=odds ratio. P_0=incidence of toxicity in non-carriers (reference value). P_C=incidence of toxicity in carriers. P=resulting incidence of toxicity in unselected cases. ΔD_{esc}=permissible dose escalation in non-carriers to maintain isotoxicity with unselected population.

Table 1: **Effect sizes, quantified as the OR between carriers and non-carriers that can be resolved with 99% power and a nominal α of $2·7\times10^{-5}$**

	SNP	MAF	Base change	HWE p value	Originally reported significantly associated endpoints	Assay
ABCA1	rs2230806	0·28	G→A	0·02	Acute radiation dermatitis[13]	Fluidigm
ALAD	rs818707	0·12	G→A	0·84	Early adverse skin reactions[32]	Fluidigm
APEX1	rs1130409	0·48	T→G	0·56	Acute skin toxicity;[29] delay in DNA strand-break rejoining;[30] mitotic delay[31]	Fluidigm
ATM	rs1800889	0·05	C→T	1·00	*ATM* tag SNP	Fluidigm
ATM	rs1800054	0·01	C→G	1·00	Frequently studied breast cancer susceptibility allele[33,34]	Fluidigm
ATM	rs664677	0·42	T→C	0·35	Early or late adverse radiotherapy reaction according to RTOG/EORTC in breast cancer[36]	Fluidigm
ATM	rs227060	0·31	T→C	0·85	Early or late adverse radiotherapy reaction according to RTOG/EORTC in breast cancer[36]	Fluidigm
ATM	rs17503908	0·11	T→G	0·51	*ATM* tag SNP	Fluidigm
ATM	rs639923	0·05	G→A	0·13	*ATM* tag SNP	Fluidigm
ATM	rs11212592	0·18	G→A	0·83	*ATM* tag SNP	Fluidigm
ATM	rs11212570	0·09	G→A	1·00	*ATM* tag SNP	Fluidigm
ATM	rs4987889	0·02	T→C	0·34	*ATM* tag SNP	Fluidigm
ATM	rs4988023	0·14	C→A	0·79	*ATM* SNP correlated with (r^2=1) rs1801516 associated with early or late adverse radiotherapy reaction according to RTOG/EORTC;[36] late toxicity according to RTOG;[38] severe radiation-induced sequelae (CTCAE version 3.0 grade ≥3);[11] in-vitro clonogenic survival fraction;[9] breast cancer susceptibility allele;[34] development of telangiectasia[37]	Fluidigm
ATM	rs1800056	0·02	T→C	0·09	Frequently studied breast cancer susceptibility allele;[33,34] increased macronuclei formation in vitro[40]	Fluidigm
ATM	rs1800058	0·02	C→T	0·0098	Frequently studied breast cancer susceptibility allele;[33,34] lung fibrosis, pleural thickening, and atrophy[37]	Fluidigm
ATM	rs1800057	0·03	C→G	0·15	Frequently studied breast cancer susceptibility allele;[33,34] increased macronuclei formation in vitro[40]	Fluidigm
ATM	rs4986761	0·01	C→T	0·30	Frequently studied breast cancer susceptibility allele[33]	Fluidigm
BAX	rs918546	0·48	G→T	0·01	Early adverse skin reactions[32]	Fluidigm
CD44	rs8193	0·34	C→T	0·82	Early adverse skin reactions[32]	Fluidigm
CDKN1A	rs1801270	0·08	C→A	0·05	Included as a risk allele associated with in-vitro clonogenic survival fraction[9]	Fluidigm
DCLRE1C	rs35441642	0·08	G→C	1·00	Radiosensitivity and a double-strand break repair defect in G2 phase[35]	Fluidigm
EPDR1	rs1376264	0·31	T→C	0·77	Dysuria at 3 months[14]	Fluidigm
ERCC2	rs1799793	0·34	G→A	0·67	Increased chromatid aberrations[42,43]	Taqman
ERCC2	rs1052555	0·32	T→C	0·74	Severe (grade ≥2) late bladder or rectal toxicity (RTOG)[45]	Fluidigm
ERCC2	rs1799787	0·30	C→T	0·76	Increased chromatid aberrations[42]	Taqman
ERCC4	rs1799801	0·29	T→C	0·65	Need for long-term percutaneous feeding gastrostomy tube[47]	Fluidigm
ERCC4	rs1800067	0·08	G→A	0·18	Need for long-term percutaneous feeding gastrostomy tube[47]	Fluidigm
GSTA1	rs3957356	0·00	T→C	N/A	Telangiectasia[41]	Failed
GSTP1	rs1695	0·35	G→A	0·65	Acute toxicity[44] and increased pleural thickening[46]	Fluidigm
HIF1A	rs12435848	0·19	G→A	0·18	*HIF1A* tag SNP	Fluidigm
HIF1A	rs2284999	0·10	T→C	<0·0005	*HIF1A* tag SNP	Fluidigm
HIF1A	rs2301106	0·12	T→C	1·00	*HIF1A* tag SNP	Fluidigm
HIF1A	rs2301113	0·23	C→A	0·10	*HIF1A* tag SNP	Fluidigm
HIF1A	rs2301111	0·21	G→C	0·03	*HIF1A* tag SNP	Fluidigm
HIF1A	rs4899056	0·11	T→C	<0·0005	*HIF1A* tag SNP	Fluidigm
IL12RB2	rs3790568	0·05	G→A	0·12	Acute radiation dermatitis	Fluidigm
LIG3	rs3744355	0·10	G→C	0·36	Early adverse skin reactions[32]	Fluidigm
LIG4	rs1805389	0	T→C	N/A	LIG4 syndrome patients[51]	Failed
LIG4	rs1805388	0·17	T→C	0·23	LIG4 syndrome patients[51]	Fluidigm
LIG4	rs1805386	0·17	T→C	0·10	Severe (grade ≥2) late bladder or rectal toxicity (RTOG)[45]	Fluidigm
LIG4	rs12856974	0·06	T→C	0·44	In strong linkage disequilibrium (r^2=1·0) with *LIG4* SNP rs1805389	Taqman
MAD2L2	rs2294638	0·48	G→C	0·61	Early adverse skin reactions[32]	Fluidigm
MAP3K7	rs3757244	0·00	A→T	1·00	Early adverse skin reactions[32]	Fluidigm
MAT1A	rs2282367	0·32	G→A	0·53	Early adverse skin reactions[32]	Fluidigm

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	SNP	MAF	Base change	HWE p value	Originally reported significantly associated endpoints	Assay
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MLH1	rs1799977	0·32	A→G	0·36	Severe (grade ≥2) late bladder or rectal toxicity (RTOG)[45]	Fluidigm
MLH1	rs1800734	0·22	A→G	0·99	Acute myeloid leukaemia after methylating chemotherapy for Hodgkin disease[53]	Fluidigm
MPO	rs2333227	0	T→C	N/A	Acute toxicity in women with increased BMI[39]	Failed
MRE11A	rs569143	0·41	G→C	0·32	Breast cancer susceptibility[52]	Fluidigm
MRE11A	rs2155209	0·33	T→C	0·93	Bladder cancer susceptibility[50]	Fluidigm
MSH2	rs2303428	0·10	T→C	0·90	Acute myeloid leukaemia after chemotherapy with alkylating agents[54]	Fluidigm
NEIL3	rs3805169	0·07	T→C	0·76	Early adverse skin reactions[32]	Fluidigm
NFE2L2	rs1806649	0·26	T→C	0·55	Early adverse skin reactions[32]	Fluidigm
NOS3	rs1799983	0·34	T→G	0·35	Telangiectasia[41] and acute toxicity in women with increased BMI[39]	Fluidigm
PAH	rs1126758	0·39	G→A	0·57	Dysuria at 3 months[14]	Fluidigm
PRKDC	rs2213178	0·24	T→C	0·18	No statistical association with chromosomal radiosensitivity[49]	Fluidigm
PTTG1	rs3811999	0·39	T→C	0·93	Early adverse skin reactions[32]	Fluidigm
PTTG1	rs2961950	0·33	A→G	0·96	Early adverse skin reactions[32]	Fluidigm
PTTG1	rs2961952	0·27	G→A	0·74	Early adverse skin reactions[32]	Fluidigm
RAD17	rs3756402	0·06	G→A	0·0006	Early adverse skin reactions[32]	Fluidigm
RAD21	rs1050838	0·17	T→C	0·45	Clinically radiosensitive patients with various tumour types;[67] severe radiation-induced sequelae[11]	Fluidigm
RAD9A	rs2286620	0·07	T→C	0·73	Early adverse skin reactions[32]	Fluidigm
RAD9A	rs917570	0·44	G→C	0·50	Early adverse skin reactions[32]	Fluidigm
REV3L	rs190246	0·11	C→A	0·83	Early adverse skin reactions[32]	Fluidigm
REV3L	rs240962	0·11	T→C	0·92	Early adverse skin reactions[32]	Taqman
SART1	rs2276015	0·00	G→A	1·00	Dysuria at 3 months[14]	Fluidigm
SH3GL1	rs73234	0·45	G→C	0·13	Early adverse skin reactions[32]	Fluidigm
SOD2	rs4880	0·49	T→C	0·0070	Subcutaneous fibrosis,[10] late rectal bleeding,[56] and severe radiation-induced sequelae[11]	Fluidigm
TGFB1	rs1466345	0·30	G→A	0·62	*TGFB1* tag SNP	Fluidigm
TGFB1	rs1800469	0·30	C→T	0·06	Subcutaneous fibrosis[10,57,58] and severe radiation-induced sequelae[11]	Fluidigm
TGFB1	rs1800470	0·38	T→C	0·08	Subcutaneous fibrosis,[10] clonogenic survival fraction[9]	Taqman
TGFB1	rs8110090	0·05	A→G	0·02	*TGFB1* tag SNP	Fluidigm
TGFB1	rs4803455	0·49	C→A	0·04	No association with toxicity in previous RAPPER study[55]	Fluidigm
TGFB1	rs1466338	0·34	G→C	0·45	*TGFB1* tag SNP	Fluidigm
TGFB3	rs2268622	0·17	T→C	0·20	Early adverse skin reactions[32]	Fluidigm
TGFBR3	rs1926261	0·24	G→A	0·52	Early adverse skin reactions[32]	Fluidigm
TP53	rs1042522	0·25	G→C	0·07	Telangiectasia,[59] acute skin reaction,[60] and clonogenic survival fraction[9]	Fluidigm
XPC	rs2228000	0·26	T→C	0·58	DNA damage repair capacity by alkaline comet assay[61]	Fluidigm
XPC	rs2228001	0·39	C→A	0·89	DNA damage repair capacity by alkaline comet assay[61]	Fluidigm
XRCC1	rs1799782	0·06	T→C	0·06	Toxicity score according to CTCAE version 3.0 after pelvic radiotherapy,[12] single-strand breaks and DNA repair rates;[64] DNA damage measured by comet assay,[30,63] adverse reaction to breast radiotherapy scored by EORTC[66]	Fluidigm
XRCC1	rs25487	0·36	G→A	0·72	Subcutaneous fibrosis[10] adverse reaction to breast radiotherapy scored by EORTC,[66] early adverse skin reactions,[29,32] single-strand breaks and DNA repair rates,[64] severe radiation-induced sequelae,[11] DNA strand-break rejoining;[30] in-vitro cell cycle G_2 delay,[31] chromosome deletions, [42] DNA repair[62]	Fluidigm
XRCC1	rs25489	0·04	G→A	0·22	Erectile dysfunction;[56] single-strand breaks and DNA repair rates[62,64]	Fluidigm
XRCC1	rs3213235	0·01	C→A	1·00	Haplotype analysis of patients with adverse radiotherapy response in the breast[68]	Fluidigm
XRCC1	rs2854496	0·19	G→A	0·45	*XRCC1* tag SNP	Fluidigm
XRCC1	rs3213282	0·44	G→C	0·52	*XRCC1* tag SNP	Fluidigm
XRCC1	rs1799778	0·36	C→A	0·72	*XRCC1* tag SNP	Fluidigm
XRCC1	rs3213266	0·08	T→C	0·02	*XRCC1* tag SNP	Fluidigm
XRCC1	rs3213334	0·23	T→C	0·35	*XRCC1* tag SNP	Fluidigm
XRCC1	rs2293036	0·07	A→G	0·0005	*XRCC1* tag SNP	Fluidigm

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	SNP	MAF	Base change	HWE p value	Originally reported significantly associated endpoints	Assay
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XRCC1	rs2023614	0·08	G→C	0·05	*XRCC1* tag SNP	Fluidigm
XRCC1	rs12611088	0·36	G→A	0·65	*XRCC1* tag SNP	Fluidigm
XRCC3	rs1799794	0·20	G→A	0·65	Severe (grade ≥2) late bladder or rectal toxicity (RTOG)[45]	Fluidigm
XRCC3	rs3212090	0·33	G→A	0·45	*XRCC3* tag SNP	Fluidigm
XRCC3	rs3212102	0·03	G→A	0·40	*XRCC3* tag SNP	Fluidigm
XRCC3	rs3212079	0·07	T→C	0·51	*XRCC3* tag SNP	Fluidigm
XRCC3	rs861534	0·37	G→A	0·37	In strong linkage disequilibrium with rs861539 (r^2=0·96), which is associated with subcutaneous fibrosis,[10] toxicity according to CTCAE version 3.0 after pelvic radiotherapy,[12] erectile dysfunction,[56] acute toxicity,[48] clonogenic survival fraction,[9] severe radiation-induced sequelae,[11] chromosomal radiosensitivity,[65] and chromosome deletions[42]	Fluidigm
XRCC5	rs3835	0·12	T→C	0·92	Chromosomal radiosensitivity[49]	Fluidigm
XRCC6	rs2267437	0·44	C→G	1·00	Dysuria at 3 months,[53] development of severe dysphagia,[48] and increased chromosomal radiosensitivity[49]	Taqman
XRCC6	rs132788	0·34	G→C	0·01	Decreased chromosomal radiosensitivity[49]	Fluidigm

SNP=single nucleotide polymorphism. MAF=minor allele frequency. HWE=Hardy–Weinberg equilibrium. RTOG=Radiation Therapy Oncology Group. EORTC=European Organisation for Research and Treatment of Cancer. CTCAE= Common Terminology Criteria for Adverse Events. BMI=body-mass index.

Table 2: **98 SNPs selected from the literature review**

plates. DNA was genotyped for 95 SNPs with the Fluidigm high-throughput platform and Fluidigm 96.96 Dynamic Arrays according to the manufacturer's instructions and read with the Fluidigm EP1 (Fluidigm Corporation, San Francisco, CA, USA). Genotypes were automatically called with the BioMark Genotyping Analysis software (version 2.1.1), but all cluster plots were also checked manually and adjusted as necessary.

Six SNP assays failed quality control on the Fluidigm system and so these, as well as three SNPs chosen later, were genotyped with the Taqman 7900HT Sequence Detection System (Applied Biosystems, Warrington, UK). Genotypes were established with Allelic Discrimination Sequence Detection software (version 2.1.1). Primer and probe details are available on request.

For both Fluidigm and Taqman genotyping, 5% of all samples were duplicated as a reproducibility control and negative controls were also included on all plates. The concordance rate between duplicate samples was 100%. The average sample call rate was 99·6% (range 98·0–99·9). Three SNPs (rs3957356, rs1805389, and rs2333227) failed quality control on both platforms and genotype distributions of a further three deviated from those expected under Hardy–Weinberg equilibrium with the Bonferroni correction for multiple testing ($p<0·00051$) and so were excluded from further analysis (rs2284999, rs2293036, rs4899056). Therefore, results from 92 SNPs were analysed.

Statistical analysis

Stata version 10.1 was used. Univariate analysis was initially done by linear regression of mean toxicity scores against genotype. Multivariable analysis of overall and individual endpoints of toxicity included all covariates, identified from univariate analyses, with p values of less than 0·05 (appendix 1).[22,23,25] Many of these factors are known to be associated with toxicity, but are unlikely to be confounders of any genotype-toxicity association. In genetics, a confounding variable is genetically determined and could lead to spurious associations with a phenotype—eg, genetic variation associated with large breast size leading to a large volume of normal tissue irradiated and so increased toxicity. Adjustment for covariates should result in any true association becoming more significant because of improvement in the signal-to-noise ratio. After multivariable analysis, residuals were calculated for each patient to quantify the toxicity not accounted for by available patient-related and treatment-related factors.[23,26] Patients with residuals of zero had toxicity entirely accounted for by the available patient-related and dose-related factors. Patients with negative or positive residuals had less or greater toxicity, respectively, than was accounted for by known factors. The means of the residuals for overall toxicity (rSTAT) and individual endpoints were correlated with genotype with linear regression. For SNPs that were significantly associated with a particular toxicity endpoint, the endpoint was dichotomised to obtain a more clinically interpretable per-allele odds ratio (OR).

A Bonferroni correction was made to adjust for the effects of multiple testing; the nominal significance threshold (p=0·05) was divided by the number of tests done (92 SNPs×20 endpoints) to give a significance threshold of $p=2·7\times10^{-5}$. Q-Q plots were created to assess the distribution of the 92 test statistics from that expected under the null hypothesis that no SNP was associated with late radiotherapy toxicity.

We undertook power calculations by specifying differences to be detected between genotypes in the mean

of the toxicity endpoint and adjusting for groups of unequal size according to minor allele frequency (MAF; appendix 1). Power of 99% and a type I error rate (p value) of $2{\cdot}7\times10^{-5}$ were selected to reduce risk of false-negative and false-positive associations, respectively, and the detectable effect sizes (ORs) were estimated with STPLAN software (version 4.3) downloaded from the Department of Biostatistics at the MD Anderson Cancer Center (Houston, TX, USA; table 1). If the incidence of toxicity in non-carriers (P_0) is assumed to be 20%, the incidence of toxicity in carriers (P_C) will depend on the effect size of that allele. The incidence of toxicity in the unselected population (P) is estimated as $MAF\times P_c+(1-MAF)\times P_0$. To estimate the clinical relevance of the detectable effects, a hypothetical scenario was considered in which the SNP was used to identify sensitive individuals and escalate the dose in the remaining, relatively more resistant, cases. The permissible dose escalation (ΔD_{esc}) that would result in the same incidence of toxicity in the non-carriers as would be seen in the unselected population was calculated. To this end, a logistic dose–response curve with a steepness of $\gamma_{50}=3$ was assumed—ie, at the steepest part of the dose–response curve, corresponding to a 50% response level, there would be a 3% increase in response in percentage points for a 1% increase in dose, a typical value for late normal tissue effects (appendix 1).[27] The γ value at the 20% response level is 1·7.[28] Using this value and the difference between P and P_0, we calculated ΔD_{esc} using a linear approximation to the dose–response curve. This approximation was deemed adequate over the fairly narrow range of response probabilities considered.

Role of the funding source

The funding sources had no role in the design of the study, collection, analysis, interpretation of the data, writing of the report, or in the decision to submit for publication. GCB had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

66 SNPs previously reported to be associated with radiotherapy toxicity, either clinically or in in-vitro assays,[9–14,29–68] were selected along with an additional 28 SNPs in *ATM*, *TGFB1*, *XRCC1*, *XRCC3*, and *HIF1A* as tagging SNPs, designed to capture all common genetic variation within these genes, or because of evidence for direct functionality (table 2). Appendix 2 shows the unadjusted results from regression analysis of all 92 analysed SNPs in 46 genes against all endpoints.

Some genetic variants, depending on their action, are likely to affect toxicity in both breast and prostate cancer. For example, polymorphisms such as those in DNA repair genes would be likely to act soon after irradiation and therefore could affect all late toxicity measures. In the combined cancer site analysis, we sought any such variants first, using the STAT score of overall late toxicity in the combined sets of patients with breast or prostate cancer. None were significant after adjustment for multiple testing. Figure 2 shows Q-Q plots of the observed versus expected p values of all 92 successfully genotyped SNPs with overall late toxicity. A quantile-quantile or Q-Q plot is obtained by ordering the p values obtained from association tests and plotting them against that which would be expected from chance alone. Deviation from the identity line (x=y) at the tail of the distribution suggests deviation from the null distribution (that expected under the null hypothesis that no SNP is associated with the trait) and the presence of true association. The distribution of observed p values does not deviate significantly from that expected by chance.

See Online for appendix 2

The strongest association in the analysis of acute toxicity in patients with breast or prostate cancer was for *ATM* SNP rs4988023 with the endpoint of acute bladder toxicity in patients with prostate cancer. However, the OR (per additional risk allele) was 1·53 (95% CI 1·08–2·18) for development of acute symptoms of grade 2 or higher (p=0·0006) and thus short of the value regarded as

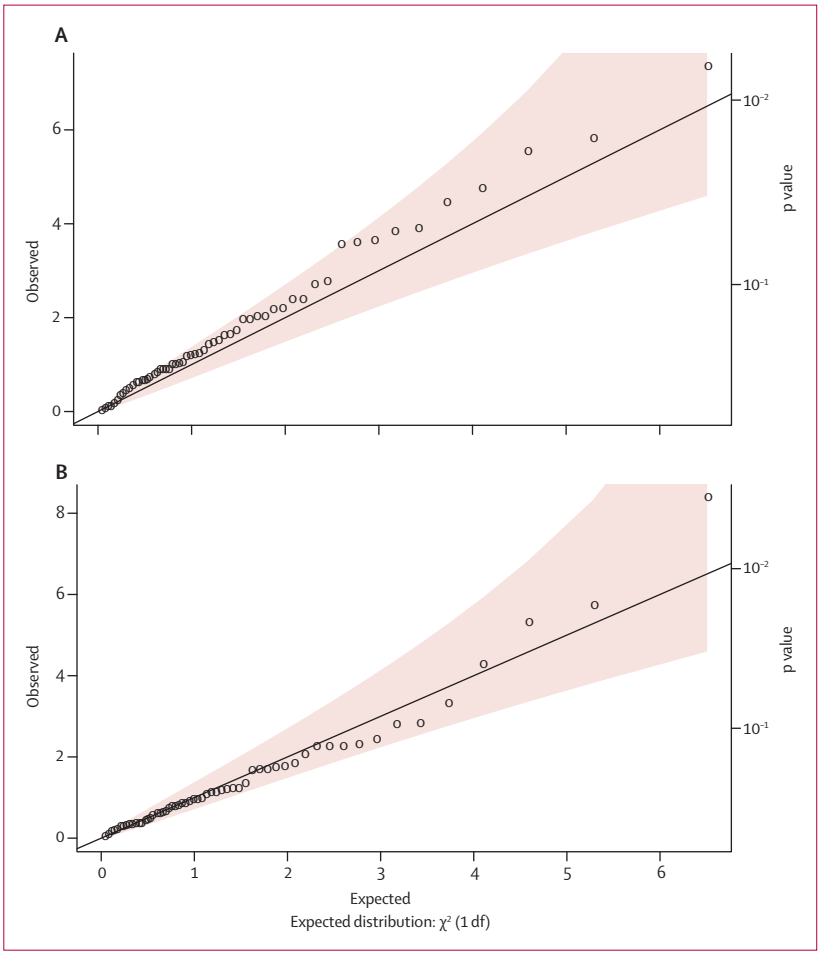

Figure 2: **Q-Q plots showing p values (and corresponding χ^2 values) obtained from (A) univariate and (B) multivariable analyses of overall toxicity against genotype at the 92 SNPs**
The solid lines represent the identity line (x=y). p values do not vary significantly from those that would be expected from chance alone. SNP=single nucleotide polymorphism. df=degree of freedom.

	SNPs included in profile	SNPs genotyped in present study	Endpoints associated with number of risk alleles in initial publication	Associations found in the present study
Andreassen and colleagues[10]	*TGFB1* rs1800470 *TGFB1* rs1800469 *TGFB1* rs1800471 *SOD2* rs4880 *XRCC1* rs25487 *XRCC3* rs861539	*TGFB1* rs1800470 *TGFB1* rs1800469 *SOD2* rs4880 *XRCC1* rs25487 *XRCC3* rs861534*	Fibrosis in 41 postmastectomy patients	No association with breast induration or breast shrinkage on univariate or multivariable analysis in 976 patients with breast cancer
Alsbeih and colleagues[9]	*ATM* rs1801516 *TP53* rs1042522 *CDKN1A* rs1801270 *XRCC1* rs25487 *XRCC3* rs861539 *TGFB1* rs1800470	*ATM* rs4988023† *TP53* rs1042522 *CDKN1A* rs1801270 *XRCC1* rs25487 *XRCC3* rs861534* *TGFB1* rs1800470	54 fibroblast strains of varying radiosensitivity measured by clonogenic survival assay	No association with overall toxicity on univariate and multivariable analysis in patients with breast or prostate cancer combined No association with breast induration or breast shrinkage on univariate or multivariable analysis in 976 patients with breast cancer
Azria and colleagues[11]	*TGFB1* rs1800470 *TGFB1* rs1800469 *ATM* rs1801516 *SOD2* rs4880 *XRCC1* rs25487 *XRCC3* rs861539 *RAD21* rs1050838	*TGFB1* rs1800470 *TGFB1* rs1800469 *ATM* rs4988023† *SOD2* rs4880 *XRCC1* rs25487 *XRCC3* rs861534* *RAD21* rs1050838	16 patients with severe radiation-induced sequelae and 18 control patients without adverse effects; RTOG/EORTC grade in various cancers	No association with overall toxicity on univariate and multivariable analysis in patients with breast or prostate cancer combined
De Ruyck and colleagues[12]	*XRCC1* rs25487 *XRCC1* rs25489 *XRCC1* rs3547‡ *XRCC3* rs1799796‡	*XRCC1* rs25487 *XRCC1* rs25489	62 women with cervical or endometrial cancer; toxicity score according to CTCAE version 3.0	No significant increase in any toxicity endpoint in patients with breast or prostate cancer
Suga and colleagues[14]	*SART1* rs2276015 *ID3* rs2742946 *EPDR1* rs376264 *PAH* rs1126758 *XRCC6* rs2267437	*SART1* rs2276015§ *EPDR1* rs376264 *PAH* rs1126758 *XRCC6* rs2267437	197 patients with prostate cancer; dysuria at 3 months	No significant association with acute bladder toxicity at 3 months
Isomura and colleagues[13]	*ABCA1* rs2230806 *IL12RB2* rs3790568	*ABCA1* rs2230806 *IL12RB2* rs3790568	156 women treated with adjuvant breast radiotherapy; acute toxicity	No significant increase in acute or late toxicity endpoints in patients with breast cancer

SNP=single nucleotide polymorphism. RTOG=Radiation Therapy Oncology Group. EORTC=European Organisation for Research and Treatment of Cancer. CTCAE=Common Terminology Criteria for Adverse Events. *In strong linkage disequilibrium with *XRCC3* rs861539 with r^2=0·96. †In strong linkage disequilibrium with *ATM* rs1801516 with r^2=1. ‡SNPs not in HapMap and therefore the linkage disequilibrium with SNPs genotypes in the present study could not be calculated; *XRCC1* and *XRCC3* genes tagged completely in present study. §No rare homozygotes in present study.

***Table 3*: Results from testing of previously proposed SNP profiles**

significant after Bonferroni correction ($p=2·7\times10^{-5}$). Similarly, for late toxicity in patients with breast or prostate cancer, no associations were significant after conservative Bonferroni correction. The strongest association was between urinary frequency and rs1800734 in *MLH1* (p=0·0010). The OR (per additional risk allele) was 1·84 (95% CI 1·26–2·70) for any increase in urinary frequency.

If we attempt to confirm only SNP associations with previously reported endpoints, a less stringent Bonferroni correction threshold could be deemed appropriate. In this analysis, 196 tests were done because many of the previous studies looked at non-specific outcomes, such as early or late adverse radiotherapy reaction, and therefore the threshold for significance after a Bonferroni correction was $2·6\times10^{-4}$. A few borderline significant associations were identified, but none reached this p-value threshold: the *RAD21* SNP rs1050838[11,67] and *SOD2* SNP rs4880[11] have previously been reported to be associated with severe radiosensitivity in various tumour types and were respectively associated with urinary incontinence (p=0·02) and proctitis (p=0·03). *SART1* rs2276015 was reported to be associated with dysuria at 3 months,[14] and here was associated with acute bladder toxicity (p=0·04). *XRCC1* SNPs rs1799782 and rs25487 were reported as being associated with adverse reactions to radiotherapy[66] and here were associated with altered pigmentation (p=0·03) and telangiectasia (p=0·01).

Where possible, we tested the associations of previously reported SNP signatures, but not all the SNPs within such signatures were successfully genotyped in this study. We were unable to confirm association of increased toxicity with increasing number of risk alleles for any of the previously published signatures (table 3).

Discussion

None of the previous reports of significant associations between radiation toxicity and SNPs in candidate genes have been confirmed with appropriate levels of confidence within the design and sample size of our study (panel). The Q-Q plots show that there are no more significant associations among the 92 SNPs tested for overall late effects than would have been expected by chance. This finding is despite the fact that all the variants had already been reported as significantly associated with radiotherapy toxicity or otherwise judged to have a high previous probability of involvement. The associations of these highly selected variants are thus no better than those expected for any set of random SNPs picked from

the genome. Our findings suggest that the published literature on this subject is dominated by false-positive associations due to small sample sizes, multiple testing, and the absence of rigorous independent validation attempts in the original studies.

For five genes thought to be particularly good candidates (*ATM*, *TGFB1*, *XRCC1*, *XRCC3*, and *HIF1A*), we not only re-examined the previously reported SNPs but also examined a more comprehensive set of tag SNPs designed to represent all the known common variants (MAF >0·05) within the genomic footprint of each gene. None of these SNPs revealed significant associations after correction for multiple testing. Our results therefore suggest that common variants within these genes are unlikely to affect the development of radiotherapy toxicity.

The strongest although not significant association identified in this study was between *ATM* SNP rs4988023 (MAF 0·14) and acute bladder toxicity in patients with prostate cancer. This SNP had been selected as a more reliably genotyped surrogate for another *ATM* SNP, rs1801516 (correlation r^2=1). rs1801516 had previously been reported to be associated with several effects including adverse radiotherapy reaction according to RTOG/European Organisation for Research and Treatment of Cancer (EORTC),[36] late toxicity according to RTOG,[38] severe radiation-induced sequelae (Common Terminology Criteria for Adverse Events version 3.0 grade ≥3),[11] in-vitro clonogenic survival fraction,[69] and development of telangiectasia.[34] It has been reported as a breast cancer susceptibility allele,[37] although not confirmed in subsequent studies.[70] In view of this additional background, the association we noted could be a true-positive association. *ATM* SNP rs1801516 results in a non-conservative aminoacid substitution of asparagine for aspartic acid at position 1853 of the protein. *ATM* codes for the main phosphatidylinositol 3-kinase related kinase responding to double-strand breaks. It is defective in the hereditary disorder ataxia telangiectasia, which is associated with hypersensitivity to radiation, immunodeficiency, and cancer predisposition. The relation between heterozygous truncating mutations in *ATM* and radiosensitivity remains equivocal.

A possible limitation of our study was our necessary reliance on 2-year follow-up toxicity data in this still maturing study. Normal tissue reactions, such as induration and telangiectasia, develop gradually after a latency period over many years.[71,72] Although there is evidence that late effects at 2 years are predictive of those at 5 years,[73] we might not have detected the full severity of developing late toxicity in some patients. A re-analysis of data is planned after longer follow-up, which will enable us to better address the effect of late toxicity timepoint assessments. A further potential limitation of this study was the inability to assess erectile dysfunction. However, only two studies examined this endpoint. The only genome-wide association study undertaken so far into late toxicity from radiotherapy has reported an association that reached genome-wide significance between SNP rs2268363 in the follicle-stimulating hormone receptor gene (*FSHR;* involved in testes development and spermatogenesis) and erectile dysfunction in a small cohort of African American men.[4] Collaborations developed through the Radiogenomics Consortium are enabling replication studies to be done.[74,75]

We have aimed to minimise both false-positive and false-negative findings in this validation study, since the need for multiple testing was generally ignored in the original hypothesis-generating studies. We used a conservative Bonferroni correction to reduce the chance of false-positive results, in view of the large number of tests we had done. In so doing, we might have missed some true associations with small effect sizes for which the power of this study was low. However, use of such a stringent p value is justified; as table 1 shows, even with this conservative Bonferroni correction, we have enough power to detect clinically relevant ORs.

If a Bonferroni correction is made, the actual p value chosen to represent statistical significance can vary slightly according to estimates of the degree of correlation between the multiple hypotheses tested. For example, there might be correlation between some of the SNPs and between some of the endpoints. Yet, the need for a stringent p value is paramount, however many tests are done. The p value is the probability of the data given that the null hypothesis is true. The probability that we are actually interested in is the probability that the null hypothesis is true given the data,[76] which depends on the p value, the power of the study to detect the effect, and the previous probability the null hypothesis is true. None of these factors are changed by the number of tests done. The previous probability that any one SNP is associated with any endpoint is, at best, very small. For example, the previous probability might have increased from 1:100 000 to 1:10 000 given previous positive association. This estimate assumes there are 10 million SNPs in the human genome; results from other

***Panel:* Research in context**

Systematic review

We undertook a comprehensive search of Medline and PubMed databases using the keywords "radiotherapy", "radiation", "toxicity", "adverse effects", "genetic variation", and "polymorphism". This search identified 69 in-vitro or in-vivo studies published up to Dec 31, 2009. Studies to date have been underpowered, including fewer than 500 samples, have tested many single nucleotide polymorphisms without adjusting for multiple comparisons, and findings have proved difficult to replicate.[6–8]

Interpretation

Our failure to replicate the associations of most variants reported from candidate gene studies suggests that common variation in the genes studied is increasingly unlikely to be clinically important in determining individual variation in response to radiotherapy.

genome-wide association studies suggest no more than 50 SNPs would be expected to account for 1% of the phenotypic variance with detectable effect sizes and the previous probability is doubled for candidate gene selection. So, a very stringent p value is needed.

Correction for multiple testing might be unnecessarily stringent when we aimed to directly confirm previous reports. However, this assumption is only true if the validation study tests one previous association. We studied 20 endpoints and included 28 new SNPs. Also, many of the previously reported endpoints were either non-specific—eg, early or late adverse radiotherapy reaction according to RTOG/EORTC[36]—or were in-vitro tests of radiosensitivity, which did not provide an effect size to be tested here, and so we felt a form of correction was necessary. Further study of *RAD21* SNP rs1050838, *SOD2* SNP rs4880, *SART1* SNP rs2276015, and *XRCC1* SNPs rs1799782 and rs25487 in even larger studies would be merited. However, our study had sufficient power to detect effect sizes needed for an individual SNP to affect clinical management. Our study was larger than the published studies reporting associations. It is, therefore, likely that the original reports were false positives. All studies have reduced power to detect associations for rare SNPs and thus an increased chance of finding false-negative associations. The detectable effect sizes increase with decreasing MAF. However, with increasing rarity of these radiosensitive cases, the clinical benefit of their removal from the risk set tends to zero—ie, identification of such rare variants in a hypothetical subgroup is likely to be increasingly irrelevant for the treatment deliverable to most patients. Table 1 shows that, if carriers of a SNP with a MAF of 5% associated with an increase in toxicity from 20% to 55·2% (equal to an OR of 4·9) could be advised not to receive radiotherapy, this approach would only allow an increase in dose to the remaining patients of 0·5 Gy if we aim for isotoxicity in the non-carriers. For a MAF of 0·35, we had 99% power to detect an OR of 2·2. Therefore we can essentially exclude the hypothesis that published SNPs individually exert a clinically relevant effect.

Our failure to confirm findings of candidate gene studies is by no means unprecedented in the field of genetic studies; in fact, this situation has been the norm for most candidate gene studies of cancer susceptibility. Before the development of genome-wide association studies, more than 500 SNPs were examined in more than 100 candidate genes for breast cancer susceptibility;[77] many of those genes were examined in our study. None of those associations were confirmed, although subsequently more than 30 genetic loci with unequivocal effects on breast cancer risk have been identified and are continuing to be discovered through ever larger genome-wide association studies.[78–80] A few of these loci lie close to genes that had already been considered in candidate studies (eg, 8q24 upstream of *MYC*, 6q25 upstream of *ESR1*, and 11q upstream of *CCND1*) and are likely to be regulatory regions for these genes. Genome-wide association studies have served to emphasise the poverty of our understanding of the biological basis of most complex traits and diseases and one of the earliest advances to come from this new technology will be the recognition of new biochemical pathways, and hence drug targets, for these traits.

Despite the failure to validate previously identified genetic determinants of radiotoxicity in our study, there remains evidence that genetic variation might account for a proportion of the patient-to-patient variability in response to radiation therapy. Several recent studies have shown that chromosomal radiosensitivity of lymphocytes is largely determined by genetic factors.[81–85] Rare radiosensitivity syndromes, such as ataxia telangiectasia, certainly affect radiosensitivity in individual mutation carriers, but the prevalence of such mutations is so low that they have little effect on radiosensitivity in most patients with cancer.

The ultimate goal of radiogenomics is to develop a genetic risk profile including many SNPs for individualisation of radiation dose prescriptions to optimise tumour control while minimising normal tissue damage. Importantly, there is interest in identification of both individuals with low and those with high risk of toxicity, and there is no conceptual difference between the two. For example, if one allele of a polymorphic genetic locus is associated with a high risk of toxicity, by definition the other allele can be regarded as a non-toxicity allele. Development of genetic risk profiles could, therefore, stratify patients into subgroups with different probabilities of developing toxicity; this approach would allow individualised dose prescription, to increase survival and decrease the morbidity associated with cancer. The first stage in development of such a profile is to identify genetic variants that are unequivocally associated with differences in radiation toxicity, even if their individual effect size is small. The establishment of the Radiogenomics Consortium in 2009 should provide a route to not only undertake large, sufficiently powered studies, but also to address methodological challenges associated with radiogenomics.[74,75]

The RAPPER study is continuing and a stage 1 genome-wide analysis of STAT score and individual late toxicity endpoints has been done. Validation and replication in an independent cohort is underway. Ultimately, the top 5–10% of SNPs showing the most significant association with toxicity will be genotyped across the Radiogenomics Consortium and included in a meta-analysis with other similar genome-wide association studies.

In conclusion, our failure to replicate the associations of most variants reported from candidate gene studies suggests that common variation in the genes studied is increasingly unlikely to be important in determining individual variation in response to radiotherapy. We cannot yet exclude a role for variants in these genes with very low MAF or small effects, both of which would limit the clinical importance of these associations.

Contributors
GCB, CMLW, NGB, PDPP, and AMD were involved in the conception and design of the study. RME and GCB were involved in data acquisition. CB, CL, DC, GCB, and RP were involved in laboratory work. CEC is the principal investigator of the Cambridge Breast IMRT Trial. JSW, CEC, and GCB were involved in patient data acquisition for the Cambridge Breast IMRT trial. SLG, MRS, and DPD were involved in data collection and analysis of the RT01 trial. EH and DPD were involved in data collection and analysis of the CHHiP trial. DPD designed, initiated and was chief investigator for the RT01 and CHHiP trials. VM was involved in data collection in the Manchester prospective toxicity study. GCB, JT, PDPP, and SMB were involved in data analysis and interpretation. All authors were involved in writing or critical review of the draft report and all approved the final version.

Conflicts of interest
We declare that we have no conflicts of interest.

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