Clinical Oncology 31 (2019) 9–16

Contents lists available at ScienceDirect

Clinical Oncology

journal homepage: www.clinicaloncologyonline.net

Original Article

Genetic Variants Predict Optimal Timing of Radiotherapy to Reduce Side-effects in Breast Cancer Patients

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Received 20 April 2018; received in revised form 7 August 2018; accepted 10 September 2018

Abstract

Aims: Radiotherapy is an important treatment for many types of cancer, but a minority of patients suffer long-term side-effects of treatment. Multiple lines of evidence suggest a role for circadian rhythm in the development of radiotherapy late side-effects.
Materials and methods: We carried out a study to examine the effect of radiotherapy timing in two breast cancer patient cohorts. The retrospective LeND cohort comprised 535 patients scored for late effects using the Late Effects of Normal Tissue-Subjective Objective Management Analytical (LENT-SOMA) scale. Acute effects were assessed prospectively in 343 patients from the REQUITE study using the CTCAE v4 scales. Genotyping was carried out for candidate circadian rhythm variants.
Results: In the LeND cohort, patients who had radiotherapy in the morning had a significantly increased incidence of late toxicity in univariate ($P = 0.03$) and multivariate analysis ($P = 0.01$). Acute effects in the REQUITE group were also significantly increased in univariate analysis after morning treatment ($P = 0.03$) but not on multivariate analysis. Increased late effects in the LeND group receiving morning radiotherapy were associated with carriage of the PER3 variable number tandem repeat 4/4 genotype ($P = 6 \times 10^{-3}$) and the NOCT rs131116075 AA genotype ($P = 5 \times 10^{-3}$).
Conclusion: Our results suggest that it may be possible to reduce toxicity associated with breast cancer radiotherapy by identifying gene variants that affect circadian rhythm and scheduling for appropriate morning or afternoon radiotherapy.

Key words: Adverse reactions; breast cancer; circadian rhythm; genetics

Introduction

Circadian rhythms are 24 h endogenous biological cycles that infiltrate every aspect of physiology, biochemistry and behaviour in higher organisms. The phasing of these rhythms can be adjusted by environmental factors such as light and temperature, feeding times and genotoxic agents. Circadian timing regulates rhythmic events in the cell cycle, DNA repair, apoptosis and in the immune system [1].

There is strong evidence that the response to xenobiotics, including drugs, varies according to circadian rhythms, both in terms of efficacy and side-effects [2]. This has been particularly demonstrated for some anti-cancer agents [3–6].

The main tissue at risk for acute and late effects of breast radiotherapy is the skin and subcutaneous connective tissue. Fibroblasts in the dermis and keratinocytes in the epidermis show diurnal variation in cell proliferation activity regulated by circadian rhythm genes [7]. The skin is

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https://doi.org/10.1016/j.clon.2018.10.001

subject to damage from the ultraviolet irradiation from the sun. It has been postulated that the pattern of skin cellular proliferation has adapted to this potentially harmful effect during daylight hours and is under circadian control [8]. Although ultraviolet irradiation only penetrates as far as the dermis it has similar effects on the epidermis as much more penetrating X or gamma rays. Proliferating cells are more radiosensitive or ultraviolet sensitive than non-dividing cells (in G0) and radiosensitivity varies throughout the cell cycle. Cells in the DNA synthetic phase (S phase) are the least sensitive and cells just before mitosis or during mitosis are the most sensitive (G2/M phases) [9].

In humans, the maximum number of dividing epidermal cells are in the radioresistant S phase at the time of maximal potential solar exposure [8]. Janich and colleagues [7] identified five peaks of circadian gene activity and proliferation events in human keratinocytes in a 24 h cycle. Three of the observed peaks were in the late evening and early morning hours and were related to keratinocyte differentiation. The other two peaks were in the afternoon and evening hours and were related to DNA replication and cell division. The family of Period genes (PER1–3) were observed to be part of the auto-regulated feedback loop involved in this process.

There has previously been mixed evidence for whether radiotherapy side-effects are affected by the time of treatment [10]. Noh *et al.* [11] examined the relationship with radiotherapy treatment time and acute skin toxicity in 395 breast cancer patients. They reported that patients treated in the afternoon had more chance of developing acute skin toxicity (Radiation Therapy Oncology Group grade 2 or more) ($P = 0.0088$). Bjarnason *et al.* [12] investigated the relationship of treatment time to grade 3 or more mucositis in head and neck patients treated with radical doses of radiotherapy but failed to show a significant difference between morning and afternoon groups. However, on subgroup analysis, when patients were divided by gender there was a trend that women had enhanced toxicity in the morning, whereas men showed the effect in the afternoon. A gender-specific circadian rhythm response has also been seen for chemotherapy response [13,14].

A variable number tandem repeat (VNTR) polymorphism in the PER3 gene has been found to be associated with sleep–wake patterns, with the 5 allele associated with morningness [15]. Earlier evidence found that the C allele of rs1801260 SNP in the CLOCK gene was associated with eveningness [16].

A genome-wide association study for breast cancer side-effects found an association between adverse reactions and a single nucleotide polymorphism (SNP) in a circadian rhythm gene called Nocturnin (NOCT), which encodes a RNA deadenylase [17]. The SNP was the top hit associated with overall radiosensitivity with a P value of 1.21×10^{-6}, which, however, does not meet the conventional significance cut-off for a genome-wide association study. These data led us to investigate potential circadian effects on radiotherapy treatment.

Materials and Methods

Patient Selection and Assessment

All patients were recruited after gaining appropriate consent and ethics approval was granted by the National Health Service research and ethics committee and health research authority.

LeND Cohort

For the assessment of late radiotherapy effects, a group of 664 breast cancer patients previously recruited to the LeND study were assessed as previously described [18–20]. Most patients received 50 Gy of radiation in 25 fractions. Participants were recruited at follow-up oncology clinics from 2008 to 2010 in Leicester, Nottingham and Derby at least 3 years after adjuvant radiotherapy treatment (median follow-up time 62 months). Radiotherapy toxicity was recorded using the Late Effects of Normal Tissue-Subjective Objective Management Analytical (LENT-SOMA) criteria [21]. At the same visit, volunteers donated a blood sample or buccal cheek swab for DNA extraction. Samples of frozen isolated DNA (1 ng/μl) obtained from whole blood were available for volunteers 150–633.

REQUITE Cohort

In total, 343 breast cancer patients were recruited at Leicester Royal Infirmary to the international European Union-funded REQUITE study [22] between 2014 and 2016. All breast cancer patients underwent a wide local excision before adjuvant whole breast radiotherapy. Most patients received 40 Gy of radiation in 15 fractions. Comorbidity data were recorded by the clinical team, with depression being defined as having a clinical diagnosis of depression.

Patients were assessed by a clinician at baseline and again within the last three fractions of radiotherapy to review any acute reactions using an adapted version of the CTCAE v4 scoring system. At the same time as the baseline assessment, a 10 ml sample of fresh whole blood was collected in an EDTA tube. This was transferred to the CIGMR Biobank (Manchester, UK) who isolated DNA using robotic magnetic bead extraction technology. The isolated DNA (20 ng/μl) was then stored at −80 °C.

Radiotherapy Timing

Scheduling of radiotherapy treatment in most cases is a result of department capacity and patient request. At the Leicester Royal Infirmary, radiotherapy records were reviewed to obtain the time of treatment for every fraction received. Patients with more than 66% of their radiotherapy before noon were classified as morning treatment; patients who received more than 66% after noon were classified as afternoon treatment; those falling outside these criteria were classified as a mixed group.

Radiotherapy had been delivered in Leicester, UK, which is at latitude 52.6°N and has daylight savings time. For the purposes of this analysis, seasons were grouped around the solstices, with the darkest half of the year being 20 September to 20 March and the lightest half of the year being 21 March to 19 September.

DNA Analysis

Of the 664 LeND patients, DNA was available for genotyping on 508 patients. In the REQUITE cohort, DNA was available for genotyping in 324 of 343 patients.

PER3

In both cohorts, the PER3 VNTR region was amplified by polymerase chain reaction (PCR) and agarose gel electrophoresis was carried out. The two alleles are differentiated by an extra 56 bp repeat in the 5 allele: 4/4 VNTR showed a single band at 639 bp, 4/5 genotype showed two bands at 639 and 685 bp 5/5 VNTR showed a single band at 685 bp.

In the LeND cohort, PCR for PER3 VNTR was successful in 476 patients; in the remaining 32 samples there was inadequate DNA to produce reliable results. In total, 225 patients were 4/4, 191 patients were 4/5 and the remaining 60 patients 5/5 genotype.

In the REQUITE cohort, PCR was successful in 309 patients. In total, 140 patients were found to have 4/4, 136 4/5 and the remaining 33 patients were 5/5 genotype. Genotyping results were tested and found to be in Hardy–Weinberg equilibrium.

NOCT rs13116075

NOCT rs13116075 was genotyped by Taqman assay. Supplementary Figure S1 shows the plots that resulted from this assay.

In the LeND cohort, PCR for NOCT rs13116075 was successful in 466 patients; in the remaining 42 samples there was inadequate DNA to produce reliable results. In total, 317 patients were AA, 141 were AG and the remaining eight were GG genotypes. In the REQUITE cohort, genotyping was successful in 323 patients; the remaining one patient sample failed to produce reliable results. Genotyping revealed that 233 patients were AA, 80 were AG and 10 were GG. Genotype frequencies were found to be in Hardy–Weinberg equilibrium.

CLOCK rs1801260

CLOCK rs1801260 was genotyped by Taqman assay in just the REQUITE cohort. Supplementary Figure S2 shows the plots that resulted from this assay.

Statistical Analysis

For acute toxicity (in REQUITE) breast erythema was taken as a surrogate marker of overall acute toxicity. Any baseline score was deducted from the score assessed within the last three fractions of radiotherapy to give a corrected acute toxicity score.

Late toxicity (in LeND) was assessed using Bivariate STAT score. Use of the STAT score was described by Barnett *et al.* [17] and has been used previously as a dichotomised variable [18]. In brief, a Z score was calculated for each patient and all toxicity end points (i.e. fibrosis, telangiectasia, atrophy, oedema) (Z = [score − mean score]/standard deviation for the whole population). A STAT score can then be calculated as an average of the patient Z scores. The population are then divided into upper quartile and lower three-quarters to form a bivariate STAT score.

Statistical analysis was carried out using IBM SPSS version 24. Differences in categorical variables were analysed by chi-squared test. Multivariable analysis was carried out using logistic regression. Covariates were selected by bidirectional elimination.

Results

Radiotherapy Treatment Time

The LeND Cohort

In total, 664 women were enrolled in the LeND cohort and of these radiotherapy treatment time was available for 536 patients. Of those with no data, 75 were recruited in subsites and 53 received radiotherapy before computerised records became available in 1998. Patients were grouped according to treatment time: 185 patients (34.5%) received radiotherapy mainly in the morning, 170 patients (31.7%) received radiotherapy mainly in the afternoon and 181 (33.8%) had a mix of treatment times. Patients received either 45 Gy X-rays in 20 fractions or 50 Gy in 25 fractions. Seventy-six patients received a 9–15 Gy electron boost in three to five fractions. Baseline characteristics for these patients by group are shown in Table 1. Chi-squared testing for categorical variables and ANOVA testing for continuous variables revealed that none of these baseline characteristics was significantly different between the treatment time groups. The only exception was if the mixed group were excluded from the analysis, then significantly more patients received a boost in the morning compared with the afternoon ($P = 0.04$).

Late toxicity (bivariate STAT score) was available on 536 of the patients. Univariate analysis using a chi-squared test was carried out and showed a significantly increased frequency of toxicity in patients treated in the mornings: 29.2% of the morning treatment group had high toxicity compared with 21.1% of the afternoon group and 17.7% of the mixed group ($P = 0.03$). Multivariable analysis was carried out using logistic regression and radiotherapy treatment time remained significant ($P = 0.01$). Results are summarised in Table 2. An interaction term for boost × treatment time was included due to the difference between the groups in the proportion receiving boosts.

Table 1
Baseline tumour, patient and treatment characteristics for the LeND cohort. Percentages are of non-missing data within each time group

Patients	Radiotherapy treatment time				P =
		AM	Mixed	PM	Total	
		185	181	170	536	
Age (mean years)		59.1	57.4	58.7		0.28
Grade	1	29 (16.9%)	40 (25.8%)	39 (24.8%)	108 (22.3%)	
	2	81 (47.1%)	73 (47.1%)	67 (42.7%)	221 (45.7%)	0.20
	3	62 (36.0%)	41 (26.5%)	51 (32.5%)	154 (31.8%)	
	Missing	13	26	13	52	
Chemotherapy	Yes	71 (38.4%)	49 (27.1%)	49 (29.0%)	169 (31.6%)	
	No	114 (61.6%)	132 (72.9%)	120 (71.0%)	366 (68.4%)	0.05
	Missing	0	0	1	1	
Oestrogen receptor positive	Yes	162 (88.5%)	147 (82.1%)	141 (82.9%)	450 (84.6%)	
	No	21 (11.5%)	32 (17.9%)	29 (17.1%)	82 (15.4%)	0.19
	Missing	2	1	0	3	
Mean radiotherapy dose (Gy)		47.8	47.8	48		0.76
Boost to tumour bed	Yes	36 (19.6%)	22 (12.2%)	20 (11.8%)	78 (14.6%)	
	No	148 (80.4%)	159 (87.8%)	150 (88.2%)	457 (85.4%)	0.06
	Missing	1	0	0	1	
Diabetes	Yes	15 (8.1%)	6 (3.3%)	12 (7.1%)	33 (6.2%)	
	No	170 (91.9%)	175 (96.7%)	158 (92.9%)	503 (93.8%)	0.14
Smoker	Current smoker	27 (14.9%)	21 (11.7%)	16 (9.5%)	64 (12.1%)	
	No	134 (74.0%)	138 (76.7%)	121 (72.0%)	393 (74.3%)	0.16
	Ex-smoker	20 (11.0%)	21 (11.7%)	31 (18.5%)	72 (13.6%)	
	Missing	4	0	2	6	
Side of treatment	Right	100 (54.1%)	102 (56.4%)	90 (52.9%)	292 (54.5%)	
	Left	79 (42.7%)	73 (40.3%)	78 (45.9%)	230 (42.9%)	0.59
	Bilateral	6 (3.2%)	6 (3.3%)	2 (1.2%)	14 (2.6%)	
Bra cup size	A–AA	13 (9.2%)	9 (6.8%)	16 (11.9%)	38 (9.3%)	
	B	53 (37.3%)	40 (30.0%)	41 (30.4%)	134 (33.7%)	
	C	34 (23.9%)	36 (27.1%)	27 (20.0%)	96 (23.6%)	0.38
	D	22 (15.5%)	16 (12.1%)	22 (16.3%)	60 (14.6%)	
	DD–GG	20 (14.0%)	32 (24.1%)	29 (21.4%)	71 (19.7%)	
	Missing	43	48	35	126	

REQUITE Cohort Baseline Tumour, Patient and Treatment Data

Radiotherapy treatment time was available for 343 of the REQUITE Leicester breast cohort. In total, 111 patients (32.4%) received radiotherapy in the morning, 152 were treated in the afternoon (44.3%) and 80 (23.3%) received a mix of morning and afternoon treatment. Table 3 summarises the baseline characteristics of the tumour, patient and treatment between the treatment times. Chi-squared testing for categorical variables and ANOVA testing for continuous variables revealed that significantly more patients in the morning group received a boost ($P = 0.01$) and had higher grade tumours ($P = 0.01$) compared with the afternoon and mixed groups.

Acute breast erythema score was available on 331 of the study participants. Univariate analysis using a chi-squared test was carried out and showed a significantly increased toxicity rate in patients treated in the mornings: 23.6% of patients had grade 2 or more erythema in the morning group compared with 11.0% in the afternoon group and 19.0% in the mixed group ($P = 0.03$). Removal of the mixed treatment time group from the analysis resulted in a P value of 7.0×10^{-3}. Multivariable analysis was carried out using

Table 2
Multivariate analysis (LeND cohort) for effect on late toxicity (bivariate STAT score)

Variable	*P* Value	Odds ratio	95% confidence interval for odds ratio	
			Lower	Upper
Boost to tumour bed	0.58	2.32	0.12	45.52
BED	0.03	70.54	1.64	3044
Cup size	5.1×10^{-7}	1.27	1.16	1.39
Afternoon radiotherapy treatment	0.01	0.61	0.41	0.90
Boost × time	1.0×10^{-3}	6.80	2.23	20.63

BED, biological equivalent dose.

Table 3
Baseline characteristics in the REQUITE cohort for tumour, patient and treatment. Percentages are of non-missing data within each time group

Patients	Radiotherapy treatment time					$P =$
		AM	Mixed	PM	Total	
		111	80	152	343	
Age (mean years)		60.0	61.4	60.5		0.60
Grade	1	25 (28%)	9 (14%)	35 (30%)	69 (25%)	
	2	36 (40%)	38 (58%)	65 (55%)	139 (51%)	
	3	28 (31%)	18 (28%)	18 (15%)	64 (24%)	0.008
	Missing	22	15	34	71	
Chemotherapy	Yes	4 (4%)	6 (9%)	11 (9%)	21 (7%)	
	No	87 (96%)	62 (91%)	111 (91%)	260 (93%)	0.40
	Missing	20	12	30	62	
Oestrogen receptor positive	Yes	50 (55%)	35 (51%)	68 (55%)	150 (54%)	
	No	41 (45%)	33 (49%)	56 (45%)	130 (46%)	0.89
	Missing	20	12	31	63	
Mean radiotherapy dose (Gy)		41.2	40.8	41.0		0.77
Boost to tumour bed	Yes	12 (13%)	5 (7%)	3 (2%)	20 (8%)	
	No	79 (87%)	63 (93%)	119 (98%)	261 (93%)	0.01
	Missing	20	12	30	62	
Diabetes	Yes	8 (7%)	7 (9%)	14 (9%)	29 (8%)	
	No	103 (93%)	72 (91%)	138 (91%)	313 (92%)	0.84
	Missing	0	1	0	1	
Smoker	Current smoker	14 (14%)	4 (7%)	19 (17%)	37 (14%)	
	No	39 (40%)	27 (44%)	45 (39%)	111 (41%)	
	Ex-smoker	44 (45%)	30 (49%)	50 (44%)	124 (46%)	0.47
	Missing	14	19	38	71	
Side of treatment	Right	48 (54%)	36 (54%)	61 (50%)	145 (52%)	
	Left	41 (46%)	31 (46%)	61 (50%)	133 (48%)	0.82
	Bilateral	0	0	0	0	
	Missing	22	13	30	65	
Body mass index		29.1	27.9	29.7		0.59
Bra cup size	AA–A	9 (8.1%)	6 (7.6%)	8 (5.3%)	23 (6.7%)	
	B	33 (29.7%)	19 (24.1%)	43 (28.5%)	95 (27.9%)	
	C	24 (21.6%)	13 (16.5%)	41 (27.2%)	78 (22.9%)	
	D	18 (16.2%)	15 (19.0%)	27 (17.9%)	60 (17.6%)	
	DD–J	27 (24.3%)	26 (32.9%)	32 (21.2%)	85 (24.9%)	0.35
	Missing		1	1	2	

logistic regression and radiotherapy treatment time was not significant ($P = 0.28$). Results are summarised in Table 4.

Genetic Variants as Effect Modifier

We tested if the time-of-day effect is modified by candidate polymorphisms in circadian rhythm genes, including SNPs in the CLOCK and NOCT genes and a VNTR in the PER3 gene.

LeND Cohort

There was no significant direct relationship between PER3 VNTR and late toxicity (bivariate STAT score). Taking the time of radiotherapy into consideration showed a significant effect of PER3 VNTR on late toxicity, with *4/4* PER3 VNTR being associated with increased toxicity if treated in the morning compared with the afternoon ($P = 6.0 \times 10^{-3}$) (Table 5). A Bonferroni correction for six tests (based on six genotypes in Table 5) would give a *P* value of 0.036.

There was no significant direct relationship with NOC rs13116075 and late toxicity (bivariate STAT score). Taking the time of radiotherapy treatment into consideration showed a significant effect of NOC rs13116075 on late toxicity. Patients carrying the AA NOC rs13116075 genotype had increased toxicity if treated in the mornings ($P = 5.0 \times 10^{-3}$) (Table 5). A Bonferroni correction for six tests would give a *P* value of 0.03.

Combination of PER3 VNTR and NOCT rs13116075 genotypes had no significant direct effect on late radiotherapy toxicity. However, taking the time of radiotherapy treatment into consideration showed a significant effect of NOCT rs13116075 and PER3 VNTR on late toxicity. Patients with AA NOCT rs13116075 genotype and 4/4 PER3 VNTR had increased late radiotherapy toxicity if treated in the mornings ($P = 4.5 \times 10^{-4}$) (data not shown).

Table 4
Multivariate analysis of the REQUITE breast cohort for the effect on acute toxicity

Variable	*P* Value	Odds ratio	95% confidence interval for odds ratio	
			Lower	Upper
Bra cup size	4.90×10^{-5}	1.71	1.32	2.23
Depression	0.02	0.07	0.01	0.62
Boost to tumour bed	0.64	1.66	0.20	14.17
Boost × radiotherapy time	0.49	1.89	0.31	11.64
Season	0.65	1.21	0.53	2.72
Afternoon radiotherapy treatment	0.28	0.76	0.46	1.25
Biological equivalent dose	1.57×10^{-7}	1.45	1.26	1.66

Table 5
Late toxicity split by PER, NOCT rs13116075 and radiotherapy treatment time in the LeND cohort

Radiotherapy treatment time	PER3 VNTR	Late toxicity (bivariate STAT score) *n* =		*P* Value*	NOCT rs13116075	Late toxicity (bivariate STAT score *n* =)		*P* Value*
		75% lowest	25% highest			75% lowest	25% highest	
Morning	4/4	43 (68.3%)	20 (31.7%)	6.0×10^{-3}	AA	61 (76.7%)	29 (23.3%)	5.0×10^{-3}
Afternoon		49 (79.0%)	13 (21.0%)			80 (85.1%)	14 (14.9%)	
Mixed		54 (91.5%)	5 (8.5%)			72 (84.7%)	13 (15.3%)	
Morning	4/5	37 (77.1%)	11 (22.9%)	0.49	AG	22 (75.9%)	7 (24.1%)	0.74
Afternoon		51 (85.0%)	9 (15.0%)			34 (75.6%)	11 (24.4%)	
Mixed		45 (77.6%)	13 (22.4%)			36 (81.8%)	8 (18.2%)	
Morning	5/5	11 (73.3%)	4 (26.7%)	0.75	GG	3 (100%)	0	0.08
Afternoon		12 (66.7%)	6 (33.3%)			1 (33.3%)	2 (66.7%)	
Mixed		14 (77.8%)	4 (22.2%)			0	0	
Total		316	85			316	85	

VNTR, variable number tandem repeat.
* Chi-squared test for significance including all treatment times.

REQUITE Cohort

There was no association between PER3 VNTR genotype and acute toxicity score in the 294 patients with data available for both ($P = 0.79$). Taking the time of radiotherapy treatment into consideration showed no significant effect of PER3 VNTR on acute toxicity with any of the genotypes (Table 6). If the mixed treatment time group was excluded from the analysis, patients with 4/5 PER3 VNTR had increased acute toxicity with morning compared with afternoon treatment ($P = 0.02$).

Acute toxicity score was available for 308 of the 323 breast patients, with genotyping data available for NOC rs13116075. The NOC rs13116075 AG genotype was significantly associated with increased toxicity ($P = 0.04$), but not after applying a correction for multiple testing. The other genotypes were not related to toxicity.

Taking the time of radiotherapy treatment into consideration showed no significant effect of NOC rs13116075 (Table 6) on acute toxicity with any of the genotypes. If the mixed treatment time group was excluded from the analysis, patients with AG NOC rs13116075 had increased acute toxicity in the mornings ($P = 0.04$). A Bonferroni correction for eight tests would render these results non-significant.

Combination of PER3 VNTR and NOCT rs13116075 had no direct association with increased late toxicity. Nor was there any evidence of the combination modifying the time-of-day effect on toxicity score. There was no effect of CLOCK genotype in any analysis.

Discussion

In this study we found some evidence that breast cancer patients treated in the morning had worse radiotherapy side-effects than those treated in the afternoon.

These results are in contrast with some earlier studies [10,11], but the differences are probably due to differences in the gender of patients, irradiated tissue type, geographical latitude, allocation of time groups and methods of booking patients into radiotherapy.

To enable the reduction of side-effects while maintaining activity in radiotherapy suites over the whole day it is necessary to be able to predict which patients would benefit from having their treatment delivered at a defined time of day (chronotherapy). To that end we carried out genotyping for some candidate circadian rhythm polymorphisms and found that the circadian effect was strongest in individuals who were homozygous for the PER3 4 repeat or NOCT A alleles. Importantly, these alleles were not found to be directly associated with radiotherapy toxicity in this study,

Table 6
Acute breast erythema split by PER3 and NOCT rs13116075 genotyping and radiotherapy treatment time in the REQUITE breast cohort

Treatment time	PER3 VNTR	Acute toxicity (breast erythema score) n =		P Value	NOCT rs13116075	Acute toxicity (breast erythema score) n =		P Value
		<2	≥2			<2	≥2	
Morning	4/4	31 (81.6%)	7 (18.4%)	0.71*/0.71†	AA	59 (80.8%)	14 (19.2%)	0.21*/0.18†
Afternoon		49 (84.5%)	9 (15.5%)			83 (88.3%)	11 (11.7%)	
Mixed		31 (88.6%)	4 (11.4%)			49 (90.7%)	5 (9.3%)	
Morning	4/5	32 (72.7%)	12 (27.3%)	0.07*/0.02†	AG	15 (65.2%)	8 (34.8%)	0.06*/0.04†
Afternoon		53 (89.8%)	6 (10.2%)			30 (88.2%)	4 (11.8%)	
Mixed		20 (76.9%)	6 (23.1%)			10 (62.5%)	6 (37.5%)	
Morning	5/5	9 (81.8%)	2 (18.2%)	0.19*/0.16†	GG	1 (100%)	0	N/A
Afternoon		10 (100%)	0			5 (100%)	0	
Mixed		7 (70.0%)	3 (30.0%)			1 (100%)	0	
TOTAL		242	49			253	51	

VNTR, variable number tandem repeat.
* Chi-squared test for significance including all treatment times.
† Chi-squared test for significance excluding the mixed treatment times group.

but only to potentiate the time-of-day effect. It is possible that larger studies would find a direct association, as the NOCT SNP was previously found to be associated with overall toxicity by genome-wide association study. The PER group of genes are intimately connected with the control and timing of keratinocyte division [7]. Nocturnin has been shown to affect the proliferation of adipocytes, an important cell type in the breast [23].

There are several potential physiological mechanisms to explain how time could affect reactions to irradiation, e.g. melatonin, cortisol, inflammatory factors or cell proliferation/DNA damage. Melatonin has antioxidant properties [24], has been shown to be radioprotective in mice [25] and reduce oral mucositis in irradiated rats [26]. Cortisol levels can be used as a marker of stress and can affect inflammatory markers [27]. Cortisol levels can influence the rate of cell division and may be associated with possible increased cellular division of skin in the morning compared with later in the day [28].

Many clinical studies have shown a weak association between acute and late toxicity, with some making a distinction between consequential and non-consequential late effects [29]. Therefore, the fact that the time-of-day effect is the same in the present study for acute and late toxicity may give a clue to the mechanism. Future work will determine how the cell cycle of keratinocytes and fibroblasts responds to circadian rhythms.

There were several limitations of this study. First, the study was conducted at a single centre and therefore the generalisability of the results will not be known until replicated at other centres. Second, acute and late toxicity were assessed in different cohorts who received radiotherapy in different calendar years with different radiotherapy protocols, which complicates interpretation and adjustment for multiple testing. Added to this is that the LeND cohort was collected retrospectively, meaning no assessment was possible of tissue changes from baseline. This will be remedied when late toxicity data become available for the REQUITE cohort. Acute toxicity in the REQUITE cohort was recorded before the end of radiotherapy, so will have missed manifestations occurring some weeks later. Third, the cohort sizes limit the statistical power to detect effects; larger cohorts are needed to confirm the data.

These genetic data potentially open the possibility of a simple test to identify the patients who would benefit from receiving their treatment in the afternoon, whereas the remaining patients could be treated at any time. This approach will need to be verified using a clinical trial that randomises between a group in which patients choose their own treatment time and a chronotherapy group. This virtually cost-free intervention would be predicted to reduce side-effects and improve quality of life for breast cancer survivors.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank the patients in both the LeND and the REQUITE studies for their involvement in this work, as well as Anusha Müller and Rebecca Elliott for excellent data and project management of the REQUITE project. Support for this study was provided by Breast Cancer Now (grant number 2007NovPR45), Hope Against Cancer (grant number RM33G0351) and the European Union (grant agreement 601826). Patient recruitment was supported through the Leicester Experimental Cancer Centre. Taqman genotyping was carried by the NUCLEUS Genomics service at the University of Leicester. The REQUITE consortium includes: David Azria, Anthony Brookes, Tom Burr, Jenny Chang-Claude, Susan Davidson, Dirk De Ruysscher, Alison Dunning, Rebecca Elliott, Sara Gutiérrez Enríquez, Philippe Lambin, Tiziana Rancati, Barry Rosenstein, Petra Seibold, R. Paul Symonds, Chris Talbot, Hubert Thierens, Riccardo Valdagni, Ana Vega, Liv Veldeman, Frederik Wenz, Martin Yuille and Catharine West.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clon.2018.10.001.

References

[1] Innominato PF, Roche VP, Palesh OG, Ulusakarya A, Spiegel D, Levi FA. The circadian timing system in clinical oncology. *Ann Med* 2014;46:191–207.

[2] Ballesta A, Innominato PF, Dallmann R, Rand DA, Levi FA. Systems chronotherapeutics. *Pharmacol Rev* 2017;69:161–199.

[3] Levi F, Okyar A, Dulong S, Innominato PF, Clairambault J. Circadian timing in cancer treatments. *Annu Rev Pharmacol Toxicol* 2010;50:377–421.

[4] Dulong S, Ballesta A, Okyar A, Levi F. Identification of circadian determinants of cancer chronotherapy through *in vitro* chronopharmacology and mathematical modeling. *Mol Cancer Ther* 2015;14:2154–2164.

[5] Zeng ZL, Luo HY, Yang J, *et al*. Overexpression of the circadian clock gene Bmal1 increases sensitivity to oxaliplatin in colorectal cancer. *Clin Cancer Res* 2014;20:1042–1052.

[6] Altinok A, Levi F, Goldbeter A. Identifying mechanisms of chronotolerance and chronoefficacy for the anticancer drugs 5-fluorouracil and oxaliplatin by computational modeling. *Eur J Pharm Sci* 2009;36:20–38.

[7] Janich P, Meng QJ, Benitah SA. Circadian control of tissue homeostasis and adult stem cells. *Curr Opin Cell Biol* 2014;31:8–15.

[8] Beri K, Milgraum SS. Rhyme and reason: the role of circadian rhythms in skin and its implications for physicians. *Future Sci OA* 2016;2:Fso115.

[9] Hall EJ, Giaccia AJ. *Radiosensitivity and cell age in the mitotic cycle. Radiobiology for the radiologist*, 7 ed. Philadelphia: Lippincott Williams and Wilkins; 2012. p. 54–66.

[10] Chan S, Rowbottom L, McDonald R, Bjarnason GA, Tsao M, Danjoux C, *et al*. Does the time of radiotherapy affect treatment outcomes? A review of the literature. *Clin Oncol* 2017;29:231–238.

[11] Noh JM, Choi DH, Park H, Huh SJ, Park W, Seol SW, *et al*. Comparison of acute skin reaction following morning versus late afternoon radiotherapy in patients with breast cancer who have undergone curative surgical resection. *J Radiat Res* 2014;55:553–558.

[12] Bjarnason GA, Mackenzie RG, Nabid A, Hodson ID, El-Sayed S, Grimard L, *et al*. Comparison of toxicity associated with early morning versus late afternoon radiotherapy in patients with head-and-neck cancer: a prospective randomized trial of the National Cancer Institute of Canada Clinical Trials Group (HN3). *Int J Radiat Oncol Biol Phys* 2009;73:166–172.

[13] Ahowesso C, Li XM, Zampera S, Peteri-Brunbäck B, Dulong S, Beau J, *et al*. Sex and dosing-time dependencies in irinotecan-induced circadian disruption. *Chronobiol Int* 2011;28(5):458–470.

[14] Giacchetti S, Dugué PA, Innominato PF, Bjarnason GA, Focan C, Garufi C, *et al*. ARTBC International Chronotherapy Group. Sex moderates circadian chemotherapy effects on survival of patients with metastatic colorectal cancer: a meta-analysis. *Ann Oncol* 2012;23(12):3110–3116.

[15] Archer SN, Robilliard DL, Skene DJ, Smits M, Williams A, Arendt J, *et al*. A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference. *Sleep* 2003;26(4):413–415.

[16] Katzenberg D, Young T, Finn L, Lin L, King DP, Takahashi JS, *et al*. A CLOCK polymorphism associated with human diurnal preference. *Sleep* 1998;21(6):569–576.

[17] Barnett GC, Thompson D, Fachal L, Kerns S, Talbot C, Elliott RM, *et al*. A genome wide association study (GWAS) providing evidence of an association between common genetic variants and late radiotherapy toxicity. *Radiother Oncol* 2014;111:178–185.

[18] Talbot CJ, Tanteles GA, Barnett GC, Burnet NG, Chang-Claude J, Coles CE, *et al*. A replicated association between polymorphisms near TNFalpha and risk for adverse reactions to radiotherapy. *Br J Cancer* 2012;107:748–753.

[19] Murray RJ, Tanteles GA, Mills J, Perry A, Peat I, Osman A, *et al*. Association between single nucleotide polymorphisms in the DNA repair gene LIG3 and acute adverse skin reactions following radiotherapy. *Radiother Oncol* 2011;99:231–234.

[20] Andreassen CN, Rosenstein BS, Kerns SL, Ostrer H, De Ruysscher D, Cesaretti JA, *et al*. Individual patient data meta-analysis shows a significant association between the ATM rs1801516 SNP and toxicity after radiotherapy in 5456 breast and prostate cancer patients. *Radiother Oncol* 2016;121:431–439.

[21] Pavy JJ, Denekamp J, Letschert J, Littbrand B, Mornex F, Bernier J, *et al*, EORTC Late Effects Working Group. Late effects toxicity scoring: the SOMA scale. *Radiother Oncol* 1995;35:11–15.

[22] West C, Azria D, Chang-Claude J, Davidson S, Lambin P, Rosenstein B, *et al*. The REQUITE project: validating predictive models and biomarkers of radiotherapy toxicity to reduce side-effects and improve quality of life in cancer survivors. *Clin Oncol* 2014;26:739–742.

[23] Kawai M, Green CB, Lecka-Czernik B, Douris N, Gilbert MR, Kojima S, *et al*. A circadian-regulated gene, Nocturnin, promotes adipogenesis by stimulating PPAR-gamma nuclear translocation. *Proc Natl Acad Sci USA* 2010;107:10508–10513.

[24] Ben-David MA, Elkayam R, Gelernter I, Pfeffer RM. Melatonin for prevention of breast radiation dermatitis: a phase II, prospective, double-blind randomized trial. *Isr Med Assoc J* 2016;18:188–192.

[25] Vijayalaxmi, Meltz ML, Reiter RJ, Herman TS, Kumar KS. Melatonin and protection from whole-body irradiation: survival studies in mice. *Mutat Res* 1999;425:21–27.

[26] Abdel Moneim AE, Guerra-Librero A, Florido J, Shen YQ, Fernandez-Gil B, Acuna-Castroviejo D, *et al*. Oral mucositis: melatonin gel an effective new treatment. *Int J Mol Sci* 2017;18:1003.

[27] Wright Jr KP, Drake AL, Frey DJ, Fleshner M, Desouza CA, Gronfier C, *et al*. Influence of sleep deprivation and circadian misalignment on cortisol, inflammatory markers, and cytokine balance. *Brain Behav Immun* 2015;47:24–34.

[28] Sporl F, Korge S, Jurchott K, Wunderskirchner M, Schellenberg K, Heins S, *et al*. Kruppel-like factor 9 is a circadian transcription factor in human epidermis that controls proliferation of keratinocytes. *Proc Natl Acad Sci USA* 2012;109:10903–10908.

[29] Dörr W, Hendry JH. Consequential late effects in normal tissues. *Radiother Oncol* 2001;61(3):223–231.