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Next-generation sequencing identifies germline *MRE11A* variants as markers of radiotherapy outcomes in muscle-invasive bladder cancer

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Background: Muscle-invasive bladder cancer (MIBC) can be cured by radical radiotherapy (RT). We previously found tumour MRE11 expression to be predictive of survival following RT in MIBC, and this was independently validated in a separate institute. Here, we investigated germline *MRE11A* variants as possible predictors of RT outcomes in MIBC, using next-generation sequencing (NGS).

Patients and methods: The *MRE11A* gene was amplified in germline DNA from 186 prospectively recruited MIBC patients treated with RT and sequenced using bar-coded multiplexed NGS. Germline variants were analysed for associations with cancer-specific survival (CSS). For validation as a prognostic or predictive marker, rs1805363 was then genotyped in a cystectomy-treated MIBC cohort of 256 individuals. *MRE11A* mRNA isoform expression was measured in bladder cancer cell lines and primary tumour samples.

Results: Carriage of at least one of six (five novel) rare variants was associated with the worse RT outcome (hazard ratio [HR] 4.04, 95% confidence interval [95% CI] 1.42–11.51, $P = 0.009$). The single-nucleotide polymorphism (SNP), rs1805363 (minor allele frequency 11%), was also associated with worse CSS (per-allele HR 2.10, 95% CI 1.34–3.28, $P_{trend} = 0.001$) following RT in MIBC, with a gene-dosage effect observed, but no effect seen on CSS in the cystectomy cohort ($P_{trend} = 0.89$). Furthermore, rs1805363 influenced relative *MRE11A* isoform expression, with increased isoform 2 expression with carriage of the rs1805363 minor A allele.

Conclusions: Germline *MRE11A* SNP rs1805363 was predictive of RT, but not of cystectomy outcome in MIBC. If successfully validated in an independent RT-treated cohort, this SNP could be a useful clinical tool for selecting patients for bladder-conserving treatment.

Key words: *MRE11A*, bladder cancer, next-generation sequencing, radiotherapy, cystectomy, biomarkers

introduction

Muscle-invasive bladder cancer (MIBC) is the sixth most common cause of male cancer death in the UK (http://info.cancerresearchuk.org/cancerstats/type/bladder). Radical radiotherapy (RT) and chemoradiotherapy (CRT) have the advantage over cystectomy of bladder preservation, with similar cure rates in selected patients [1, 2]. With the failure of a recent trial randomising cystectomy versus RT (SPARE) [3], there is an urgent need to find a predictive biomarker to aid treatment choice in MIBC.

MRE11, as part of the MRE11–RAD50–NBS1 complex, detects and binds to DNA double-strand breaks (DSBs), the lethal lesions caused by RT, processes damaged DNA ends and activates the ATM protein, cell cycle checkpoints and apoptotic responses [4]. In MIBC, we found tumour MRE11 protein expression to be predictive of survival following RT but not cystectomy [5, 6]. Germline single-nucleotide polymorphisms (SNPs) in DNA damage response genes, including *MRE11A*, have been associated with an increased bladder cancer risk [7, 8]. Candidate gene studies have found associations with RT response in several cancer sites, and in MIBC coding *ERCC2* and *XRCC1* SNPs were associated with platinum-based CRT outcomes [9]. Acute and late normal tissue toxicity following RT has also been studied, including at the

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genome-wide level, but these initial candidate SNP hits have not yet been replicated [10].

There is a high negative selection pressure against coding variants of *MRE11A*; dbSNP (http://www.ncbi.nlm.nih.gov/snp/) contains only nine non-synonymous coding variants, all rare with minor allele frequencies (MAFs) <1%. Rare germline variants are more likely to be functional than the more common SNPs; they can only be detected by DNA sequencing, made affordable by next-generation sequencing (NGS) technology [11].

We hypothesised that NGS could identify germline SNPs and rare variants in *MRE11A* predictive of both tumour response and toxicity following radical RT in MIBC.

methods

patients

MIBC RT patients ($N = 201$) were recruited prospectively having given written informed consent with local ethical approval, as previously described [2, 12] and treated using three-dimensional conformal external beam RT (52.5–55 Gy in 20 fractions over 4 weeks) between August 2002 and October 2009 in Leeds, UK. Toxicity data were collected as outlined in supplementary Methods and supplementary Table S9, available at *Annals of Oncology* online. Blood samples were collected before RT and germline DNA extracted using standard salting-out protocols. Cystectomy patients ($N = 256$) were treated at Aarhus University Hospital, Denmark, between 1992 and 2008 [13] without neoadjuvant or adjuvant chemotherapy. Time of death was provided by the Danish Central Personal Registry and cause of death by hospital charts.

germline MRE11A sequencing, sequence analysis, variant calling and genotyping

The *MRE11A* reference genomic sequence (NCBI Accession NG_007261.1 HG19 assembly accessed January 2011, size 76.6 kb) was used. For each RT-treated MIBC individual, all exons plus flanking introns (minimum 200 bases) were amplified as long PCR products in 12 amplicons (total 28.2 kb) (supplementary Table S1, available at *Annals of Oncology* online), then normalised in equimolar quantities. Tagged library preparation was carried out as previously described [14] using an unique indexed adaptor for each individual (supplementary Table S2, available at *Annals of Oncology* online). Twenty to 24 indexed libraries were pooled together in equimolar quantities and each pool was single-end sequenced for 90 cycles on an Illumina GAII. In-house software (Illuminator) [15] was used for indexed read sorting and sequence alignment against the reference *MRE11A* genomic sequence, and variants called if coverage was >200× and the variant allele seen in >20% of reads. Poor confidence 'variants' were excluded by visual inspection of sequence alignment and read coverage data. High-confidence variants were confirmed by conventional Sanger sequencing (supplementary Table S3, available at *Annals of Oncology* online). In the cystectomy cohort, rs1805363 was genotyped using standard pre-designed Taqman SNP genotyping assays (Applied Biosystems, UK).

MRE11A mRNA isoform expression profiling

MRE11A isoforms were amplified using primers flanking *MRE11A* rs1805363 and conventionally Sanger sequenced (see supplementary File 1, available at *Annals of Oncology* online).

statistical analysis

Cancer-specific survival (CSS) analysis was carried out using Kaplan–Meier and Cox proportional hazard models for all confirmed SNPs and rare variants. Assuming an overall 50% 5-year CSS, at a 5% significance level, the RT-treated MIBC cohort had >80% power to detect a hazard ratio (HR) of 2.00 for an MAF of 0.20 and >85% for a HR of 3.00 at an MAF of 0.05 (see supplementary File 1, available at *Annals of Oncology* online).

results

MIBC patient demographics

At 76-month median follow-up (range 20.3–104.6 months), there were 70 cancer-specific events in the 186 RT-treated MIBC patients where PCR amplification was successful. Clinical details of the cystectomy-treated MIBC cohort ($N = 256$) have been previously published [13] (Table 1). No patient had distant metastases.

MRE11A variants identified from next-generation sequencing

RT patient samples were sequenced in nine pools, with 2507× median coverage per sample (range 673–9891); 95% of target regions were sequenced at over 221× per sample. Following filtering for high-confidence variants, 85 variants were selected for confirmatory sequencing and were all successfully validated (supplementary Table S4, available at *Annals of Oncology* online). Thirty of 33 common SNPs (MAF >0.05) were in high linkage disequilibrium with each other ($R^2 > 0.80$, data not shown); nine common haplotypes were seen (supplementary Figure S1, available at *Annals of Oncology* online).

genetic associations with survival in the RT-treated MIBC cohort

In Cox proportional hazards analysis, none of the demographical and histological/clinico-pathological variables were significantly associated with CSS (Table 2), perhaps reflecting the sample size and relative variable frequencies. Using the data-adaptive sum test, collapsed analysis of the uncommon and rare variants (MAF <0.05) did not reveal any significant associations with CSS for all the rare variants ($P = 0.45$) or just rare exonic variants ($P = 0.59$). As the 3′ UTR is involved in gene post-transcriptional regulation and hence could influence MRE11 expression, we carried out simple collapsed analysis of the six rare *MRE11* 3′ UTR variants seen in seven individuals. Carriage of at least one 3′ UTR rare variant was significantly associated with worse CSS ($P = 0.009$; Table 2) in carriers versus non-carriers (5-year CSS: 42.9% versus 54.8%, respectively; Figure 1A).

Individual variant analysis identified two common SNPs to be significantly associated with CSS, rs1805363 ($P_{trend} = 0.001$) and rs13447623 ($P_{trend} = 0.05$) (Table 2), with the respective variant A and G alleles in complete linkage disequilibrium ($D' = 1.00$, $R^2 = 0.38$). A two-locus model of both SNPs showed that only rs1805363 was significant, indicating that the effect seen with the rs13447623 G allele was secondary to concomitant carriage of the rs1805363 A allele. The rs1805363 A minor allele was associated with worse CSS following RT treatment (per-allele HR 2.10, 95% confidence interval [95% CI] 1.34–3.28, $P_{trend} = 0.001$), and a gene-dosage effect was seen: GG 5-year CSS 58.3%, GA 42.0% and AA 0% (Figure 1B). No significant interaction was detected between rs1805363 and baseline prognostic factors, again possibly due to insufficient power. Corrections for multiple testing were not carried out. For a

Table 1. Clinical demographics of the MIBC study cohorts

Variable	RT-treated cohort ($N = 186$), *n* (%)	Cystectomy-treated cohort ($N = 256$), *n* (%)
Age (years)		
Median (range)	79 (55–93)	65 (34–85)
Gender		
Male	139 (74.7)	187 (73.0)
Female	47 (25.3)	69 (27.0)
Tumour stage[a]		
T2	118 (63.5)	94 (36.7)
T3	51 (27.5)	112 (47.6)
T4	9 (4.9)	40 (15.7)
Tx	8 (4.4)	0 (0.0)
Nodal stage		
N0	179 (96.3)	184 (71.9)
N1	4 (2.2)	32 (12.5)
N2+	2 (1.1)	39 (15.2)
Nx	1 (0.6)	1 (0.4)
Histological grade		
High grade	167 (89.8)	251 (98.0)
Low grade	14 (7.6)	5 (2.0)
Unrecorded	5 (2.7)	0 (0.0)
Hydronephrosis		
No	135 (72.6)	Data not available
Yes	51 (27.5)	Data not available
Neoadjuvant chemotherapy		
Not received	175 (94.1)	256 (100.0)
Received	11 (6)[b]	0 (0.0)
Concurrent chemotherapy/radiosensitizer		
Not received	171 (91.9)	256 (100.0)
Received	15 (8.1)[c]	0 (0.0)
Salvage/adjuvant chemotherapy[c]		
Not received	177 (95.2)	256 (100.0)
Received	9 (4.9)	0 (0.0)
Salvage cystectomy		
Not received	167 (89.8)	Not applicable
Received	19 (10.3)	Not applicable

[a]Tumour stage in the RT-treated cohort was the highest of the pre-treatment biopsy and radiological stage; in the cystectomy-treated cohort, the highest of the pre-treatment biopsy and post-cystectomy pathological stage.
[b]All received platinum-based combination chemotherapy.
[c]Ten patients received concurrent gemcitabine (100 mg/m^2) weekly ×4 as part of a phase II clinical trial and five patients received concurrent carbogen and nicotinamide as part of the BCON phase III clinical trial.

simple Bonferroni correction, the significance threshold would be 6×10^{-4}, making rs1805363 non-significant. However, this correction would be over-conservative, not accounting for the extensive linkage disequilibrium in the region. A haplotype analysis (supplementary Figure S1, available at *Annals of Oncology* online) results in a Bonferroni significance threshold of 0.006 with a significant association for the only haplotype containing rs1805363 (*P*-value unchanged). Carriage of the rs1805363 A allele and 3′ UTR rare variants was mutually exclusive.

genetic associations with late radiation bladder toxicity

Thirty-four patients developed ≥Grade 2 CTC bladder toxicity and three developed ≥Grade 2 CTC late rectal toxicity. Neither the 3′ UTR rare variants nor rs1805363 were associated with developing late bladder toxicity ($P = 0.09$ and 0.33, respectively). Rare variants were not associated with toxicity on collapsed analysis. However, rs13447623 and the common 3′ UTR SNP rs2155209 (MAF = 0.38) [7] were associated with late bladder toxicity (per-allele odds ratio [OR] 2.12, 95% CI 1.30–3.45, $P_{trend} = 0.003$; and per-allele OR 0.57, 95% CI 0.35–0.92, $P_{trend} = 0.02$, respectively); only rs13447623 contributed independently to bladder toxicity risk on conditional analysis ($P = 0.03$). There was no association seen between severity of acute radiation bladder toxicity and late radiation bladder toxicity ($N = 134$, $P = 0.33$) or of rs13447623 with severity of acute radiation bladder toxicity ($P = 0.41$, supplementary Table S5, available at *Annals of Oncology* online).

rs1805363: prognostic versus predictive marker

To explore the prognostic versus predictive value of rs1805363 on MIBC CSS, 256 MIBC patients treated by radical cystectomy were genotyped for rs1805363. No association was found between the rs1805363 genotypes and CSS (per-allele HR 0.99, 95% CI 0.61–1.60, $P_{trend} = 0.89$); GG 5-year CSS of 59.7%, GA 54.0% and AA 75.0% (Figure 1C). Multivariate analysis, adjusting for age, gender, tumour stage, nodal stage and histological grade, did not reveal any significant associations of the rs1805363 genotypes with CSS ($P = 0.88$). This suggests that MRE11 is functionally involved in the RT response (i.e. predictive) rather than a general prognostic marker in MIBC.

rs1805363 influences MRE11A isoform expression

rs1805363's location is either intronic in *MRE11A* isoform 1, five bases 3′ to the Exon 1 AG donor splice site, or within the 5′ UTR of *MRE11A* isoform 2 (Figure 2A). We hypothesised that rs1805363 may influence gene splicing and/or relative isoform expression. Tumour RNA was obtained from pre-treatment samples in three RT-cohort patients, one AA and two GA, and six wild-type bladder cancer cell lines (supplementary Figure S2, available at *Annals of Oncology* online). The relative expression of *MRE11A* isoform 1 (139 bp) and isoform 2 (246 bp) was determined (Figure 2B). There was no difference in overall *MRE11A* expression between genotypes (supplementary Table S6, available at *Annals of Oncology* online), and the percentage of *MRE11A* isoform 1 relative to overall *MRE11A* fell with each rs1805363 minor A allele (GG 61.7%, GA 49.6% and AA 30.3%). The isoforms were confirmed on sequencing (supplementary Figure S3, available at *Annals of Oncology* online).

rs1805363 and tumour MRE11 protein expression

MRE11 immunohistochemical protein expression data were available in 72 RT patients [5]. There was no evidence of significant correlation between the number of rs1805363 A alleles carried or 3′ UTR rare variants and a tumour MRE11 semi-quantitative score ($P = 0.48$ and 0.61, respectively; supplementary Table S7, available at *Annals of Oncology* online).

Table 2. Cox proportional hazards multivariate analysis of cancer-specific survival in demographical, histological/clinic-pathological variables, and in rs1805363, rs13447623 and 3′ UTR rare variants

Covariates			HR (95% CI)	*P*-value[a]
Age at diagnosis (years)			1.01 (0.98–1.04)	0.61
Gender (male versus female)			0.72 (0.39–1.31)	0.28
T stage (increasing stage)			1.50 (0.98–2.30)	0.06
N stage (increasing stage)			1.96 (0.88–4.37)	0.10
Histological grade (increasing grade)			1.12 (0.50–2.48)	0.79
Hydronephrosis (yes versus no)			1.21 (0.66–2.22)	0.53
Neoadjuvant/concurrent chemotherapy (yes versus no)			0.52 (0.22–1.20)	0.12
Variant (observed MAF)	Genotype	*N*		
rs1805363 G > A (0.11)	GG	152	1	**0.001**
	AG	28	1.49 (0.80–2.78)	
	AA	6	8.00 (2.93–21.90)	
rs13447623 A > G (0.25)	AA	110	1	**0.05**
	AG	62	0.86 (0.50–1.49)	
	GG	14	3.68 (1.78–7.60)	
Joint two-SNP analysis				
rs1805363 G > A			2.19 (1.12–4.30)	**0.02**
rs13447623 A > G			0.95 (0.54–1.66)	0.86
Rare variants collapsed analysis				
No 3′ UTR rare variants		179	1	**0.009**
1+ 3′ UTR rare variants		7	4.04 (1.42–11.51)	

[a]Significant *P*-values of <0.05 are in bold.

discussion

Predictive biomarkers are clearly needed to aid clinical decision-making in MIBC, but radiogenetics remains in its infancy despite successes in pharmacogenetics.

MRE11A has a key role in DNA DSB detection and repair signalling, with low tumour MRE11 protein expression predictive of worse RT outcome in MIBC patients [5, 6]. Ricceri *et al.* [16] identified five intronic *MRE11A* SNPs significantly associated with increased DSB repair efficiency, in peripheral blood mononuclear cells from 118 healthy individuals. We thus looked for *MRE11A* genetic variants associated with tumour response and radiation toxicity. Previous radiogenetics studies on RT clinical outcomes have focused on candidate coding DNA repair gene SNPs. Although a candidate gene approach was used here, NGS technology allowed in-depth investigation of all *MRE11A* variants in the study population, including novel rare ones, rather than only known variants or tag SNPs.

We found, for the first time, germline *MRE11A* SNPs and rare variants associated with survival and late radiation normal tissue toxicity following RT in MIBC. *MRE11A* 3′ UTR rare variant carrier status and the common SNP rs1805363 were associated with poor RT outcomes, and rs13447623 with increased late radiation bladder toxicity, thus potentially identifying patients not best served by bladder-conserving RT treatment. In cystectomy-treated patients, rs1805363 was not associated with prognosis, suggesting that it is a predictive genetic RT marker in MIBC. From dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP), 20% of the European population carry at least one allele of this SNP. In our RT and cystectomy cohorts, 18.2% and 15.5% carried at least one allele, respectively. The SNP could have significant clinical impact in identifying RT poor responders, with homozygous carriers doing particularly badly (all dead within 24 months).

No published data are available on the relative expression or functional differences between the two expressed isoforms of *MRE11A* transcripts (http://genome.ucsc.edu/): isoform 1 (4772 bases: shorter 5′ UTR and exon 16 expressed) and isoform 2 (4668 bases: longer 5′ UTR and exon 16 not expressed). We hypothesised that rs1805363 could influence isoform expression as it was situated only five bases from the isoform 1 3′ terminal of exon 1. Expression was reduced with each minor allele carried. Isoform 2 may result in more efficient DSB repair thus promoting cancer cell survival, or isoform 1 may be critical for initiating DSB repair and cell death pathways, thus reduction in isoform 1 results in failure of cancer cells to initiate apoptosis after RT. Alternatively, as the MRN complex exists as a heterotetramer containing two MRE11 molecules [4], a critical balance of isoforms may be required for complex stability or DSB detection.

There was no evidence of correlation between rs1805363 genotype and MRE11 protein expression, so its effects may be mediated at the mRNA level.

To our knowledge, this is the first study investigating an association between germline rare variants and RT outcomes. We identified one known and five novel rare variants in the 3′ UTR of *MRE11A* (supplementary Table S8, available at *Annals of Oncology* online), associated with survival following RT (Figure 1A). Only one of these six rare variants was predicted to

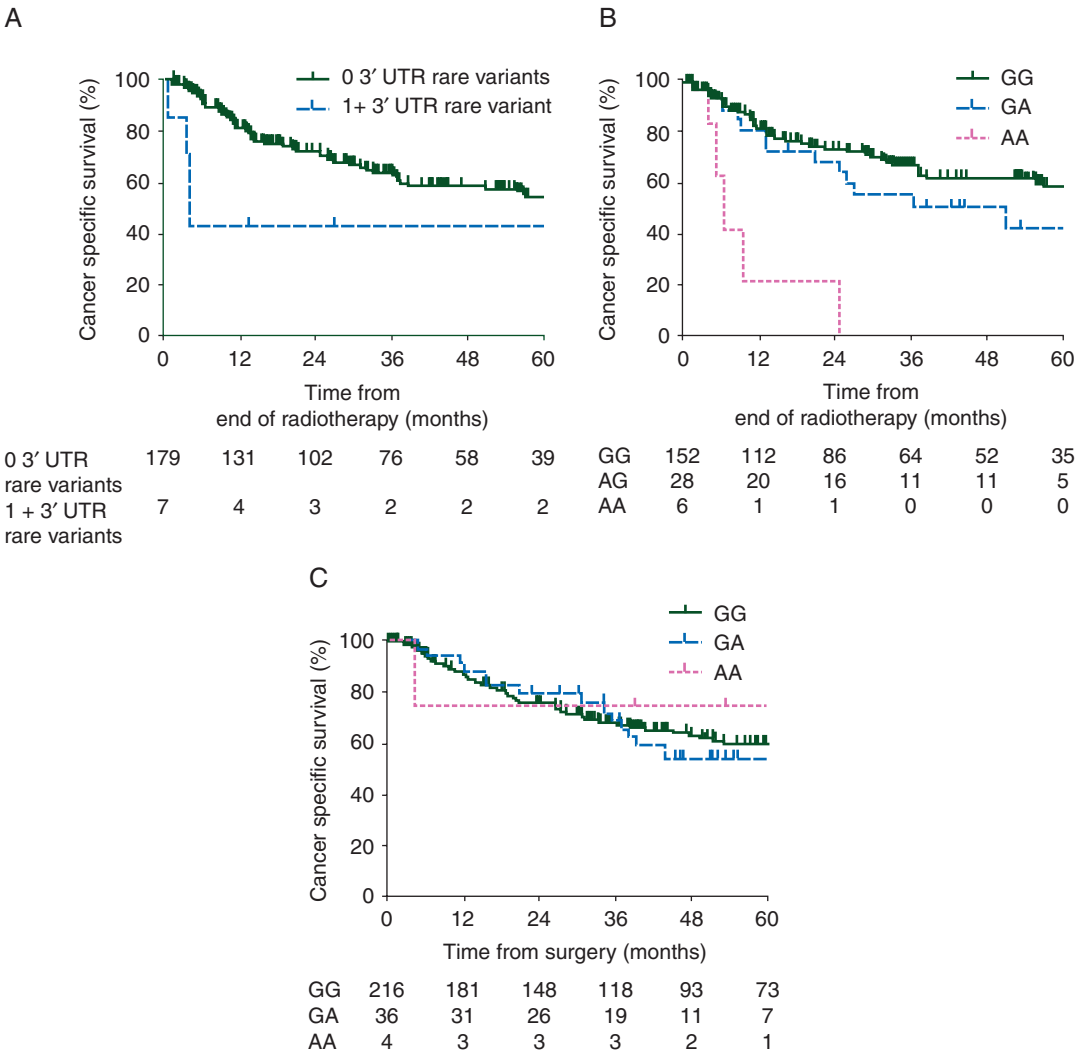

Figure 1. Kaplan–Meier graphs of (A) cancer-specific survival (CSS) for carriers of at least one 3′ UTR *MRE11A* rare variant allele in the RT-treated MIBC cohort; (B) CSS for *MRE11A* rs1805363 genotypes in the RT-treated MIBC cohort and (C) CSS for *MRE11A* rs1805363 genotypes in the cystectomy-treated MIBC cohort.

affect microRNA binding (supplementary Table S8, available at *Annals of Oncology* online).

This study demonstrates the first use of NGS technology for the in-depth investigation of germline common and rare variants within a candidate gene for potential biomarkers of RT outcomes. To our knowledge, only four other studies, in haematological malignancies, have applied NGS technology to search for potential prognostic biomarkers [17–20]. With rapid advances in NGS technology, larger studies are now possible, thus accelerating the discovery of new predictive and prognostic biomarkers [21, 22], and diagnostic laboratories already use NGS technology for clinical genetic testing. Also, whole-genome sequencing can be carried out on germline DNA and tumour DNA, and the first reports in bladder cancer are eagerly awaited.

Germline genetic SNP markers have several advantages over tumour immunohistochemistry markers: a blood sample is easily obtainable for DNA extraction, SNP genotyping is rapid, so patient treatment is not delayed and the result is clearly binary (SNP present/absent).

The main limitations of this study were the relatively small sample sizes and the lack of an available independent RT-treated MIBC validation study population. Whether rs1805363 is a true predictive marker of RT response still needs to be validated in a second independent cohort. Unfortunately, none of the recent or on-going phase III RT clinical trials in bladder cancer collected germline DNA samples for translational research [3, 23, 24], which must be seen as a research priority. We cannot tell if neoadjuvant chemotherapy treatment or concurrent chemotherapy/radiosensitisers would influence the predictive value of the *MRE11A* variants, but our previous RT-alone MRE11 immunohistochemistry findings were subsequently validated in a CRT cohort [5, 6].

In summary, using NGS technology for the first time in MIBC, we have identified germline *MRE11A* SNPs and rare variants as potential markers of RT outcomes and toxicity in MIBC. Carriage of *MRE11A* rs1805363A was predictive of poor RT outcomes but not surgery in MIBC patients and was shown to affect relative *MRE11A* isoform expression. Further validation

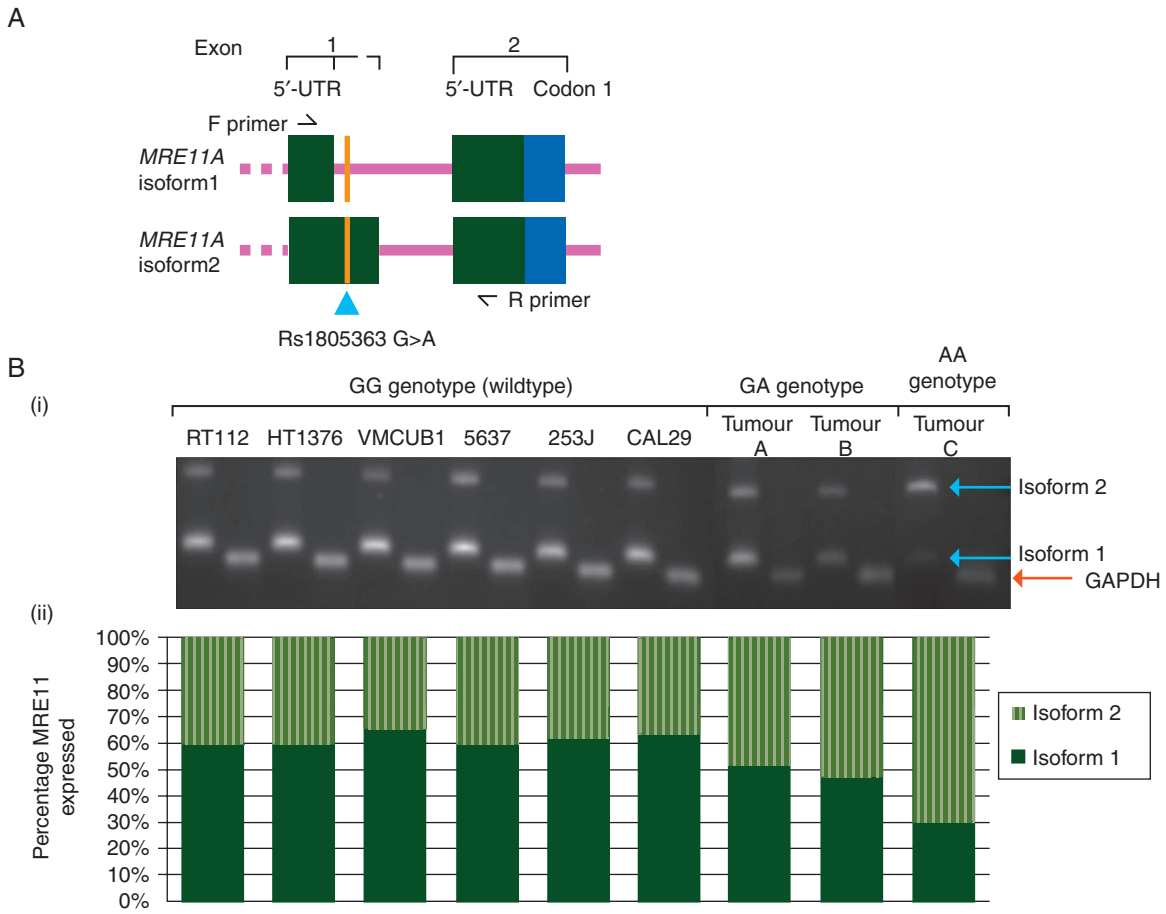

Figure 2. (A) *MRE11A* isoform 1 and isoform 2 alternative splicing and the relative position of common SNP rs1805363 with sequencing forward (F) and reverse (R) primer relative positions indicated. (B) *MRE11A* isoform expression in bladder cancer cell lines and primary bladder tumours with different genotypes for rs1805363: (i) gel electrophoresis bands for *MRE11A* isoforms 1 and 2, *GAPDH* PCR amplified from cDNA from six bladder cancer cell lines (all wild-type GG genotype for rs1805363), primary bladder tumours A and B (heterozygote GA genotype for rs1805363) and primary bladder tumour C (homozygote variant AA genotype for rs1805363) and (ii) percentage expression of *MRE11A* for each isoform relative to overall *MRE11A* expression for each sample.

of rs1805363 is urgently needed for its translation into clinical practice as a predictive tool for personalised medicine.

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disclosure

The authors have declared no conflicts of interest.

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Do quality of life or physical function at diagnosis predict short-term outcomes during intensive chemotherapy in AML?

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Background: Intensive chemotherapy (IC) used to treat acute myeloid leukemia (AML) is associated with toxicity, particularly in older adults. Emerging data suggest that baseline quality of life (QOL) and physical function may predict outcomes in oncology, although data in AML are limited. We investigated the association between baseline QOL and physical function with short-term treatment outcomes in adults and elderly AML patients.

Materials and methods: We conducted a prospective, longitudinal study of adults (age 18+) AML patients undergoing IC. Before starting IC, patients completed the European Organisation for the Research and Treatment of Cancer (EORTC) 30-item questionnaire (QLQ-C30) and Functional Assessment of Cancer Therapy Fatigue subscale (FACT-Fatigue) in addition to physical function tests (grip strength, timed chair stands, 2-min walk test). Outcomes included 60-day mortality, intensive care unit (ICU) admission and achievement of complete remission (CR). Logistic regression was carried out to evaluate each outcome.

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