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Sequencing of *DICER1* in sarcomas identifies biallelic somatic *DICER1* mutations in an adult-onset embryonal rhabdomyosarcoma

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Background: Sarcomas are rare and heterogeneous cancers. We assessed the contribution of *DICER1* mutations to sarcoma development.

Methods: The coding region of *DICER1* was sequenced in 67 sarcomas using a custom Fluidigm Access Array. The RNase III domains were Sanger sequenced in six additional sarcomas to identify hotspot *DICER1* variants.

Results: The median age of sarcoma diagnosis was 45.7 years (range: 3 months to 87.4 years). A recurrent embryonal rhabdomyosarcoma (ERMS) of the broad ligament, first diagnosed at age 23 years, harboured biallelic pathogenic somatic *DICER1* variants (1 truncating and 1 RNase IIIb missense). We identified nine other *DICER1* variants. One somatic variant (p.L1070V) identified in a pleomorphic sarcoma and one germline variant (c.2257-7A>G) may be pathogenic, but the others are considered to be benign.

Conclusions: We show that deleterious *DICER1* mutations underlie the genetic basis of only a small fraction of sarcomas, in particular ERMS of the urogenital tract.

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Sarcomas are a rare group of histologically and genetically heterogeneous tumours of mesenchymal origin (Fletcher *et al*, 2013). Most sarcomas arise sporadically. However, a small number of cases manifest in individuals with germline mutations in genes associated with cancer predisposition syndromes, such as *TP53*, *NF1*, *RB1*, *APC*, *RECQL4*, and *HRAS* (Fletcher *et al*, 2013; Ballinger *et al*, 2016; Thomas and Ballinger, 2016). The age of onset of sarcomas is often lower than that observed for most epithelial cancers and, as such, the heritable proportion of sarcomas is likely to be higher than is currently documented (Fletcher *et al*, 2013; Thomas and Ballinger, 2016). Along with translocations, intra-exonic somatic mutations may also contribute to sarcoma development. In a heterogeneous series of 811 next-generation-sequenced sarcomas, the Cancer Genome Atlas Research Network identifies *TP53*, *PIK3CA*, *ATRX*, *PCLO*, and *LRP1B* to be the five most frequently somatically mutated genes (Supplementary Tables S1a and S1b) (cBioPortal for Cancer Genomics).

There are rare reports of sarcomas arising in the context of the DICER1 syndrome (Foulkes *et al*, 2011; Rio Frio *et al*, 2011; Kim *et al*, 2013; Doros *et al*, 2014; Schultz *et al*, 2016), a rare paediatric tumour predisposition syndrome caused by germline mutations in *DICER1* (OMIM 601200). Priest *et al* (1996) noted the occurrence of paediatric-onset sarcomas co-occurring with pleuropulmonary blastoma, a tumour now known to be prototypic of the syndrome. Hill *et al* (2009) further substantiated the association by reporting sarcomas in germline *DICER1* mutation carriers. Subsequent reports of sarcomas in *DICER1* germline-mutated patients include a para-spinal rhabdomyosarcoma in a 20-year-old (Rio Frio *et al*, 2011) and a pleomorphic sarcoma of the thigh (consistent with a leiomyosarcoma) in a 26-year-old (Foulkes *et al*, 2011). A cervical primitive neuroectodermal tumour (Ewing/cPNET) was also reported in a germline *DICER1*-mutated patient (Foulkes *et al*, 2011). However, as testing for characteristic second somatic *DICER1* RNase IIIb mutations (Foulkes *et al*, 2014) was not performed, it is not possible to discern whether the lesions are manifestations of the syndrome or co-incidental occurrences. In contrast, an Askin/Ewing family tumour that arose in a 13-year-old germline *DICER1* mutation carrier (for more details, see de Kock *et al*, 2014b) was not found to harbour a characteristic RNase IIIb hotspot mutation (Foulkes, unpublished data). There are also several reports of somatic *DICER1* RNase IIIb hotspot mutations in uterine carcinosarcoma (Table 1 and Supplementary Table S1c).

More recently, *DICER1* mutations have been strongly implicated in the pathogenesis of embryonal rhabdomyosarcoma (ERMS) of the uterine cervix (cERMS) (Tomiak *et al*, 2014; de Kock *et al*, 2016) the ovary (de Kock *et al*, 2015), and anaplastic sarcoma of the kidney (D1ASK) (Doros *et al*, 2014; Wu *et al*, 2016). Characteristic hotspot *DICER1* RNase IIIb mutations were identified in the three aforementioned lesions. Biallelic somatic *DICER1* mutations were similarly detected in a case of adult-onset cERMS (de Kock *et al*, 2016).

Despite the above evidence, the true contribution of *DICER1* mutations to sarcomas is not yet known. In this study, we aimed to uncover the contribution of *DICER1* mutations to a convenience sample of 61 predominantly adult-onset sarcomas of various subtypes. We recruited an additional 12 Ewing sarcomas consequent to the observation of a cPNET/Ewing and Askin/Ewing family tumour in DICER1 kindred, as described above, for a total of 73 sarcomas.

MATERIALS AND METHODS

Patients and samples. We collected 73 sarcomas of 24 different subtypes, as detailed in the Supplementary Materials and Methods. Age of diagnosis ranged from ages 3 months to 87.4 years (median age 45.7 years), and 38 of the patients were female and 35 were male. This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine of McGill University, Montreal, Quebec, Canada, number A12-M117-11A, and patients signed consent forms in accordance with the IRB approval.

DICER1 screening

Fluidigm access array. We screened the full *DICER1* coding region and exon–intron boundaries in tumour gDNA from 67 (of 73) sarcomas (Supplementary Tables S2a and S2b) using a custom Fluidigm Access Array, which targets all exons and exon–intron boundaries of *DICER1*, followed by next-generation sequencing on an Illumina (San Diego, CA, USA) MiSeq, as previously described (de Kock *et al*, 2014a). All identified mutations were validated by Sanger sequencing and matched-normal gDNA, if available, was used to determine whether mutations were germline or somatic in origin.

Sanger sequencing. For the six remaining sarcomas (all FFPE-derived) (Supplementary Tables S2a and S2b), we focused our investigation on the RNase domains of *DICER1* to identify known hotspot mutations (Foulkes *et al*, 2014). The regions encoding the RNase III domains were PCR amplified and Sanger sequenced (de Kock *et al*, 2014b). Other regions of *DICER1* were not sequenced in these six samples.

MLPA assay. We screened for deletions or duplications of *DICER1* in the germline of 53 patients from whom good quality non-tumour DNA was available (cases 1–52 and 56) using an in-house multiplex ligation-dependent probe amplification (MLPA) assay, as described previously (Sabbaghian *et al*, 2014).

Details of bioinformatics methods, cloning experiments, mosaicism experiments, TruSight Tumour 15 panel sequencing of case 1 (including *TP53* gene, Illumina), and *DICER1* copy number variation (CNV) experiments are provided in the supplement (Materials and Methods section).

RESULTS

We identified multiple *DICER1* variants in an ultimately fatal case of abdominal ERMS that arose in a 23-year-old female following a short history of abdominal pain (case 1) (Figure 1 and Supplementary Tables S2a and S3). Two of these variants are likely to be pathogenic (discussed below). The ERMS was detected on ultrasound as a mixed solid and cystic pelvic mass in the broad ligament, measuring $\sim$20 cm in its longest diameter with a 10–11 cm solid component (Figure 1). The ERMS, obtained following chemo- and radiotherapy (see Figure 1), harboured a *DICER1* RNase IIIb hotspot mutation in exon 25 (c.5439G>T; p.E1813D), which co-occurred with a predicted-truncating *DICER1* mutation in exon 11 (c.1785_1786insA; p.T596Nfs*3), both of which were not detected by regular sequencing techniques in the patient's germline. The patient carried an additional germline insertion (c.2040+53_2040+54insT) in intron 12 of *DICER1* (Figure 1C). Experiments to investigate a potential mosaic origin of the exon 25 and exon 11 mutations suggest that neither are likely to be mosaic in nature (Supplementary Table S4). Given the young age of sarcoma onset, we also screened the patient's germline and tumour samples for *TP53* mutations and did not identify any pathogenic *TP53* alterations (Supplementary Table S5). Further characterisation of the *DICER1* mutations revealed that the exon 11 mutation was *in trans* with both the intron 12 and exon 25 mutations. The latter two were therefore present *in cis* (Figure 2 and Supplementary Figure S1). Cloning of a cDNA fragment encompassing all three mutations revealed that the transcript bearing the

Table 1. Literature review—sarcomas with somatic *DICER1* mutations

Sarcoma type	Site	Sex	Age of Sarc. Dx	Case	Somatic *DICER1* mutation(s)	Germline *DICER1* mutation	Mutations *in cis* or *in trans*?	Clinically suspicious at time of sarcoma Dx?[a]	Evidence of DICER1 syndrome (age of Dx)	Reference
ERMS	Ovary	F	6y	1	c.5425G>A; p.G1809R	c.1196_1197dupAG; p.W400Sfs*59	Not known	No[b]	CN (12y); MNG (13y)	de Kock *et al*, 2015
	Uterine cervix	F	13y	2	c.5113G>A; p.E1705K	c.3907_3908delCT; p.L1303Vfs*4	Not known	Yes	MNG (11y); LC (13y)	Foulkes *et al*, 2011 and de Kock *et al*, 2016
		F	14y	3	c.5438A>G; p.E1813G	c.3611_3616delACTACAinsT	Not known	Yes	MNG (14y)	Foulkes *et al*, 2011 and de Kock *et al*, 2016
		F	53y	4	c.5439G>T; p.D1813D	c.2457C>G; p.I813_Y819del	Not known	Yes	MNG (17y)	Rio Frio *et al*, 2011 and de Kock *et al*, 2016
		F	—	5	c.5428G>T; p.D1810Y	None identified	NA	No	—	Heravi-Moussavi *et al*, 2012
		F	13y	6	c.5437G>A; p.E1813K	c.3535_3538delTCTT; p.S1179Tfs*12	Not known	No	LC, likely PPB Type Ir	Tomiak *et al*, 2014
		F	Adult	7	c.5125G>A; p.D1709N[c]	Not done	NA	—	—	Conlon *et al*, 2015
		F	44y	8	c.2062C>T; p.R688* & c.5438A>G; p.E1813G	None identified	Not known	No	None	de Kock *et al*, 2016
	Uterus	F	12y	9	c.5365-1G>T	None identified	NA	No	—	Doros *et al*, 2012
	Abdomen	—	—	10	c.4259_4261delGAG; p.1418_1420delE	Not done	NA	No	—	Doros *et al*, 2012
	Brain stem	F	21y	11	c.5125G>A (& LOH)	c.4050+1G>A	Not known	Yes	cERMS[d]	de Kock *et al*, 2014a
	Lower genital tract[e]	F	14y	12	c.5428G>C; p.D1810H	c.5387C>T, p.Q1783*	*In trans*	No[b]	MNG (20y)	Fernández-Martínez *et al*, 2017
Anaplastic sarcoma	Kidney	F	21y	1	c.2233C>T; p.R745* & c.5437G>A; p.E1813K	None identified	Not known	No	—	Doros *et al*, 2014
		F	1.75y	2	c.5425G>A; p.G1809R	None identified	NA	No	—	Doros *et al*, 2014
		F	9y	3	c.5425G>A; p.G1809R	c.2062C>T; p.R688*	*In trans*	Yes	PPB Type I (8mo)	Wu *et al*, 2016a
		—	12y	4	c.5125G>A; p.D1709N & c.5138A>T; p.D1713V	Negative	Not known	No	—	Wu *et al*, 2014 meeting
		F	7mo	5	c.5438A>G; p.E1813G	c.2450delC; p.P817Lfs*15	*In trans*[f]	Yes	ASK in CN (7mo)	Wu *et al*, 2016b
Liposarcoma	Site not stated	—	—	1	p.E1797D[g]	Not done	NA	No	—	Kim *et al*, 2013
		—	—	2	p.E1797D[g]	Not done	NA	No	—	Kim *et al*, 2013
Carcinosarcoma	Uterus	F	—	1	c.5425G>A; p.G1809R	c.2516C>T; p.S839F	*In trans*	No	—	Chen *et al*, 2015
		F	Adult	2	c.5437G>C; p.E1813Q[h]	Not done	NA	—	—	Conlon *et al*, 2015
	Ovary	F	—	3	c.5438A>G; p.E1813G	Suspected inactivation	*In trans*	No	—	Chen *et al*, 2015; Heravi-Moussavi *et al*, 2012
Undifferentiated sarc.	Ovary	F	10y	1	c.5125G>A; p.D1709N	c.5096-12G>A	Not known	No[b]	SLCT (14y)	Schultz *et al*, 2016
STS (unknown subtype)	Site not stated	F	30-39y	1	p.G1809R	c.3665dupT; p.L1222fs*13	Not known	—	—	Schrader *et al*, 2016

Abbreviations: ASK = anaplastic sarcoma of kidney; CN = cystic nephroma; ERMS = embryonal rhabdomyosarcoma; F = female; LC = lung cysts; MNG = multinodular goitre; mo = months; NA = not applicable; PPB = pleuropulmonary blastoma; Sarc. = sarcoma; SLCT = Sertoli–Leydig cell tumour; STS = soft tissue sarcoma; y = years.

[a] Clinically suspicious for DICER1 syndrome.

[b] DICER1 syndrome was not clinically suspected at time of sarcoma diagnosis, but later development of DICER1-associated lesion with or without germline mutation identification led to identification of the syndrome in these patients.

[c] Mutation identified in a metastasis from a primary cervical RMS.

[d] cERMS from this patient not included in the table as no somatic testing was performed.

[e] The site of the ERMS was not reported in the publication; personal communication with the authors revealed the site to be in the lower genital tract.

[f] Not definitive.

[g] Mutation found in tumour, but not confirmed to be somatic.

[h] Mutation identified within a rhabdomyosarcomatous component of a uterine carcinosarcoma.

—*No data.*

The asterisks shown at the end of certain mutations is HGVS nomenclature indicating a termination codon.

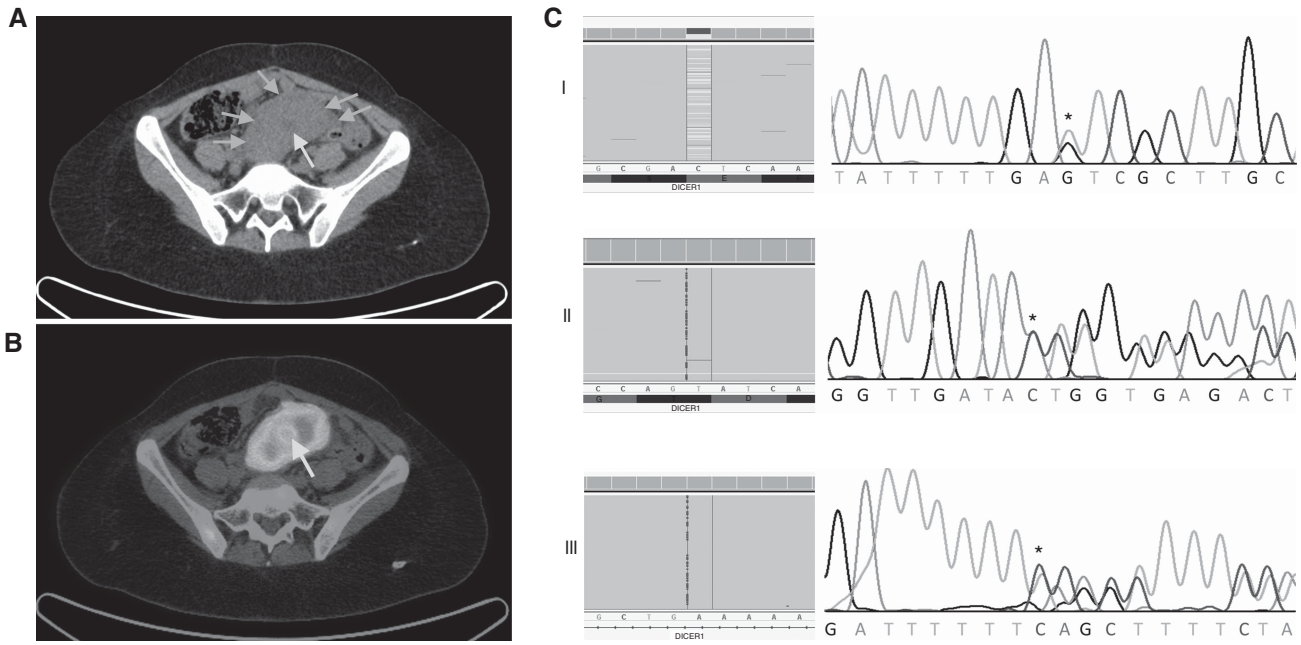

Figure 1. Diagnostic images and mutations for case 1. (**A**) Axial computed tomography (CT) of pelvis demonstrating a solid pre-sacral soft tissue mass (arrows) with low attenuation signal suggesting central cystic/necrotic change (bottom right arrow). (**B**) Fused positron emission tomographic and CT image of pre-sacral mass demonstrating high metabolic activity as reflected by F-18 flourodeoxyglucose avidity. Central area of reduced activity coincides with area of central tumor necrosis (arrow). Following surgical resection of the tumour, the patient underwent chemotherapy (vincristine, doxorubicin, and cyclophosphamide) for 4 months and radiotherapy of the abdomen and pelvis (24 Gy). Recurrent pelvic disease was detected after an 18-month disease-free interval. Surgical resection was attempted, but complications were incurred. Three months later, recurrent disease was again noted on positron emission tomography imaging. Two cycles of irinotecan/temozolamide chemotherapy were administered. Fifty-two months after initial diagnosis, the patient succumbed to her disease. (**C**) The exon 25 c.5439G>T somatic mutation (Panel I), exon 11 c.1785_1786insA somatic mutation (Panel II) and intron 12 c.2040+53_2040+54insT germline mutation (Panel III) as seen in Fluidigm-derived data (left) and chromatogram (right). The mutations are indicated by an asterisk and the wild-type sequence is provided below each chromatogram. A full color version of this figure is available at the *British Journal of Cancer* journal online.

exon 11, c.1785_1786insA insertion was almost always degraded by nonsense-mediated decay as only 3 of 48 sequenced clones expressed the mutation. No cDNA clones were found to exhibit aberrant splicing as a consequence of the intron 12, c.2040+53_2040+54insT variant, indicating that this variant is most likely to be non-contributory (Supplementary Figure S1).

Because of *DICER1*'s involvement in the above-mentioned ERMS, we sequenced a further 72 sarcomas (60 sarcomas of various subtypes and 12 Ewing sarcomas; Supplementary Tables S2a and S2b) and an additional 9 *DICER1* variants were identified (Supplementary Table S3). Of the nine variants, six were established to be germline in origin, two were somatic, and for one variant, the germline *vs* somatic origin remains undetermined (no germline DNA sample available). One somatic variant, c.3208C>G (p.L1070V), identified in a pleomorphic sarcoma with giant cells (case 46), is predicted to be damaging by both PolyPhen2 and SIFT with a score of 1 and 0.01, respectively. However, no additional characteristic RNase IIIb hotspot mutation was found within this sarcoma and therefore its causal role remains speculative. An intronic *DICER1* variant, c.2257-7A>G, had previously been identified in the germline of patient 73. However, no RNase IIIb mutation was identified in the Ewing sarcoma. Based on mutation frequency data and *in silico* effect predictions, the remaining seven variants are unlikely to be involved in the pathogenesis of the sarcomas in question. Germline deletions in *DICER1* have also been found to predispose to the DICER1 syndrome (Sabbaghian *et al*, 2014). We therefore screened for deletions or duplications in the germline of 53 patients from whom good quality non-tumour DNA was available (cases 1–52 and 56) and no such alterations were identified (Supplementary Figure S3). Copy number alterations of *DICER1* have been identified in various cancers including breast cancer, ovarian cancer and melanoma (Zhang *et al*, 2006; Pugh *et al*, 2014). We screened for CNVs of *DICER1* in 59 sarcomas using a ddPCR experiment (chosen due to low DNA input requirement) and detected copy number changes involving the *DICER1* locus in 5 cases (8.5%), each of which was a unique subtype (Supplementary Table S6). However, the extent of the CNVs is not accurately definable using the ddPCR system.

DISCUSSION

DICER1 is an RNase III endoribonuclease responsible for processing hairpin precursor microRNAs (miRNAs) into mature miRNAs, which in turn, regulate the expression of messenger RNAs (Foulkes *et al*, 2014). Germline mutations in *DICER1* predispose to several early childhood or adolescent-onset phenotypes, including pleuroplumonary blastoma, Sertoli-Leydig cell tumour and paediatric cystic nephroma (Foulkes *et al*, 2014). Genetically, DICER1 syndrome-associated tumours are most often characterised by a predisposing germline *DICER1* mutation that inactivates one allele, coupled with a highly distinctive second somatic missense mutation affecting one of the RNase IIIb metal ion-binding sites on the other allele (Foulkes *et al*, 2014). The biallelically mutated recurrent ERMS in our study (case 1) demonstrates that such mutations may contribute to the development of ERMS, even if both mutations are acquired somatically. Although most *DICER1*-related lesions manifest in early childhood (Foulkes *et al*, 2014), it is becoming increasingly evident that the acquisition of two somatic *DICER1* mutations can lead to a

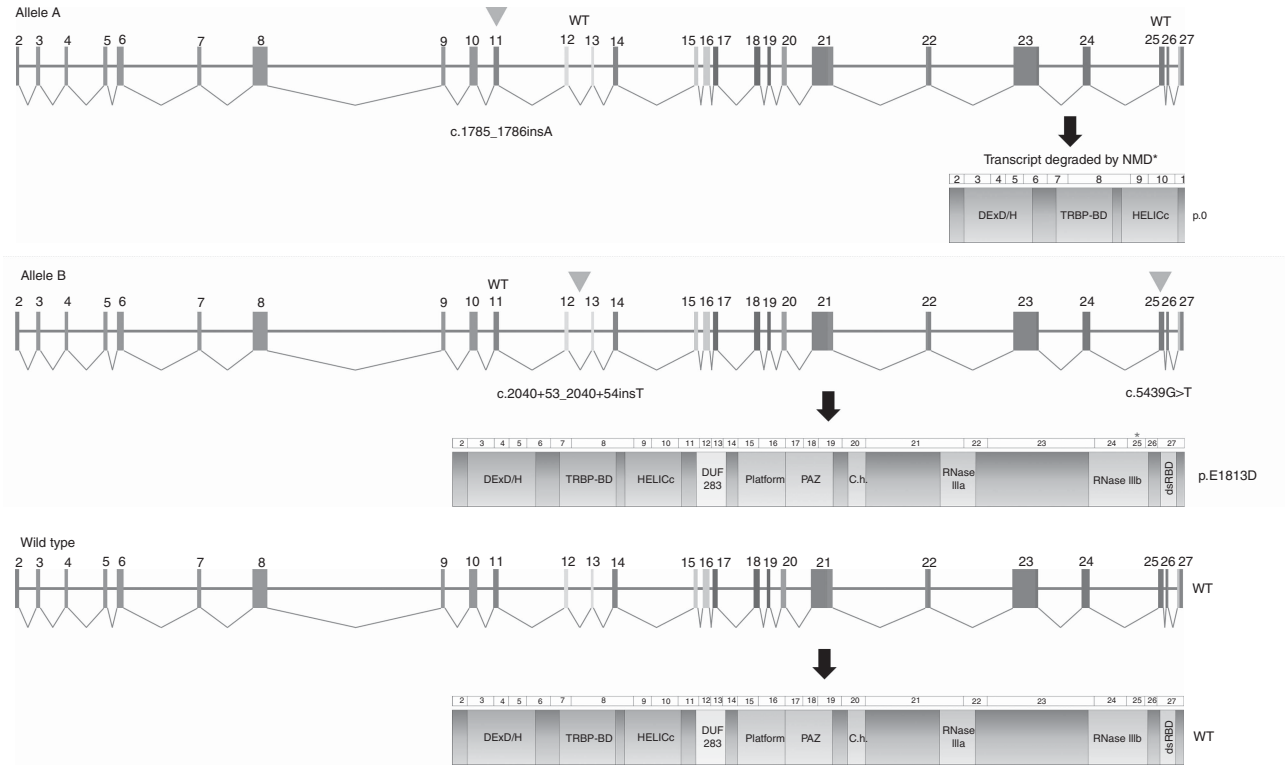

Figure 2. Graphic depiction of biallelic nature of *DICER1* mutations identified in the recurrent ERMS of case 1. The exon 11 c.1785_1786insA mutation is *in trans* (Allele A, top panel) with the intron 12 c.2040 + 53_2040 + 54insT and exon 25 c.5439G > T mutations (Allele B, middle panel). The mutations are indicated by a triangle. Only 3 of 48 clones were found to express the exon 11 mutation, suggesting that the mutated transcript is almost always degraded by nonsense mediated decay (NMD) and thus, no protein is likely to be produced from this allele (p.0). No clones were found to exhibit aberrant splicing as a consequence of the intronic c.2040 + 53_2040_54insT mutation. As such, the resulting protein is predicted to be normal, except for the single amino acid substitution at position p.E1813 (asterisk, Allele B, middle panel). The wild-type (WT) scenario is depicted in the bottom panel. A full color version of this figure is available at the *British Journal of Cancer* journal online.

later-onset of neoplasia (de Kock *et al*, 2016), as was observed in case 1.

Most ERMS that arise in the context of *DICER1* mutations involve the urogenital system and interestingly, the biallelically mutated ERMS from our study arose in the broad ligament, which is the peritoneal fold that attaches the uterus, fallopian tubes and ovaries to the pelvis. Although a limited number of other sarcoma subtypes have been found to carry both truncating and/or RNase IIIb hotspot somatic *DICER1* mutations (Table 1 and Supplementary Table S1c) (de Kock and Foulkes, 2016), ERMS appears to be the subtype that is most commonly *DICER1* mutated. Clinicians should be mindful of the association between ERMS and *DICER1* syndrome. Genetic testing should be performed particularly if ERMS are seen to arise in constellation with one or more known DICER1 syndrome phenotypes, as the identification of germline *DICER1* mutations has important implications for the screening and counselling of patients and their families.

In summary, our study demonstrates that likely-pathogenic *DICER1* mutations underlie the genetic basis of only a small fraction of sarcomas, with ERMS the most likely sarcoma subtype to harbour such mutations. Conversely, the occurrence of a sarcoma at a site other than the genito-urinary system and of a type other than an ERMS (with the exception of anaplastic sarcoma of the kidney) is not suggestive of the DICER1 syndrome.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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