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ORIGINAL ARTICLE

PRAME protein expression in DICER1-related tumours

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Abstract

DICER1 syndrome is an autosomal dominant tumour predisposition syndrome usually affecting persons under 30 years of age. Many of the associated benign and malignant lesions occur almost exclusively in DICER1 syndrome. One such tumour, pituitary blastoma (pitB), overexpresses *PRAME* 500x above control levels. PRAME (PReferentially expressed Antigen in MElanoma) is expressed in malignancies that are not DICER1-related (e.g. melanoma). To address whether PRAME expression is part of the DICER1 phenotype, or simply a feature of pitB, a series of 75 *DICER1*-mutated specimens and 33 non-mutated specimens was surveyed using immunohistochemistry for PRAME, together with EZH2, which complexes with PRAME. In *DICER1*-mutated specimens, positive staining for PRAME was only seen in malignant tumours; 7 of 11 histological types and 34/62 individual tumours were positive, while non-tumourous lesions were always negative. Pleuropulmonary blastoma (PPB) showed a continuum in staining, with type I lesions being PRAME negative ($n = 7$) but all type II and type III lesions PRAME positive ($n = 7$). Similarly, cystic nephroma (CN) was negative ($n = 8$), with anaplastic sarcoma of the kidney being positive ($n = 2$). However, one atypical CN with mesenchymal cell proliferation was PRAME-positive. Embryonal rhabdomyosarcoma (RMS) with *DICER1* pathogenic variants (PVs) was positive for PRAME (5/6), but the same tumour type without *DICER1* PVs was also positive (9/15). Staining for EZH2 corresponded to that seen with PRAME, validating the latter. This study leads us to conclude that (1) PRAME expression occurs in two-thirds of DICER1-related malignancies; (2) PRAME may be a marker for the progression that certain DICER1-related lesions are thought to undergo, such as PPB and CN; and (3) PRAME expression in some tumours, such as RMS, appears to be an intrinsic feature of the tumour, rather than specifically related to *DICER1* PVs. Therapy directed against PRAME may offer novel treatment options in patients with the DICER1 syndrome.

Keywords: DICER1; PRAME; pleuropulmonary blastoma; cystic nephroma; anaplastic sarcoma of the kidney

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No conflicts of interest were declared.

Introduction

DICER1 syndrome is an autosomal dominant tumour predisposition syndrome usually affecting individuals from birth to age 30 years, but typically with low penetrance [1–3]. There is a female predominance amongst DICER1 syndrome patients [3,4]. The syndrome is associated with numerous benign and malignant lesions, many of which occur almost exclusively in DICER1 syndrome [5,6]. Lesions characteristic of DICER1 syndrome are: pleuropulmonary blastoma (PPB) [7,8], cystic nephroma (CN) [9,10], anaplastic sarcoma of the kidney (ASK) [10,11], multinodular goitre [12], ovarian sex cord-stromal cell tumours (especially Sertoli–Leydig cell tumours [13]), embryonal rhabdomyosarcoma (RMS) of the uterine

cervix [14], ciliary body medulloepithelioma [15], nasal chondromesenchymal hamartoma (NCMH) [16], pituitary blastoma (pitB) [17], pineoblastoma [18], and intracranial spindle cell sarcoma [19].

DICER1 is a cytoplasmic endoribonuclease that cleaves precursor molecules into mature micro-RNAs (miRNAs). These miRNAs then hybridise to targeted mRNAs, leading to post-transcriptional gene silencing. DICER1 possesses RNase cleavage domains IIIa and IIIb that cleave the 3′ and 5′ arms of the hairpin-shaped precursor molecules, respectively, to yield 3p miRNAs and 5p miRNAs. Individuals with DICER1 syndrome typically have a germline loss-of-function *DICER1* pathogenic variant (PV) affecting one allele. The tumours arising in these individuals have a somatic mutation in the second allele, typically in the IIIb domain ('hot-spot') [6,13]. The resulting DICER1 protein is dysfunctional, leading to impaired 5p strand production but maintaining 3p synthesis [1,8,13,20]. This imbalance between the 3p and 5p miRNAs disturbs which mRNAs are modulated, and is permissive to tumour development, the exact mechanism of which remains to be elucidated. This sequence of molecular events was recently confirmed in a series of pitBs, with dysregulation of numerous mRNA targets [21]. There was activation of the RAR (retinoic acid receptor), WNT, and NOTCH and PI3K pathways. PitBs expressed higher level of genes associated with undifferentiated cells compared to the normal pituitary. The most significantly upregulated gene was *PRAME*, with a 500-fold increase, and PRAME protein expression was detectable by immunohistochemistry.

PRAME (PReferentially expressed Antigen in MElanoma) is a tumour-associated antigen that was identified as an antigen targeted by T-cells obtained from a patient with metastatic melanoma [22]. PRAME is normally only expressed in testis, ovary, placenta, adrenal gland, and endometrium [22,23]. PRAME expression is best known in cutaneous melanoma [24–27] and uveal melanoma [28]. However, PRAME expression has also been documented in a wide variety of other malignancies, including non-small cell lung cancer, squamous cell carcinoma of the head and neck, squamous cell carcinoma of the oesophagus, gastric carcinoma, hepatocellular carcinoma, breast carcinoma, renal cell carcinoma, urothelial bladder carcinoma, ovarian carcinoma, endometrial carcinoma, seminoma, various leukaemias, medulloblastoma, neuroblastoma, synovial sarcoma, myxoid liposarcoma, dedifferentiated liposarcoma, angiosarcoma, malignant peripheral nerve sheath tumour, and osteosarcoma [29–44]. PRAME expression is associated with adverse biology of some nature, such as higher tumour grade, higher clinical stage, increased rate of metastases, poor response to chemotherapy, and decreased patient survival. PRAME expression is almost exclusively restricted to malignancies. A notable exception is expression (almost universally focal) in up to a quarter of benign melanocytic nevi [24,25,27].

The only DICER1-associated tumour thus far reported to express PRAME is pitB [21]. Clearly, *DICER1* PVs are not required for PRAME expression; in the list of malignancies mentioned above, none are considered to be associated with DICER1 syndrome. However, the observation of PRAME expression in pitB raises the question whether PRAME expression is also seen in other constituent tumours of DICER1 syndrome, or is peculiar to pitB. This question can be addressed by examining PRAME expression in other tumours from DICER1 patients. Such a survey will also test the hypothesis that PRAME expression is a marker of malignancy, as several DICER1-related tumours are benign. The results are presented in this report.

Materials and methods

Samples used in this study were acquired following appropriate IRB approvals that include the ability to conduct further studies such as the one described in this manuscript. A total of 108 cases, obtained from 92 patients, were included in the study. Ten patients had 2–4 specimens analysed, accounting for 26 of the specimens (supplementary material, Table S1). The remainder of the specimens were from individual patients. All specimens had been referred to one of the authors (WDF) for *DICER1* variant analysis. Analysis of the patients' lesions and peripheral blood mononuclear lymphocyte cells had been performed over a 10-year period using a variety of techniques including Fluidigm Access Array, whole-exome sequencing, and Sanger sequencing [6]. The details of the results for all cases with PVs are presented in supplementary material, Table S2. Cases with germline ± somatic PVs were considered to have DICER1 syndrome. For cases in which no PVs were detected, the techniques used on each case are presented in Table 1. Immunohistochemical staining for PRAME and EZH2 was performed on a tissue microarray constructed from formalin-fixed paraffin-embedded blocks that included a series of 73 lesions with confirmed *DICER1* PVs and 20 without; 15 samples were not available at the

Table 1. Summary of results of immunohistochemical staining for PRAME and EZH2

DICER1-mutated lesions				
Diagnosis	Number of cases	PRAME staining	EZH2 staining	Specimen number(s) for *DICER1* PVs*
Multinodular goitre	11	Negative (5)	Negative (5)	3, 4, 9, 10, 11
		Negative (3)	1+ moderate (3)	2, 5, 7
		Negative (3)	2+ moderate (3)	1, 6, 8
Thyroid carcinoma (follicular variant of papillary carcinoma)	2	Negative	2+ moderate	1
		1+ moderate	2+ moderate	2
Ciliary body medulloepithelioma**	1	4+ strong	4+ strong	1
Intracranial spindle cell sarcoma	1	2+ weak	4+ moderate	1
Nasal chondromesenchymal hamartoma	2	1+ weak	3+ moderate	2
		2+ weak	3+ moderate	1
Pineoblastoma**	2	3+ moderate	3+ moderate	2
		4+ strong	4+ strong	1
Sertoli–Leydig cell tumour of the ovary, moderately differentiated**	13	Negative	Negative	5
		2+ weak	3+ moderate	7
		2+ weak	4+ moderate	3
		3+ weak (3)	3+ moderate (3)	2, 6, 8
		3+ moderate	4+ moderate	4
		3+ strong (2)	3+ strong (2)	10, 11
		4+ moderate	3+ moderate	12
		4+ strong	3+ moderate	1
		4+ strong (2)	4+ strong (2)	9, 13
Sertoli–Leydig cell tumour of the ovary, poorly differentiated**	5	1+ weak	3+ strong	2
		2+ weak	3+ strong	5
		3+ strong	3+ strong	3
		4+ weak	4+ strong	1
		4+ moderate	4+ strong	4
Adult pulmonary blastoma**	3	3+ moderate	3+ strong	2
		3+ moderate	4+ strong	1
		4+ strong	4+ strong	3
PPB type I	4	Negative	2+ moderate	4
		Negative	3+ strong	1
		1+ weak	4+ strong	2
		2+ moderate	2+ moderate	3
PPB type Ir	3	Negative	1+ moderate	1
		Negative (2)	2+ moderate (2)	2, 3
PPB type II**	3	3+ moderate	3+ strong	2
		4+ moderate	4+ strong	3
		4+ strong	4+ strong	1
PPB type III**	4	3+ weak	4+ strong	4
		3+ moderate	4+ strong	3
		4+ weak	4+ strong	1
		4+ moderate	4+ strong	2
CN	9	Negative (5)	2+ moderate (5)	1, 3, 4, 6, 7
		Negative (2)	3+ moderate (2)	5, 8
		2+ weak	3+ moderate	2
		3+ moderate	2+ moderate	9
ASK**	2	3+ strong	4+ strong	2
		4+ strong	4+ strong	1
Wilms tumour	2	Negative	1+ moderate	2
		2+ weak	4+ moderate	1
Cystic hepatic neoplasm	1	Negative	2+ moderate	1
Paratesticular tumour of probable Müllerian origin	1	2+ moderate	3+ moderate	1
Embryonal RMS of the ovary**	1	4+ strong	4+ strong	1
Embryonal RMS of the cervix**	5	2+ weak	3+ moderate	3
		3+ weak	2+ moderate	5
		4+ strong (3)	4+ strong (3)	1, 2, 4

(Continues)

DICER1-non mutated lesions				
Diagnosis	Number of cases	PRAME staining	EZH2 staining	Testing for *DICER1* PVs[†]
Multinodular goitre	8	Negative Negative (2) Negative (5)	Negative 1+ moderate (2) 2+ moderate (5)	Negative by (a), (b), and (c)
Thyroid carcinoma (follicular variant of papillary)	2	Negative Negative	1+ moderate 2+ moderate	Negative by (a) and (b)
Sertoli–Leydig cell tumour of the ovary, well differentiated	3	Negative 2+ weak 2+ weak	2+ moderate 2+ moderate 3+ moderate	Negative by (a) and (c)
Infantile pulmonary teratoid tumour	1	3+ moderate	4+ strong	Negative by (c)
Congenital lung cyst	3	Negative	2+ moderate	Negative by (a)
Paratesticular embryonal RMS	13	2+ weak 2+ moderate (2) 2+ moderate 3+ moderate (2) 3+ strong (2) 3+ strong 4+ strong 4+ strong (3)	2+ moderate 2+ moderate (2) 3+ strong 3+ moderate (2) 3+ strong (2) 4+ strong 3+ strong 4+ strong (3)	Negative by (a)
Embryonal RMS of the vagina	2	Negative 2+ strong	3+ moderate 3+ strong	Negative by (a) and (c)
Neuroblastoma	1	Negative	2+ moderate	Negative by (a)[^^]

The grading system for immunohistochemical staining is detailed in Materials and methods.
*For data on detected PVs, see supplementary material, Table S1.
**DICER1-related malignant tumours immunopositive for PRAME.
[†]Negative for likely pathogenic or PVs in all coding exons of *DICER1* by the techniques indicated as: (a) Fluidigm, (b) Sanger, and (c) whole-exome sequencing.
[^^]This case did contain a germline *DICER1* PV but the absence of a second hit led us to consider this tumor to be unrelated to the *DICER1* PV.

time the tissue microarray was constructed and these were tested separately. All samples on the tissue microarray were tested in duplicate. All tumours in the tissue microarray were reviewed by LF and the array was built by NB. The lesions that contained *DICER1* PVs were: multinodular goitre (11); follicular variant of papillary thyroid carcinoma (2); ciliary body medulloepithelioma (1); intracranial spindle cell sarcoma (1); NCMH (2); pineoblastoma (2); Sertoli–Leydig cell tumour of the ovary (18) including moderately and poorly differentiated subtypes; adult *DICER1*-mutated pulmonary blastoma (3); PPB (14) with 4 type I, 3 type Ir (type I, regressed form), 3 type II, and 4 type III; CN (9); Wilms tumour (2); a DICER1-related cystic hepatic neoplasm (1); embryonal RMS of the ovary (1) and cervix (5); and a paratesticular tumour of probable Müllerian origin (1). Non-*DICER1*-mutated cases included: multinodular goitre (8); follicular variant of papillary thyroid carcinoma (2); Sertoli–Leydig cell tumour of the ovary, well differentiated (3); infantile pulmonary teratoid tumour with biallelic *SMARCA4* PVs (1) [45]; congenital lung cyst (3); embryonal RMS of the vagina (2); and neuroblastoma (1). Normal testis (2 samples) was used as a positive control. Negative controls included omission of the primary antibody and normal tissues not known to express PRAME including thyroid (2 samples), colon (3), oesophagus (1), ciliary body (1), nasal sinus mucosa (1), kidney (2), liver (1), lung (2), ovary (1), cervix, vagina (1), placenta (1), brain (1), pineal (1), and anterior pituitary (1). As noted above, 15 samples not in the tissue microarray were also included: ASK (2, both with *DICER1* PVs) [46] and paratesticular embryonal RMS (13, none with *DICER1* PVs) [4].

Immunohistochemistry was performed by NB at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital) as previously described [21]. Formalin-fixed paraffin-embedded sections were cut at 4 μm and the slides were dried overnight at 37°C. Staining was performed using the Discovery XT Autostainer (Ventana Medical Systems, Oro Valley, AZ, USA). Slides underwent heat-induced epitope retrieval and were stained for PRAME (rabbit monoclonal anti-PRAME, diluted at 1:200) (Abcam, Boston MA, USA). Because PRAME complexes with EZH2 to alter the transcription of genes [28,47], immunostaining was also performed for EZH2 (rabbit monoclonal anti-EZH2, ready-to-use) (Roche, Mississauga ON, Canada). Both PRAME and EZH2 antibodies were applied for 32 min at 37°C followed by OmniMap anti-Rabbit-horseradish peroxidase and ChromoMap-diaminobenzidine (Roche). Slides were counterstained with haematoxylin. Only nuclear staining was considered positive [39] and the proportion of positive cells was scored as follows: 0, negative;

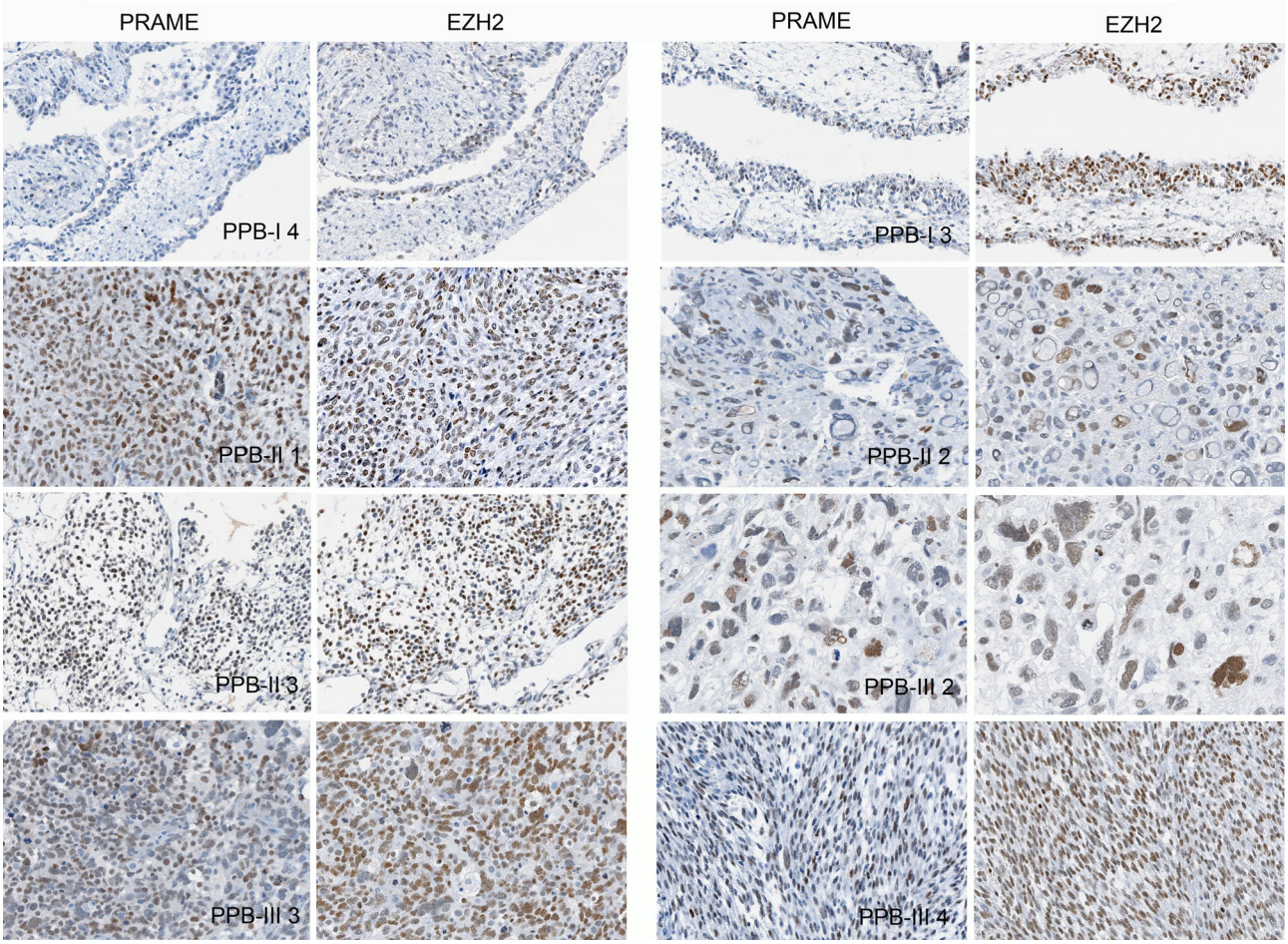

Figure 1. PRAME and EZH2 staining in PPB. All cases shown had *DICER1* PVs. For each case, fields are matched for the two antibodies. PPB type I was usually negative for PRAME (PPB-I 4) but occasionally showed focal staining of epithelial cells (PPB-I 3). In contrast, type II (PPB-II 1, PPB-II 2, and PPB-II 3) and type III (PPB-III 2, PPB-III 3, and PPB-III 4) lesions were PRAME-positive, with diffuse staining of mesenchymal cells. Immunostaining for EZH2 generally matched that of PRAME, but with greater staining of epithelial cells in PPB type 1 (PPB-I 4 and PPB-I 3) and more intense staining in some cases (e.g. PPB-III 3 and PPB-III 4) (original magnifications ×200). Specimen numbers correspond to cases listed in Table 1 and supplementary material, Tables S1 and S2.

1+, <10% positive cells; 2+, 10–50% positive cells; 3+, >50–90% positive cells; 4+, > 90% positive cells [21]. Tumours were considered to be PRAME- or EZH2-positive if >50% of cells showed expression. Tumours were also scored by staining intensity (weak, moderate, and strong), but staining intensity was not used to stratify samples as this has not been shown to have any significance [25,39]. All slides were read by a single pathologist (PST).

Results

The results of immunostaining for PRAME and EZH2 are summarised in Table 1. DICER1-related lesions showed a range of staining for PRAME. Negative lesions included multinodular goitre and follicular variant of papillary thyroid carcinoma. Cases with these same pathological diagnoses but lacking *DICER1* PVs were also negative for PRAME. A single DICER1-related cystic hepatic neoplasm was negative. Focal staining was noted in some lesions, including intracranial spindle cell sarcoma, Wilms tumour, and NCMH. Positive tumours included ciliary body medulloepithelioma, pineoblastoma, a paratesticular tumour of probable Müllerian origin, and adult pulmonary blastoma. Overall, 7/11 malignant tumour types were PRAME-positive (Table 1), including 34 of the individual specimens. Of the 75 specimens with *DICER1* PVs, 13 were considered non-neoplastic

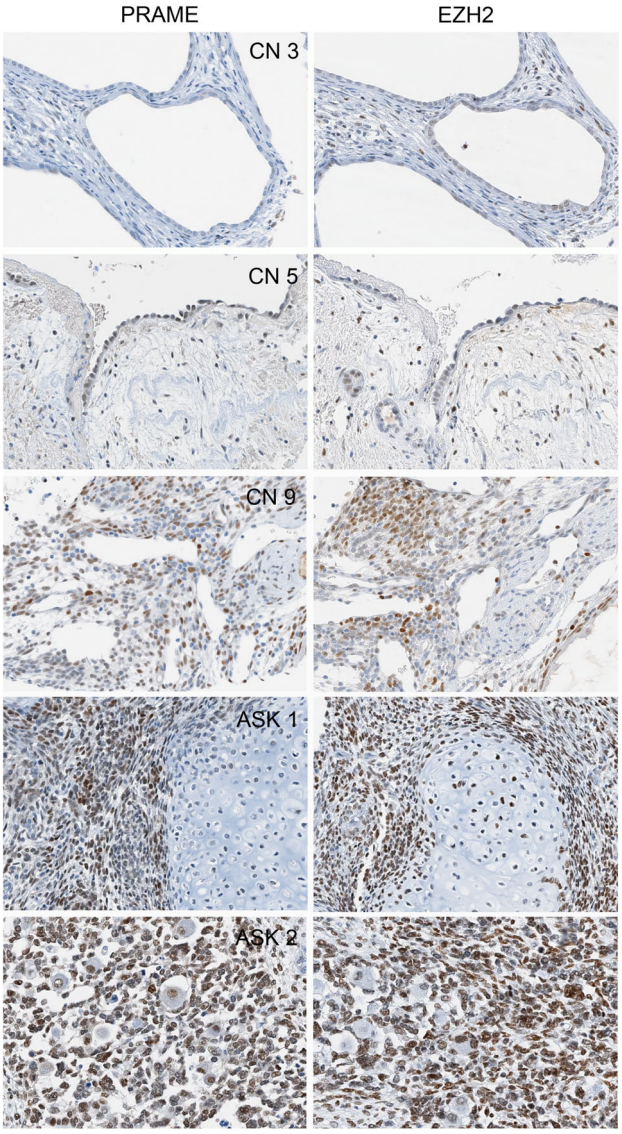

Figure 2. PRAME and EZH2 staining in CN and ASK. All cases shown had *DICER1* PVs. For each case, fields are matched for the two antibodies. Most cases of CN were PRAME-negative (CN 3) or showed focal staining of some epithelial cells (CN 5). One CN was unusual with proliferation of mesenchymal cells in the septa; these cells were PRAME-positive (CN 9). ASK was strongly PRAME-positive, particularly in the undifferentiated areas (ASK 1) and rhabdomyoblastic areas (ASK 2). Immunostaining for EZH2 generally matched that of PRAME except for additional staining of some epithelial and stromal cells in CNs (CN 3 and CN 5) (original magnifications ×200). Specimen numbers correspond to cases listed in Table 1 and supplementary material, Tables S1 and S2.

(multinodular goitre and NCMH); thus, overall, 34 of 62 neoplastic specimens were PRAME-positive. For Sertoli–Leydig cell tumours with *DICER1* PVs, there was a range of staining, with 13/18 cases considered as positive. The *DICER1*-PV-positive group included moderately and poorly differentiated tumours, but there was no correlation between PRAME expression and the degree of differentiation, with 10/13 moderately differentiated cases considered as positive and 3/5 poorly differentiated cases positive. On the other hand, the three cases of well-differentiated Sertoli–Leydig cell tumour were all DICER1-PV-negative and none were PRAME-positive.

In the PPBs, there was a spectrum of staining for PRAME, with types I and Ir either negative (5/7 cases) or with focal staining in occasional epithelial cells (2/7 cases, both type I). In contrast, all seven cases of PPB types II and III were positive (Figure 1). A parallel situation was noted in CN and ASK (Figure 2). CN was usually PRAME-negative (7/9 cases) or with focal staining (1 case). The anaplastic sarcomas were both PRAME-positive. Of note, one CN was positive. This case (#9 in supplementary material, Table S2) was unusual with groups of mesenchymal stromal cells that were PRAME-positive; such cells are not present in typical CN, and their presence was interpreted to indicate progression beyond the classic benign lesion. This patient also had a separate CN (#5) scored as PRAME-negative and a pineoblastoma (#1) that was PRAME-positive. Embryonal RMS associated with *DICER1* PVs was usually PRAME-positive (5/6 cases; and the remaining case showed focal staining) (Figure 3). A survey of this same tumour without *DICER1* PVs showed similar results, with 9/15 cases being positive and the remaining cases showing focal staining with one negative case. The one case of infantile pulmonary teratoid tumour was positive, while the other non-DICER1-related lesions were negative, namely the one neuroblastoma and the three congenital lung cysts.

Staining for EZH2 generally corresponded to that seen with PRAME, with PRAME-positive cells also EZH2-positive. Two differences, however, were noted. First, staining intensity of EZH2 was often greater than that seen for PRAME in the same areas of a particular lesion (Figures 1–3). Second, EZH2 positivity was sometimes noted in cells that were PRAME-negative. This was seen in multinodular goitre, neuroblastoma, Wilms tumour, the DICER1-related cystic hepatic neoplasm, and in the epithelial components of PPB (types I, II, and III) (Figure 1) and CN (Figure 2).

Discussion

This survey examined PRAME expression by immunohistochemistry, enabling us to study a set of archival

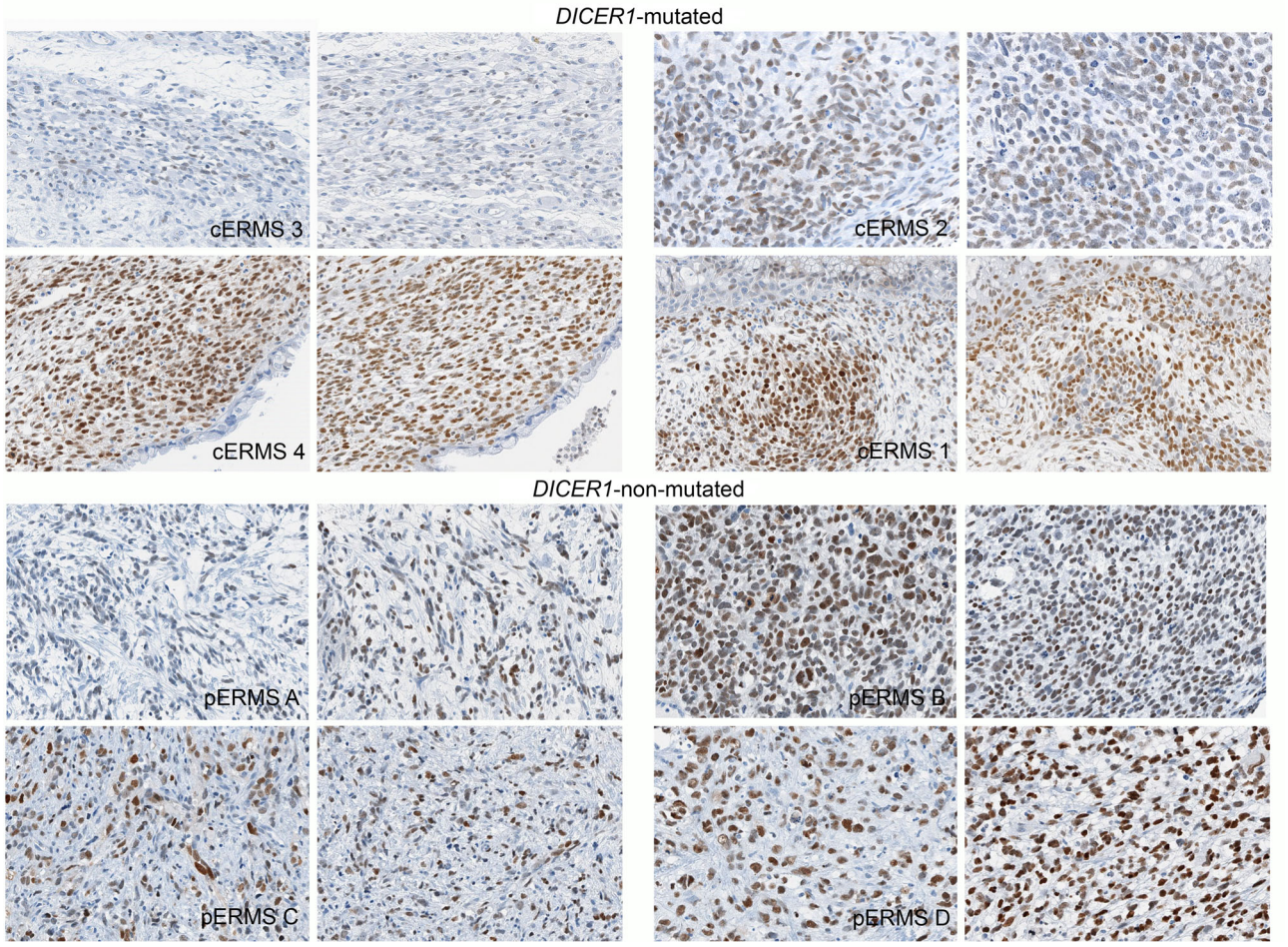

Figure 3. PRAME and EZH2 staining in embryonal rhabdomyosarcoma (ERMS). For each case, fields are matched for the two antibodies (PRAME left, EZH2 right). In *DICER1*-mutated cases, two-thirds of cases were PRAME-positive (cERMS 2, cERMS 4, and cERMS 1) with the others showing only focal staining (cERMS 3). In cases without *DICER1* PVs, two-thirds of cases were positive (pERMS B, pERMS C, and pERMS D) with the others showing focal (pERMS A) or negative staining (original magnifications ×200). Specimen numbers for RMS specimens with PVs correspond to cervical (c) cases listed in Table 1 and supplementary material, Tables S1 and S2. RMS without PVs are paratesticular (p), and only appear in Table 1 as a group; they are therefore not numbered.

specimens of DICER1-related lesions, to determine if PRAME expression is a feature of the DICER1 phenotype, or only occurs in a subset of associated lesions. Some lesions were negative, such as multinodular goitre and carcinoma of the thyroid, while other lesions sometimes showed weak and/or focal staining (<50% positive cells), such as NCMH, intracranial spindle cell sarcoma, Wilms tumour, CN, and PPB type I. The significance of focal staining is unclear but, for the purposes of this study, we elected to consider these lesions as not significant expressors of PRAME, similar to criteria proposed by others [25,39]. The list of positive tumours in our study included pineoblastoma, ciliary body medulloepithelioma, adult pulmonary blastoma, Sertoli–Leydig cell tumour of the ovary, embryonal RMS (cervix and ovary), a paratesticular tumour of probable Müllerian origin [4], PPB types II and III, and ASK. For the Sertoli–Leydig cell tumours, staining was variable, with 13/18 cases being positive. There was a correlation with the presence of a *DICER1* PV. Well-differentiated tumours lacked *DICER1* PVs, and none were PRAME-positive. However, for the moderately and poorly differentiated cases, all had *DICER1* PVs but only ∼70% of cases were PRAME-positive, regardless of the degree of differentiation. Thus, while PRAME expression is not a consistent feature of the DICER1 phenotype, it does occur in 7/11 DICER1-related malignancies and, adding pitB from our previous study [21], makes this proportion two-thirds (8/12). These results are in keeping with the

concept of PRAME expression as a marker of malignancy.

Of particular interest is PPB, a tumour that in some cases progresses over time from a cystic lesion (type I) to a mixed cystic and solid lesion (type II) and then to a highly aggressive solid neoplasm (type III) [8,48]. The mesenchymal component in the cyst walls of type I PPB represents an early malignant population that can develop sarcomatous overgrowth in types II and III PPB, manifesting the characteristic DICER1 mixed sarcoma phenotype including RMS, chondrosarcoma, fibrosarcoma, primitive blastemal cells, and large anaplastic cells. However, not all type I cases follow this sequence, and some type I cases may regress (type Ir). There is a significant correlation between histological type and outcome, with very good outcomes reported for type I, but the 5-year overall survival for types II and III are 71 and 53%, respectively [48]. We noted a strong correlation between PPB histological subtype and PRAME expression. PPB types I (n = 4) and Ir cases (n = 3) were immuno-negative, except for two type I cases that showed focal staining and were still scored as "negative" (Table 1). In contrast, all PPB types II and III (n = 7) were PRAME-positive, suggesting that PRAME expression may be related to malignant progression.

Further support for this concept comes from the results obtained for CN and ASK. CN is a multicystic renal lesion, composed of epithelial cysts separated by a scanty fibrous stroma. This lesion can evolve into a solid anaplastic sarcoma composed of undifferentiated spindle cells, with varying amounts of chondroid, blastemal, rhabdomyoblastic, and osteoid tissue [11,46]. An intervening stage between CN and solid sarcoma has been described in a cystic lesion showing cellular foci with anaplastic nuclei [49]. These stages of evolution are comparable to PPBs progressing from type I to type III [10], and PPB and ASK can be viewed as analogous DICER1-related tumours occurring in different organs [46]. In the present study, there was no PRAME expression in CN, which is benign, and strongly positive expression in ASK, which is a malignancy. However, two cases of CN showed positive staining for PRAME in stromal cells, and one of these showed focal proliferation of such stromal cells. This histological finding is not expected in CN, suggesting that this lesion is progressing beyond a CN and perhaps PRAME expression can serve as a marker of this progression. Additional cases need to be studied to test this hypothesis.

The results for RMS warrant comment as RMS is one of the tumours in the DICER1 phenotype that occurs more commonly outside of this clinical situation. For this reason, we studied a series of embryonal RMSs that lacked *DICER1* PVs, including two vaginal tumours, and a series of 13 paratesticular RMSs [4]. All DICER1-related cases were of embryonal subtype, all showed some PRAME staining, and 83% were considered to be PRAME-positive. For non-mutated RMS, all but one case showed some staining and 60% were scored as positive. Thus, PRAME expression appears to be an intrinsic feature of embryonal RMS, rather than specifically related to *DICER1* mutation. PRAME expression has been recently reported in RMS, including both embryonal and alveolar subtypes, but the *DICER1* status of these cases was not provided [50].

The PRAME protein forms a heterotrimeric complex with EZH2 that can alter the transcription of genes [28,47]. Logically, EZH2 should then co-localise with PRAME by immunostaining. This was true for all cases studied, validating the PRAME staining results. In addition, expression of EZH2 was occasionally seen in areas where PRAME was not expressed (e.g. epithelial components of PPB and epithelial and stromal cells of CN). The significance of this expression in the absence of PRAME is not clear. However, it is known that EZH2 is expressed in a variety of malignancies, including breast, prostate, and bladder [51]. EZH2 mediates post-translational histone modifications and promotes oncogenesis by epigenetic activation of oncogenic signalling pathways and silencing of tumour suppressor genes [52]. EZH2 expression is involved in the development of pre-malignant states in both squamous cell carcinoma of the lung [52] and breast cancer [53]. Thus, expression of EZH2 might also play a role in the progression of PPB from type I to type III, and CN to ASK.

The only other potential marker of progression that has been studied in DICER1-related tumours is p53 in PPBs. In one study, 33% of cases overall were immunopositive [8] while, in another study, immunostaining was matched to histological type: PPB type I showed no or focal p53 expression by immunostaining, whereas PPB types II and III showed positive staining (>25% positive cells) in 67 and 70% of cases, respectively [48]. We have carried out limited immunostaining for p53 in ASK and found that two-thirds of these tumours are positive [46], especially in anaplastic cells [11,49], something that has also been noted for PPBs [48]. PPB cases immunopositive for p53 have *TP53* PVs [8,48]. Thus, p53 expression is not related to alterations in wild-type *TP53* expression resulting from *DICER1* PVs, but represents a separate mechanism for oncogenesis in these tumours. Overexpression of PRAME has been reported to upregulate *TP53* expression in leukaemic cells [54], but decrease *TP53* expression in hepatocellular carcinoma [44]. In our study on pitB, of the many genes with expression altered by *DICER1* PVs, *TP53* was not one of them [21].

For PRAME to be a marker of malignancy, and/or malignant progression, there should be downstream effects from overexpression of PRAME that would favour malignant transformation. Increased PRAME expression is associated with low expression of p27 and loss of cell cycle control in osteosarcoma [42] and leukaemic cells [43]. The protein TRAIL (Tumour necrosis factor-related Apoptosis-Inducing Ligand) participates in anti-cancer surveillance by immune cells. There is an inverse correlation between PRAME and TRAIL expression. Thus, PRAME could promote oncogenesis by blocking tumour suppressor pathways mediated by TRAIL [32]. Reduced TRAIL expression was noted in our study of pitB [21] but whether this applies to other DICER1-related malignancies is not known. In melanoma cells *in vitro*, PRAME, in conjunction with EZH2, represses the transcription of genes containing RAR-binding sites [28,47], whereas our previous study on PitB showed that the RAR pathway was activated, with overexpression of all but a few genes in that pathway [21]. Thus, the downstream effects of PRAME may be different in the setting of *DICER1* PVs. In pitB, there was low expression of *CALR* (calreticulin) and reduced expression of this gene is known to favour an oncogenic phenotype [55,56]. In addition, there was increased expression of several genes known to be overexpressed in various cancers, including: *SNW1* (SNW domain containing 1) [57], *CRABP2* (cellular retinoic acid binding protein 2) [58], and *CYP26A1* (cytochrome P450 family 26 subfamily A member 1) [59]. What roles these genes might play in other DICER1-related tumours remain to be determined.

To conclude, PRAME expression is seen in several malignancies associated with the DICER1 syndrome. How its expression is brought about by *DICER1* PVs is not yet clear, nor are the downstream effects in oncogenesis in the different tumour types that express PRAME. PRAME expression increases as PPB and CN progress to a malignant phenotype, providing a histological marker for this, as well as a biological avenue to pursue for better understanding of this progression. The restricted expression of PRAME in malignancies has given rise to the concept of using PRAME as a target for immunotherapy of cancers that express this protein [60–62]. This approach may offer new therapeutic options to patients with DICER1 syndrome.

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Author contributions statement

PST interpreted the results and drafted the manuscript. A-SC acquired the data and revised the manuscript. JN analysed the data and revised the manuscript. NB and PM provided technical support. RC and LF provided technical and material support. WDF conceived the work that led to the submission, interpreted the results, and revised the manuscript.

Data availability statement

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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SUPPLEMENTARY MATERIAL ONLINE

Table S1. Patients with multiple lesions included in the study

Table S2. *DICER1* variants identified in the lesions included in the study