PRAME protein expression in DICER1-related tumours

Paul S Thorner1, Anne-Sophie Chong2,3,4, Javad Nada2,3, Naciba Benlimame5, Paula Marrano6, Rose Chami1,6, Lili Fu7 and William D Foulkes2,3,4,8*

1Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada
2Department of Human Genetics, McGill University, Montreal, QC, Canada
3Cancer Axis, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, QC, Canada
4Cancer Research Program, Research Institute of the McGill University Health Centre, Montreal, QC, Canada
5Research Pathology Facility, Lady Davis Institute, Jewish General Hospital, Montreal, QC, Canada
6Division of Pathology, Hospital for Sick Children, Toronto, ON, Canada
7Department of Pathology, McGill University Health Centre, McGill University, Montreal, QC, Canada
8Gerald Bronfman Department of Oncology, McGill University, Montreal, QC, Canada

*Correspondence to: William D Foulkes, The Program in Cancer Genetics, Division of Medical Genetics, Department of Specialized Medicine, The Lady Davis Institute, Jewish General Hospital, 3755 Cote St. Catherine Road, Montreal, QC H3T 1E2, Canada. E-mail: william.foulkes@mcgill.ca

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 MEtastatic 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

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...
PRAME in DICER1 syndrome

PRAME (PRReferentially 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 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.

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

| 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+ weak                          | 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                      | 3                                 |
| 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                                 |
| Wilm’s 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                           |
### DICER1 non-mutated lesions

| Diagnosis                                      | Number of cases | PRAME staining | EZH2 staining | Testing for DICER1 PVs† |
|------------------------------------------------|-----------------|----------------|---------------|-----------------------|
| Multinodular goitre                           | 8               | Negative       | Negative      | Negative by (a), (b), and (c) |
| Thyroid carcinoma (follicular variant of papillary) | 2               | Negative       | 1+ moderate   | Negative by (a) and (b) |
| Sertoli–Leydig cell tumour of the ovary, well differentiated | 3               | Negative       | 2+ 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   | Negative by (a)       |
| Embryonal RMS of the vagina                   | 2               | Negative       | 3+ moderate   | 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.

††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.

|               | Number of cases | PRAME staining | EZH2 staining | Testing for DICER1 PVs†   |
|---------------|-----------------|----------------|---------------|--------------------------|
| Multinodular goitre | 8               | Negative       | Negative      | Negative by (a), (b), and (c) |
| Thyroid carcinoma (follicular variant of papillary) | 2               | Negative       | 1+ moderate   | Negative by (a) and (b) |
| Sertoli–Leydig cell tumour of the ovary, well differentiated | 3               | Negative       | 2+ 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   | Negative by (a)       |
| Embryonal RMS of the vagina                   | 2               | Negative       | 3+ moderate   | Negative by (a) and (c)|
| Neuroblastoma                                 | 1               | Negative       | 2+ moderate   | Negative by (a)††      |

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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;
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.
(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
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 NCMM, 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

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.
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. Over-expression 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].

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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 on 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