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Teratoma-associated and so-called pure Wilms tumour of the ovary represent two separate tumour types with distinct molecular features

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Teratoma-associated and so-called pure Wilms tumour of the ovary represent two separate tumour types with distinct molecular features

Aims: Ovarian Wilms tumour (WT)/nephroblastoma is an extremely rare neoplasm that has been reported to occur in pure form or as a component of a teratomatous neoplasm. We hypothesized that teratoma-associated and pure ovarian WT may represent different tumour types with diverging molecular backgrounds. To test this hypothesis, we comprehensively characterized a series of five tumours originally diagnosed as ovarian WT.

Methods and Results: The five cases comprised three teratoma-associated (two mature and one immature) and two pure WTs. Two of the teratoma-associated WTs consisted of small nodular arrangements of "glandular"/epithelial structures, while the third consisted of both an epithelial and a diffuse spindle cell/blastemal component. The pure WTs consisted of "glandular" structures, which were positive for sex cord markers (including inhibin and SF1) together with a rhabdomyosarcomatous component. The two pure WTs harboured *DICER1* pathogenic variants (PVs), while the three associated with teratomas were *DICER1* wildtype. Panel-based DNA sequencing of

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four of the cases did not identify PVs in the other genes investigated. Analysis of the HA19/IGF2 imprinting region showed retention of imprinting in the pure WTs but loss of heterozygosity with hypomethylation of the ICR1 region in two of three teratoma-associated WTs. Furthermore, copy number variation and clustering-based whole-genome DNA methylation analyses identified divergent molecular profiles for pure and teratoma-associated WTs.

Conclusion: Based on the morphological features, immunophenotype, and molecular findings (*DICER1* PVs, copy number, and DNA methylation profiles), we suggest that the two cases diagnosed as pure primary ovarian WT represent moderately to poorly differentiated Sertoli Leydig cell tumours (SLCTs), while the tumours arising in teratomas represent true WTs. It is possible that at least some prior cases reported as pure primary ovarian WT represent SLCTs.

Keywords: *DICER1*, DNA methylation, nephroblastoma, ovary, Sertoli-Leydig cell tumour, Wilms tumour

Introduction

Wilms tumour/nephroblastoma (WT) is the most common renal tumour in children and is thought to arise through disrupted embryogenesis during kidney development.[1] During kidney development, intermediate mesoderm gives rise to the metanephric mesenchyme, which further differentiates into the nephron structures and associated kidney tissues through mesenchymal to epithelial transition. In WT, embryogenesis may be disrupted at various stages, leading to a variable histological appearance that usually includes a triphasic pattern consisting of undifferentiated blastema, epithelial, and stromal components. However, some tumours only exhibit biphasic or monophasic differentiation. Microscopically, the blastemal component consists of undifferentiated, small- to medium-sized cells with scant cytoplasm and monotonous nuclei often displaying brisk mitotic activity. Epithelial components may include "well differentiated" areas with glomerular-like structures, as well as "poorly differentiated" components, and rarely heterologous elements, such as squamous or mucinous epithelium. Stromal components comprise spindled tumour cells, sometimes exhibiting heterologous elements such as rhabdomyoblasts or cartilage.[2]

The molecular background of WT is diverse and frequently includes alterations affecting *WT1*, *CTNNB1*, and *WTX*, as well as less common driver genes such as *MYCN*. Epigenetic modifiers, such as *BCOR*, and genes involved in microRNA biogenesis (*DROSHA*, *DICER1*, and *DGCR8*), are also implicated.[3] More than half of WTs show epigenetic abnormalities at the imprinted *H19-IGF2* loci on 11p15, leading to increased IGF2 expression.[4] Although WT usually shows a stable genome with only minimal mutational load, the occurrence of oncogenic *TP53* variants in WT is associated with anaplasia and chromosomal instability, including chromothripsis.[5,6]

Individuals affected by WT usually present with a unilateral renal mass, although bilateral involvement occurs in up to 7% of patients. Extrarenal WT is extremely rare and the most common sites include the retroperitoneum, the lumbosacral and pelvic area, the mediastinum, the paratesticular region, and the female genital tract.[7] In the female genital tract, few cases of primary WT have been reported, including in the uterine corpus and cervix.[8–14] Primary ovarian WT represents an extremely rare neoplasm, with only 13 prior cases reported in the literature.[15–26] In the ovary, WT either occurs as a pure neoplasm, or in association with a teratomatous neoplasm (teratoma-associated WT).

We hypothesized that ovarian teratoma-associated and pure WT may represent different tumour types, with diverging molecular foundations. In testing this hypothesis, we report five cases of so-called primary ovarian WT, two pure and three teratoma-associated, the largest reported series to date. Based on the morphological features and immunophenotype, as well as molecular features, we suggest that some cases diagnosed as pure primary ovarian WT represent *DICER1*-associated moderately to poorly differentiated Sertoli Leydig cell tumours (SLCTs), while cases arising in teratomas represent true WTs. In reporting these cases, we review prior reports of primary ovarian WT.

Materials and Methods

PATIENT SAMPLES AND PATHOLOGY REVIEW

We collected five cases diagnosed as primary ovarian WT from the in-house material and consult

cases of the pathology departments with which the authors are affiliated. Clinical and pathological information was retrieved from the medical records, the pathology reports, and the referring pathologists. The available histological sections and immunohistochemical slides were reviewed during the preparation of this article; in the consult cases, sometimes not all the slides were available. Cases 3 and 5 have previously been published.[2,3] In addition, three WTs of the kidney and seven mature cystic teratomas of the ovary were used for comparative DNA methylation analyses. All samples were collected in accordance with the Ethics Review Board regulations. Differences between group averages were evaluated using one-way analysis of variance (ANOVA). A *P*-value of <0.05 was considered statistically significant.

DNA EXTRACTION AND ARRAY-BASED DNA METHYLATION PROFILING

A minimum of 100 ng genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumour tissue. FFPE tumour tissue was enriched for the WT components of Cases 1 and 2 (teratoma-associated WTs) by macrodissection. The Maxwell 16 FFPE Plus LEV DNA Kit or the Maxwell 16 Tissue DNA Purification Kit (for frozen tissue) was applied on the automated Maxwell device (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA was subject to bisulfite conversion and processed on the Illumina Infinium EPIC (850 k) BeadChip (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

COPY NUMBER ANALYSIS

The copy number alterations of genomic segments were calculated from the methylation array data and plotted using the R-package *conumee*.[27] Gene amplifications and deletions were identified by manual inspection.

11P15 ICR1 AND ICR2 METHYLATION ANALYSIS

Analysis of DNA methylation of imprinting control regions ICR1 (IGF2/H19) and ICR2 (KCNO1/CDKN1C) were performed based on Gadd *et al.*[3] In brief, methylation levels for all probes in the imprinting control regions ICR1 and ICR2 were averaged. Retention of imprinting was defined as 0.33–0.66 for both ICR1 and ICR2; loss of imprinting as >0.66 for ICR1 and 0.33–0.66 for ICR2; and loss of heterozygosity as <0.33 or >0.66 for ICR1 and <0.33 or >0.66 for ICR2.

GENOME-WIDE DNA METHYLATION ANALYSIS

DNA methylation data of the five ovarian WTs were analysed in the context of three WTs of the kidney, seven mature cystic teratomas of the ovary, and a larger dataset of *DICER1*-associated and *DICER1* wildtype neoplasms previously published.[28] Data analysis was performed in R v. 4.1.2, using packages from Bioconductor.[29] Illumina EPIC and 450 k methylation array data were merged to a combined dataset by selecting the intersection of probes present on both arrays (combineArrays function, minfi). DNA methylation data were normalized by applying background correction and dye bias correction. Probes targeting sex chromosomes, probes containing multiple single nucleotide polymorphisms, and those which could not be uniquely mapped were removed. For unsupervised hierarchical clustering of DNA methylation data, 20,000 probes with the highest median absolute deviation across beta values were selected. Samples were hierarchically clustered using Euclidean distance and Ward's linkage method.

DNA SEQUENCING AND VARIANT CALLING

DNA of Cases 1–4 was sequenced using a customized SureSelect XT technology (Agilent, Santa Clara, CA, USA) panel covering the coding regions of 201 genes (Table S1). Library preparation, quality control, sequencing on a NextSeq or HiSeq sequencer (Illumina), and data processing were performed as previously described.[30] Reads were aligned to the reference human genome hg19 and variants were annotated using ANNOVAR software.[31] Synonymous and stop-loss variants, variants with a frequency exceeding 1% in the healthy population, as well as variants described as known polymorphisms in the single nucleotide polymorphism database were excluded. In Case 5 the entire *DICER1* (NM_177438.3) coding sequence and exon boundaries were sequenced using a Fluidigm Access array as previously described.[32]

LITERATURE REVIEW

We performed a literature review to identify all primary ovarian WTs previously reported. We conducted a systematic search in most appropriate publications and in the electronic database PubMed using a combination of keywords (e.g. Wilms

tumour + ovary). Furthermore, we reviewed the reference lists of identified articles manually.

Results

CASE HISTORIES

The ages of the patients with the teratoma-associated WTs (Cases 1–3) were 28, 32, and 36 years and the patients with pure WT (Cases 4 and 5) were aged 4 and 16 years. In all cases, the patients presented with symptoms related to an ovarian mass. Three of four tumours, where this information was available, were unilateral, two involving the right ovary and one the left ovary. One tumour (Case 5) was bilateral; the main tumour mass was in the left ovary, but microscopic involvement of the right ovary was identified when further surgery was undertaken 18 weeks into chemotherapy. Where information was available (four of five cases), all patients initially underwent salpingo-oophorectomy or oophorectomy alone. Follow-up was available in three cases, with two patients showing no evidence of recurrence after 17 (Case 2) and 48 (Case 4) months. In Case 5, there was early recurrence in the abdomen and pelvis and 18 weeks into chemotherapy, the patient underwent resection of the right ovary and tube, a right pelvic mass, peritoneum, omentum, and appendix, all of which contained tumour. Brief clinicopathological features of the cases reported herein and previously reported primary ovarian WTs are presented in Table 1. Cases 3 and 5 have been previously reported and detailed clinical and pathological details are provided there.[23]

PATHOLOGICAL FINDINGS

Teratoma-associated ovarian WTs

Case 1. The specimen in Case 1 consisted of multiple pieces of tan, yellow-pink tissue, measuring 7 cm in the aggregate. The cut surface showed solid and cystic areas, the latter containing hair. Histologically, the tumour consisted of a mature teratoma including thyroid tissue, with areas of mucinous cystadenoma and a small, microscopic focus of WT measuring 0.1 cm (present in 1 out of 14 tumour blocks). The WT consisted of nodular arrangements of "glandular" structures composed of regular cells with hyperchromatic nuclei with dispersed chromatin and scant, eosinophilic cytoplasm; there was low mitotic activity. The glandular structures were diffusely positive for PAX8, WT1, and CD10 and negative for inhibin.

Case 2. The specimen in Case 2 comprised an ovarian mass measuring 26 cm in maximum dimension with an intact capsule. Sectioning revealed solid and cystic components with sebaceous material and calcification. Histologically, the tumour comprised a grade III immature teratoma containing skin with appendage structures, respiratory epithelium, intestinal-type mucinous epithelium, cartilage, bone, smooth muscle, and mature and immature glial tissue. There was a small focus of WT measuring 0.5 cm (present in 1 out of 18 tumour blocks) and consisting of nodular arrangements of glandular/tubular elements, some of which were slit-like. The glandular structures consisted of uniform cells with regular nuclei, scant cytoplasm, and low mitotic activity. The glandular structures were diffusely positive for PAX8, WT1, and CD10, and negative for inhibin, calretinin, and SF1. The tumour was FIGO stage IA. No tumour was present in the omentum or the appendix. Figure 1 shows representative images of this neoplasm.

Case 3. The specimen in Case 3 comprised a 16.1-cm ovarian mass with focal disruption of the capsule. The cut surface revealed a multiloculated mass consisting of cysts, as well as multiple firm to gelatinous nodules. Histologically, the tumour comprised a mature cystic teratoma consisting of skin with appendage structures, respiratory epithelium, gastric-type epithelium, and cartilage. In addition, there was a neoplasm composed of cells with various architectural growth patterns, including epithelial arrangements with glands/tubules and nests, as well as a diffuse blastemal and spindled cell component. The detailed immunohistochemical profile has been reported elsewhere.[23] In brief, the epithelial and blastemal components were diffusely positive for PAX8 and WT1, while showing variable positivity for oestrogen and progesterone receptors and CD10. Tumour cells were negative for inhibin and calretinin. Figure 2 shows representative images of this neoplasm.

Pure ovarian WTs

Case 4. The specimen in Case 4 consisted of an ovarian mass weighing 296 g and measuring 8.6 cm in maximum dimension. Histologically, the tumour showed a low-power lobulated architecture with cellular areas separated by an oedematous stroma. Within the cellular areas, the tumour was composed of nests, cords, and tubules, some of which were dilated. There were also retiform foci. The tumour cells had hyperchromatic nuclei and scant cytoplasm and were diffusely positive for WT1, CD56, and inhibin, with focal immunoreactivity for calretinin and negative staining for epithelial membrane antigen (EMA). Occasional collections of cells with abundant eosinophilic cytoplasm, in keeping with Leydig cells, were present. There were

Table 1. Clinicopathological features of reported cases of primary ovarian Wilms tumour

References	This study	Year	Age (years)	Clinical presentation	Treatment	Tumour size, (cm)	Laterality	Histology	Outcome	Follow-up (months)
Current study	Case 1	2023	28	NA	Right oophorectomy	NA	Right	Teratoma-associated Wilms tumour	Unknown	Unknown
Current study	Case 2	2023	32	Pelvic mass	Left salpingo-oophorectomy, appendicectomy, omentectomy	26	Left	Teratoma-associated Wilms tumour	No recurrence	17
Current study and Turashvili *et al.*[23]	Case 3	2020	36	Abdominal pain, ovarian torsion	Right salpingo-oophorectomy	16.1	Right	Teratoma-associated Wilms tumour	Unknown	Unknown
Current study	Case 4	2023	4	NA	NA	8.6	NA	Pure Wilms tumour/ SLCT *DICER1*-associated	No recurrence	48
Current study and Turashvili *et al.*[23]	Case 5	2020	16	Abdominal pain, ovarian torsion, fever	Left salpingo-oophorectomy	18	Bilateral (see text)	Pure Wilms tumour/ SLCT *DICER1*-associated	Widespread recurrence in the abdomen and pelvis (see text)	18
Wu *et al.*[25]		2022	38	Pelvic mass	Right oophorectomy	NA	Right	Teratoma-associated Wilms tumour	Peritoneal dissemination	Unknown
Nakabayashi *et al.*[26]		2019	33	Abdominal pain	Left cystectomy	7.7	Left	Teratoma-associated Wilms tumour	Peritoneal dissemination	12
Alexander *et al.*[24]		2017	26	Abdominal pain	Right salpingo-oophorectomy	13	Right	Teratoma-associated Wilms tumour	No recurrence	11
Marwah *et al.*[22]		2012	1	Abdominal pain, vomiting	Right salpingo-oophorectomy, chemotherapy	10	Right	Pure Wilms tumour	No recurrence	3
Liang *et al.*[21]		2008	22	Abdominal pain and distension	Right oophorectomy	9	Right	Pure Wilms tumour	Unknown	Unknown
Öner *et al.*[20]		2002	3.5	Abdominal pain, vomiting	Left salpingo-oophorectomy, appendicectomy, partial omentectomy, peritoneal biopsies, retroperitoneal lymphadenectomy, chemotherapy	13	Left	Pure Wilms tumour	No recurrence	7
Pereira *et al.*[19]		2000	3.5	Abdominal pain and distension	Right oophorectomy, left ovarian biopsy, chemotherapy	13	Right	Pure Wilms tumour	No recurrence	78

Table 1. (*Continued*)

References	This study	Year	Age (years)	Clinical presentation	Treatment	Tumour size, (cm)	Laterality	Histology	Outcome	Follow-up (months)
Isaac *et al.*[18]		2000	21	Abdominal pain, menorrhagia	Right oophorectomy, wedge resection of left ovary, chemotherapy	19	Right	Pure Wilms tumour	No recurrence	6
O'Dowd and Ismail[17]		1990	20	Amenorrhea, elevated androgen	Right salpingo-oophorectomy	2.5	Right	Wilms tumour associated with juvenile granulosa cell tumour	No recurrence	Unknown
Sahin and Benda[16]		1988	56	Bilateral calf pain due to deep vein thrombosis, pelvic mass	Total abdominal hysterectomy, bilateral salpingo-oophorectomy, appendicectomy, pelvic radiotherapy, chemotherapy	12	Left	Pure Wilms tumour	No recurrence	108
Nicod[15]		1965	35	Menorrhagia	Left oophorectomy, radiation therapy	10	Left	Pure Wilms tumour	No recurrence	24

also focal areas consisting of small cellular nests within an oedematous stroma. In these areas, the tumour cells had hyperchromatic nuclei and scant cytoplasm and exhibited prominent mitotic and apoptotic activity. Occasional cells with abundant eosinophilic cytoplasm were present, in keeping with rhabdomyoblasts. In these areas, there was focal positivity for desmin, myogenin, and MyoD1. No teratomatous elements were identified. Figure 3 shows representative images of this neoplasm.

Case 5. The specimen in Case 5 consisted of multiple pieces of red-yellow gelatinous tissue, the largest measuring 14 cm in maximum dimension and weighing 500 g in the aggregate. Histologically, the tumour consisted of glandular/tubular elements that were diffusely positive for WT1 and focally positive for SF1 and FOXL2, while inhibin was negative. In addition, there were sheets of small, spindled to round cells with hyperchromatic nuclei and scant cytoplasm, which were focally positive for myoD1, in keeping with areas of rhabdomyosarcomatous differentiation. There were also nodules composed of foetal-type cartilage. No teratomatous elements were identified.

DNA SEQUENCING

DNA sequencing identified *DICER1* pathogenic variants (PVs) in the two pure ovarian WTs (Figure 4F). Cases 4 and 5 each harboured a *DICER1* loss-of-function PV (c.1174C>T, p.R392X and c.947G>A, p.W316X, respectively) alongside a *DICER1* RNase IIIb hotspot mutation (c.5425G>A, p.G1809R and c.5428G>T, p.D1810Y, respectively). For Case 4, the truncating PV is germline in origin, and subsequent pedigree examination revealed a cousin with a pleuropulmonary blastoma (not previously tested for *DICER1*). Several family members carry the p.R392X PV. For Case 5, we do not know the provenance of the truncating variant. In contrast, all three teratoma-associated WTs showed wildtype *DICER1* alleles. Panel-based sequencing did not identify any additional PVs in the included genes. Variants of unknown significance identified are summarized in Table S2.

COPY NUMBER VARIATIONS (CNVS)

Array-based CNV analysis identified gains of chromosome 3 in two of the three teratoma-associated WTs and gain of chromosome 12 in one of them (Case 3) (Figure 4A–C). On the other hand, both pure ovarian WTs showed gain of chromosome 8 (Figure 4D,E). Case 5 also showed an amplification of the *TERT* locus.

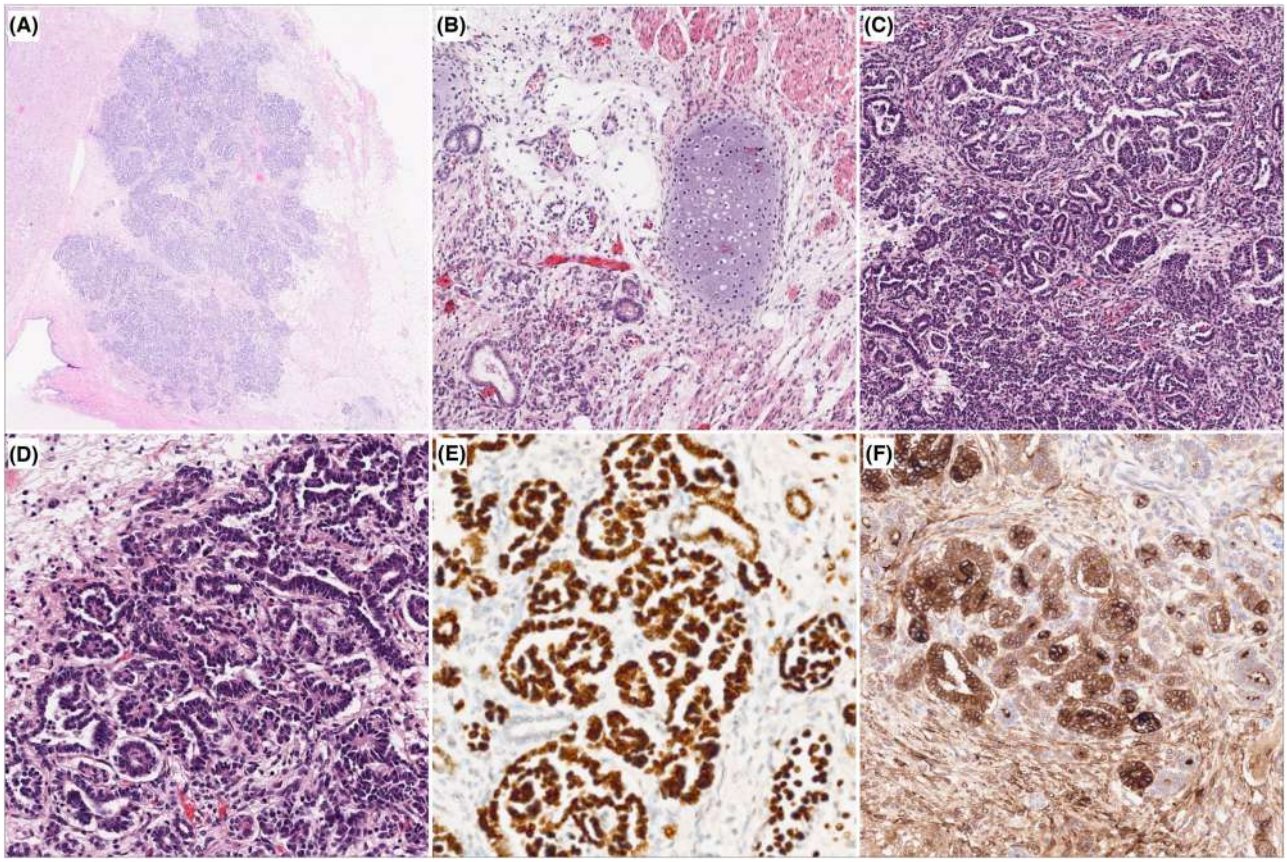

Figure 1. Representative images of a teratoma-associated ovarian Wilms tumour (WT) (Case 2) showing nodular arrangements of "glandular" structures (**A**) adjacent to teratomatous elements, including cartilage (**B**). The glandular structures consist of uniform cells with regular nuclei, scant cytoplasm, and low-mitotic activity (**C**,**D**). The glandular structures show diffuse nuclear positivity for WT1 (**E**) and diffuse cytoplasmic and membranous positivity for CD10 (**F**).

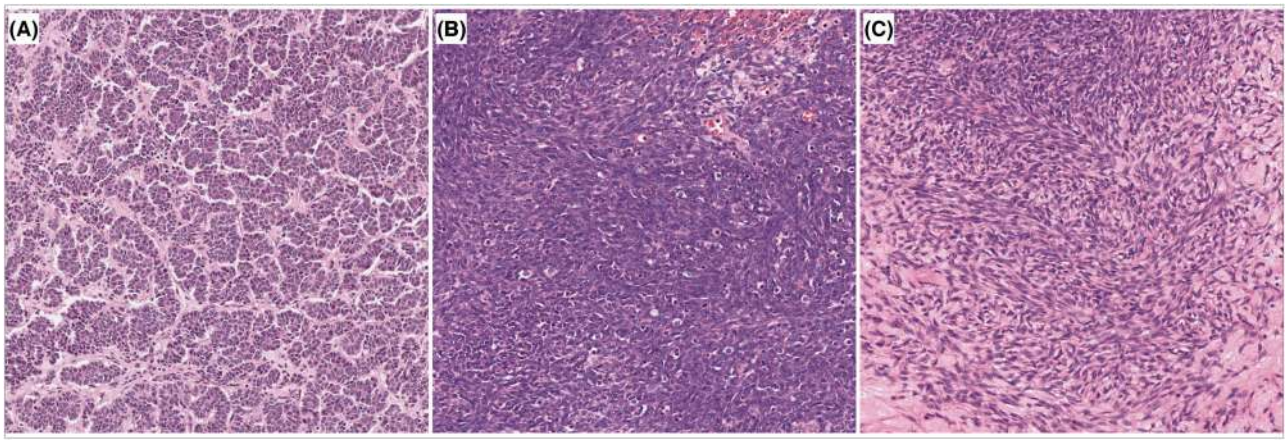

Figure 2. Representative images of a teratoma-associated ovarian Wilms tumour (Case 3) showing cells with various architectural growth patterns, including glands/tubules arranged in a nested pattern (**A**), as well as a diffuse blastemal (**B**), and spindle cell component (**C**).

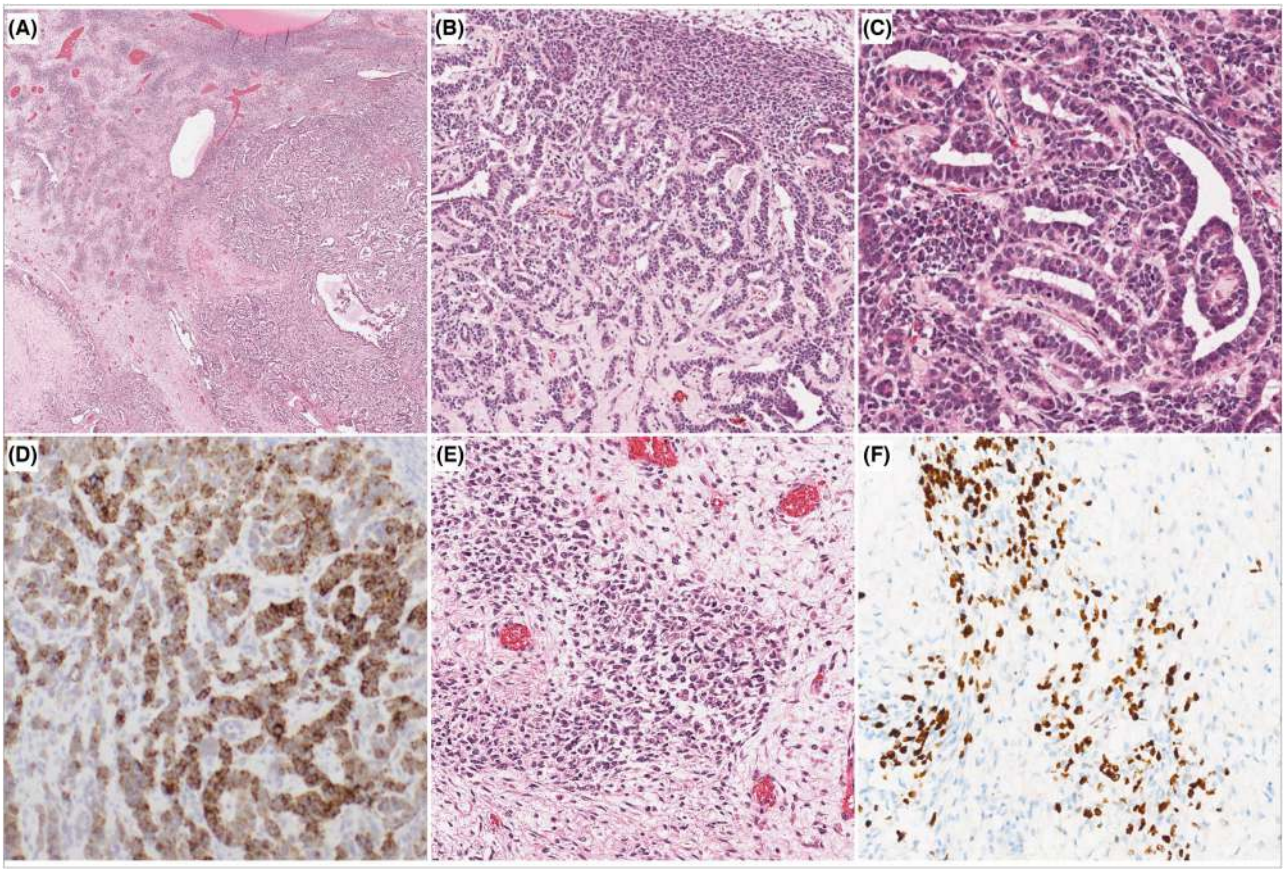

Figure 3. Representative images of a so-called pure ovarian Wilms tumour representing a morphological variant of Sertoli Leydig cell tumour (Case 4) displaying lobulated architecture with cellular areas separated by an oedematous stroma (**A**,**B**). The tumour is composed of nests, cords, and tubules (**C**), which are diffusely positive for inhibin (**D**). There are also focal areas of rhabdomyosarcomatous differentiation with cellular nests of tumour cells with hyperchromatic nuclei and scant cytoplasm (**E**) exhibiting focal positivity for myogenin (**F**).

11P15 ICR1 AND ICR2 METHYLATION

Analysis of the methylation levels of the ICR1 and ICR2 region of 11p15 suggested retention of imprinting in both pure WTs and indicated loss of heterozygosity with hypomethylation of the ICR1 region in two of three teratoma-associated WTs (Cases 2 and 3) (Figure 4G). The third teratoma-associated WT (Case 1) showed methylation levels suggesting retention of imprinting.

GENOME-WIDE DNA METHYLATION PATTERNS

Genome-wide DNA methylation analysis showed that the teratoma-associated and pure ovarian WTs were defined by diverging DNA methylation profiles, distinct from renal WTs (Figure 5). While both pure WTs were assigned to the cluster of sarcomas with *DICER1* alteration (SARC DICER1), two of three teratoma-associated WTs (Cases 1 and 2) were assigned to a cluster that included mature cystic ovarian teratomas and low-grade mesenchymal tumours with *DICER1*-alterations (LGMT DICER1). The third teratoma-associated WT (Case 3) clustered in a neighbouring cluster with miscellaneous tumours, including various uterine sarcoma types.

REVIEW OF PRIOR REPORTS OF OVARIAN WILMS TUMOUR

There have been 13 previously reported cases of primary ovarian WT[15–26] and, including the cases detailed herein, 16 altogether (see Table 1), since two of the cases we report have been reported previously.[23] Patients ranged in age from 1 to 56 years. All but one neoplasms were unilateral (the bilateral neoplasm was Case 5 in the current study, as detailed previously). Ten cases have been pure ovarian WT (including two in the current study) and six arose in a teratoma (including three in the

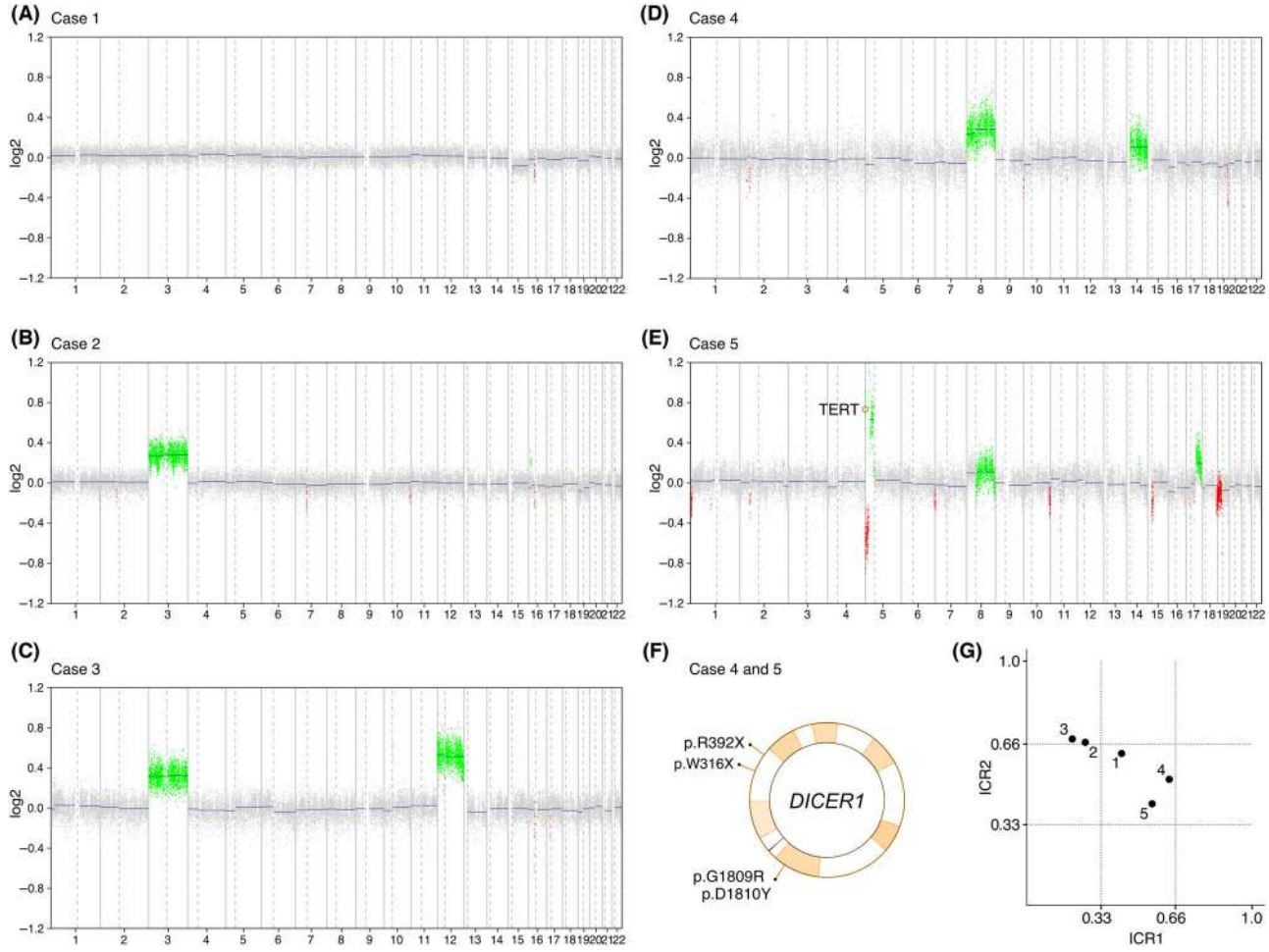

Figure 4. Copy number variation (CNV) profiles of three teratoma-associated Wilms tumour (WT) harbouring only a few CNVs, among them gains of chromosomes 3 and 12 (**A**–**C**), as well as two so-called pure ovarian WTs representing morphological variants of Sertoli Leydig cell tumour, showing gain of chromosome 8 in both tumours and amplification of the TERT locus in one case (**D**,**E**). **F:** *DICER1* PVs identified in Cases 4 and 5. **G:** DNA methylation levels for imprint control regions ICR1 (IGF2/H19) and ICR2 (KCNO1/CDKN1C), indicating loss of heterozygosity with hypomethylation of the ICR1 region in Cases 1 and 2, and retained heterozygosity in Cases 4 and 5. Case 3 shows a borderline heterozygous signature.

current study). One of the pure ovarian WTs previously reported was associated with a component of juvenile granulosa cell tumour, but herein this is included in the group of pure tumours, since there were no teratomatous elements.[17] The median age of patients with pure ovarian WTs was 16 years (range: 1–56 years) and for teratoma-associated WTs was 32 years (range: 28–38 years); this difference was not statistically significant ($P = 0.1$).

Discussion

We report five tumours that were originally designated primary ovarian WT. Given the morphological, immunohistochemical, and molecular features, we suggest that the three teratoma-associated neoplasms represent bona-fide WT, but the two pure cases represent moderately to poorly differentiated SLCT (*DICER1*-associated) with heterologous rhabdomyosarcomatous elements, resulting in close morphological mimicry of WT.

SLCT is an uncommon ovarian neoplasm. Well-differentiated SLCT is typically composed of a population of Sertoli cells arranged in tubules, along with Leydig cells with abundant eosinophilic or clear cytoplasm.[33] Although rare, these cases are relatively straightforward to diagnose, given their characteristic morphology and positivity with sex cord markers and they are not associated with *DICER1* PVs.[34,35] In

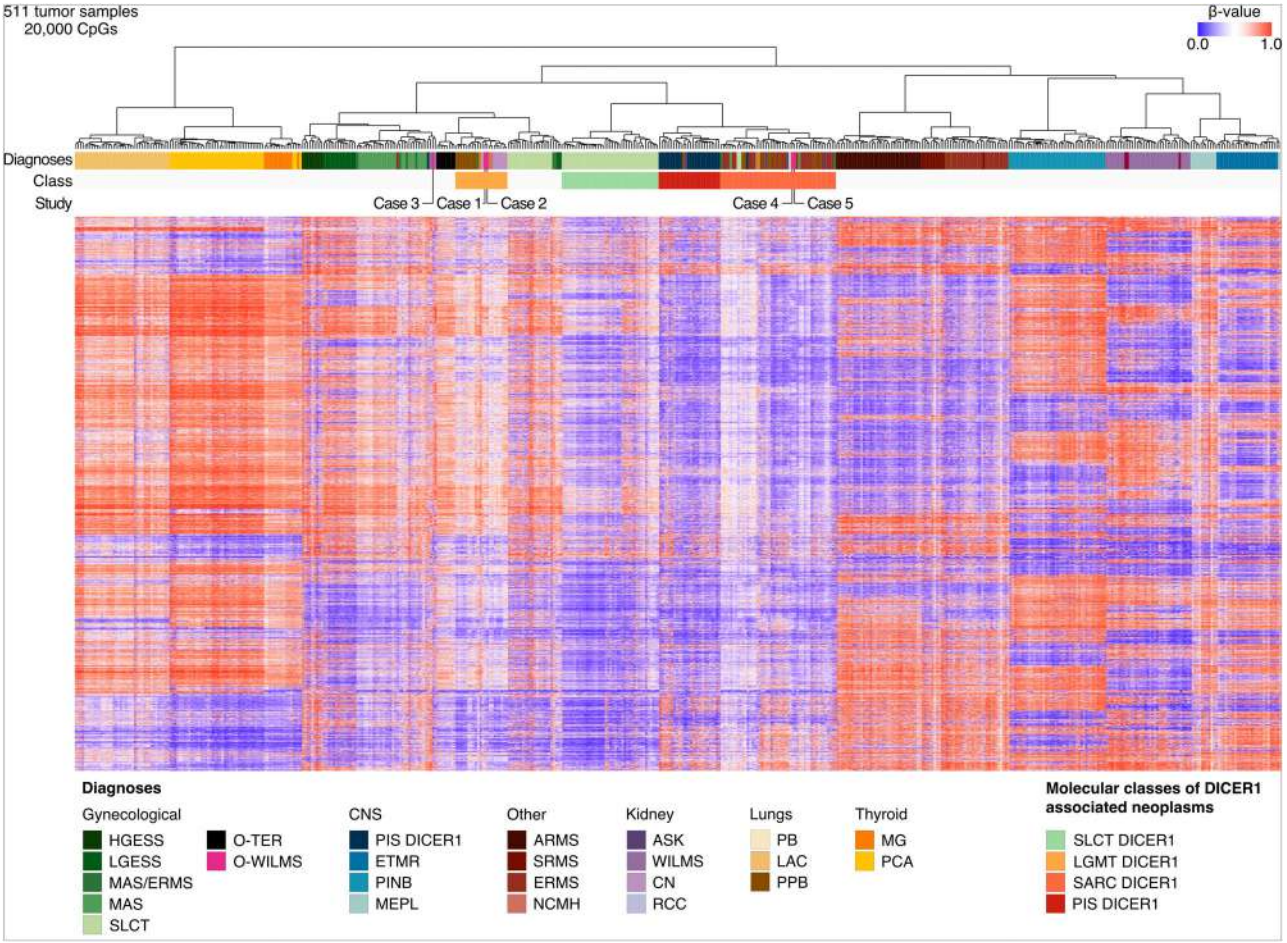

Figure 5. Unsupervised hierarchical clustering (Euclidean ward) of the 20,000 most differentially methylated CpGs in ovarian WTs (O-WILMS), ovarian cystic teratoma (O-TER), and a previously reported, large cohort of *DICER1*-associated neoplasms[28] by DNA methylation analysis. Samples are coloured according to their institutional diagnoses and belonging to molecular classes of *DICER1*-associated mesenchymal neoplasms.

contrast, moderately to poorly differentiated SLCTs are very commonly associated with somatic and/or germline *DICER1* PVs (patients with germline PVs have DICER1 syndrome) and represent a fundamentally different tumour type to well-differentiated SLCT.[36] Moderately and poorly differentiated SLCTs form part of a spectrum, with a wide variation in morphology and the differential diagnosis may be wide. The combination of Sertoliform tubules, nests, and cords (resembling "epithelial" formations) together with solid areas (resembling blastema or stromal elements) and even truly sarcomatous elements exhibiting rhabdomyoblastic differentiation may overlap significantly with WT; features in favour of SLCT include the presence of a component of Leydig cells and positive staining with sex cord markers, while features in favour of WT include glomeruloid structures, an absence of Leydig cells, and negative staining with sex cord markers. In both cases in our study, which were initially reported as pure ovarian WT (one of which has been previously reported[23]), there were rhabdomyosarcomatous elements, positive staining with sex cord markers (inhibin and calretinin positive in Case 4; SF1 and FOXL2 positive in Case 5, although inhibin was negative) and *DICER1* PVs were identified on molecular testing. On reviewing these cases, we also felt the morphology was in keeping with moderately to poorly differentiated SLCT, with close mimicry of WT. Rhabdomyosarcomatous elements are uncommon but well-described in moderately to poorly differentiated SLCT[37]; in one of the cases we report, there were also heterologous cartilaginous elements. In contrast, the WTs associated with teratomas were negative for the sex cord

marker inhibin and were *DICER1* wildtype. Although SLCT are usually positive for the most commonly used sex cord markers (inhibin, calretinin, FOXL2, SF1), one or more of these markers may be negative in individual cases. Expression of these markers in renal WTs has rarely been studied, but in one study all 45 WTs were inhibin-negative.[38] While some renal WTs harbour defects in genes associated with miRNA biogenesis, including *DICER1* PVs in a small subset,[3] we believe the presence of these alterations in an ovarian tumour with morphology compatible with SLCT, argues for the diagnosis of SLCT. As far as we are aware, *DICER1* PVs have not been looked for in extrarenal, including ovarian, WT.

Although there was no statistically significant age difference between patients with pure and teratoma-associated WT in our literature review, the age at diagnosis of pure ovarian WTs (median 16 years) is similar to that of SLCT. While renal WT usually affects young children, *DICER1*-associated SLCT typically arises in adolescentes and young adults.[1,34,39]

Our hypothesis was further supported by the results of DNA methylation analysis. Cancer cells harbour DNA methylation profiles that reflect both the cell of origin and somatically acquired DNA methylation changes.[40] We have previously shown that this technique allows the tracing of the distinct cellular origins of various cancers in the gynaecological tract.[41–43] In the current study, we show that teratoma-associated ovarian WTs mostly share a DNA methylation cluster with mature cystic teratomas, which is distinct from primary renal WT (including three renal WT with *DICER1* alterations). Furthermore, the two so-called pure ovarian WTs clustered with the recently described DNA methylation cluster of SARC DICER1, which is distinct from both the clusters of primary renal WT and conventional *DICER1*-associated SLCT.[28] These results are in line with previous findings that SLCT displaying a DNA methylation signature closely resembling that of SARC DICER1 exhibit heterologous elements, such as rhabdomyosarcoma.[28] This is likely to reflect the fact that in both cases of so-called pure ovarian WT we report (as discussed, reclassified as moderately to poorly differentiated SLCT), there were areas of rhabdomyosarcoma. Moreover, in one of these two cases, the tumour block used for molecular testing was predominantly composed of rhabdomyosarcomatous elements. Interestingly, this hypothesis is further supported by the findings of our CNV analysis, where we identified gain of chromosome 8 in both pure ovarian WTs, a feature characteristic of SARC DICER1.[28] Another novel finding in our study is that, as far as we are aware, this is the first report of an SLCT harbouring a *TERT* amplification.

As mentioned above, renal WT frequently shows a loss of imprinting at the HA19/IGF2 locus, which is associated with upregulation of IGF2. Analysis of the HA19/IGF2 imprinting region did not show loss of imprinting in any of the cases studied herein. However, while the two pure ovarian WTs we report showed retention of imprinting, two of the three teratoma-associated WTs showed loss of heterozygosity with hypomethylation of the ICR1 region, which is in keeping with these neoplasms being of germ-cell origin. Ovarian teratomas are believed to develop from a retained oocyte and show loss of heterozygosity, a feature which can be used to identify somatic ovarian neoplasms of teratomatous origin, for example, some ovarian mucinous neoplasms.[44]

The panel-based DNA sequencing did not identify mutations sometimes found in renal WT, such as *CTNNB1*, *TP53*, *BCOR*, *MYCN*, *DROSHA*, or *DGCR8*, in the three teratoma-associated WTs.[3] However, our study is limited in that the panel did not include *WT1* or *WTX*, the two genes most frequently altered in WT. Analysis of CNVs showed gain of chromosome 3 in two of three teratoma-associated WTs and one also exhibited chromosome 12 gain. Renal WTs have been reported to harbour various CNVs, among them gains of chromosome 8 and 12 in a subset (18%).[45] Of note, we did not identify chromosomal aberrations in any of the seven mature cystic teratomas of the ovary analysed for comparative DNA methylation analyses (data not shown).

Given our findings that the two pure ovarian WTs in our study actually represent moderately to poorly differentiated SLCT and are associated with *DICER1* PVs, it is possible that some of the previously reported ovarian WTs not associated with a teratoma represent SLCTs. In fact, one of the previously reported cases was associated with a component of juvenile granulosa cell tumour[17]; we suspect this may represent a sex cord-stromal tumour of some type with foci resembling WT, since the combination of a sex cord-stromal tumour and a WT would be most unusual. Expanding on this theme, WT-like morphology may occur in other sex cord tumours of the gynaecological tract, such as uterine tumour resembling ovarian sex cord tumour (UTROSCT), where a tubular/glomeruloid component may be present, as well as spindle cell foci.[46,47] It is theoretically possible that some cases reported as primary uterine WT represent UTROSCT. Therefore, we feel a diagnosis of primary pure WT of the gynaecological tract should only be considered if other differentials, especially SLCT and UTROSCT, are excluded; if

necessary, by means of molecular testing. However, it is also possible that occasional true pure (not associated with teratoma) WTs occur in the ovary and other sites in the gynaecological tract, either representing total overgrowth of the WT elements within a teratoma or arising *de novo*. Recently, Willis *et al*. reported on a pure extrarenal anaplastic WT, arising in the lower abdomen and upper pelvis of a 10-year-old boy that harboured various chromosomal gains and losses, as well as a PV in *TP53*.[48] Interestingly, their whole-exome sequencing did not identify PVs in *DICER1*.

The cases we report herein highlight the value of specialist pathology review and integration of morphological, immunohistochemical, and molecular parameters to help avoid the risk of rare tumours being erroneously associated with a specific molecular event. If the two pure WTs in the series we report (one of which has been previously published) had not been reclassified as SLCTs, ovarian WT could have erroneously been added to the list of rare tumour types associated with *DICER1* PVs and potentially DICER1 syndrome. Scenarios such as this have been highlighted previously in an editorial published by two of us (W.G.M., W.D.F.).[49] We suggest that when a diagnosis of a pure ovarian WT is made, this should result in referral for a specialist opinion to exclude an SLCT and tumour testing to look for a *DICER1* PV; in the presence of the latter, referral to genetics is advised to exclude DICER1 syndrome.

In summary, we report five cases of so-called primary ovarian WT, including two pure and three teratoma-associated. The two pure WTs contained *DICER1* PVs, while the three teratoma-associated neoplasms were *DICER1* wildtype. Based on the morphological features, immunophenotype, and molecular findings (*DICER1* PVs, copy numbe,r and DNA methylation profiles), we suggest that some cases diagnosed as pure primary ovarian WT represent morphological variants of SLCT, while cases arising in teratomas likely represent true WT.

Author contributions

Felix K F Kommoss, W Glenn McCluggage, and William D Foulkes conceived the project. Felix K F Kommoss and William D Foulkes coordinated data generation. Felix K F Kommoss and Andreas von Deimling performed DNA methylation and panel-based sequencing analysis. Felix K F Kommoss, Anne-Sophie Chong, Maria Apellaniz-Ruiz, and William D Foulkes interpreted data. Felix K F Kommoss, Gordan Vujanic, and W Glenn McCluggage reviewed histology. Data were visualized by Felix K F Kommoss. Gulisa Turashvili, Kay Park, Krisztina Hanley, Elvis Terci Valera, and W Glenn McCluggage provided tumours samples and meta data. Felix K F Kommoss, W Glenn McCluggage, and William D Foulkes wrote the article with input from all authors. The final article was reviewed and approved off by all coauthors.

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Conflict of interest

AvD is the recipient of a research grant from Illumina Inc. No other conflicts of interest are declared.

Data availability statement

Data available on request from the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of genes that were included in targeted DNA sequencing.

Table S2. Variants of unknown significance called by targeted DNA sequencing.