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ARTICLE

Genomic profiling of primary and recurrent adult granulosa cell tumors of the ovary

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Abstract

Adult-type granulosa cell tumor (aGCT) is a rare malignant ovarian sex cord-stromal tumor, harboring recurrent *FOXL2* c.C402G/p.C134W hotspot mutations in 97% of cases. These tumors are considered to have a favorable prognosis, however aGCTs have a tendency for local spread and late recurrences, which are associated with poor survival rates. We sought to determine the genetic alterations associated with aGCT disease progression. We subjected primary non-recurrent aGCTs ($n = 7$), primary aGCTs that subsequently recurred ($n = 9$) and their matched recurrences ($n = 9$), and aGCT recurrences without matched primary tumors ($n = 10$) to targeted massively parallel sequencing of ≥410 cancer-related genes. In addition, three primary non-recurrent aGCTs and nine aGCT recurrences were subjected to *FOXL2* and *TERT* promoter Sanger sequencing analysis. All aGCTs harbored the *FOXL2* C134W hotspot mutation. *TERT* promoter mutations were found to be significantly more frequent in recurrent (18/28, 64%) than primary aGCTs (5/19, 26%, $p = 0.017$). In addition, mutations affecting *TP53*, *MED12*, and *TET2* were restricted to aGCT recurrences. Pathway annotation of altered genes demonstrated that aGCT recurrences displayed an enrichment for genetic alterations affecting cell cycle pathway-related genes. Analysis of paired primary and recurrent aGCTs revealed that *TERT* promoter mutations were either present in both primary tumors and matched recurrences or were restricted to the recurrence and absent in the respective primary aGCT. Clonal composition analysis of these paired samples further revealed that aGCTs display intra-tumor genetic heterogeneity and harbor multiple clones at diagnosis and relapse. We observed that in a subset of cases, recurrences acquired additional genetic alterations not present in primary aGCTs, including *TERT*, *MED12*, and *TP53* mutations and *CDKN2A/B* homozygous deletions. Albeit harboring relatively simple genomes, our data provide evidence to suggest that aGCTs are genetically heterogeneous tumors and that *TERT* promoter mutations and/or genetic alterations affecting other cell cycle-related genes may be associated with disease progression and recurrences.

Introduction

Adult-type granulosa cell tumors (aGCTs) of the ovary are a rare form of ovarian cancer (<5%) characterized by rather simple genomes and by the presence of recurrent *FOXL2* p.C134W somatic missense mutations in ≥97% of cases [1–3]. Despite their indolent growth and overall good prognosis, recurrences occur in 10–30% of aGCTs [4–7]. These rare tumors exhibit long latency periods, with a median time to first recurrence of 4–7 years [4–6], with late recurrences reported up to 20–30 years following initial diagnosis [8]. Hence, the natural history of aGCTs poses therapeutic challenges, requiring long-term follow-up [5, 9, 10].

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Somatic *TERT* promoter mutations (C228T and C250T), associated with telomerase activation, have been reported at high frequency in cancers (12% overall) [11], including gynecologic malignancies such as ovarian clear cell carcinomas (16%) [12, 13]. In addition, recent studies have reported a significantly higher frequency of the *TERT* C228T promoter hotspot mutations in recurrent (41–67%) than in primary aGCTs (22–29%) [14, 15]. Patients with primary aGCTs harboring *TERT* promoter hotspot mutations were also found to have a significantly worse overall survival than those with wild-type *TERT* [14]. Furthermore, *KMT2D* inactivating mutations have been reported to be associated with recurrences in aGCTs [16].

Although *TERT* promoter and *KMT2D* mutations appear to provide the basis for recurrences in a subset of aGCTs, the genetic basis of the clinical behavior in a substantial proportion of recurrent aGCTs has not been defined to date. Here, we sought to compare the repertoire of somatic genetic alterations of (1) primary aGCTs that did not recur within at least four years of follow-up, (2) primary aGCTs that recurred and (3) aGCT recurrences. Furthermore, given that samples from primary aGCTs and their respective relapses were available, we have also compared the *TERT* mutation status between paired primary and recurrent aGCTs, and investigated whether aGCTs would display intra-tumor genetic heterogeneity and if specific genetic alterations would be selected during progression from primary tumor to recurrence.

Materials and methods

Subjects and samples

Following approval by the Institutional Review Board (IRB) of the authors' institutions, we retrieved representative hematoxylin and eosin and unstained tissue sections from formalin-fixed paraffin embedded aGCTs from Memorial Sloan Kettering Cancer Center (MSKCC, NY, USA), Fudan University Cancer Center (Shanghai, China), Hospital Universitario de Bellvitge (Barcelona, Spain), Hospital Universitario Arnau de Vilanova (Lleida, Spain), and Cleveland Clinic (OH, USA). Patient consents were obtained according to the protocols approved by the local IRBs of the authors' institutions. Samples were anonymized prior to analysis. Samples from 40 cases were reviewed by eight pathologists (RB, SES, MV, CGP, XM-G, BPR, JSR-F, and DFD) following the criteria put forward by the World Health Organization [17]. Only cases where a consensus diagnosis of aGCT was achieved were included in this study ($n = 38$), and two cases were excluded. Patients were defined as having primary non-recurrent aGCTs if no recurrence was detected within at least 48 months of follow-up, based on the median and range of time-to-recurrence of aGCTs previously reported [4–6]. Our final series included 47 samples from 38 patients: (1) primary non-recurrent aGCTs ($n = 10$), (2) primary aGCTs that subsequently recurred ($n = 9$) and their matched recurrences ($n = 9$ from nine patients), and aGCT recurrences without matched primary tumors ($n = 19$; Table 1 and Supplementary Table S1). Surgical staging was performed according to the 2014 International Federation of Gynecology and Obstetrics system [18].

Targeted capture massively parallel sequencing using the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) [11, 19] was performed on tumor-normal pairs from 26 patients, including seven primary non-recurrent aGCTs, nine primary recurrent aGCTs and their matched recurrences, and ten aGCT recurrences. The remaining three primary non-recurrent aGCTs and nine aGCT recurrences from 12 additional patients did not yield sufficient DNA for MSK-IMPACT sequencing, and, therefore, were subjected to Sanger sequencing analyses to assess the presence of *FOXL2* and *TERT* promoter hotspot mutations (see below; Supplementary Table S1).

Microdissection and DNA extraction

Representative sections of tumor tissue samples were microdissected under a stereomicroscope (Olympus SZ61) to ensure a tumor cell content >80%, as previously described [20, 21]. DNA from tumor and matched normal tissues was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturers' instructions. DNA of sufficient quantity/ quality was obtained for Sanger sequencing for all 38 cases (47 samples) and for targeted massively parallel sequencing for 26 cases (35 samples; see below).

Assessment of *FOXL2* and *TERT* promoter hotspot mutations by Sanger sequencing

PCR amplification of *FOXL2* and *TERT* promoter hotspot loci was performed using the AmpliTaq Gold 360 Master Mix kit (Life Technologies, ThermoFisher Scientific) using previously described primers [22, 23]. PCR fragments were cleaned using ExoSAP It (ThermoFisher Scientific) and Sanger sequenced as previously described [22].

Targeted massively parallel sequencing

Microdissected tumor and matched normal DNA samples from primary non-recurrent aGCTs ($n = 7$), primary recurrent aGCTs ($n = 9$), and matched recurrences ($n = 9$), and aGCT recurrences ($n = 10$) were subjected to

Table 1 Clinico-pathological features of ovarian adult-type granulosa cell tumors patients included in this study.

	Primary non-recurrent aGCTs ($n = 10$)	p value[a]	Primary recurrent aGCTs ($n = 9$)	aGCT recurrences without matched primary tumors ($n = 19$)
Median age (years)	52.5 (34–87)	0.74	62 (41–83)	56 (34–89)
Median tumor size (cm)	6.0 (1.7–14.0)	0.07	12.0 (3.0–25.0)	4.7 (0.7–17.0)
Surgical stage diagnosis				
I	10 (100%)	0.087	6 (67%)	N/A
II	0 (0%)		1 (11%)	
III	0 (0%)		2 (22%)	
IV	0 (0%)		0 (0%)	
Menopausal status				
Pre	2 (20%)	0.40	4 (44%)	5 (26%)
Post	6 (60%)		5 (56%)	9 (48%)
Unknown	2 (20%)		0 (0%)	5 (26%)

aGCT adult-type granulosa cell tumor, N/A not applicable.

[a]Primary non-recurrent aGCTs versus primary recurrent aGCTs, using Student's t test and Fisher's exact test.

MSK-IMPACT sequencing of 410–468 cancer-related genes, as previously described [19, 24]. Sequencing data were processed and analyzed as previously described [21, 24]. In brief, reads were aligned to the reference human genome GRCh37 using the Burrows–Wheeler Aligner (v0.7.15) [25]. Local realignment, duplicate removal, and base quality recalibration were performed using the Genome Analysis Toolkit (v3.7) [26]. Somatic single-nucleotide variants (SNVs) were detected by MuTect (v1.0) [27], and small insertions and deletions (indels) were detected using a combination of Strelka (v2.0.15) [28], VarScan2 (v2.3.7) [29], Lancet (v1.0.0) [30], Scalpel (v0.5.3) [31], and Platypus [32]. Pathogenic mutations were defined as variants that were deleterious and/or mutational hotspots. In addition, mutations that were identified in the primary or recurrent tumor from a given patient were subsequently interrogated in the matched respective primary or recurrent sample using mpileup from SAMtools mpileup (version 1.2 htslib 1.2.1) [33]. Allele-specific copy number alterations (CNAs) and loss of heterozygosity (LOH) were defined using FACETS [34], as previously described [20, 21]. The fraction of the genome altered was computed from the CNAs obtained from FACETS. The cancer cell fraction of each mutation was determined using ABSOLUTE (v1.0.6) [35], as previously described [20, 24]. A combination of mutation function predictors was employed to define the potential functional impact of each missense SNV, as previously described [20, 21, 36]. Mutational hotspots were assigned according to Chang et al. [37]. The median depth of coverage of tumor and normal samples was 518x (range 120x–1223x) and 366x (range 117x−510x), respectively (Supplementary Table S2).

Pathway analyses

A MSigDB and DAVID pathway analysis was performed based on genes affected by nonsynonymous pathogenic somatic mutations, amplifications, or homozygous deletions in primary non-recurrent aGCTs ($n = 7$), primary aGCTs ($n = 9$) and matched recurrences ($n = 9$) and aGCT recurrences ($n = 10$) [38]. Pathways found to be significantly enriched ($p < 0.01$) were selected as previously reported [39]. In addition, a mutual exclusivity analysis was performed using combinations of mutually exclusive alterations (CoMET) with the use of a pair-wise Fisher's exact test to detect the presence of significant pairs of genes [40].

Mutation-based tree construction

The mutation-based trees of the primary and matched aGCT recurrences were constructed using Treeomics [41] based on all synonymous and nonsynonymous mutations identified, as previously described [24]. For these analyses, a given mutation was considered "shared" if it was present in both the primary and matched aGCT recurrences. We defined mutations present only in the primary tumor or only in the recurrence as "private to the primary aGCT" and "private to the aGCT recurrence", respectively.

Statistical analysis

The frequencies of somatic mutations affecting cancer genes in primary and recurrent aGCTs were compared using two-tailed Fisher's exact test. Mutual exclusivity was tested using two-tailed Fisher's exact test. The fraction of genome affected by CNAs in primary and recurrent aGCTs was

evaluated using a Mann-Whitney *U* test. All *p* values were two-tailed, and 95% confidence intervals were adopted for all analyses.

Results

Clinico-pathologic features of primary and recurrent aGCTs

Our series encompassed aGCTs from 38 patients, including 10 patients with non-recurrent aGCTs (primary non-recurrent aGCTs) and 28 patients with recurrent disease. Of the 28 patients with recurrent disease, we analyzed samples from the primary tumor (primary recurrent aGCT) and matched recurrences from nine patients, and from the recurrent tumors only from 19 patients (aGCT recurrences; Supplementary Table S1).

The median age of patients at aGCT diagnosis was 52.5 years (range 34–87 years) in primary non-recurrent aGCTs ($n = 10$), 62 years (range 41–83 years) for patients with primary aGCT that developed recurrences ($n = 9$) and 56 years (range 34–89 years) for patients with aGCT recurrences without available matched primary tumors ($n = 19$). No significant differences in age at diagnosis were observed between primary non-recurrent aGCTs and primary aGCTs that recurred ($p = 0.74$, Student's *t* test, Table 1, Supplementary Table S1). All patients (10/10, 100%) with primary non-recurrent aGCTs had early stage disease (IA and IC) at the time of diagnosis, whereas 1 (11%) and two patients (22%) with primary aGCTs that recurred had stage II and stage III disease at the time of diagnosis, respectively. No significant differences in stage were observed between primary non-recurrent aGCTs and primary aGCTs that recurred ($p = 0.087$, Fisher's exact test), or other clinic-pathologic factors assessed (Table 1, Supplementary Table S1).

aGCT recurrences display distinct genomic profiles from primary aGCTs

Consistent with previous reports [2, 14, 15], all aGCTs analyzed in this study harbored *FOXL2* p.C134W missense mutations as defined by MSK-IMPACT and/or Sanger sequencing (Fig. 1a). In addition, we identified recurrent *TERT* promoter mutations by Sanger and/or MSK-IMPACT sequencing, affecting not only the previously described C228T hotspot locus but also the C250T locus (Fig. 1a). In these 38 aGCTs, a significantly higher frequency of *TERT* promoter mutations was observed in aGCT recurrences (18/28, 64%) than in primary non-recurrent and primary recurrent aGCTs (5/19, 26.3%, $p = 0.017$, Fisher's exact test, Fig. 1a, Table 2). While there was a stepwise increase in the frequency of *TERT* promoter mutations from primary non-recurrent aGCTs (2/7; 29%) to primary recurrent aGCTs (3/ 9; 33%) and aGCT recurrences (10/19; 52%; Fig. 1b), no significant differences in the *TERT* hotspot mutation frequency between primary aGCTs with ($n = 9$) and without ($n = 10$) recurrences were found (33 vs. 20% $p = 0.434$, Fisher's exact test; Fig. 1a, Table 2).

Analysis of the somatic mutation data obtained from MSK-IMPACT further revealed that aGCTs overall displayed a relatively low mutation burden, with a median of 3 (range 1–7) somatic mutations in the genes analyzed, of which two (range 1–6) were nonsynonymous (Supplementary Table S3). Despite the numerically higher number of somatic mutations identified in aGCT recurrences (median 3, range 2–7), no statistically significant differences in the mutational burden were observed when compared with primary non-recurrent aGCTs (median 2, range 1–5; $p = 0.47$, Fisher's exact test) or primary recurrent aGCTs (median 2, 1–5; $p = 0.86$, Fisher's exact test). Recurrent mutations affecting known cancer-related genes such as *GNAQ* and *KMT2C* were identified, however none of these was statistically different between the groups (Fig. 1b). Also, inactivating *KMT2D* mutations, which have been reported to be associated with recurrence in aGCTs [16], were only found in a single sample in our study and affected a primary non-recurrent aGCT (aGCT35-P; Fig. 1b, Supplementary Table S3). In contrast, we identified *TP53* pathogenic mutations only in aGCT recurrences (16%). Of note, the *TP53*-mutant aGCT recurrences did not harbor *TERT* promoter mutations (Fig. 1b); however formal mutual exclusivity analysis using CoMET showed that *TERT* promoter and *TP53* mutations were not significantly mutually exclusive in aGCT recurrences ($p = 0.062$, Fisher's exact test, Fig. 2a), likely due to the low number of samples/*TP53* mutations. We further found a subclonal pathogenic mutation affecting *TET2* (5%) that was restricted to an aGCT recurrence lacking a *TERT* promoter mutation (aGCT82-R; Fig. 1b, Supplementary Fig. S1), and *STAG2* and *IDH1* pathogenic mutations in primary recurrent aGCTs (11%) and their matched aGCT recurrences (5%) but not in primary non-recurrent aGCTs (0%) (Fig. 1b). None of these differences reached statistical significance, however, likely due to the small sample size.

When assessing the CNAs in the aGCTs subjected to MSK-IMPACT sequencing, we found primary aGCTs and aGCT recurrences to display overall similar copy number profiles with similar fractions of the genome altered (primary non-recurrent aGCTs, median 11%, range 5–48%; primary recurrent aGCTs, median 9%, range 0–91%; aGCT recurrences, median 8%, range 0–52%, Fig. 2b). Nevertheless, a numerically higher frequency of *CDKN2A/B* homozygous deletions was observed in aGCT recurrences (3/19, 16%) and in primary recurrent aGCTs (1/9, 11%) as

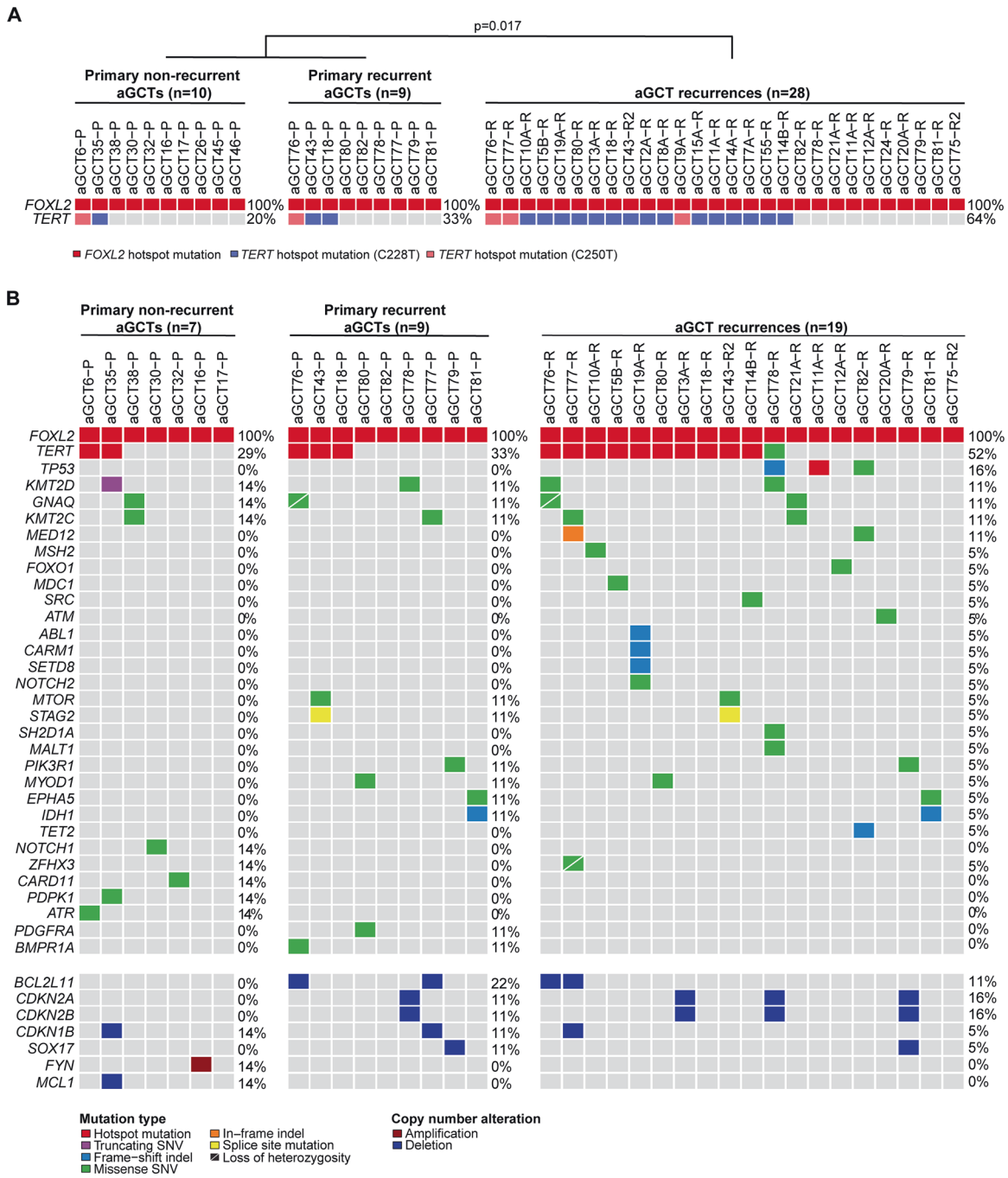

Fig. 1 Landscape of somatic genetic alterations in cancer-related genes in primary and recurrent adult-type granulosa cell tumors of the ovary. a *FOXL2* and *TERT* promoter hotspot mutations in 38 adult-type granulosa cell tumors of the ovary (aGCTs) subjected to MSK-IMPACT and/or Sanger sequencing. Statistical significance was evaluated by Fisher's exact test. **b** Nonsynonymous somatic mutations, amplifications, and homozygous deletions identified in primary adult-type granulosa cell tumors (aGCT) using MSK-IMPACT sequencing, including those without (non-recurrent, $n = 7$, left) and with (recurrent, $n = 9$, middle) subsequent recurrences, and in aGCT recurrences ($n = 19$, right). Cases are shown in columns and genes in rows. Genetic alterations are color-coded according to the legend. Indel small insertion and deletion, SNV single-nucleotide variant.

compared with primary non-recurrent aGCTs (0%). Similarly, homozygous deletions of *BCL2L11* were also identified in primary recurrent aGCTs (2/9, 22%) and in aGCT recurrences (2/19, 11%) but not in primary non-recurrent aGCTs (Fig. 1b).

Recurrent aGCTs harbor somatic genetic alterations affecting the cell cycle pathway

Given the distinct genetic alterations observed in primary non-recurrent aGCTs and aGCT recurrences, we sought to

Table 2 *TERT* promoter mutational status in primary and recurrent adult-type granulosa cell tumors of the ovary.

Clinical presentation aGCTs	*TERT* promoter mutant, *n* (%)	*TERT* promoter wild-type, *n* (%)	*p* value[a]
Primary non-recurrent ($n = 10$)	2 (20%)	8 (80%)	0.434
Primary recurrent ($n = 9$)	3 (33%)	6 (67%)	
Primary non-recurrent and primary recurrent ($n = 19$)	5 (26%)	14 (74%)	**0.017**
Recurrences ($n = 28$)	18 (64%)	10 (36%)	

aGCT adult-type granulosa cell tumor.

[a]Fisher's exact test.

Bold values indicate statistical significance *p* values.

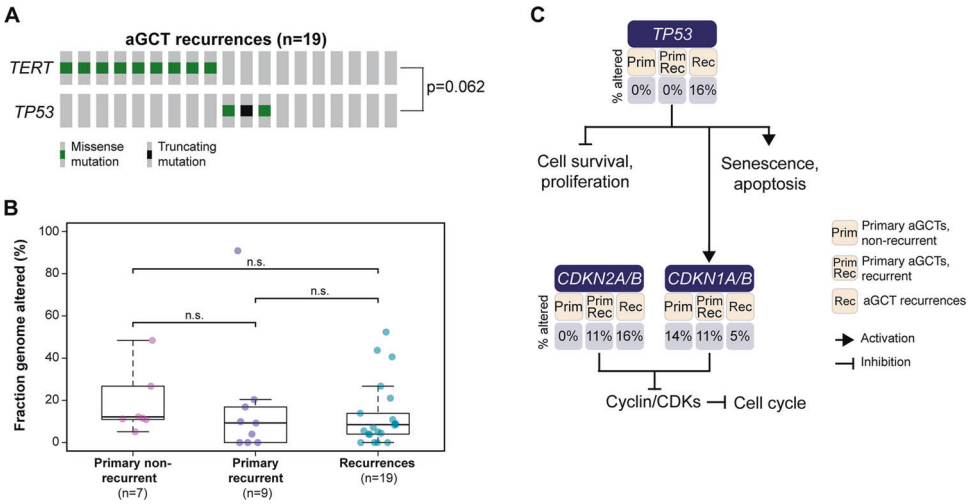

Fig. 2 Mutual exclusivity analysis, fraction of the genome altered and genetic alterations affecting the cell cycle pathway in primary and recurrent adult-type granulosa cell tumors of the ovary. **a** Mutual exclusivity analysis between *TERT* promoter hotspot mutations and *TP53* mutations in adult-type granulosa cell tumor (aGCT) recurrences. The type of mutations is color-coded according to the legend. Mutual exclusivity analysis was performed using combinations of mutually exclusive alterations (CoMET) and Fisher's exact test. **b** Fraction of the genome altered in primary non-recurrent aGCTs, primary recurrent aGCTs, and aGCT recurrences. **c** Frequency of loss-of-function somatic genetic alterations affecting genes in the canonical cell cycle pathway. Genes are depicted in blue rectangles, and the percentage of primary non-recurrent aGCTs (Prim), primary recurrent aGCTs (Prim Rec), and aGCT recurrences (Rec) altered is shown below each gene.

determine the signaling pathways that are enriched in aGCT recurrences. For this, we performed a pathway analysis using the genes that were either affected by nonsynonymous pathogenic somatic mutations, amplifications, or homozygous deletions. This analysis revealed that there was an enrichment in cell cycle pathway genes that were altered in aGCT recurrences but not in primary aGCTs (Supplementary Table S4). As mentioned above, pathogenic *TP53* alterations were solely found in aGCT recurrences (Fig. 2c, top), whereas *CDKN2A/B* homozygous deletions were identified in both aGCT recurrences and primary recurrent aGCTs but not in primary non-recurrent aGCTs (Fig. 2c, bottom left). In contrast, *CDKN1B* homozygous deletions were identified at different frequencies in primary non-recurrent aGCTs (14%), primary aGCTs that recurred (11%) and in aGCT recurrences (5%; Fig. 2c, bottom right). These findings suggest that in addition to the described *TERT* promoter mutations [14, 15] alterations in cell cycle-related genes and apoptosis might also play a role in the progression of aGCTs.

TERT promoter mutations in primary aGCTs and matched recurrences

To investigate the role of *TERT* promoter mutations in the progression of aGCTs, we next assessed the *TERT* promoter mutation status in primary aGCTs and their matched recurrences using MSK-IMPACT and Sanger sequencing. Of the nine pairs of primary and recurrent aGCTs included in this study, five harbored *TERT* mutations in at least one of the samples of a given patient based on MSK-IMPACT sequencing (Fig. 3). We found that in two cases (aGCT77 and aGCT80), the primary lesion lacked *TERT* promoter mutations, but that the matched aGCT recurrences harbored a clonal C250T mutation (aGCT77) or a subclonal C228T

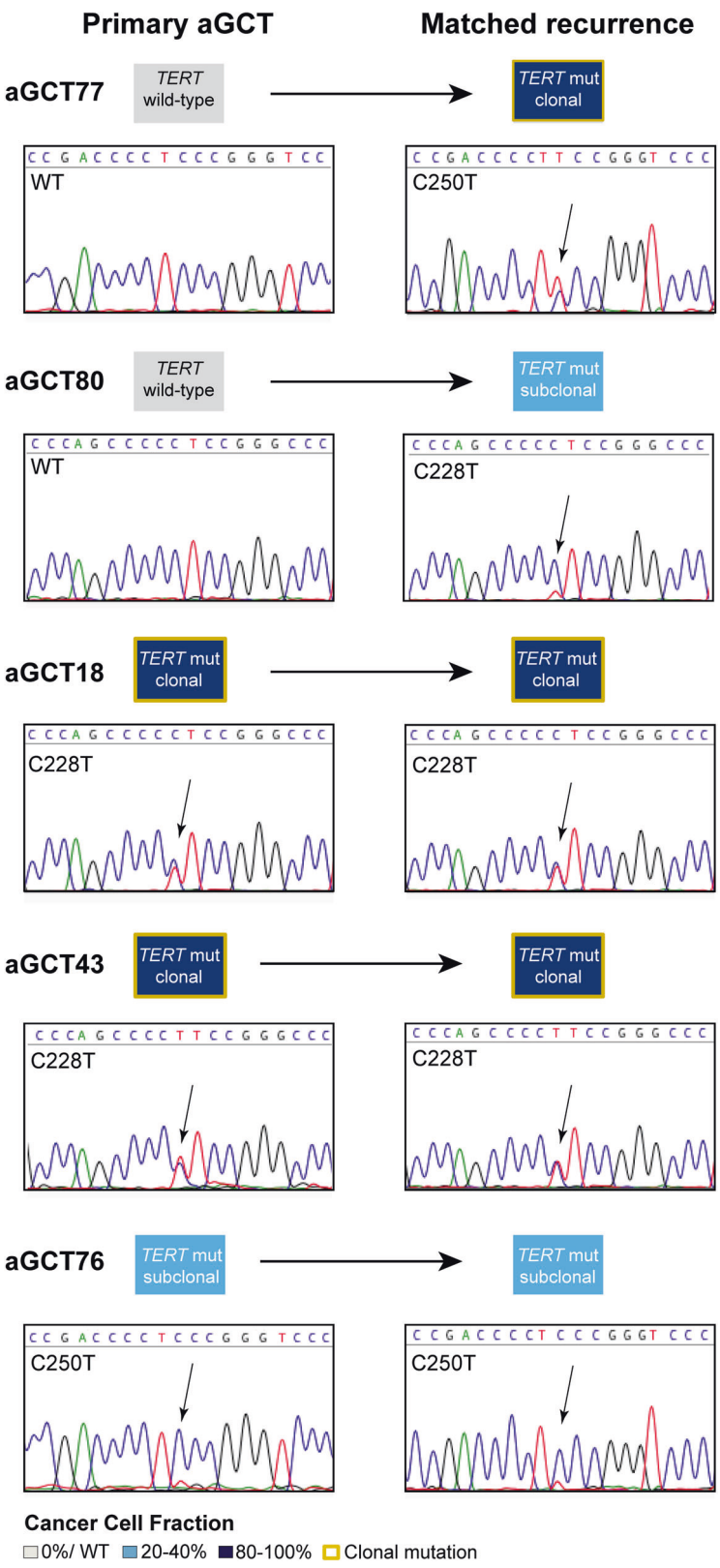

Fig. 3 *TERT* promoter hotspot mutations in paired primary aGCTs and recurrences. *TERT* promoter hotspot mutations and their clonality identified in primary aGCTs (left) and their matched recurrences (right) using targeted MSK-IMPACT sequencing. The *TERT* mutations were validated by Sanger sequencing and the electropherograms of all samples are shown. Cancer cell fractions are color-coded according to the legends and clonal mutations are depicted by a yellow box. Arrows in electropherograms indicate *TERT* promoter mutations.

mutation (aGCT80). This finding provides evidence to suggest that, in these cases, *TERT* mutations were either selected from a minor subclone not detected in the sequencing of the primary tumor or were acquired during disease progression. In contrast, the primary aGCT18 and aGCT43 both harbored clonal C228T *TERT* promoter

hotspot mutations, which were preserved in the respective recurrences. Finally, a subclonal C250T was found in both the primary and matched recurrence of case aGCT76 (Fig. 3).

Clonal composition analysis of paired primary and aGCT recurrences

To interrogate the genetic alterations in addition to *TERT* promoter mutations that might be associated with clinical progression of aGCTs, we performed a clonal composition analysis of the nine primary aGCTs and their matched recurrences. Our analyses revealed that both the primary aGCTs and their matched recurrences shared clonal mutations in *FOXL2* (p.C134W) in all nine cases analyzed (Fig. 4, Supplementary Fig. S2). Furthermore, primary tumors and their matched recurrences also shared somatic mutations affecting *KMT2C* (p.A1685S), *MYOD1* (p.S260F), *KMT2D* (p.C5481F), *PIK3R1* (p.W624R), and the *TERT* promoter (Fig. 4, Supplementary Fig. S2). We observed, however, that in a subset of cases, the recurrences acquired additional somatic mutations or CNAs not present in the primary aGCT: we identified clonal *MED12* (aGCT77, p.Q2076dup), clonal *SH2D1A* (aGCT78, p.P97S), and subclonal *TET2* (aGCT82, p.C1281Vfs*82) mutations restricted to the recurrences. Furthermore, two aGCT recurrences acquired alterations in cell cycle-related genes such as *TP53* mutations (aGCT78, p.F338Lfs*7; aGCT82, p.Y236H) or *CDKN2A/B* homozygous deletions (aGCT79), which were not detected in their respective primary tumors (Fig. 4).

Discussion

Despite their relatively indolent behavior, management of a subset of aGCTs remains challenging due to their unpredictable behavior and late relapses. Identification of markers predictive of disease recurrences/metastases has been the subject of considerable interest. Here we not only confirm the presence of *TERT* promoter hotspot mutations in aGCTs but also find that somatic genetic alterations affecting cell cycle progression and apoptosis-related genes may play a role in the progression from primary aGCTs to recurrences.

Recent studies have reported a highly recurrent somatic mutation (C228T) in the promoter region of *TERT* in aGCTs [14, 15]. In our study, in addition to the C228T mutation (80%), we also identified recurrent C250T *TERT* hotspot mutations in 20% of the aGCTs harboring *TERT* promoter mutations. Whilst *TERT* promoter mutations have been reported to be an early genetic event in several cancer types [42–44], in aGCTs one study has suggested that these mutations are a late event [14]. The overall frequency of *TERT* promoter hotspot mutations in our series was significantly higher in aGCT recurrences than in primary aGCTs. *TERT* promoter mutations have been reported to lead to increased *TERT* expression and telomerase activation, to overcome the proliferative barrier imposed by telomere shortening, and to promote both immortalization and tumorigenesis [45]. Of note, the analysis of paired primary and recurrent aGCTs revealed that *TERT* promoter mutations were likely acquired during disease progression in two cases (aGCT77 and aGCT80), whereas in three cases (aGCT18, aGCT43 and aGCT76) these mutations were present in both primary tumors and matched recurrences. These findings suggest that depending on the context and activation of other signaling pathways, *TERT* promoter mutations may either be an early event and play a role in the development of more aggressive primary disease, that has the potential to spread and recur; or, in other contexts, *TERT* promoter mutations may be a late event and acquired during progression, disease spread or recurrence [14, 15]. Further studies are required to understand the interplay between *TERT* promoter mutations and other genetic or epigenetic alterations, and TERT's activation and role in aGCT maintenance and progression. It is unlikely, however, that *TERT* promoter mutation status alone would be the sole genetic predictor of recurrence. In our series, only one third of primary aGCTs with recurrences harbored a *TERT* hotspot mutation, as did two primary aGCTs (20%) without subsequent recurrences after more than 8 years of follow-up.

In our series, *TP53* mutations were identified in 16% of aGCT recurrences but were not present in primary aGCTs. Importantly, we observed that these *TP53* mutations were acquired during disease progression in cases that did not harbor *TERT* promoter mutations. Alexiadis et al. also reported a *TP53* mutation in a recurrent aGCT that also lacked *TERT* promoter mutations but they had a lower frequency of *TP53* mutations overall in their series (4%, 1/22) [15]. Our findings suggest that *TP53* may play a role in the progression of aGCTs, in particular in those lacking *TERT* promoter mutations.

Apart from *TP53*, we observed an enrichment of alterations in cell cycle genes in aGCT recurrences, including *CDKN2A/B* homozygous deletions. Given the small numbers assessed, further studies are warranted to define the frequency and role of cell cycle-related genes in the progression of aGCTs. Whilst homozygous deletions or loss-of-function mutations of *CDKN2A/B* are frequent events in various human primary solid and hematopoietic neoplasias [46, 47], these have not been previously reported in aGCTs. Gene expression analyses comparing wild-type *FOXL2* and mutant *FOXL2* C402G (p.C134W) transfected COV434 aGCT cells in vitro revealed the presence of differentially expressed genes associated with cell death and cell proliferation in mutant *FOXL2* cells, such as *CDKN1A*,

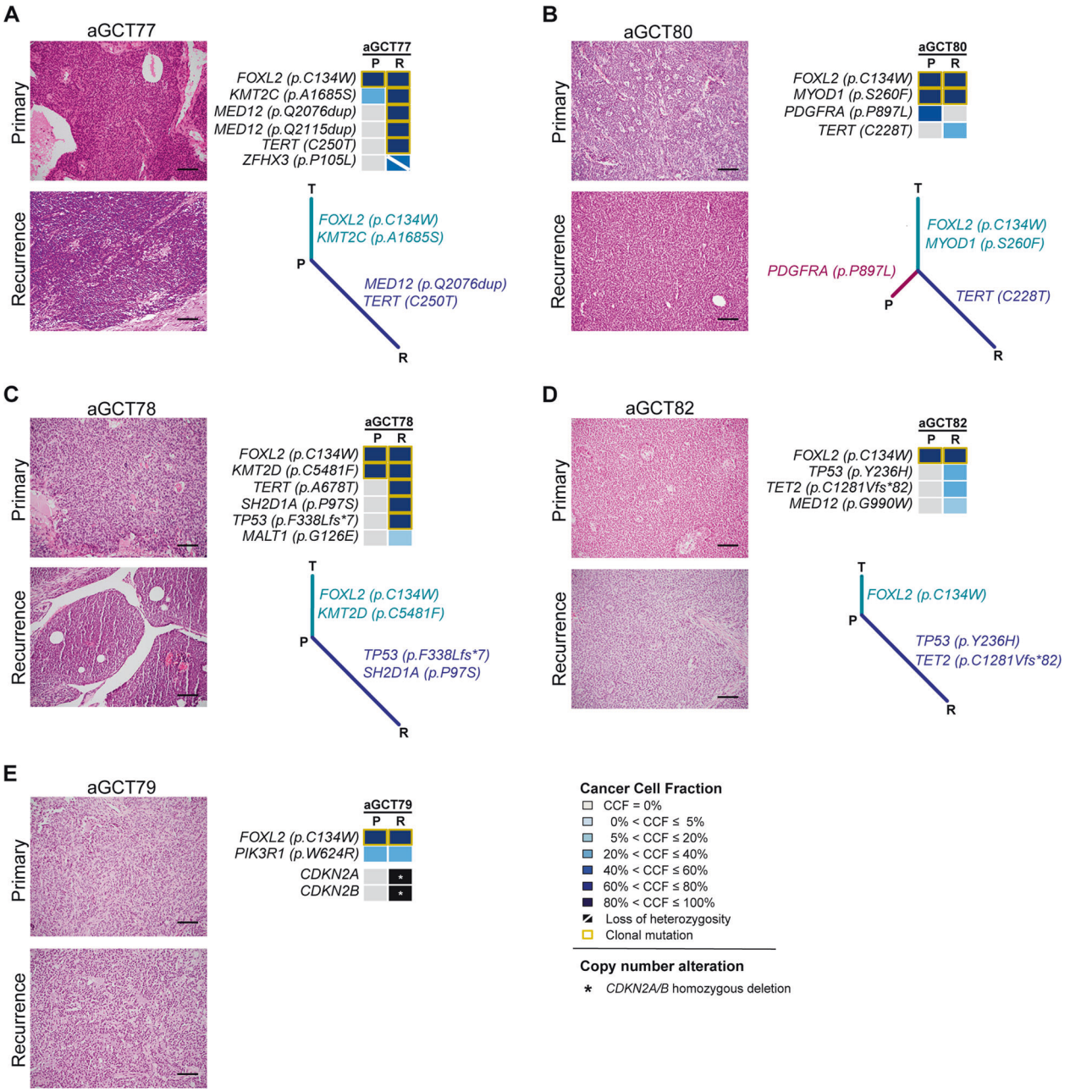

Fig. 4 Clonal composition of primary and matched recurrent adult-type granulosa cell tumors of the ovary. Representative hematoxylin and eosin micrographs (magnification, 200×; left), cancer cell fractions (clonal frequency) of mutations (top right) and mutation-based trees (bottom right) depicting the clonal evolution of matched primary and recurrent adult-type granulosa cell tumors (aGCTs) of the ovary for (**a**) aGCT77, (**b**) aGCT80, (**c**) aGCT78, (**d**) aGCT82, and (**e**) aGCT79. Cancer cell fractions are color-coded according to the legend. Clonal mutations are depicted by a yellow box. The length of the trunk and branches of the phylogenetic trees is proportional to the number of shared and private mutations identified in primary and recurrent aGCTs. Scale bars, 200 µM. P primary tumor, R recurrence, T truncal.

CDKN2A, and *CDK6* [48]. Inactivating mutations in the chromatin remodeling gene *KMT2D* have been recently reported to be strongly associated with aGCT recurrences [16]. The frequency of *KMT2D* loss-of-function mutations was very low in the aGCT analyzed here (one truncating mutation identified in one primary non-recurrent aGCT35). Furthermore, even when taking all nonsynonymous *KMT2D* mutations into account, no difference in frequency between primary aGCTs with/without recurrences and aGCT recurrences could be identified.

Clonal composition analysis revealed that primary aGCTs and their matched recurrences, despite their generally simple genomes with few mutations and CNAs, display intra-lesion heterogeneity harboring clonal and subclonal mutations. In the progression from primary to metastatic disease, the acquisition of additional mutations,

LOH of the wild-type allele of a given mutated gene as well as clonal shifts of genes affected by somatic mutations have been described [49–51]. Through the analysis of paired primary and matched recurrent aGCTs, we found that the acquisition of mutations, including those affecting *TP53*, *TET2*, *MED12*, and *SH2D1A*, was most commonly associated with disease progression.

Our study has important limitations. The sample size of the study is small, given the rarity of aGCTs, and larger validation studies are warranted. In addition, given the multi-institutional nature of our study, survival analyses could not be performed. Furthermore, our findings may not be applicable to the general population, given that the majority of the patients included in our cohort were treated at tertiary referral centers that tend to see higher risk populations. The recurrence rates observed here were similar to those reported in the literature, however [5, 7]. Given the limited amounts of DNA available from these lesions, we restricted our sequencing analysis to 410–468 cancer-related genes. We cannot rule out, however, that there are other genes which may play a role in the progression of aGCTs. Nonetheless, our findings provide support to the notion that *TERT* promoter hotspot mutations are the most recurrent genetic events affecting aGCTs and might be associated with disease progression in a subset of cases. We further identified genetic alterations affecting cell cycle-related genes, which may be associated with aGCT progression. Finally, our data suggest that whilst aGCTs harbor simple genomes, intra-tumor heterogeneity is present in this rare subtype of pure sex cord tumor.

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Author contributions SES, BW, and DFD conceived the study. RB, AV, SG, XM-G, BPR, and DFD provided tissue samples. RB, SES, MV, CGP, XM-G, BPR, JSR-F, and DFD conducted pathology review. RB, ADCP, and EMdS performed sample processing. ADCP, LF, and PS performed bioinformatics analyses. Data acquisition, interpretation, and analysis was performed by RB, ADCP, EMdS, FP, PS, LF, RAS, NRA-R, JSR-F, DFD, and BW. ADCP, EMdS, FP, SHK, JSR-F, and BW drafted the manuscript, and all authors edited and approved the final draft of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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