Adult-type granulosa cell tumor of the ovary: a FOXL2-centric disease

Jessica A Pilsworth1,2, Dawn R Cochrane1, Samantha J Neilson1, Bahar H Moussavi1, Daniel Lai1, Aslı D Munzur1, Janine Senz1, Yi Kan Wang1, Sina Zareian1, Ali Bashashati3,4, Adele Wong5, Jacqueline Keul6, Annette Staebler7, Hannah S van Meurs8, Hugo M Horlings9, Friedrich Kommoss10, Esther Oliva5, Anniina EM Färkkilä11, Blake Gilks3 and David G Huntsman1,3*

1Department of Molecular Oncology, British Columbia Cancer Research Centre, Vancouver, BC, Canada
2Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada
3Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada
4School of Biomedical Engineering, University of British Columbia, Vancouver, BC, Canada
5Department of Pathology, Massachusetts General Hospital, Boston, MA, USA
6Department of Women’s Health, Tübingen University Hospital, Tübingen, Germany
7Institute of Pathology and Neuropathology, Tübingen University Hospital, Tübingen, Germany
8Department of Gynecology, Center for Gynecologic Oncology Amsterdam, Academic Medical Center, Amsterdam, The Netherlands
9Department of Pathology, The Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, The Netherlands
10Institute of Pathology, Medizin Campus Bodensee, Friedrichshafen, Germany
11Research Program for Systems Oncology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

*Correspondence to: David G Huntsman, Department of Molecular Oncology, British Columbia Cancer Research Centre, 4th Floor, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada. E-mail: dhuntsma@bccancer.bc.ca

Abstract

Adult-type granulosa cell tumors (aGCTs) account for 90% of malignant ovarian sex cord-stromal tumors and 2–5% of all ovarian cancers. These tumors are usually diagnosed at an early stage and are treated with surgery. However, one-third of patients relapse between 4 and 8 years after initial diagnosis, and there are currently no effective treatments other than surgery for these relapsed patients. As the majority of aGCTs (>95%) harbor a somatic mutation in FOXL2 (c.C402G; p.C134W), the aim of this study was to identify genetic mutations besides FOXL2C402G in aGCTs that could explain the clinical diversity of this disease. Whole-genome sequencing of 10 aGCTs and their matched normal blood was performed to identify somatic mutations. From this analysis, a custom amplicon-based panel was designed to sequence 39 genes of interest in a validation cohort of 83 aGCTs collected internationally.

KMT2D inactivating mutations were present in 10 of 93 aGCTs (10.8%), and the frequency of these mutations was similar between primary and recurrent aGCTs. Inactivating mutations, including a splice site mutation in candidate tumor suppressor WNK2 and nonsense mutations in PIK3R1 and NLR5, were identified at a low frequency in our cohort. Missense mutations were identified in cell cycle-related genes TP53, CDKN2D, and CDK1. From these data, we conclude that aGCTs are comparatively a homogeneous group of tumors that arise from a limited set of genetic events and are characterized by the FOXL2 C402G mutation. Secondary mutations occur in a subset of patients but do not explain the diverse clinical behavior of this disease. As the FOXL2 C402G mutation remains the main driver of this disease, progress in the development of therapeutics for aGCT would likely come from understanding the functional consequences of the FOXL2 C402G mutation.

Keywords: adult-type granulosa cell tumor of the ovary; FOXL2; TERT promoter; KMT2D; targeted sequencing; mutation profiling; cell cycle genes; sex cord-stromal tumor; ovarian cancer

Received 18 June 2020; Revised 16 November 2020; Accepted 26 November 2020

Conflict of interest statement: DGH is a cofounder and shareholder of Contextual Genomics Inc. The other authors declare no conflicts of interest.
Introduction

Adult-type granulosa cell tumors (aGCTs) are the most common type of malignant ovarian sex cord-stromal tumor (SCST) [1]. They account for 2–5% of all ovarian malignant tumors and 90% of malignant SCSTs [2]. aGCTs occur in perimenopausal women with a median age of diagnosis of between 50 and 54 years [3,4]. These tumors arise from the granulosa cells of the ovarian follicles and have distinct clinical features, such as their ability to secrete estrogen and inhibins [5]. Due to their indolent growth and unique hormonal activity, these tumors are usually diagnosed at an early stage and are treated with surgery [6–8]. A somatic missense mutation (c.C402G; p.C134W) in the transcription factor FOXL2 was identified in over 95% of aGCTs and can be used as a clinical diagnostic assay for differential diagnosis [9–12]. In our previous study by McConkey et al., we showed that, in a molecularly defined cohort of aGCTs (FOXL2 C402G mutation positive), the overall survival did not differ from age-matched, population-based controls [13]. However, one-third of aGCT patients relapse, and there are no effective treatments for relapsed patients with inoperable tumors [14,15].

Due to the propensity of aGCT to recur years after initial diagnosis (typically 4–8 years, with the median being 7.2 years in molecularly defined cohorts), the challenge is identifying patients who are at high risk for relapsed disease and require prolonged surveillance [13,14,16]. As the FOXL2 C402G mutation is present in essentially all aGCTs, there has been much research on secondary mutations to explain the diverse clinical behavior of these tumors. Our research group was the first to describe TERT C228T promoter mutations in 51 of 229 (22%) primary and 24 of 58 (41%) recurrent aGCTs [17]. Likewise, Alexiads et al. performed targeted TERT promoter sequencing and found that a higher frequency of recurrent tumors harbor the TERT C228T promoter mutation [18]. Hillman et al. were the first to identify KMT2D truncating mutations in aGCT and suggested that KMT2D inactivation may increase the risk of tumor recurrence [19]. Da Cruz Paula et al. used targeted sequencing of known cancer genes and identified genetic alterations in cell cycle-related genes, including TP53, TERT promoter, MED12, and CDKN2A/B, suggesting that these mutations may play a role in recurrence [20,21]. Furthermore, Roze et al. suggest that patients with TP53 mutations represent a high-grade subgroup of aGCT [21].

In this study, we used whole-genome sequencing (WGS) on 10 aGCTs and their matched peripheral blood to identify novel somatic mutations in aGCT. Validation was performed using targeted sequencing on an independent cohort of 83 primary and recurrent aGCTs collected from four international institutions. We identified KMT2D inactivating mutations in 10 of 93 aGCTs (10.8%). A splice site mutation was identified in the candidate tumor suppressor WNK2, and nonsense mutations were identified in NLRC5 and PIK3R1. Missense mutations were identified in cell cycle-related genes TP53, CDKN2D, and CDK1.

Materials and methods

Clinical specimens for exploratory WGS

Ten representative fresh-frozen aGCTs were selected from OVCARE’s Gynecological Tissue Bank in Vancouver, Canada, for WGS. Patient tumor and peripheral blood samples were collected at diagnosis during standard-of-care debulking surgery. For DNA isolation from banked samples, frozen tissue samples were cryosectioned, and sections adjacent to the scrolls submitted for sequencing were stained with hematoxylin and eosin to assess tumor cell content. These slides were reviewed by two of the following gynecologic pathologists: HMH, ANK, HLC, and BG, and the final cases with >80% tumor cellularity were selected by BG. All cases were positive for the FOXL2 (c.C402G; p.C134W) mutation. In brief, WGS was performed using Illumina HiSeq 2500 v4 chemistry (Illumina Inc., Hayward, CA, USA) with the polymerase chain reaction (PCR)-free protocol to eliminate PCR-induced bias and to improve coverage across the genome. The average read depth was 46.6X per sample (range: 36–60X). WGS analysis comparing tumor tissue to matched peripheral blood was performed to identify somatic alterations in each case, including single-nucleotide variants (SNVs), small insertions and deletions (indels), copy number alterations (CNAs), and structural variants (SVs). This cohort was previously published by our research team, and detailed methods are available [22].

Validation cohort description

Formalin-fixed paraffin-embedded (FFPE) aGCTs (n = 83) were collected from Helsinki University Hospital (Helsinki, Finland; n = 39), Massachusetts General Hospital (Boston, MA, USA; n = 27), Tübingen University Hospital (Tübingen, Germany; n = 13), and Netherlands Cancer Institute (Amsterdam, The Netherlands; n = 4). Sections prepared from FFPE tumor blocks were reviewed by one of the following
pathologists (BG, FK, HMH, ANK, RB, HLC, BT-C, and LH) to confirm aGCT diagnosis. DNA was extracted for each FFPE tumor block, and the FOXL2 C402G mutation was confirmed with a single-nucleotide polymorphism (SNP) genotyping assay using two allele-specific fluorescent labelled probes. The respective institutional research ethics board approved the waiver for patient consent. The University of British Columbia and BC Cancer’s Research Ethics Boards approved the overall project methods.

Targeted sequencing
To explore the frequency of variants identified in the WGS study of 10 aGCTs, a custom amplicon-based panel was designed to sequence a larger validation cohort of FFPE cases. The genes included in the custom panel were prioritized by (1) genes with frame-shift (small insertions/deletions) or nonsense or splice site mutations, (2) genes with SNVs in known cancer genes, and (3) genes with SNVs involved in granulosa cell biology. The full list of genes is given in supplementary material, Table S1. Two sets of custom oligo capture probes flanking each gene of interest were designed for the construction of two different sequencing libraries (A and B). These custom probes amplified the entire coding region of 39 genes selected in the custom panel. TruSeq Custom Amplicon Dual Strand Library Preparation was performed to generate 1536 amplicons (150 base pairs each) per sample in a single multiplex reaction using the manufacturer’s protocol (Illumina Inc.). PCR was used to incorporate both a unique index for each sample and sequencing primers. For each sequencing run, 24 samples were pooled into a single library with a final concentration of 4 nM. The pooled library was sequenced on an Illumina MiSeq using the MiSeq Reagent Kit V2. The average read depth was 1254X per sample (range: 296–3212X).

Case inclusion
All cases were tested for FOXL2 C402G mutation using an SNP genotyping assay and were confirmed to contain the FOXL2 C402G mutation. As the validation cohort consisted of FFPE material with no matched normal tissue available, the cases included in the final cohort reported were selected if they satisfied the following two conditions: (1) two libraries (A and B) per case were constructed successfully and sequenced, and (2) FOXL2 C402G mutation was detected using the targeted sequencing variant analysis pipeline to call mutations in the gene panel. Condition 1: Two libraries (A and B) per case are important to eliminate any sequencing artifacts that may have resulted from the formalin fixation process. Libraries A and B were constructed with different probes to generate amplicons covering the 39 genes selected in our custom gene panel. Both libraries A and B were sequenced separately and processed independently through the variant analysis pipeline. After the variants were called for both libraries A and B, an intersection function was performed that generated the list of variants that were present in both libraries A and B. The assumption that formalin fixation introduces SNVs at random enabled the removal of these randomly generated SNVs that were only present in one of the two libraries. The mutations reported in the Results section were present in both libraries A and B for each case. Condition 2: The detection of the FOXL2 C402G mutation using the targeted sequencing variant analysis pipeline was employed to ensure that the targeted sequencing pipeline was highly sensitive. The aim of the study was to report on a molecularly defined aGCT cohort, where all cases harbor the FOXL2 C402G mutation. Therefore, cases were tested beforehand using an SNP genotyping assay to confirm the presence of the FOXL2 C402G mutation, and only cases where the FOXL2 C402G mutation was called using the targeted sequencing variant analysis pipeline were included in the validation cohort. This ensured that the pipeline was highly sensitive in detecting variants in the remaining genes in the panel. Due to the stringency required to remove FFPE artifacts and the fact that some of the cases are over 50 years old with no matched normal tissue available, it was expected that there would be a high number of cases excluded from the validation cohort. A less rigorous approach would lead to the inclusion of an overwhelming number of false-positive mutations. A flowchart outlining the number of cases excluded at each step is illustrated in Figure 1.

Variant calling and filtering
SNVs were detected by MutationSeq version 4.3.7 (Vancouver, Canada) and Strelka version 1.0.14 (Illumina Inc., San Diego, CA, USA). Small insertions and deletions (indels) were detected using Strelka version 1.0.14 and Mutect2 version 4.1.8.1 from GATK (The Broad Institute, Cambridge, MA, USA). SNVs were removed if they were not detected in both libraries A and B and by both MutationSeq and Strelka. Indels were removed if they were not detected in both libraries A and B and by both Mutect2 and Strelka. Variants with a read depth <100 and SNVs from the Single Nucleotide Polymorphism Database (dbSNP) were removed. The presence of the FOXL2 C402G mutation in all the cases included in validation cohort
was confirmed prior to targeted sequencing. The lowest variant allele frequency (VAF) in which FOXL2 C402G was detected was 8.5% using the variant analysis pipeline on the targeted sequencing data. This established the limit of the detection for identifying the FOXL2 C402G mutation and was thus chosen as the VAF for variant reporting of the remaining genes in the panel. Furthermore, the FOXL2 C402G mutation is expected to be clonal with secondary subclonal mutations. For missense mutations, three tools (PolyPhen-2, SIFT, and MutationAssessor) were selected to predict the pathogenicity of each variant using Ensembl’s Variant Effect Predictor. Variants that were predicted by two or more tools to have no effect on protein function were removed. Therefore, only missense mutations that were predicted as pathogenic by two or more tools were reported. A flowchart describing the steps of variant filtering is illustrated in supplementary material, Figure S1.

Results

WGS on exploratory cohort
Ten aGCTs (eight primary and two recurrent) and their matched normal DNA samples were subjected to WGS as previously described [22]. The median age of diagnosis was 51 years for aGCT, and the clinical characteristics are described in Table 1. All aGCTs were heterozygous for the FOXL2 C402G mutation.

Somatic variations were detected in the tumor genome of each patient, including SNVs, small indels, CNAs, and SVs. The overall mutational burden for these 10 aGCTs was low as previously described in the Wang et al’s study [22]. SNVs and small indels are described in Table 1. The majority of mutations reported was identified in only 1 of 10 aGCTs (10%) except for the TERT C228T promoter mutation that was present in 5 of 10 aGCTs (50%). Missense mutations in BMP7 and MPHOSPH8 were identified in 2 of 10 aGCTs (20%). Copy number analysis revealed loss of chromosome 22 in 6 of 10 aGCTs (60%) and gain of chromosome 14 in 2 of 10 aGCTs (20%). Concurrent loss of chromosome 22 and gain of chromosome 14 was observed in 1 of 10 aGCTs (10%). No gain of chromosome 12 was found. These results confirm previous reports of CNAs in aGCTs, including loss of chromosome 22 in 30–56% and gain of chromosome 14 in 20–27% [18,21,23–25]. Other structural rearrangements were sporadic and infrequent (see supplementary material, Figure S2).

Targeted sequencing on validation cohort
Our validation cohort encompassed 83 aGCTs, including 61 primary and 22 recurrent tumors, collected from four international institutions. For the 58 patients with age data, the median age at diagnosis was 52 years (range: 26–94 years). The clinical features for each cohort are described in Table 2. All cases included in the validation cohort have confirmed FOXL2 C402G mutation.
### Table 1. Clinical features of patients and mutations in the exploratory cohort.

| Patient | Age | Diagnosis | Stage | Disease | Menopause status | Tumor size | Adjuvant treatment | Mutations (protein change) |
|---------|-----|-----------|-------|---------|------------------|------------|--------------------|---------------------------|
| DG1331  | 63  | aGCT      | Unk   | Recurrent| Unk              | Unk        | No                 | DOCK3 S1702F, TSKU W237*, DNMBP R340fs |
| DG1332  | 76  | aGCT      | Unk   | Recurrent| Post             | 7 × 4 × 4 cm | Palliative radiation | CDKN2D V24G, KDR R299W, NLRC5 R1161K, PIK3CA G1007R, SLITRK2 R713*, WNK2 L1004F, ACSS1 I134fs, GANC Y249fs, TERT C228T promoter† |
| DG1333  | 73  | aGCT      | I     | Primary  | Post             | Unk        | Chemotherapy       | CENPE L2378W, KDMSC W555R, MPHOSPH8 L590F, TP53 K121N, KMT2D S1398fs |
| DG1334  | 61  | aGCT      | IA    | Primary  | Peri              | 5.5 × 3.5 × 2 cm | No                 | KCMF1 Y28*, MPHOSPH8 L590F, NDUFA10 K285N, NDUFA10 Q286*, FOXL2 A243fs, POLR1B P1084fs |
| DG1335  | 59  | aGCT      | I     | Primary  | Pre              | Unk        | No                 | BMP7 R188W, NOX1 F185L, GRIA3 W137fs, TERT C228T promoter† |
| DG1336  | 60  | aGCT      | IC    | Primary  | Post             | 15 × 8 × 8 cm | No                 | KANSL3 Q372*, SMAAD3 L135P, ZP1 R552fs, TERT C228T promoter† |
| DG1337  | 64  | aGCT      | Unk   | Primary  | Unk              | 10 × 7.5 × 3.5 cm | Unk                | GLU1 G544E, PIK3R1 M582_splice, WRDR72 D201fs, TERT C228T promoter† |
| DG1338  | 37  | aGCT      | I     | Primary  | Pre              | Unk        | No                 | BMP7 N289K, CDK1 M223V, WRDR52 R1655*, TRO I543fs, TERT C228T promoter† |
| DG1339  | 43  | aGCT      | IA    | Primary  | Pre              | 24 × 11 × 9 cm | No                 | BMP7 N289K, CDK1 M223V, WRDR52 R1655*, TRO I543fs, TERT C228T promoter† |
| DG1340  | 66  | aGCT      | IC    | Primary  | Post             | 17 × 13 × 6 cm | No                 | BMP7 N289K, CDK1 M223V, WRDR52 R1655*, TRO I543fs, TERT C228T promoter† |

fs, frameshift mutation; splice, splice site mutation; Unk, unknown.
*Stop codon, nonsense mutation.
†DNA sequence change.

### Table 2. Clinical features of patients in the validation cohort.

| Cohort       | Finland | USA | Germany | The Netherlands |
|--------------|---------|-----|---------|-----------------|
| Number of patients | 39      | 27  | 13      | 4               |
| Median age   | 52      | Unknown | 46      | 53.5            |
| Stage        |         |       |         |                 |
| I            | 36      | 0   | 8       | 3               |
| II           | 2       | 0   | 1       | 0               |
| III–IV       | 1       | 0   | 1       | 1               |
| Unknown      | 0       | 27  | 3       | 0               |
| Disease      |         |       |         |                 |
| Primary      | 32      | 17  | 8       | 4               |
| Recurrent    | 7       | 10  | 5       | 0               |
| Adjuvant treatment |       |       |         |                 |
| No           | 34      | 0   | 9       | 4               |
| Chemotherapy | 4       | 0   | 1       | 0               |
| Radiation    | 1       | 0   | 0       | 0               |
| Unknown      | 0       | 27  | 3       | 0               |
| FOXL2 C402G mutation status |       |       |         |                 |
| Homozygous/hemizygous | 1      | 1   | 0       | 0               |
| Heterozygous | 38      | 26  | 13      | 4               |
| TERT C228T promoter mutation |       |       |         |                 |
| Heterozygous | 8       | 7   | 2       | 1               |
| Wildtype     | 27      | 13  | 11      | 3               |
| Unknown      | 4       | 6   | 0       | 0               |
mutation determined by an SNP genotyping assay, and this mutation was detected in the targeted sequencing for all cases reported (Figure 1). Two cases harbor FOXL2 C402G hemizygous mutations, which appear as a homozygous state. The remaining cases were FOXL2 C402G heterozygous. For the 73 aGCTs with available TERT C228T promoter mutation data, the frequency was 18 of 73 aGCTs (24.7%), which is similar to our original TERT C228T promoter mutation publication that contains additional cases along with 73 cases of the current validation cohort (75/287; 26.1%). The following missense mutations were mutated in two aGCTs: KMT2D (c.C3893T; p.S1298F) and NLRC5 (c.G823A; p.E275K). All genes mutated in our panel are shown in Figure 2 and in supplementary material, Table S2. KMT2D inactivating mutations (nonsense, indel, or splice site mutations) were present in 9 of 83 aGCTs in our validation cohort, and an indel (frameshift mutation) was reported in 1 case from the exploratory cohort (10/93; 10.8%). Missense mutations in KMT2D were observed in 9 of 93 aGCTs (9.7%); however, these mutations have not previously been reported, and therefore, their relevance to aGCT development is unknown. The missense mutations annotated in dark blue in Figure 2 are putative driver events and include mutations in FOXL2, TP53, and KDR. The

Figure 2. Mutations in aGCTs. OncoPrint showing the distribution of genetic alterations in aGCTs. Genetic alterations in genes (rows) on targeted sequencing panel are shown in each aGCT case (columns). The nonsense, indels (insertions/deletions), splice site, and missense mutations are shown in red, yellow, purple, and blue, respectively. Missense mutations that are putative driver mutations (hot-spot mutations) are colored in dark blue. The percentage of cases harboring mutations in each gene is annotated on the far right. The country from which each case was collected from, the disease status (primary or recurrent), and TERT C228T promoter mutation status are annotated on the tracks above the OncoPrint.
significance of the remaining missense mutations annotated in light blue remains unknown. Splice site mutations were identified in KMT2D, WNK2, DOCK3, and GLI1. Nonsense mutations were identified in KMT2D, NLRC5, PIK3R1, POLR1B, WDR52, WDR72, NDUFA10, NOX1, and STK31. Moreover, genes involved in cell cycle regulation were mutated at a low frequency in aGCT, including TP53 (4/93; 4.8%), CDKN2D (2/93; 2.1%), and CDK1 (1/93; 1.2%).

Mutual exclusivity of TP53 and TERT C228T promoter mutations was observed for the three cases with available TERT C228T promoter mutation data.

Discussion

Here, we report the genomic analysis of 93 aGCTs, a malignant SCST of the ovary associated with late recurrence. The current study is a targeted genomic analysis of a molecularly defined aGCT cohort, where all tumors harbor the FOXL2 C402G mutation. Our research team initially described TERT C228T promoter mutations in 51 of 229 primary (22%) and 24 of 58 recurrent (41%) aGCTs, suggesting that these mutations occur later in tumorigenesis. The majority of the previously described cohort is included in this study, and TERT C228T promoter mutations are annotated in Figure 2 and Table 2. Multiple research groups have also observed TERT promoter mutations with a higher frequency of mutations in recurrent aGCTs (6/9, 67%; 1/28, 64%) compared to primary aGCTs (5/17, 29%; 5/19, 26%) [18,20]. In our study and that of Alexiadis et al, seven and five patients whose tumors were initially wild-type for the TERT promoter acquired the C228T mutation in their recurrent tumors, respectively [17,18]. These data support our statement that TERT promoter mutations are acquired during tumor progression after the initial FOXL2 C402G driver mutation. Although targeting telomerase as an anticancer treatment may represent an attractive strategy for recurrent aGCT, the development of efficacious cancer-specific telomerase inhibitors remains an ongoing challenge [26].

Using a combination of WGS and targeted sequencing, the results of the current study demonstrate that KMT2D undergoes frequent inactivating mutations in aGCTs. We report a similar frequency of KMT2D inactivating mutations (10/93, 10.8%) as the Hillman et al’s study, which reported that 11 of 79 (13.9%) aGCTs harbored KMT2D inactivating mutations. Hillman et al described inactivating mutations in KMT2D as a recurrent event (10/43, 23%) in these tumors compared to primary tumors (1/35, 3%) [19]. However, in our dataset, we observed a similar frequency of inactivating mutations in KMT2D in primary (8/69, 11.5%) and recurrent (2/24, 8.3%) aGCTs. Aligned with our current results, Da Cruz Paula et al identified only one inactivating KMT2D mutation in a primary nonrecurrent aGCT in a cohort consisting of 10 primary nonrecurrent aGCTs (defined by no recurrent tumor within 4 years of initial diagnosis), 28 recurrent aGCTs, and 9 matched primary aGCTs with known recurrences [20]. The occurrence of KMT2D inactivating mutations in 10.8% of our aGCT cohort not only suggests that they are a pathogenic driver mutation in a subset of aGCTs but also that these mutations do not correlate with recurrence. We also observed KMT2D missense mutations in a subset of aGCTs (9/93; 9.7%), but these variants are of unknown significance. A limitation of this study was that the validation cohort did not have matched normal tissue, and thus, some reported missense mutations may be rare SNPs. Further research into the role of KMT2D in aGCT development is warranted, specifically exploring the epigenetic landscape of these tumors to identify aberrant histone marks and methylation patterns.

Additional inactivating mutations, including a splice site mutation in the candidate tumor suppressor WNK2 and nonsense mutations in the newly discovered protein NLRC5, are reported. WNK2 was recently discovered to be epigenetically silenced through promoter hypermethylation in gliomas and meningioma, suggesting its role as a growth suppressor [27-29]. WNK2 was also found to be downregulated via epigenetic silencing in early pancreatic ductal adenocarcinoma and may support cell proliferation through the Mitogen-activated protein kinase (MAPK) signaling pathway [30]. WNK2 somatic mutations and copy number loss were reported in hepatocellular carcinoma, resulting in lower WNK2 protein levels, which were associated with early tumor recurrence linked to enhanced ERK1/2 signaling [31]. Moreover, WNK kinases have been linked to Wnt/β-catenin signaling, a known pathway involved in granulosa cell development, and loss-of-function WNK genes resemble canonical Wnt pathway mutants [32]. NLRC5 has recently been linked to the regulation of cancer immune evasion, but its role in tumor development is controversial [33]. Some research studies report that NLRC5 elicits antitumor immunity, while other studies found that it promoted tumorigenesis and progression [33]. In fact, NLRC5 has been shown to regulate the Wnt/β-catenin signaling pathway to promote cell proliferation and migration in both clear cell renal carcinoma and hepatocellular carcinoma [34,35].

© 2021 The Authors. The Journal of Pathology: Clinical Research published by The Pathological Society of Great Britain and Ireland & John Wiley & Sons, Ltd.
Various signaling pathways have been explored in the development of aGCT, including transforming growth factor beta (TGF-β), phosphatidylinositol-3-kinase; serine/threonine kinase (PI3K/AKT), GATA4, and vascular endothelial growth factor (VEGF). These factors have been shown to play important roles in granulosa cell proliferation, apoptosis, or angiogenesis. In our aGCT cohort, we observed a small number of mutations in members of these pathways including GLI1, BMP7, SMAD3 (TGF-β pathway), PIK3R1 (PI3K/AKT pathway), GATA4, and KDR/VEGFR2. However, these mutations occurred in a small percentage of aGCT patients and do not provide any prognostic or predictive value. In addition, mutations in cell cycle-related genes, including TP53, CDKN2D, and CDK1, were identified.

Da Cruz Paula et al used targeted screening of over 400 cancer-related genes on a cohort of 38 aGCT patients. They identified mutations present in recurrent aGCTs that were not present in primary aGCT, including TERT promoter mutations (C228T and C250T), MED12, and TP53, as well as CDKN2A/B homozgyous deletions, suggesting that genetic alterations in cell cycle-related genes may be associated with recurrence [20]. In the current cohort, TP53 mutations were observed in three primary and one recurrent aGCTs, CDKN2D mutations were observed in one primary and one recurrent aGCT, and a CDK1 mutation was observed in one primary aGCT. The frequency of mutations in cell cycle-related genes is low in our cohort; thus, it is difficult to draw conclusions regarding their association with recurrence.

Most recently, Roze et al used WGS of 33 aGCT patients and identified TP53 mutations in three patients (3/33; 9.1%) with higher tumor mutational burden and mitotic activity. They propose that tumors with TP53 mutations define a high-grade subgroup of aGCT and suggest that a personalized medicine approach is required for treatment of aGCTs. Furthermore, they state that the absence of the FOXL2 C402G mutation does not exclude an aGCT diagnosis and that TP53, TERT C228T promoter, and DICER1 mutations may drive tumorigenesis [21]. The data presented here also show that aGCT can occasionally harbor other driver mutations, including KMT2D (10/93; 10.8%) and TP53 (4/93; 4.8%). However, only a small minority of patients would benefit from a genomic-driven approach to treatment. The FOXL2 C402G mutation is the dominant genetic event, and greater benefits would be derived from targeting the downstream mutations.

Inclusively, this study and other research studies have not identified any common actionable features in aGCT. Although it is still possible that features such as promoter methylation could be discovered in noncoding regions, it appears that aGCT is not a disease that could be stratified into different treatment groups by genomic features. Our data and those of others suggest that this is a highly specific clinical entity driven by the FOXL2 C402G mutation, and treatment strategies focusing on targeting FOXL2 or the downstream consequences are more likely to be successful in treatment compared to a precision medicine approach. Two recent publications have shown initial evidence that the FOXL2 C402G mutation alters DNA-binding specificity of the FOXL2 C134W protein (FOXL2C134W). Carles et al identified unique targets of FOXL2C134W, including SLC35F2, a solute transporter that transports sepantronium bromide, which is a transcriptional suppressor of survivin. They found that the immortalized granulosa cell line SVOG3e transduced with inducible FOXL2C134W showed an increased sensitivity to YM155, a small molecule inhibitor of survivin, compared to FOXL2wild-type or empty vector. The authors suggest YM155 as a potential therapeutic strategy for aGCT [36]. Weis-Banke et al show that the mutant FOXL2C134W acquires the ability to bind SMAD4, which forms a FOXL2C134W/SMAD4/SMAD2/3 complex that is able to bind to a novel hybrid DNA motif and induces the transcription of genes involved in epithelial-to-mesenchymal transition (EMT). They show that the ablation of SMAD4 or SMAD2/3 in the immortalized granulosa cell line HGrCl reduces binding of FOXL2C134W to these hybrid sites and decreases expression of these EMT-related genes, suggesting the possibility of targeting this FOXL2C134W–SMAD4 interaction for therapeutic purposes [37]. These studies may lead to the first generally applicable, biologically informed therapeutic strategies for aGCT of the ovary.

Acknowledgements

We thank all the women who generously donated the samples used in this study. This work is supported by the Terry Fox Research Institute New Frontiers Program Project Grant #1082, the Canadian Institutes of Health Research Foundation Grant #154290, the BC Cancer Foundation, and the Vancouver General Hospital (VGH) & University of British Columbia (UBC) Hospital Foundation (to OVCARE: BC’s Ovarian Cancer Research Team, Vancouver). JAP is supported by the University of British Columbia Four-Year Doctoral Fellowship. DGH is supported by the Dr. Chew Wei Memorial Professorship in Gynecologic Oncology and the Canada Research Chairs Program (Research Chair in Molecular and Genomic Pathology). We are grateful to the clinicians from each institution for patient recruitment and the OVCARE
Gynecologic Tissue bank for providing patient tissues. We also thank Drs. Blake Gilks, Friedrich Kommoss, Hugo M. Horlings, Ralf Butzow, Anthony N. Kamezis, Hector Li Chang, Basile Tessier-Cloutier, and Lien Hoang for expert pathology review. We are grateful to Winnie Yang and Amy Lum for their technical support.

Author contributions statement

JAP, DRC and DGH conceived and designed the study. AEMF, HMM, HSVM, AW, EO, JK, SK, FK, BG and DGH provided study materials or patients. JAP, DRC, SJN and JS collected and assembled data. JAP, DRC, YKW, DL, AB, BHM and ADM analyzed and interpreted data. JAP, DRC and DGH wrote the manuscript. All authors approved the final manuscript.

References

1. Jamieson S, Fuller PJ. Molecular pathogenesis of granulosa cell tumors of the ovary. Endocr Rev 2012; 33: 109–144.
2. Young RH. Sex cord-stromal tumors of the ovary and testis: their similarities and differences with consideration of selected problems. Mod Pathol 2005; 18 (Suppl 2): S81–S98.
3. Schumer ST, Cannistra SA. Granulosa cell tumor of the ovary. J Clin Oncol 2003; 21: 1180–1189.
4. Fuller PJ, Leung D, Chu S. Genetics and genomics of ovarian sex cord-stromal tumors. Clin Genet 2017; 91: 285–291.
5. Farkkila A, Haltia UM, Tapper J, et al. Pathogenesis and treatment of adult-type granulosa cell tumor of the ovary. Ann Med 2017; 49: 435–447.
6. Bryk S, Farkkila A, Butzow R, et al. Clinical characteristics and survival of patients with an adult-type ovarian granulosa cell tumor: a 56-year single-center experience. Int J Gynecol Cancer 2015; 25: 33–41.
7. Mangili G, Ottolina J, Gadducci A, et al. Long-term follow-up is crucial after treatment for granulosa cell tumours of the ovary. Br J Cancer 2013; 109: 29–34.
8. van Meurs HS, Bleeker MC, van der Velden J, et al. The incidence of endometrial hyperplasia and cancer in 1031 patients with a granulosa cell tumor of the ovary: long-term follow-up in a population-based cohort study. Int J Gynecol Cancer 2013; 23: 1417–1422.
9. Jamieson S, Butzow R, Andersson N, et al. The FOXL2 C134W mutation is characteristic of adult granulosa cell tumors of the ovary. Mod Pathol 2010; 23: 1477–1485.
10. Shah SP, Kohel M, Senz J, et al. Mutation of FOXL2 in granulosa-cell tumors of the ovary. N Engl J Med 2009; 360: 2719–2729.
11. Al-Agha OM, Huwaiit HF, Chow C, et al. FOXL2 is a sensitive and specific marker for sex cord-stromal tumors of the ovary. Am J Surg Pathol 2011; 35: 484–494.
12. Schrader KA, Gorbatcheva B, Senz J, et al. The specificity of the FOXL2 c.402C>G somatic mutation: a survey of solid tumors. PLoS One 2009; 4: e7988.
13. McConney MK, Farkkila A, Horlings HM, et al. Molecularly defined adult granulosa cell tumor of the ovary: the clinical phenotype. J Natl Cancer Inst 2016; 108: djw134.
14. Sun HD, Lin H, Jao MS, et al. A long-term follow-up study of 176 cases with adult-type ovarian granulosa cell tumors. Gynecol Oncol 2012; 124: 244–249.
15. Wilson MK, Fong P, Mesnage S, et al. Stage I granulosa cell tumours: a management conundrum? Results of long-term follow up. Gynecol Oncol 2015; 138: 285–291.
16. Lee IH, Choi CH, Hong DG, et al. Clinicopathologic characteristics of granulosa cell tumors of the ovary: a multicenter retrospective study. J Gynecol Oncol 2011; 22: 188–195.
17. Pilsworth JA, Cochrane DR, Xia Z, et al. TERT promoter mutation in adult granulosa cell tumor of the ovary. Mod Pathol 2018; 31: 1107–1115.
18. Alexiadis M, Rowley SM, Chu S, et al. Mutational landscape of ovarian adult granulosa cell tumors from whole exome and targeted TERT promoter sequencing. Mol Cancer Res 2019; 17: 177–185.
19. Hillman RT, Celestino J, Terranova C, et al. KMT2D/MLL2 inactivation is associated with recurrence in adult-type granulosa cell tumors of the ovary. Nat Commun 2018; 9: 2496.
20. Da Cruz Paula A, da Silva EM, Segura SE, et al. Genomic profiling of primary and recurrent adult granulosa cell tumors of the ovary. Mod Pathol 2020; 33: 1606–1617.
21. Roze J, Monroe G, Kutzer J, et al. Whole genome analysis of ovarian granulosa cell tumors reveals tumor heterogeneity and a high-grade TP53-specific subgroup. Cancers (Basel) 2020; 12: 1308.
22. Wang YK, Bashashati A, Anglesio MS, et al. Genomic consequences of aberrant DNA repair mechanisms stratify ovarian cancer histotypes. Nat Genet 2017; 49: 856–865.
23. Lindgren V, Waggoner S, Rotmensch J. Monosomy 22 in two ovarian granulosa cell tumors by comparative genomic hybridization. Gynecol Oncol 2015; 136: 241–245.
24. Lin YS, Eng HL, Jan YJ, et al. Molecular cytogenetics of ovarian granulosa cell tumors by comparative genomic hybridization. Gynecol Oncol 2005; 97: 68–73.
25. Cabaret S, Anttonen M, Todeschini AL, et al. Combined comparative genomic hybridization and transcriptomic analyses of ovarian granulosa cell tumors point to novel candidate driver genes. BMC Cancer 2015; 15: 251.
26. Wu L, Fidan K, Um JY, et al. Telomerase: key regulator of inflammation and cancer. Pharmacol Res 2020; 155: 104726.
27. Hong C, Moorefield KS, Jun P, et al. Epigenome scans and cancer genome sequencing converge on WNK2, a kinase-independent suppressor of cell growth. Proc Natl Acad Sci U S A 2007; 104: 10974–10979.
28. Jun P, Hong C, Lal A, et al. Epigenetic silencing of the kinase tumor suppressor WNK2 is tumor-type and tumor-grade specific. Neuro Oncol 2009; 11: 414–422.
29. Moniz S, Martinho O, Pinto F, et al. Loss of WNK2 expression by promoter gene methylation occurs in adult gliomas and triggers Rac1-mediated tumour cell invasiveness. Hum Mol Genet 2013; 22: 84–95.
30. Dutruel C, Bergmann F, Rooman I, et al. Early epigenetic down-regulation of WNK2 kinase during pancreatic ductal adenocarcinoma development. *Oncogene* 2014; **33**: 3401–3410.

31. Zhou SL, Zhou ZJ, Hu ZQ, et al. Genomic sequencing identifies WNK2 as a driver in hepatocellular carcinoma and a risk factor for early recurrence. *J Hepatol* 2019; **71**: 1152–1163.

32. Serysheva E, Berhane H, Grumolato L, et al. Wnk kinases are positive regulators of canonical Wnt/beta-catenin signalling. *EMBO Rep* 2013; **14**: 718–725.

33. Tang F, Xu Y, Zhao B. NLRC5: new cancer buster? *Mol Biol Rep* 2020; **47**: 2265–2277.

34. Wang Q, Ding H, He Y, et al. NLRC5 mediates cell proliferation, migration, and invasion by regulating the Wnt/beta-catenin signalling pathway in clear cell renal cell carcinoma. *Cancer Lett* 2019; **444**: 9–19.

35. Peng YY, He YH, Chen C, et al. NLRC5 regulates cell proliferation, migration and invasion in hepatocellular carcinoma by targeting the Wnt/beta-catenin signaling pathway. *Cancer Lett* 2016; **376**: 10–21.

36. Carles A, Trigo-Gonzalez G, Cao Q, et al. The pathognomonic FOXL2 C134W mutation alters DNA-binding specificity. *Cancer Res* 2020; **80**: 3480–3491.

37. Weis-Banke SE, Lerdrup M, Kleine-Kohlbrecher D, et al. Mutant FOXL2(C134W) hijacks SMAD4 and SMAD2/3 to drive adult granulosa cell tumors. *Cancer Res* 2020; **80**: 3466–3479.

**SUPPLEMENTARY MATERIAL ONLINE**

**Figure S1.** Variant calling and filtering of targeted sequencing data

**Figure S2.** Circos plots of aGCTs from the WGS exploratory cohort

**Table S1.** Design and selection of genes for custom panel sequencing

**Table S2.** Mutations in the aGCT validation cohort