Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

Oncogenes and Tumor Suppressors

Molecular Cancer Research

Mutational Landscape of Ovarian Adult Granulosa Cell Tumors from Whole Exome and Targeted *TERT* Promoter Sequencing

Maria Alexiadis[1], Simone M. Rowley[2], Simon Chu[1], Dilys T.H. Leung[1], Colin J.R. Stewart[3,4], Kaushalya C. Amarasinghe[2], Ian G. Campbell[2], and Peter J. Fuller[1]

Abstract

Adult granulosa cell tumor (aGCT), the most common malignant ovarian sex cord-stromal tumor, is characterized by the forkhead transcription factor FOXL2 p.C134W somatic mutation. Late recurrences are relatively common but the molecular mechanisms of relapse or aggressive behavior are not known. The mutational landscape of FOXL2 p.C134W mutation–positive tumors ($n = 22$) was determined using whole-exome sequencing (WES). An average of 64 coding and essential splice-site variants were identified per tumor. As the TERT promoter region is poorly covered by the WES, targeted sequencing identified the TERT -124C>T promoter mutation as the only recurrent mutation (~40% of cases). Pathway analysis suggested an association with DNA replication/repair and the EGFR family canonical pathways. Copy number analysis confirmed that gains of chromosomes 12 and 14 occur in approximately 30% of aGCT and loss of chromosome 22 occurs in approximately 40% of cases. In summary, exome-wide analysis of the mutational landscape of aGCT revealed that, except for the TERT promoter mutation, recurrence and/or aggressive behavior is not defined by activation or loss of specific genes.

Implications: This study found that although aGCTs are defined by the presence of a common *FOXL2* gene mutation, recurrence and/or aggressive behavior cannot be attributed to subsequent mutation of specific gene(s) or pathways; however, there is a high frequency of the TERT -124C>T promoter mutation, which is associated with more aggressive disease.

Introduction

Granulosa cell tumors (GCT) are the most common type of malignant ovarian sex-cord stromal tumor and represent approximately 3%–5% of all primary ovarian malignancies (1, 2); they are subclassified as adult (95%) or juvenile (5%) types. The somatic missense mutation in the *FOXL2* gene (c.402 C>G; p.C134W), which is found in approximately 97% of aGCT (3, 4) argues strongly that this mutation has an etiologic role in these tumors. Although the majority of aGCTs are stage 1 and are cured by surgery, patients presenting with high-stage disease or who later develop recurrence are likely to succumb to their disease (2). Because virtually all aGCTs contain the *FOXL2* mutation, it would seem logical that recurrence and/or aggressive behavior would reflect other subsequent somatic genetic changes in the tumor. The identification of mutations that predict recurrence and/or aggressive behavior would inform the management of women with aGCT and potentially also identify therapeutic targets. Current treatment options, other than surgery, are in general of limited efficacy despite occasional responses in individual patients (2, 5).

Studies that have sought mutations in aGCT by analyzing known oncogenes, tumor suppressor genes, and key signaling pathways have largely been unrewarding (2). To our knowledge, unbiased studies using large-scale sequencing to determine the somatic landscape of aGCT have been limited to the original study of Shah and colleagues (3), which identified the *FOXL2* p.C134W mutation from RNA-seq data, using only four aGCTs. This technology has therefore not previously been applied to an expanded, well-curated panel of aGCTs to address the critical issue of which mutation(s) are responsible for recurrence and/or aggressive behavior. To gain insight into the somatic changes that may determine pathogenesis in aGCT beyond the ubiquitous *FOXL2* p.C134W mutation, we have applied whole-exome sequencing (WES) to DNA extracted from 22 aGCTs. The data have been analyzed for potentially oncogenic and/or novel single nucleotide variants (SNV) and insertions or deletions (indels) as well as for copy number variation. We used a targeted approach to examine the promoter region of the *TERT* gene that encodes the catalytic subunit of telomerase, for two hotspot mutations, -124C>T and -146C>T (sometimes designated C228T and C250T, respectively), which are commonly found in many cancer types (6, 7) one of which, -124C>T, has recently been reported by Pilsworth and colleagues (8) in aGCT.

We report a significant and consistent rate of mutation across the aGCTs including a small number of known oncogenic events but without recurrent mutations in either genes or pathways. The copy number changes observed are consistent with previous

[1]Hudson Institute of Medical Research and Monash University Department of Molecular Translational Science, Clayton, Victoria, Australia. [2]Cancer Genetics Laboratory, Peter MacCallum Cancer Centre, Melbourne, Australia. [3]Department of Histopathology, King Edward Memorial Hospital, Perth, Australia. [4]The School for Women's and Infants' Health, University of Western Australia, Crawley, Western Australia, Australia.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: Peter J. Fuller, Hudson Institute of Medical Research, 27–31 Wright Street, Clayton, Victoria 3168, Australia. Phone: 613-8572-2535; Fax: 613-9594-6125; E-mail: peter.fuller@hudson.org.au

doi: 10.1158/1541-7786.MCR-18-0359

©2018 American Association for Cancer Research.

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

analyses using cytogenetics or comparative genomic hybridization. A high frequency of the *TERT* -124C>T mutation was found in the recurrent aGCT.

Materials and Methods

Patient tissue samples

The GCT samples analyzed are summarized in Table 1. All samples were collected with the patient's informed consent and the study was approved by the Monash Health Human Research and Ethics Committee (Project: 02119B). DNA for WES was extracted from 22 fresh frozen aGCTs from 21 patients (14 stage 1 aGCTs and 8 recurrent aGCTs) collected sequentially and predominantly at our institution (4, 9); two of the samples, a stage 1 (sample 1) and a recurrent (sample 24) were from the same patient, at initial diagnosis and then 3 years later. Five additional previously described samples (10) were used for the *TERT* promoter analysis (Table 1) together with an additional cohort of formalin-fixed paraffin-embedded (FFPE) aGCT blocks from Western Australia (Table 2). Stage is defined according to the FIGO (International Federation of Gynecology and Obstetrics) criteria used for ovarian cancer (11). The aGCTs that were all collected at a surgery subsequent to their initial surgery may be interpreted as either a recurrence or progression of a known aGCT.

Two patients (samples 1 and 18) had matching peripheral blood samples available; one (sample 1) was the patient with two tumor samples (samples 24 and 26; Table 1). The tumor DNA was extracted using the QIAamp DNA Mini Kit and from peripheral blood samples, DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's protocol.

The DNA from the Western Australian cohort of FFPE samples (Table 2) was extracted using the Invitrogen RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Extracted DNA concentration was assessed by spectrophotometry (NanoDrop ND1000, Labtech) and fluorometry (Invitrogen Qubit, Thermo Fisher Scientific).

Exome capture, DNA sequencing, and bioinformatic analyses

Exome enrichment for all samples was performed using SureSelect XT2 Human All Exon V5 capture system (Agilent). Paired-end 100bp reads were sequenced using a HiSeq 1500 instrument (Illumina) by the Monash Health Translational Precinct (MHTP) Medical Genomics Facility. The Peter MacCallum Cancer Centre Bioinformatics Core performed sequence alignment and variant calling. Raw reads were first quality checked using FastQC and trimmed using cutadapt to ensure high read qualities. Filtered reads were then aligned to the human reference genome (GRCH37/hg19) using BWA-MEM (v.0.7.12-r1039) and duplicates were marked using Picard.

Two separate analyses were then performed. All 22 tumor samples were run through an unmatched pipeline using GATK UnifiedGenotyper (3.6), VarScan (2.3.7), and Platypus (0.8.1). The two germline exomes [from samples 1 (and 24) and 18] were analyzed using GATK HaplotypeCaller (3.6).

Annotation of variants was performed using a local copy of the Ensembl version R73 database and a customized version of Ensembl Variant Effect Predictor. Variants that were predicted to

Table 1. Clinical information for the aGCT studied

Sample	Stage	Surgery	Menopausal status	Age at surgery	*TERT* Status
1[a,b]	1	Primary	Pre	53	-124C>T
2[d]	1	Primary	Pre	54	WT
3[d]	1	Primary	Post	50	-124C>T
4[a]	1	Primary	Post	54	WT
5[d]	1	Primary	Post	79	-124C>T
6[b]	2b	Primary	Post	61	WT
7[b]	1a	Primary	Pre	48	-124C>T
8[b]	1	Primary	Pre	29	WT
9[b]	1	Primary	Unknown	50	WT
10[b]	1	Primary	Pre	43	WT
11[b]	1	Primary	Pre	32	WT
12[b]	1	Primary	Unknown	50	WT
13[b]	1	Primary	Pre	41	WT
14[b]	1	Primary	Unknown	49	-124C>T
15[c]	1a	Primary	Pre	43	WT
16[c]	1	Primary	Pre	31	WT
17[c]	1c	Primary	Post	50	WT
18[a,d]	R	Secondary	Pre	48	-124C>T
19[d]	R	Secondary	Post	58	-124C>T
20[d]	R	Secondary	Post	45	WT
21[d]	R	Secondary	Post	54	-124C>T
22[b]	R	Secondary	Post	70	WT
23[b]	R	Secondary	Pre	47	-124C>T
24[a,b]	R	Secondary	Post	56	-124C>T
25[b]	R	Secondary	Post	76	-124C>T
26[a,c]	R	Secondary	Post	54	-124C>T
27[c]	R	Secondary	Post	84	WT

NOTE: Samples 1, 24, and 26 are from the same patient; 24 and 26 are both recurrent samples but obtained at different surgeries.
Abbreviation: R, recurrent.
[a]Matched blood.
[b]WES only.
[c]Microarray (11) only.
[d]WES and Microarray (11).

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

Table 2. TERT analysis on FFPE samples from a Western Australian aGCT cohort

Patient #	Age	Tissue stage/status	*TERT* Status -124	Follow-up/Interval (yr. month)	Follow-up tissue stage/status	TERT Status -124
1	55	1	WT	8.05	Disease free	—
2	43	1	HET	17.06	Disease free	—
3	71	1	WT	11.03	Disease free	—
4	76	1	HET	19.06	Disease free	—
5	37	1	HET	16.03	Disease free	—
6	63	1	HET	18.11	Disease free	—
7	50	1	WT	14.04	Disease free	—
8	51	1	HET	16.11	Disease free	—
9	64	1	WT	5.05	Disease free	—
10	35	1	WT	6.02	Disease free	—
11	54	1	HET	12.10	Disease free	—
12	43	1	WT	10.07	Disease free	—
13	44	1	HET	10.10	Disease free	—
14	84	1	Homozygous	5.01	Disease free	—
15	52	1	HET	9.05	Disease free	—
16	76	Primary	HET	12	Recurrent	Homozygous
17	65	Primary	WT	10.06	Recurrent	WT
18	78	Primary	WT	6.03	Recurrent	HET
19	67	Primary	WT	9.09	Recurrent	HET
20	62	Primary	WT	5.03	Recurrent	HET
21	55	Primary	HET	2.10	Recurrent	HET
22	49	Primary	HET	2.01	Recurrent	HET
23	42	Primary	WT	7.04	Recurrent	WT
24	47	Primary	WT	12.02	Recurrent	WT
25	55	Primary	WT	4.07	Recurrent	HET
26	40	Primary	WT	11.04	Recurrent	HET
27	54	Primary	HET	13.08	Recurrent	HET
28	45	Primary	WT	5	Recurrent	WT
29	50	Primary	HET	18.07	Recurrent	HET
30	46	Primary	WT	6.10	Recurrent	WT
31	68	Primary	WT	4.02	—	—
32	58	Primary	HET	9.10	—	—

affect transcripts (truncating, frameshift, nonsynonymous, and essential splice-site variants) were considered for further analysis. Variants identified in the two germline samples were filtered for read depth ($\geq$10), variant allele frequency ($\geq$0.15), and a minor allele frequency (MAF) of $\leq$0.005 in the Exome Aggregation Consortium (ExAC) database.

Variants identified in the 22 unmatched tumors were filtered as follows: common germline variants and exome sequencing artefacts were eliminated by requiring that the variant allele was not observed in any of the 1000 Genome, Exome Variant Server [EVS (ESP6500 SI-v2)] or ExAC databases, or in an in-house list of variants found in 518 germline exomes. Variants present in genes listed in the top 100 Frequently Mutated Genes (FLAGS) described by Shyr and colleagues (12) were also excluded. Variants were required to be high quality (identified by all three callers), with a total read depth $\geq$10 and a variant allele frequency of $\geq$0.1 and $\leq$0.9. Genes that had more than 26 individual variants identified in multiple samples were removed from the analysis as they are likely to represent misalignments, which are generally seen in large homologous gene families. Where a complex mutation was identified and called as two or more events in one sample, only the most deleterious event was counted in the variant frequency, but all identified events are included in the final variant list in Supplementary Table S1.

The identification filter was relaxed for variants in known cancer driver genes, as we only required variants to be identified by two of the three callers. A filtered list of 1,404 variants in 22 tumors was generated (Table 3). The complex events eliminated are shaded in blue in Supplementary Table S1.

The list of 1,404 variants was then filtered against variants identified in the two matching germline exomes. This reduced the number of variants in those three tumors by approximately 80%, but had no effect on the remaining 19 tumors. Variants eliminated are seen in gray in Supplementary Table S1.

The two matched tumor/normal sets (sample 18 and samples 1 and 24 with their respective germline DNA) were also analyzed using a somatic pipeline in which SNVs and indels were called using MuTect2 (GATK 3.6), VarDict (1.4.6), and VarScan (2.3.7). For these samples, a $\geq$8 total read depth was required and that the mutant allele was not present in germline DNA. Variants were also required to be identified by two or more callers and have a quality score of $\geq$50 (Table 4).

All somatic coding mutations in the three samples with matched germline were manually reviewed by examination of BAM files using the Integrative Genomics Viewer (13, 14).

Validation of candidate driver mutations

Two representative variants (*XIAP* K448Q and *SMAD 3* S264C) identified in tumor samples with germline DNA available were validated and confirmed as somatic using Sanger sequencing. The two variants were chosen for their potential significance as discussed subsequently. Primers were designed with Primer3 software for *XIAP* (forward: 5′-ATATCTCCCGGCACTTGTTG-3′; reverse: 5′-TCCACAAGGAACA AAAACGA–3′); and *SMAD3* (forward: 5′– CACAGACCTGCAGCCAGTTA – 3′; reverse: 5′–CCTGTTGACATTGGAGAGCA–3′).

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

Table 3. Distribution of truncating, ESS, nonsynonymous, and stop loss variants across each sample

	Number of variants by consequence rank				
Sample	**1**	**2**	**3**	**4**	**Total**
1	2	1	34		37
2	4	2	46		52
3	2	2	45		49
4	8		38		46
5	7	2	43		52
6	7	3	72		82
7	6		46		52
8	7	1	56		64
9	11	1	81		93
10	7		151		158
11	4		37		41
12	10	2	91		103
13	9		60		69
14	7	2	62	1	72
18	3		26		29
19	7		35		42
20	4	1	37		42
21	9	1	56		66
22	5	3	57		65
23	6	3	96		105
24	2	1	34		37
25	4		44		48
Total	131	25	1,247	1	1,404

NOTE: Variants are listed according to their consequence: 1, truncating mutation (stop gained or frameshift); 2, ESS (essential splice-site mutation); 3, nonsynonymous mutation; 4, stop/loss mutation.

Table 4. Mutated genes identified in samples with matched germline DNA: 1, 18, and 24

Sample	Symbol	Chromosome	Protein variation	Consequence
1 & 24	ALPL	1	Trp270Ter	Stop gained
1 & 24	CNNM1	10	Val887Ile	Missense
1 & 24	COL3A1	2	Pro983Ser	Missense
1 & 24	DNAH11	7	Phe4641le	Missense
1 & 24	FBXW10	17	Thr248Asn	Missense
1 & 24	RHOT2	16	Ala394Ser	Missense
1 & 24	SMAD3	15	Ser264Cys	Missense
1 & 24	SPRTN	1	Glu37Asp	Missense
1 & 24	TXNRD3	3	Met220Ile	Missense
1 & 24	USP7	16	Met499Val	Missense
1	CILP2	19	Ala491Thr	Missense
1	CLNK	4	Pro274Leu	Missense
1	CTB-186H2.3	17	Leu45Met	Missense
1	SHKBP1	19	Arg657Ser	Missense
1	SPTBN5	15	Ala3417Ser	Missense
24	TTLL11	9	Gly516Val	Missense
24	XIAP	X	Lys448Gln	Missense
18	ANKRD52	12	Pro128His	Missense
18	ATP8B2	1	Asp476Asn	Missense
18	DCDC2C	2	His100Tyr	Missense
18	GJA3	13	Ala290Ser	Missense
18	ITPR3	6	Asp2170Asn	Missense
18	KCND1	X	Asp457Asn	Missense
18	LRP2	2	.	Splice donor
18	MEFV	16	His596Gln	Missense
18	PLA2G16	11	Ile14Phe	Missense
18	SLC4A4	4	Asp960Tyr	Missense
18	STX6	1	Gln148Arg	Missense
18	TMEM134	11	Arg122Gln	Missense
18	UROC1	3	Gly10Ala	Missense
18	ITPR3	6	Asp2170Asn	Missense
18	KCND1	X	Asp457Asn	Missense
18	LRP2	2	.	Splice donor
18	MEFV	16	His596Gln	Missense
18	PLA2G16	11	Ile14Phe	Missense
18	SLC4A4	4	Asp960Tyr	Missense
18	STX6	1	Gln148Arg	Missense
18	TMEM134	11	Arg122Gln	Missense
18	UROC1	3	Gly10Ala	Missense
18	VPS11	11	Gly346Trp	Missense
1, 18, and 24	FOXL2	3	Cys134Trp	Missense

Pathway analyses

The ontogeny of the genes identified as being mutated between stage 1 and recurrent tumors was examined using the Ingenuity Pathway Analysis (IPA) suite (www.ingenuity.com).

Copy number analysis

Copy number analysis from the exome data was performed using the program ADTEx (Aberration Detection in Tumor Exome; ref. 15) with the peripheral blood–derived germline DNA (cases 1 and 18) used as the comparator.

TERT mutation status

The *TERT* promoter region is not well targeted in the Agilent exome capture system so the *TERT* gene promoter region was amplified from the tumor DNA and additional samples using nested PCR (167 with first round primers: forward 5′-ACGAACGTGGCCAGCGGCAG-3′; reverse 5′-CTGGCGTCCC-TGCACCCTGG-3′ with an annealing temperature of 62°C and second round primers: forward 5′–CAGCGCTGCCTGAAACTC-3′; reverse 5′-GTCCTGCCCCTTCACCTT-3′ with an annealing temperature of 55°C (16). Sanger sequencing was used to identify the known hotspot mutations in the *TERT* promoter, -124C>T and -146C>T.

Results

Exome sequencing

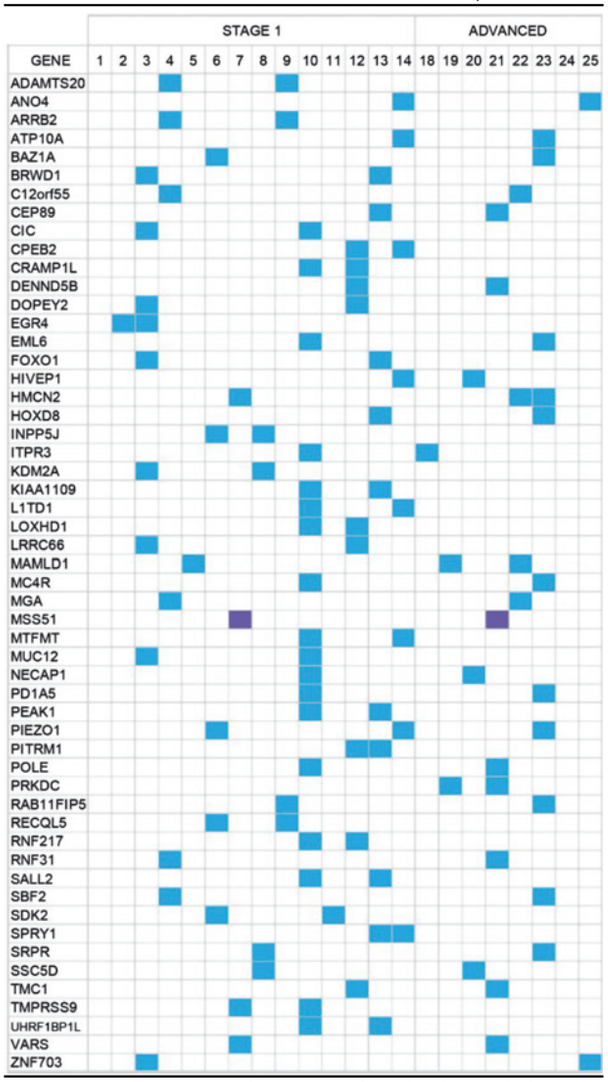
Exomes were sequenced to an average read depth of 67× (range: 40.67–105.63), with an average of 96.5% of target bases with > 10-fold coverage (range: 91.40%–98.82%). In all cases, the *FOXL2* p.C134W mutation was confirmed, a *de facto* positive control. The initial analysis of the 22 samples identified on average 64 variants in each tumor (truncating, nonsynonymous, inframe insertions/deletions, and essential splice-site variants; Table 3; Supplementary Table S1). The absolute numbers of variants identified were on average 69 in stage 1 and 54 in recurrent tumors. The matched germline data were used as reference for the 3 paired samples in a separate analysis and as a filter for the initial analysis above. These analyses identified 16, 15, and 13 coding variants in samples 1, 18, and 24, respectively, noting that samples 1 and 24 are from the same patient (Table 4; Supplementary Table S2).

A limited number of recurrent mutations was identified in individual genes. Fifty-four genes were recurrently mutated; 3 genes were mutated in 3 different patients, and 51 genes were mutated in 2 different patients (Table 5). Only one of the recurrent genes contained the same variant across 2 patients; all other variants were unique (Table 5). When the variants identified in samples 1, 18, and 24 post-germline filtering are considered, none of these variants occurred in both patients, that is, samples 1/24 and 18.

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

Table 5. Genes identified as mutated across more than 1 sample in WES cohort



NOTE: Blue squares represent the presence of a variant in a particular gene. A purple square represents the presence of the same variant across genes.

The genes that contained the 970 variants identified in stage 1 and the 434 variants in the recurrent tumors that were identified to contain truncating (stop gained or frameshift) mutations, essential splice-site mutations, nonsynonymous mutations, and stop/loss mutations were subject to variant effect pathway analysis using the Ingenuity Pathway Analysis suite (www.ingenuity.com). The significant ($P < 0.01$) pathways are shown in Table 6. The canonical pathways identified were linked to DNA replication and/or repair as might be expected in malignancy; and to signaling through the EGFR family.

Potential driver mutations in genes of known oncogenic potential or those known to play a critical role in granulosa cell function were verified in individual tumors. These included XIAP (X-linked inhibitor of apoptosis), which is overexpressed in aGCT (17), and represents a potential therapeutic target and SMAD3, which mediates TGFβ/activin signaling and therefore plays a central role in granulosa cell biology (2).

Table 6. Ingenuity pathway analysis of genes mutated in stage 1 and recurrent aGCT

Ingenuity canonical pathways	−Log (*P*)	Ratio
Cell cycle control of chromosomal replication	2.65	0.179
HER-2 signaling in breast cancer	2.5	0.148
Endometrial cancer signaling	2.21	0.156
DNA double-strand break repair by homologous recombination	2.04	0.286
ErbB2-ErbB3 signaling	2.03	0.147
EGF signaling	2.03	0.147

NOTE: $-\log$ (*P* value) $= -\log_{10} = P$ value. Ratio ($\times 100$) = % of genes analyzed that are present in pathway.

Copy number analysis

Copy number analysis achieved adequate resolution in 15 of the 22 aGCTs (Fig. 1). Of these 15, four (27%) showed gains of chromosome 12 and the same number showed gains at chromosome 14; one of these four aGCTs had both (Fig. 1C). This confirmed previous cytogenetic observations that gains of chromosomes 12 and 14 occur in approximately 30% of aGCTs (18–20). Loss of chromosome 22 has been reported in approximately 40% of aGCTs (18–20); in our cases, we found this loss in 8 of 15 (53%); other large-scale changes are more random and less frequent (Fig. 1C).

TERT promoter mutation status

Two hotspot mutations in the telomerase promoter, -124C>T and -146C>T, are commonly found in many cancer types (6). This region is not adequately covered in the WES so we used targeted PCR on the samples in the whole-exome analysis as well as additional overlapping samples from a previous transcriptomic study (Table 1). Eleven of 26, that is, 42% of the aGCTs in our analysis were heterozygous for the -124C>T *TERT* promoter mutation; a frequency that is at least as high as that seen in those cancers known to commonly contain *TERT* promoter mutations (21). That only the -124C>T *TERT* promoter mutation was found is unusual (6). Five of 17 (29%) of the aGCTs classified as stage 1 were heterozygous for the mutation, whereas 6 of 9 recurrent aGCTs (67%) contained the mutation. The -124C>T *TERT* promoter mutation is also present in the human GCT–derived *FOXL2* p.C134W mutation–positive KGN cell line (4). The initial analysis included a GCT at first presentation, which was stage 1 (sample 1), which had then recurred at a later date (samples 24 and 26); both the primary and metastatic tumors contained the -124C>T *TERT* promoter mutation. This finding, together with an apparent correlation of the presence of this mutation with tumor recurrence, suggested that *TERT* promoter mutation in a stage 1 GCT may be of prognostic significance.

To further explore the possibility that the *TERT* promoter mutation may predict recurrence we used a separate cohort of aGCT samples from Western Australia where patient follow-up data were available. We performed a comparison of 15 stage 1 tumors with no recurrence over a mean follow-up period of 12 years and 17 stage 1 tumors with subsequent metastases with a mean time interval from presentation to metastasis of 8 years and 5 months: in the latter group, 15 of the 17 samples had metastatic tumor available for analysis. All tumors were confirmed to be *FOXL2* mutation positive. The non-recurrent samples had a mean follow-up period of 12 years (9 years for those that did not contain a *TERT* promoter mutation and 14 years for those containing the -124C > T *TERT* promoter mutation). The recurrent samples

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

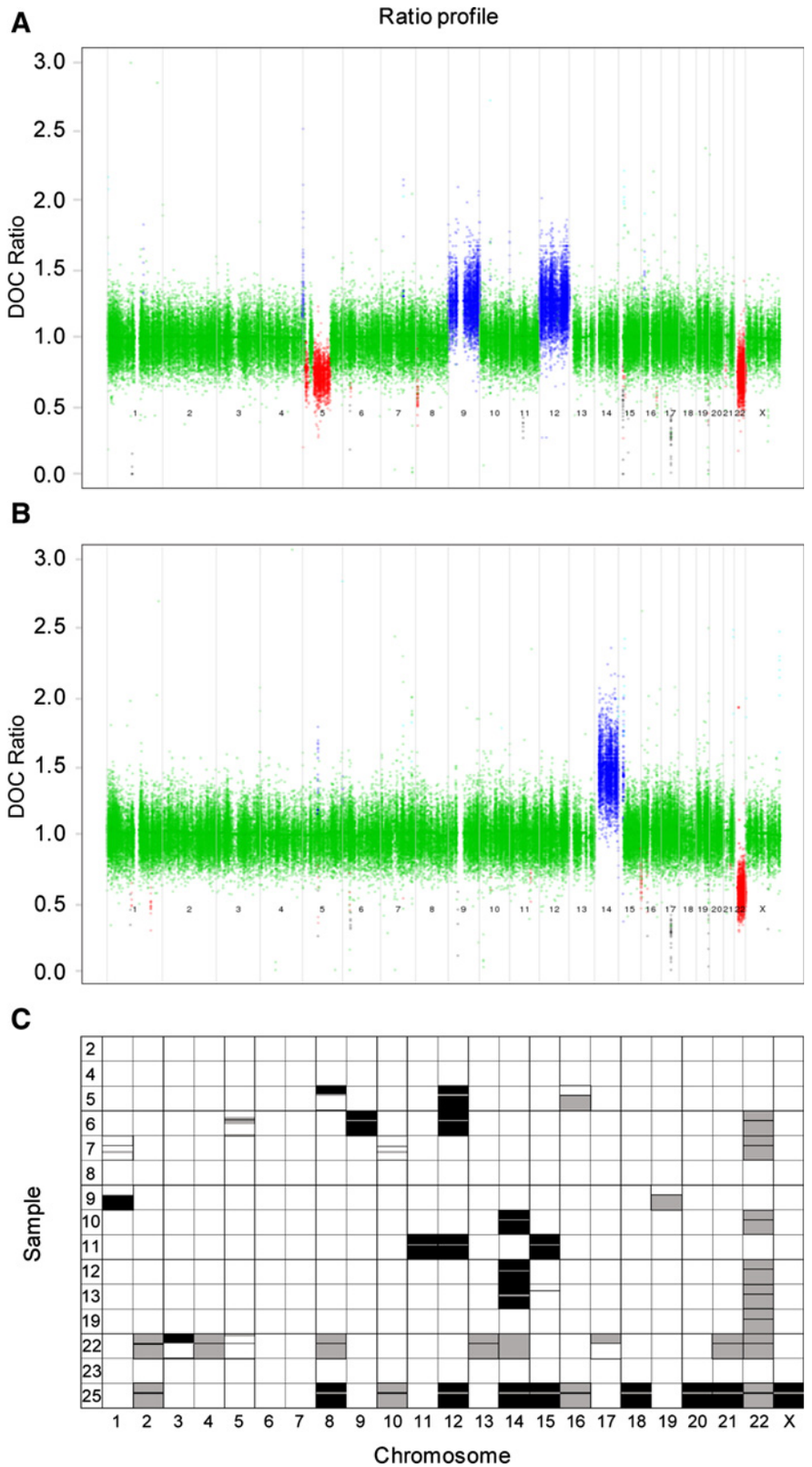


Figure 1.
Representative copy number profiles for 2 tumors (6 and 12) demonstrating the three common changes: copy number gain of chromosomes 12 (**A**) & 14 (**B**) and loss of chromosome 22 (**A** and **B**). Other changes are also seen. A schematic representation of the changes observed in those samples with adequate copy number resolution is shown in **C**. In each box, the short arm is at the top. Gray represents loss and black gain.

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

developed metastases at an average of 8 years and 5 months (7.5 years for those that did not contain a *TERT* promoter mutation and 9 years and 9 months for those that contained the -124C > T *TERT* promoter mutation; Table 2). Of the nonrecurrent tumors, 9 of 15 (60%) contained the -124C > T *TERT* promoter mutation compared with the recurrent group, where at presentation, 11 of 17 (65%) contained the -124C > T *TERT* promoter mutation. Analysis of the available metastases shows that 10 of 15 (67%) contained the -124C > T TERT promoter mutation. Five tumors that were initially wild-type had developed the mutation and one sample that was originally heterozygous had become homozygous.

In our previous transcriptomic study, we examined 12 *FOXL2* p.C134W mutation positive aGCTs. We established the *TERT* mutation status of the 12 aGCTs (Table 1) to enable reanalysis of previous transcriptomic data (10) by -124C>T *TERT* promoter mutation status. Six of 12 tumors contained the -124C>T *TERT* point mutation (2 stage 1 and 4 recurrent). Of the approximately 9,000 expressed genes, the expression levels of 124 genes differed by $\geq$ 2-fold at P value of $\leq$ 0.05 and passed a Westfall Young Permutative multiple correction test (Supplementary Table S3). Of these, 92 of 124 genes were upregulated in the aGCTs demonstrating a *TERT* promoter mutation. This contrasts with our finding of only 24 genes that differed according to tumor stage (10); evidence that *TERT* promoter status provides a more powerful discrimination between aGCT than stage. The 124 genes that differed by *TERT* promoter mutation status were also subjected to Ingenuity Pathway Analysis (Supplementary Table S4); a significant association was observed in three specific canonical pathways associated with protein kinase c-inositol phosphate signaling.

Discussion

This first comprehensive WES analysis of the mutational landscape of aGCT indicates a significant rate of unique genetic variants (SNPs and indels) per tumor. Although an average of 64 variants including the *FOXL2* mutation were identified per tumor, a limitation of this study is the lack of germline DNA for all but two of the patients whose tumors were examined. These 2 cases are, however, informative in that where the somatic pipeline is applied to the samples with matched germline DNA there are 16 (13 in the patient's recurrent sample number 24) and 15 somatic mutations in samples 1 (and 24) and 18, respectively, suggesting that approximately 15 mutations is the likely frequency for aGCT. This frequency compares with 2 to 6 driver genes mutations per tumor identified by Kandoth and colleagues (22) across 12 major cancer types using data from the Cancer Genome Atlas (TCGA). Lawrence and colleagues (23), who analyzed exome sequences from 4,742 cancers of 21 types using paired tumor/normal DNA, found between 1 and 58 mutations per tumor; 7 tumor types having < 10 and two having > 30.

There was not a significant difference in the rate of variant identification when the aGCTs were segregated by stage 1 versus recurrent tumor (69 vs. 54 variants) nor was there a meaningful difference between the stage 1 and recurrent tumor samples (1 and 24) that came from the same patient. The expanded variant numbers resulting from the limited availability of germline filtering does not however detract from the rather unexpected lack of shared potential driver mutations across the cohort; this applies whether the analysis is by mutation, gene affected, or pathway.

Kandoth and colleagues (22) identified 127 significantly mutated putative driver genes, associated with 20 cellular processes. The most commonly mutated gene was *TP53* (42% of samples) as in many studies and tumor types, whereas we identified only one *TP53* mutation (sample 22) as with previous studies of *TP53* status in aGCT (2, 21). *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) was the second most commonly mutated gene in the Kandoth and colleagues (22) analysis, but there were no mutations in our cohort. Of the other 125 putative driver genes (22), variants in 19 (15%) of these genes were also identified in one or more of our samples (Supplementary Table S5; Supplementary Fig. S1). Lawrence and colleagues (23) identified 260 significantly mutated cancer genes across 21 types of cancer; 22 were significant in 3 or more tumor types. Twenty-eight genes of the 260 genes (11%) were found to be mutated in at least one of our cases (Supplementary Table S5; Supplementary Fig. S1). Nik-Zainal and colleagues (24) identified 93 "cancer genes" in 560 breast cancers; we found 20 (22%) of these genes to be mutated (Supplementary Table S5; Supplementary Fig. S1). In combined comparative genomic hybridization and transcriptomic analyses of 10 aGCTs, Caburet and colleagues (20) identified 25 broken, amplified, and deleted candidate driver genes; of these, only 5 (20%) have mutations in 1 or more of our samples. Zehir and colleagues (21) determined the mutational landscape in tumors from 10,000 patients using their targeted MSK-IMPACT panel of 341 cancer-associated genes; 46 (13%) of these genes were mutated in 19 of the aGCT samples in our series and 27 of these overlap with the genes demonstrating mutations in the studies of Lawrence and colleagues (23), Kandoth and colleagues (22), Nik-Zainal and colleagues (24), and Caburet and colleagues (20). Within the Zehir and colleagues (21) study, there were 11 FOXL2 mutation–positive aGCTs (2 primary tumors and 9 "metastasis"; Supplementary Table S6); they identified mutations in 17 (5%) of the 341 cancer-associated genes on the array in these aGCT samples (Supplementary Table S6). In only 4 (24%) of these genes was a mutation also found in this study. In a WES study of non-serous ovarian cancers, Teer and colleagues (25) examined a single *FOXL2* p.C134W mutation–positive aGCT. They described 6 mutations, only 1 of which (ATM serine/threonine kinase) was identified in our analyses. The lack of overlap in the mutational variants identified in these various studies, is curious. Also somewhat surprising is the very limited number of recurrent mutations in specific genes, given that, by many criteria (2, 10) including the pathognomonic mutation in the *FOXL2* gene, aGCTs are remarkably homogenous. Our initial premise was that there was likely to be a small number of "second and third hit" driver mutations that would define tumors presenting at high-stage or developing later metastases but our exome-wide analysis does not, with the exception of the *TERT* promoter mutation, demonstrate specific mutations that define tumor recurrence and/or aggressive behavior. This suggests that "second-hit" mutations are totally random events or that, as in other cancer types, translocation or other copy number variant events including duplications and deletions, may be relevant. In the study of Zehir and colleagues (21), which involved 10,000 tumors of various types, putative gene fusions were identified in 15% of patients and 35% of these involved kinase genes. The associations of the mutated genes identified in the pathway analysis (Table 6) may broadly suggest pathways that may be targeted therapeutically. The association with EGFR signaling genes is particularly intriguing given first that the

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

aGCT-derived KGN cell line exhibits constitutive activity of the MAPK pathway (26, 27) through mechanisms that remain to be defined, and second that a high frequency of the *TERT* promoter mutation has been associated with tumor types that exhibit activation of MAPK signaling (6). The functional significance of the copy number changes requires further characterization, although the observed changes have previously been reported not to correlate with tumor stage or behavior (2, 18–20).

The high frequency of a heterozygous -124C>T *TERT* promoter mutation, identified in 42% of the aGCTs in our cohort and 47% at presentation in the Western Australian cohort, is similar to that seen in thyroid carcinoma and melanoma with only bladder carcinoma and glioma exhibiting higher rates of mutation (21). Pilsworth and colleagues (8) recently published a similar rate of *TERT* promoter mutation in aGCTs. The higher frequency of mutation that we observed in recurrent tumors [5 of 17 of the stage 1 (29%) versus 6 of 9 of recurrent aGCTs (67%)] raises the possibility that *TERT* promoter mutation may be associated with more aggressive clinical behaviour, as reported in papillary thyroid carcinoma (28, 29). This is supported by analysis of our gene expression data (10) where segregation by *TERT* mutation status results in greater separation of the transcriptomic profiles than when the analysis is by stage. Analysis of the Western Australian cohort samples (Table 2) supports an association of the *TERT* promoter mutation with recurrent and/or aggressive disease as also recently reported by Pilsworth and colleagues (8). The prognostic value would, however, appear to be limited in that a number of mutation-negative tumors (65%) recurred. Evidence from Pilsworth and colleagues (8) and our cohorts does, however, provide evidence of an association between *TERT* promoter mutation and progression and/or recurrence in aGCT. In this regard, it is noteworthy that Zehir and colleagues (21) found that 10 of the 11 FOXL2 mutation–positive aGCTs in their 10,000 patient study contained a *TERT* promoter mutation, and that the majority of cases (9/11) were described as metastases.

More recently, it has been shown that *TERT* promoter mutations are subclonal in well differentiated papillary thyroid carcinoma but clonal in poorly differentiated and anaplastic thyroid carcinomas (29). This analysis using Sanger sequencing although at best semiquantitative, provided no suggestion of subclonality; this suggests that, even in the stage 1 tumors examined, the mutation was present early in the development of the tumor.

The *TERT* gene encodes the catalytic subunit of telomerase. It has been noted that melanoma, glioma, and papillary thyroid and bladder carcinomas, all of which have a high frequency of *TERT* promoter mutations, are characterized by activation through *BRAF* or *EGFR* mutation of the MAPK signaling pathway (6). The association of a subset of mutations with this pathway, as noted above, is intriguing given this high frequency of the *TERT* promoter mutation in aGCT.

Elongation and/or preservation of telomere length is regarded as a hallmark of cancer. Two mutually exclusive, hotspot mutations in the telomerase promoter, -124C>T and -146C>T, are common in many cancer types. Although the ratio of these two mutations may vary, in only a few tumor types including another endocrine tumor, adrenocorticol carcinoma (30), has the -124C>T promoter mutation been identified exclusively (6). The mutations are thought to create a binding motif for the GABP (GA-binding proteins), members of the ETS family of transcription factors, which bind as a heterotetromeric complex of GABPA and GABPB; this interaction is required for promoter activation (31). There are *in vitro* data that the two mutations are not equivalent (32), suggesting that in GCT there is a tumorigenic advantage only for the -124C>T promoter mutation given the complete absence of the -146C>T mutation. Increased telomerase activity appears also to be associated with cell proliferation independent of telomere lengthening (7). TERT has been reported to interact with major oncogenic signaling pathways including c-MYC, NFκB, and Wnt/β-Catenin. Of these, activation of NFκB signaling has been reported in the KGN cell line (9, 26) and p65 nuclear localization has been reported in GCT (33), although previous studies (2) and the current studies have not identified mutations in these pathways.

This first comprehensive whole-exome analysis of the mutational landscape of aGCT suggests that recurrence and/or aggressive behavior is not defined by activation or loss of specific genes or pathways. Studies are however needed to exclude a role for splice-variants or genomic rearrangements. The functional significance of the copy number changes also requires further characterization, although they appear not to correlate with stage and/or tumor behavior. The high incidence of the *TERT* -124C>T promoter mutation is likely to be of significance in the development of more aggressive disease perhaps in concert with activation of EGFR-associated signaling pathways that contribute to the risk of recurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Alexiadis, S. Chu, I.G. Campbell, P.J. Fuller
Development of methodology: M. Alexiadis, P.J. Fuller
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Alexiadis, C.J.R. Stewart
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Alexiadis, S.M. Rowley, S. Chu, K.C. Amarasinghe, I.G. Campbell, P.J. Fuller
Writing, review, and/or revision of the manuscript: M. Alexiadis, S.M. Rowley, S. Chu, D.T.H. Leung, C.J.R. Stewart, I.G. Campbell, P.J. Fuller
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Alexiadis
Study supervision: S. Chu, P.J. Fuller

Acknowledgments

The authors wish to thank Nicole Fairweather, Prof. Martin Oehler and Zdenka Prodanovic (Victorian Cancer Biobank) for assistance with collection of the tissue samples and clinical information; Trevor Wilson for advice on the WES; Haroon Naeem from Monash Bioinformatics Platform for assisting us with the pathway analysis using Ingenuity Pathway Analysis (IPA) tool and Sue Panckridge for preparation of the figures. This work is supported by grants from the Cancer Council Victoria; the Ovarian Cancer Research Foundation (to S. Chu); the Granulosa Cell Tumor of the Ovary Foundation; the National Health & Medical Research Council of Australia through a project grant (#1058334) and a Senior Principal Research Fellowship (to P.J. Fuller; #1002559); and the Endocrine Society of Australia through a Research Higher Degree Scholarship (to D.T.H. Leung). The Hudson Institute and the Peter McCallum Cancer Centre are supported by the Victorian Government's Operational Infrastructure Scheme.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 11, 2018; revised June 11, 2018; accepted August 17, 2018; published first August 30, 2018.

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

References

1. Fuller PJ, Leung D, Chu S. Genetics and genomics of ovarian sex cord-stromal tumors. Clin Genet 2017;91:285–91.
2. Jamieson S, Fuller PJ. Molecular pathogenesis of granulosa cell tumors of the ovary. Endocr Rev 2012;33:109–44.
3. Shah SP, Köbel M, Senz J, Morin RD, Clarke BA, Wiegand KC, et al. Mutation of FOXL2 in granulosa-cell tumors of the ovary. N Engl J Med 2009;360;2719–29.
4. Jamieson S, Butzow R, Andersson N, Alexiadis M, Unkila-Kallio L, Heikinheimo M, et al. The FOXL2 C134W mutation is characteristic of adult granulosa cell tumors of the ovary. Mod Pathol 2010;23: 1477–85.
5. Seagle BL, Ann P, Butler S, Shahabi S. Ovarian granulosa cell tumor: a national cancer database study. Gynecol Oncol 2017;146:285–91.
6. Bell RJ, Rube HT, Xavier-Magalhães A, Costa BM, Mancini A, Song JS, et al. Understanding TERT promoter mutations: a common path to immortality. Mol Cancer Res 2016;14:315–23.
7. Pestana A, Vinagre J, Sobrinho-Simões M, Soares P. TERT biology and function in cancer: beyond immortalisation. J Mol Endocrinol 2017;58: R129–46.
8. Pilsworth JA, Cochrane DR, Xia Z, Aubert G, Färkkilä AEM, Horlings HM, et al. TERT promoter mutation in adult granulosa cell tumor of the ovary. Mod Pathol 2018;31:1107–15.
9. Jamieson S, Fuller PJ. Characterization of the inhibitor of kappaB kinase (IKK) complex in granulosa cell tumors of the ovary and granulosa cell tumor-derived cell lines. Horm Cancer 2013;4:277–92.
10. Alexiadis M, Chu S, Leung D, Gould JA, Jobling T, Fuller PJ. Transcriptomic analysis of stage 1 versus advanced adult granulosa cell tumors. Oncotarget 2016;7:14207.
11. Prat J, Belhadj H, Berek J, Bermudez A, Bhatla N, Cain J, et al. Abridged republication of FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum. Eur J Gynaecol Oncol 2015;36:367–9.
12. Shyr C, Tarailo-Graovac M, Gottlieb M, Lee JJ, Karnebeek CV, Wasserman WW. FLAGS, frequently mutated genes in public exomes. BMC Med Genomics 2014;7:64.
13. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol 2011;29:24–6.
14. Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high performance genomics data visualisation and exploration. Brief Bioinform 2013;14:178–92.
15. Amarasinghe KC, Li J, Hunter SM, Ryland GL, Cowin PA, Campbell IG, et al. Inferring copy number and genotype in tumour exome data. BMC Genomics 2014;15:732.
16. Bullock M, Ren Y, O'Neill C, Gill A, Aniss A, Sywak M, et al. TERT promoter mutations are a major indicator of recurrence and death due to papillary thyroid carcinomas. Clin Endocrinol 2016;85:283–90.
17. Chu S, Alexiadis M, Fuller PJ. Proteasome inhibition by bortezomib decreases proliferation and increases apoptosis in ovarian granulosa cell tumors. Reprod Sci 2009;16:397–407.
18. Van den Berghe I, Dal Cin P, De Groef K, Michielssen P, Van den Berghe H. Monosomy 22 and trisomy 14 may be early events in the tumorigenesis of adult granulosa cell tumor. Cancer Genet Cytogenet 1999;112:46–8.
19. Lin YS, Eng HL, Jan YJ, Lee HS, Ho WL, Liou CP, et al. Molecular cytogenetics of ovarian granulosa cell tumors by comparative genomic hybridization. Gynecol Oncol 2005;97:68–73.
20. Caburet S, Anttonen M, Todeschini AL, Unkila-Kallio L, Mestivier D, Butzow R, 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.
21. Zehir A, Benayed R, Shah RH, Syed A, Middha S, Kim HR, et al. Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. Nat Med 2017;23:703–13.
22. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational landscape and significance across 12 major cancer types. Nature 2013;502:333–9.
23. Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. Nature 2014;505:495–501.
24. Nik-Zainal S, Davies H, Staaf J, Ramakrishna M, Glodzik D, Zou X, et al. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature 2016;534:47–54.
25. Teer JK, Yoder S, Gjyshi A, Nicosia SV, Zhang C, Monteiro ANA. Mutational heterogeneity in non-serous ovarian cancers. Sci Rep 2017;7:9728.
26. Chu S, Nishi Y, Yanase T, Nawata H, Fuller PJ. Transrepression of estrogen receptor β signaling by nuclear factor-κB in ovarian granulosa cells. Mol Endocrinol 2004:18:1919–28.
27. Jamieson S, Fuller PJ. Tyrosine kinase inhibitors as potential therapeutic agents in the treatment of granulosa cell tumors of the ovary. Int J Gynecol Cancer 2015;25:1224–31.
28. Melo M, da Rocha AG, Vinagre J, Batista R, Peixoto J, Tavares C, et al. TERT promoter mutations are a major indicator of poor outcome in differentiated thyroid carcinomas. J Clin Endocrinol Metab 2014;99:E754–65.
29. Landa I, Ibrahimpasic T, Boucai L, Sinha R, Knauf JA, Shah RH, et al. Genomic and transcriptomic hallmarks of poorly differentiated and anaplastic thyroid cancers. J Clin Invest 2016;126:1052–66.
30. Liu T, Brown TC, Juhlin CC, Andreasson A, Wang N, Bäckdahl M, et al. The activating TERT promoter mutation C228T is recurrent in subsets of adrenal tumors. Endocr Relat Cancer 2014;21:427–34.
31. Bell RJ, Rube HT, Kreig A, Mancini A, Fouse SD, Nagarajan RP, et al. Cancer. The transcription factor GABP selectively binds and activates the mutant TERT promoter in cancer. Science 2015;348:1036–9.
32. Li Y, Zhou QL, Sun W, Chandrasekharan P, Cheng HS, Ying Z, et al. Non-canonical NF-κB signalling and ETS1/2 cooperatively drive C250T mutant TERT promoter activation. Nat Cell Biol 2015;17:1327–38.
33. Bilandzic M, Chu S, Wang Y, Tan HL, Fuller PJ, Findlay JK, et al. Betaglycan alters NFκB-TGFβ2 cross talk to reduce survival of human granulosa tumor cells. Mol Endocrinol 2013;27:466–79.

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.

Published OnlineFirst August 30, 2018; DOI: 10.1158/1541-7786.MCR-18-0359

AACR American Association for Cancer Research

Molecular Cancer Research

Mutational Landscape of Ovarian Adult Granulosa Cell Tumors from Whole Exome and Targeted *TERT* Promoter Sequencing

Maria Alexiadis, Simone M. Rowley, Simon Chu, et al.

Mol Cancer Res 2019;17:177-185. Published OnlineFirst August 30, 2018.

Updated version	Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-18-0359
Supplementary Material	Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2018/08/30/1541-7786.MCR-18-0359.DC1

Cited articles	This article cites 33 articles, 5 of which you can access for free at: http://mcr.aacrjournals.org/content/17/1/177.full#ref-list-1
Citing articles	This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/17/1/177.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/17/1/177. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

Downloaded from mcr.aacrjournals.org on January 20, 2021. © 2019 American Association for Cancer Research.