Laboratory Investigation

The molecular landscape of 227 adult granulosa cell tumors of the ovary: Insights into the progression from primary to recurrence

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Abstract:	Adult granulosa cell tumors (AGCTs) of the ovary are characterized by their propensity

	for late recurrences and are primarily managed surgically due to the limited efficacy of systemic treatment. The FOXL2 p.C134W somatic mutation has been identified in ~95% of AGCT cases, and TERT promoter alterations have been linked to worse overall survival. Additionally, KMT2D mutations have been observed more frequently in recurrences compared to primary tumors. This study analyzed a total of 183 primary AGCTs and 44 recurrences without corresponding primary tumors. The primary AGCTs were categorized into three groups: 77 non-recurrent tumors, 18 tumors which later recurred (including 9 cases with matched primary-recurrence pairs), and 88 tumors with unknown recurrence status. Targeted next-generation sequencing was conducted on 786 cancer-related genes to investigate their genetic profile. The study aimed to identify the molecular alterations associated with AGCT pathogenesis and recurrence rate, comparing primary vs. recurrent tumors, and primary-recurrent vs. primary non-recurrent cases. Our findings confirmed the high prevalence (99%) of the FOXL2 p.C134W mutation in AGCTs. Secondary truncating FOXL2 mutations were observed in 5% of cases. Two cases with typical AGCT morphology were FOXL2 wild-type, harboring mutations in KRAS or KMT2D instead, suggesting alternative genetic pathways. TERT promoter mutations were found in 43% of cases, more frequently in recurrences. However, survival analyses indicated only a non-significant trend towards worse overall survival in patients with TERT promoter mutations. Other recurrent mutations detected in the cohort included KMT2D (10%), FOXO1 (7%), CHEK2 (5%), TP53 (3.5%), PIK3CA (3.5%), and AKT1 (3%). Two recurrent, FOXL2-mutated cases also carried DICER1 mutations. One tumor exhibited MSI-High status and a TMB of 19 mut/Mb. Survival analysis indicated that FOXO1 mutations could be associated with poorer overall survival and shorter time to recurrence, suggesting its potential as a prognostic marker, warranting further investigation.
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Laboratory Investigation
Dear Editor,

We would appreciate it if you would consider our manuscript " The molecular landscape of 227 adult granulosa cell tumors of the ovary: Insights into the progression from primary to recurrence" for publication in your journal.

Our study provides a comprehensive characterization of the molecular landscape of AGCTs. We confirmed a high prevalence of the FOXL2 p.C134W mutation in our dataset, while also demonstrating that the absence of this mutation does not exclude AGCT diagnosis confirmed by morphology. Additionally, we emphasized the prognostic value of FOXO1 mutations and identified the role of TERT promoter mutations in tumor recurrences. For the first time, we detected an MSI-High, TMB-High sample within the cohort of AGCTs based on NGS.

These findings highlight the importance of further research to validate potential biomarkers and assess their therapeutic relevance. Future studies should focus on incorporating molecular profiling into the clinical management of AGCTs, with the potential to advance personalized and more effective treatment approaches.

We hope that our manuscript would by beneficial for the readership.

Thank you for receiving our manuscript and considering it for review and publication in your Journal. We appreciate your time and look forward to your response.

Yours sincerely,
Kristýna Němejcová

DECLARATIONS

Ethical approval: The study was approved by the Ethics Committee of the General University Hospital in Prague in compliance with the Helsinki Declaration (No. 2140/19 S-IV). The Ethics Committee waived the requirement for informed consent as according to the Czech Law (Act. no. 373/11, and its amendment Act no. 202/17), it is not necessary to obtain informed consent in fully anonymized studies.

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The molecular landscape of 227 adult granulosa cell tumors of the ovary: Insights into the progression from primary to recurrence

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ABSTRACT

Adult granulosa cell tumors (AGCTs) of the ovary are characterized by their propensity for late recurrences and are primarily managed surgically due to the limited efficacy of systemic treatment. The *FOXL2* p.C134W somatic mutation has been identified in ~95% of AGCT cases, and *TERT* promoter alterations have been linked to worse overall survival. Additionally, *KMT2D* mutations have been observed more frequently in recurrences compared to primary tumors.

This study analyzed a total of 183 primary AGCTs and 44 recurrences without corresponding primary tumors. The primary AGCTs were categorized into three groups: 77 non-recurrent tumors, 18 tumors which later recurred (including 9 cases with matched primary-recurrence pairs), and 88 tumors with unknown recurrence status. Targeted next-generation sequencing was conducted on 786 cancer-related genes to investigate their genetic profile. The study aimed to identify the molecular alterations associated with AGCT pathogenesis and recurrence rate, comparing primary vs. recurrent tumors, and primary-recurrent vs. primary non-recurrent cases.

Our findings confirmed the high prevalence (99%) of the *FOXL2* p.C134W mutation in AGCTs. Secondary truncating *FOXL2* mutations were observed in 5% of cases. Two cases with typical AGCT morphology were *FOXL2* wild-type, harboring mutations in *KRAS* or *KMT2D* instead, suggesting alternative genetic pathways. *TERT* promoter mutations were found in 43% of cases, more frequently in recurrences. However, survival analyses indicated only a non-significant trend towards worse overall survival in patients with *TERT* promoter mutations. Other recurrent mutations detected in the cohort included *KMT2D* (10%), *FOXO1* (7%), *CHEK2* (5%), *TP53* (3.5%), *PIK3CA* (3.5%), and *AKT1* (3%). Two recurrent, *FOXL2*-mutated cases also carried *DICER1* mutations. One tumor exhibited MSI-High status and a TMB of 19 mut/Mb.

Survival analysis indicated that *FOXO1* mutations could be associated with poorer overall survival and shorter time to recurrence, suggesting its potential as a prognostic marker, warranting further investigation.

Keywords: adult-type granulosa cell tumor of the ovary, *FOXL2*, *TERT* promoter, *KMT2D*, *FOXO1*, *DICER1*, TMB, MSI

INTRODUCTION

Granulosa cell tumor (GCT) of the ovary represents a subset of estrogen-producing sex-cord stromal tumors, constituting 2-5% of all ovarian malignancies [1-3]. These tumors can be classified into two main subtypes based primarily on the clinical symptoms, patient age, and histopathological features: juvenile granulosa cell tumors (JGCTs) and adult granulosa cell tumors (AGCTs), with the AGCT being predominant and representing approximately 95% of all GCT cases.

AGCTs can manifest at any age, however, patients are commonly diagnosed during the perimenopausal or early postmenopausal stages, typically between 50 and 54 years of age [4]. These tumors often exhibit a slow and indolent growth pattern and are diagnosed as stage I disease, resulting in a favorable prognosis. However, aggressive recurrences, typically emerging > 5 years after the initial diagnosis, are observed in up to one third of cases and can be fatal [5,6].

Current treatment strategies involve surgical resection, with adjuvant therapy considered in cases where surgery is not a feasible option [7]. The prognosis of patients with AGCTs is predominantly determined by the FIGO stage, while the role of adjuvant chemotherapy remains unclear [8,9]. However, the management of recurrent disease is still clinically challenging due to the limited systemic treatment efficacy [10]. The lack of effective systematic therapies emphasizes the need for novel therapeutic

approaches and highlights the importance of molecular characterization in identifying actionable targets.

A recurrent somatic missense mutation in the *FOXL2* gene (c.402 C>G; p.C134W) was identified in 97% of AGCTs, elucidating the underlying oncogenic mechanisms [1,11]. *FOXL2*, a transcription factor involved in ovarian function and granulosa cell differentiation, emerged as a key driver in GCT pathogenesis [12]. The p.C134W variant has since been recognized as a potential molecular hallmark of AGCTs, as it has been detected only rarely in other tumor types [1,13]. Subsequent independent investigations have confirmed the high prevalence of this mutation in AGCTs, distinguishing them from other sex cord-stromal tumors [14]. Cases of AGCTs lacking this mutation raise questions about potential misdiagnoses and warrant further investigation [11,14,15].

More recently, somatic *TERT* promoter hotspot mutations, recognized as biomarker for the prognosis of various cancer type [16,17] , have been frequently observed in AGCT patients experiencing a recurrence [18-21]. Beyond the *TERT* mutation, recent studies have reported the occurrence of altered cancer-related genes, including *TP53*, *PIK3CA*, *CTNNB1*, and *KMT2D* [18-20,22,23]. Some of those have suggested that inactivating mutations in *KMT2D* may serve as potential pathogenic drivers in AGCTs [24].

Moving forward, additional focus on the molecular landscape of AGCTs may provide valuable insights into their pathogenesis, identify novel therapeutic targets, and refine diagnostic criteria for improved patient management. Unfortunately, due to the rarity of AGCT diagnosis, molecular studies on AGCTs are naturally limited to a small number of cases [25].

This study aims to comprehensively characterize the mutational profile, tumor mutational burden (TMB), and microsatellite instability (MSI) status of 227 adult granulosa cell tumors (AGCTs), including 183 primary tumors and 44 recurrences. The objective is to provide novel insights into the molecular pathogenesis and mechanisms driving the recurrence of this rare malignancy.

METHODS

Samples

The sample set of 290 AGCT cases represents a dataset already used in our previous immunohistochemical study [26]. Initially, 319 cases diagnosed as AGCT were retrieved from the archives of the co-operating institutions. Following a central review by two experienced gynecological pathologists (KN and PD), the final dataset was refined to 290 cases, of which 227 cases had successful DNA analysis. In 9 cases, tumor tissue from both the primary tumor and subsequent recurrences was available. The *FOXL2* mutation was present in all but two cases. Both *FOXL2*-negative cases exhibited typical AGCT morphology and were therefore retained in the dataset.

Patient clinical characteristics

Clinical data on the patient and tumor characteristics were obtained retrospectively from the medical records. The date of primary surgical procedure was reported as the date of diagnosis. Deaths due to unknown causes or unrelated to the diagnosis were classified as "dead of other causes". Clinical data availability was limited, with follow-up information available for only 63% (143/227) of patients.

NGS analyses of DNA

To describe the spectrum and frequency of genomic alterations and Copy Number Variations (CNV), targeted capture next-generation sequencing (NGS) analysis of the DNA was performed on 227 qualitatively sufficient FFPE samples, comprising 183 cases of primary origin, and 44 recurrences

without matched primary tumors. The DNA panel covered 786 genes or gene parts (2440 kbp of target sequence; 1992 kbp of coding sequence; Supplementary Table S1).

The DNA library preparation was conducted as previously described [27]. Libraries were paired-end sequenced using the NextSeq 500 instruments (Illumina) with the NextSeq 500/550 High Output Kit v2.5 (150 cycles). Bioinformatic evaluation, annotation, interpretation, and classification of detected variants were processed as described previously [27]. Only pathogenic and likely pathogenic variants were reported. Truncating variants in known oncogenes were not evaluated as pathogenic. The analysis does not allow for distinguishing between somatic and germline origins of the variants.

Copy Number Variations (CNVs) for each sample were calculated using the mean of four non-tumor sample controls as a baseline, with recommended loss vs. gain cut-offs for individual tumor sample purity levels (manual GW 23.0.5; https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current).

Homozygous deletions (both gene level and exon level) were defined as losses adjusted by tumor purity. Amplifications were designated if the calculated number of gene copies was > 10.

Due to the variable integrity of FFPE DNA, the quality of each CNV sample analysis was evaluated using a CNV scoring system. This system involved adding up all absolute differences between the adjusted fold change of each region and its regional fold change, then dividing by the total number of CNV regions. Samples with a CNV score of ≤ 0.20 were further analyzed for all CNV events, moreover, the detected events were manually checked for potential miscall of hybridization errors. Samples with a higher CNV score were evaluated individually, with only high-level amplifications or homozygous deletions designated as events when applicable. Homozygous deletions were evaluated only for known tumor suppressor genes, while amplifications were evaluated only for known oncogenes according to the OncoKB database [28].

Microsatellite instability (MSI) was assessed for all samples using the NGS bioinformatics pipeline based on 17 microsatellite loci, standardized for evaluating MSI in colorectal and endometrial carcinomas. These loci were included in the DNA gene panel as described previously [29]. A sample was classified as microsatellite unstable if ≥ 25% of its microsatellite markers were determined to be unstable. Additionally, immunohistochemical (IHC) analysis of mismatch repair (MMR) proteins, including MLH1, PMS2, MSH6, and MSH2, was conducted according to previously described protocol [29].

Statistical analyses

Standard descriptive statistics were employed to summarize the dataset: categorical variables were reported as frequencies and percentages, while continuous variables were expressed as means with standard deviation (SD), or medians with interquartile range.

Associations between patient characteristics and tumor origin were assessed using Pearson's chi-square test or Fisher's exact test for categorical variables, and the Mann-Whitney U-test for continuous variables. For matched sample comparisons, the paired t-test was used.

For survival analyses, data on the dates of primary excision, first recurrence, and mortality were collected in 143 cases. Overall survival (OS) was calculated from the date of surgery to the date of recorded death or to the last known follow-up date (censoring). Recurrence-free survival (RFS) was calculated from the date of primary surgery to the date of the first recurrence.

The univariate and multivariate Cox proportional hazard (Cox PH) regression models (*survival::coxph*) were used to estimate the hazard ratio associated with molecular pattern (e.g. *TERT*-mutated vs. *TERT* wild-type, *FOXL2* one hit vs. *FOXL2* two hits), adjusting for age, binarized FIGO stage, and adjuvant therapy. Cox PH were described by regression coefficients (β), hazard ratios (HR) with 95% confidence

intervals (95%CI), and corresponding statistical significance (*p*-value). A backward stepwise elimination from the full model was employed to obtain the minimal adequate model.

Survival curves were constructed using the Kaplan-Meier method (*survminer::ggsurvplot*) and differences between the compared groups were assessed using the log-rank test.

All statistical analyses were performed using the R software version 4.3.3. (2024-02-29). All tests were two-sided and a *p*-value <0.05 was considered statistically significant.

RESULTS

Clinico-pathological features of primary and recurrent AGCTs

Detailed clinico-pathological characteristics of the 227 AGCT cases are described in Table 1.

The median age of the entire patient cohort was 58 years (range 24-83), with a significant difference observed between the primary (median = 59 years) and recurrent cases (median = 47 years; $p < 0.001$). This could suggest that younger patients are more susceptible to subsequent recurrence. However, younger patients might also be monitored for a longer average duration, which increases the probability of detecting a late relapse.

Among patients with a known FIGO stage, 89% (132/148) presented with stage IA-IC. Notably, only 7% of primary cases had higher stages, whereas 25% of recurrences were diagnosed as FIGO II-IV ($p = 0.014$). Furthermore, a significant difference was observed between primary and recurrent tumors regarding the FIGO stage I classification, with primaries predominantly manifesting as stage IA (76%), while recurrences exhibited a distribution of 48% in stage IA and 52% in stages IB-IC ($p = 0.009$).

The follow-up data was available for 143 cases, with an average length of 100 months (median = 68; range 1-432). Primary cases had significantly shorter follow-up time (median = 45 months) compared to recurrences (median = 183 months; $p < 0.001$). Similarly, the primary non-recurrent cases had notably shorter follow-up time (median = 40 months) than the primary recurrent cases (median = 150 months; $p < 0.001$).

The recurrence rate in the dataset with available follow-up data was 40% (57/143), with 6% (9/143) of patients dying of the disease, all of whom experienced recurrence. The time to recurrence was an average of 100 months, with a median of 82 months.

When comparing the subset of primary cases only, based on subsequent recurrences, primary recurrent cases did not differ from primary non-recurrent cases in any of the tested parameters, except for the duration of follow-up (Table 1).

Mutational landscape of primary and recurrent AGCTs

The comprehensive mutation profile of genes recurrently altered in the entire cohort is visualized in Fig. 1. All detected pathogenic and likely pathogenic mutations are listed in Supplementary Table S2.

A comparison of the main molecular characteristics between the primary and recurrent cases is summarized in Table 2.

Briefly, 99% (225/227) of cases in our dataset harbored the *FOXL2* mutation p. C134W, with no significant difference between the primary and recurrent cases. Additionally, truncating mutations representing a secondary hit of *FOXL2* were detected in 12 cases (5%), both in primaries and recurrences (Supplementary Table S3). Notably, two tumors with typical histological and immunohistochemical features of AGCTs, which were both non-recurrent primary cases, were found to be *FOXL2* wild-type. These two cases were diagnosed at FIGO I stage, exhibited no evidence of disease, and were found to be mutated in *KRAS* or *KMT2D*, respectively.

The second most frequently alteration was *TERT* promoter mutation, observed in 43% (98/227). Mutations in the *TERT* promoter were significantly more frequent in recurrences compared to primary cases (p = 0.007). The predominant alteration observed was *TERT* c.-124C>T (known in literature as C228T), detected in 90% of mutated primary and 74% of mutated recurrent cases. The less frequent mutation, c.-146C>T (known as C250T), was present in 10% of mutated primaries and 26% of mutated recurrences. The frequency of specific TERT promoter mutations in mutated cases did not differ significantly between the primary and recurrent cases (p = 0.055).

In 10% of samples, an inactivating *KMT2D* variant was identified, with a comparable occurrence rate observed in both primary (9% mutated) and recurrent cases (14% mutated; p = 0.406).

FOXO1 truncating variants were identified in 7% of cases, with a similar frequency of mutated cases observed in primaries and recurrences (p = 0.314). Notably, all patients with *FOXO1* mutations also harbored the *FOXL2* p.C134W variant and all of them were *KMT2D* wild-type and *TP53* wild-type.

Among other frequently altered genes, *CHEK2* mutations were found in 5% of all cases, followed by *TP53* and *PIK3CA* mutations, each observed in 3.5% of cases and *AKT1* mutations in 3% of cases. *TP53* mutations were present in all cases with *FOXL2* mutations. Three cases with *TP53* mutation also harbored *TERT* promoter mutations. No mutual exclusivity was detected between these genes.

Additionally, some pathogenic or likely pathogenic mutations were detected in other genes, albeit at a much lower frequency (Fig. 1; Supplementary Table S2).

Interestingly, two cases of *DICER1* mutations were detected in our cohort of AGCTs. Both cases originated from recurrences and presented with a low FIGO stage. One of the patients harbored a frameshift variant (c.1447_1469delins, p.G483fs), while the other had two pathogenic mutations in *DICER1* (c.1630C>T, p.T55* and c.4031C>T, p.S1344L). A sufficient quantity of non-tumor tissue was not available to distinguish between a germline mutation and a somatic mutation in *DICER1*-mutated cases. Both cases were also found to harbor mutations in the *FOXL2* and *TERT* promoter and were *TP53* wild-type.

Comparison between the subset of primary recurrent and primary non-recurrent cases did not reveal any significant differences in selected molecular features between the groups. However, there was an increasing trend in the frequency of *TERT* mutations and *KMT2D* mutations from primary non-recurrent to primary recurrent cases (Table 2).

TMB, MSI, and CNV of primary and recurrent AGCTs

The mean tumor mutation burden (TMB) of the cohort was 5.8 mut/Mb (median = 6; range 2-19). There was no significant difference in TMB between the primary and recurrent cases (p = 0.937). The TMB also did not differ based on the presence of mutation in either *TERT* promoter (p = 0.399), *KMT2D* (p = 0.524), *FOXO1* (p = 0.332), *CHEK1* (p = 0.361), or *TP53* (p = 0.188).

A single case with TMB-High status (TMB = 19 mut/Mb) was identified. This case was a primary, non-recurrent tumor and was the only sample in the dataset that also exhibited an MSI-High status by NGS. However, IHC of MMR proteins was evaluable in 220 out of 227 cases, and all these tumors, including the MSI-High sample identified by NGS, were MMR proficient [26]. (Němejcová et al. 2024). No pathogenic or likely pathogenic mutations in MMR genes were found in the MSI-High sample; however, duplication of exon 7 of the *PMS2* gene (NM_000535.7) was observed.

Results of CNV were available in 58 % of cases (132/227). No amplification of oncogene was detected. Homozygous deletion of *CDKN2A* and *CDKN2B* was observed in one recurrent case. Homozygous deletion of *NOTCH1* was detected in one primary non-recurrent tumour and homozygous deletion of *POLD1* gene was detected in one primary case without information of recurrence (Fig. 1).

Comparison between matched primaries and recurrences

The mutational landscape was evaluated in primary tumors and their matched recurrences from nine patients. Mutational differences between these samples are detailed in Table 3.

Briefly, all cases harbored the *FOXL2* mutation p.C134W in both the primary and recurrence samples. *TERT* promoter mutations were identified in at least one sample of a given patient in five cases. In three pairs, the mutation was present in both the primary tumor and matched recurrence, while in one case, only the recurrence harbored the mutation, suggesting it may have been acquired during disease progression. Interestingly, in one pair, only the primary tumor exhibited the mutation, while the recurrence lacked the alteration in a specific hotspot. In this case, the *TERT* mutation was probably weeded out by selection during the ongoing tumorigenesis.

Additionally, *KMT2D* truncating mutations were detected in three pairs, with two of them sharing the mutation in both the primary and recurrence, and in one case, only the recurrence harbored the *KMT2D* mutation. Other genes, in which pathogenic mutations were detected only in the recurrence and not in the primary tumor, included *PIK3CA*, *SMARCA4*, and *NAV3* (all in one pair). Matched primaries and recurrences did not differ in TMB score ($p > 0.995$).

Association of *TERT* promoter alterations with recurrences and survival

A higher prevalence of *TERT* mutations was observed in recurrent cases compared to primary cases ($p = 0.007$; Table 2). When comparing only the subset of primary AGCTs according to known subsequent recurrences, primary non-recurrent cases did not significantly differ in the rate of *TERT* promoter mutations from the primary recurrent cases ($p = 0.393$; Table 2).

For survival analyses, we classified *TERT* mutations in two ways: first, we grouped cases with either C250T or C228T mutations as having a mutated phenotype, while the rest were considered wild-type. Second, we focused specifically on the C228T mutation, treating it as mutated and all others, including C250T, as wild-type. This was performed because C228T is the predominant mutation, and other studies have suggested its association with worse prognosis. Finally, only *TERT*-mutated cases were compared according to specific hotspot variant.

Survival analyses showed no significant difference in tested clinical outcomes between patients with and without *TERT* mutations, whether considering C228T/C250T together or C228T alone (Supplementary Table S4). However, in univariate analyses, there was a slight but non-significant trend towards worse prognosis in term of overall survival for cases with C228T mutation compared to wild-type cases ($p = 0.064$). In contrast, recurrence-free survival in patients with the *TERT* promoter mutation was similar to patients without it. When comparing only *TERT*-mutated cases in relation to specific type of alterations (C228T vs. C250T), there was no difference in any of the tested outcomes (Supplementary Table S4).

Association of *KMT2D* alterations with recurrences and survival

The prevalence of *KMT2D* truncating mutations did not differ between the primary and recurrent cases in our study ($p = 0.406$), nor between the primary non-recurrent and primary recurrent cases ($p = 0.394$; Table 2). Furthermore, no significant difference was observed in overall or recurrence-free survival based on *KMT2D* mutation status (Supplementary Table S4).

Association of other recurrent altered genes with survival

Besides the *TERT* promoter and *KMT2D* alterations, univariate survival analyses were performed based on mutation status of other frequently altered genes: *FOXO1*, *TP53*, *CHEK2,* and based on the status of *FOXL2* (C134W vs. second hit).

Only *FOXO1* mutations appeared to have prognostic value, indicating poorer overall survival in mutated cases compared to those without mutation, with a median OS of 35.8 years in *FOXO1* wild-type cases versus 11.6 years in *FOXO1*-mutated cases (p = 0.009), and shorter time to recurrence, with a median RFS of 10.3 years in wild-type cases compared to 5.7 years in mutated cases (p = 0.045; Supplementary Table S4). In multivariate Cox PH analyses, *FOXO1* status remained significant, along with the patients age for overall survival and FIGO stage for recurrence-free survival (Table 4). However, clinical data on *FOXO1*-mutated cases are highly limited, as shown in the Kaplan-Meier curves (Supplementary Fig. S1), which precludes definitive conclusions.

The mutation status of *TP53*, *CHEK2,* and *FOXL2* had no impact on survival in any of the clinical parameters tested (Supplementary Table S4).

DISCUSSION

This study provides a comprehensive analysis of the clinico-pathological and molecular characteristics of primary and recurrent adult granulosa cell tumors. The NGS analysis has reaffirmed the previously reported limited number of recurrent mutations in individual genes within AGCTs and confirmed the high prevalence of the *FOXL2* p.C134W mutation, consistent with earlier findings which have established this mutation as a hallmark of AGCTs [1]. Interestingly, two primary non-recurrent cases were *FOXL2* wild-type, harboring mutations in *KRAS* or *KMT2D* instead, suggesting that alternative genetic pathways might drive tumorigenesis in a small subset of AGCTs. Moreover, truncating mutations in *FOXL2*, in addition to p.C134W, were detected as secondary hits in 5% of samples.

A higher prevalence of *TERT* promoter mutations was observed in recurrent AGCT cases compared to primary cases, which aligns with previous findings [18-20]. Although our survival analysis did not show a statistically significant impact of *TERT* mutations on overall or recurrence-free survival, there was a non-significant trend towards worse overall survival in patients with *TERT* promoter mutations. This trend, particularly for the c.-124C>T (C228T) mutation, is consistent with prior study suggesting association of C228T with poorer overall survival in a set of 186 primary cases [18]. Our findings, based on a dataset of 143 AGCTs, support the hypothesis that *TERT* promoter mutations may contribute to poorer overall survival in AGCT patients. However, the non-significant results highlight the need for further research to fully elucidate the prognostic value of *TERT* promoter mutations in AGCTs.

The role of *KMT2D* mutations in AGCTs remains less clear. No significant difference in the prevalence of *KMT2D* mutations between the primary and recurrent cases was found in our cohort. This contrasts with previous research by Hillman et al. [24], which has suggested that *KMT2D* mutations might contribute to tumor progression and recurrence on a subset of 79 AGCT patients. On the contrary, this finding was not confirmed by other studies [20,23], nor by our study. Additionally, our survival analyses showed no significant impact of *KMT2D* mutational status on survival outcomes.

Interestingly, a *KRAS* mutation was identified in only one case in our dataset, which was the *FOXL2* wild-type case, corresponding to a frequency of 0.4%. Similarly, Roze et al. reported a *KRAS* mutation in just one patient (2%); however, in their study, this mutation co-occurred with alterations in both *FOXL2* and *KMT2D* [22]. In contrast, Rowland et al. reported a higher incidence of KRAS mutations in AGCT patients, with an occurrence rate exceeding 4% [30].

A recent study has highlighted the role of the *FOXO1* gene, an antagonist of *FOXL2*, in AGCT pathogenesis [31,32] and has suggested an interaction between the *FOXO1* and *FOXL2* pathways, proposing that *FOXO1* alterations may represent a secondary genetic event in AGCT development. These alterations could potentially contribute to tumor progression or aggressiveness, particularly in cases where *FOXO1* co-occurs with *FOXL2* [31,32]. Our findings identified *FOXO1* truncating variants in 7% of cases, all of which were *FOXL2*-mutated, *KMT2D* wild-type, and *TP53* wild-type. The presence of *FOXO1* mutations in our study was associated with significantly poorer overall survival and shorter time to recurrence. The identification of *FOXO1* as a possible prognostic marker aligns with recent research suggesting its involvement in AGCT pathogenesis and its interaction with the *FOXL2* pathway [31,32]. While it is possible that the *FOXO1* gene could serve as a potential prognostic marker for AGCT diagnosis, this hypothesis requires more robust, independent investigation.

Our study also reported rare occurrences of *DICER1* mutations in recurrent AGCTs. Mutations in *DICER1* have been identified primarily in Sertoli-Leydig cell tumors [33,34]. Although *DICER1* mutations have been reported in the juvenile type of granulosa cell tumors (JGCTs), they were not commonly observed in AGCTs [33-35], except for findings published by Roze et al. [22]. They suggested *DICER1* as a potential alternative driver in the development of this tumor type, as they detected alterations in *DICER1* only in the *FOXL2* wild-type cases [22]. On the contrary, our study detected both the *DICER* mutations in *FOXL2*-mutated recurrent cases. This could suggest a potential role for *DICER1* mutations particularly in recurrent AGCTs, but further exploration is needed.

Moreover, our findings did not confirm mutually exclusivity of *TP53* and *FOXL2* mutations. All eight *TP53*-mutated cases in our cohort harbored *FOXL2* mutation p.C134W, and all exhibited low tumor mutation burden. Contrary to a previous report [36], *TP53*-mutated cases did not demonstrate higher TMB compared to *TP53* non-mutated cases. This contradicts the hypothesis of a “high-grade” pattern proposed by some researchers, who defined a new subgroup with aggressive behavior and high TMB of *TP53*-mutated cases [22,36,37].

In AGCTs, there is limited evidence regarding the prevalence of predictive biomarkers associated with FDA-approved therapies, such as tumor mutation burden (TMB) or microsatellite instability (MSI). To our knowledge, this study is the first to report the occurrence of MSI-High status in an AGCT cohort. Previous studies have classified all AGCT cases as MMR-proficient [38,39] or MSS [36,38]. In our cohort, one tumor (0.4%) was identified as MSI-High based on NGS analysis. This case showed a high TMB of 19 mut/Mb, no pathogenic MMR genes mutations, but *PMS2* exon 7 duplication was observed. Despite this, the expression of all MMR proteins was retained. Discrepancies between NGS-MSI testing and IHC have been reported, where protein expression may be preserved, but the function of the protein is lost [40,41]. Therefore, studies based solely on IHC may underestimate the presence of MSI status in AGCTs.

Although our findings provide new insights into the genetic background of AGCTs, several limitations must be addressed. Firstly, the clinical data on patient’s follow-up and survival status were highly limited in our dataset. Secondly, CNV analysis was conducted on only 132/227 samples.

CONCLUSION

In conclusion, our study provides a comprehensive characterization of the molecular landscape of AGCTs. We confirmed a high prevalence of the *FOXL2* p.C134W mutation in our dataset, while also demonstrating that the absence of this mutation does not exclude AGCT diagnosis confirmed by morphology. Additionally, we emphasized the prognostic value of *FOXO1* mutations and identified the

role of *TERT* promoter mutations in tumor recurrences. For the first time, we detected an MSI-High, TMB-High sample within the cohort of AGCTs based on NGS.

These findings highlight the importance of further research to validate potential biomarkers and assess their therapeutic relevance. Future studies should focus on incorporating molecular profiling into the clinical management of AGCTs, with the potential to advance personalized and more effective treatment approaches.

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Author contribution: KN and PD designed the study concept. RM drafted the initial manuscript and performed all statistical analyses. RM, KN, NH and JH interpreted data. All authors contributed to material preparation and data collection. All authors provided critical feedback on the manuscript drafts and approved the final version of the manuscript.

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Data availability: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Supplementary information: The online version contains supplementary material.
Table S1: List of genes included in the NGS panel.
Table S2: List of all pathogenic/likely pathogenic variants detected in dataset of 227 AGCTs.
Table S3: List of detected *FOXL2* mutation other than p.C134W in dataset of 227 AGCTs.
Table S4: Results of univariate Cox Proportion Hazard models based on mutational status of selected genes.
Figure S1: Kaplan-Meier curves for patients with AGCT stratified by *FOXO1* status. *FOXO1*-mutated cases are associated with A) worse overall survival and B) shorter time to recurrence. Numbers at risk demonstrate the cumulative number of events, *p*-values are based on log-rank test.

DECLARATIONS

Ethical approval: The study was approved by the Ethics Committee of the General University Hospital in Prague in compliance with the Helsinki Declaration (No. 2140/19 S-IV). The Ethics Committee waived the requirement for informed consent as according to the Czech Law (Act. no. 373/11, and its amendment Act no. 202/17), it is not necessary to obtain informed consent in fully anonymized studies.

Informed consent: Not applicable.

Conflict of interests: The authors declare no conflict of interests.

TABLES AND FIGURES:

Table 1: Clinicopathological characteristics of 227 AGCT patients stratified by tissue origin (Primary vs. Recurrence) and 95 primary AGCT patients stratified by known recurrence status (P non-recurrent vs. P recurrent)

Table 2: Selected molecular characteristics of 227 AGCT patients stratified by tissue origin (Primary vs. Recurrence) and 95 primary AGCT patients stratified by known recurrence status (P non-recurrent vs. P recurrent)

Table 3: Selected clinico-pathological features and mutational status of primaries and their matched recurrences in 9 AGCT patients.

Table 4: Results of minimal adequate models of multivariate Cox Proportional Hazard regression models for overall survival and recurrence-free survival

Fig.1: OncoPrint showing the landscape of genetic alterations in cancer-related genes in primary and recurrent AGCTs.

Cases are shown in columns and genes in rows. Primary cases are categorized based on recurrence status. All clinical and molecular categories are color-coded according to the legend.

N/A – data not available, NED – no evidence of disease, AWD – alive with disease, DOC – death of other cause, DOD – death of disease, n. – number

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Figure

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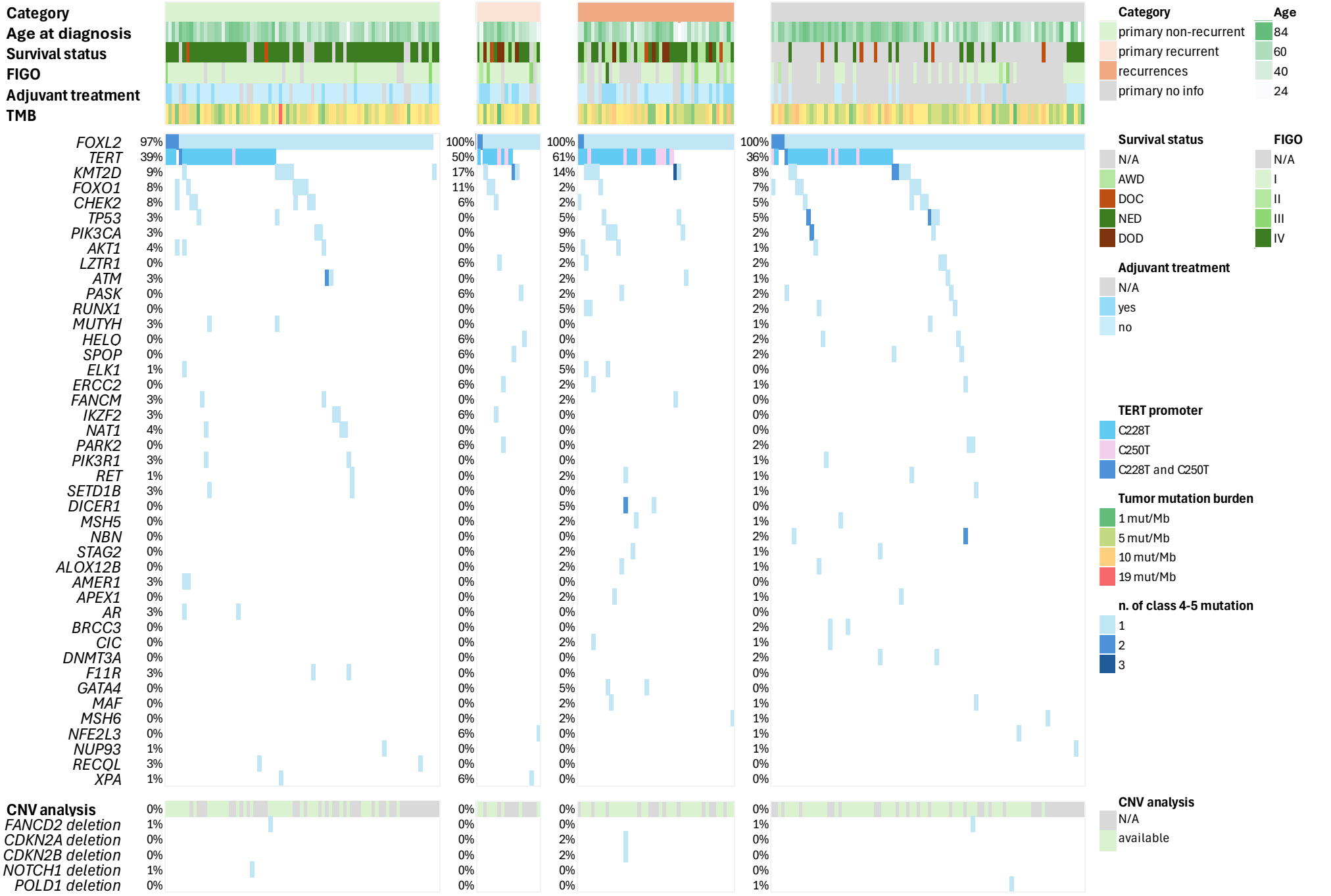

Table 1

Table 1: Clinicopathological characteristics of 227 AGCT patients stratified by tissue origin (Primary vs. Recurrence) and 95 primary AGCT patients stratified by known recurrence status (P non-recurrent vs. P recurrent)

Variables	Primary (n = 183)	Recurrence (n = 44)	*p*-value	P non-recurrent (n = 77)	P recurrent (n = 18)	*p*-value
Age at diagnosis (years)			**<0.001[m]**			0.066[m]
mean (SD)	57 (13.6)	49 (13.4)		57 (12.4)	51 (13.5)	
median (range)	59 (24-83)	47 (27-77)		59 (25-79)	51 (29-77)	
FIGO stage			**0.014[f]**			0.073[f]
low (I)	111 (93%)	21 (75%)		68 (96%)	13 (81%)	
high (II-IV)	9 (7%)	7 (25%)		3 (4%)	3 (19%)	
N/A	*63*	*16*		*6*	*2*	
FIGO I			**0.009[p]**			0.088[f]
IA	84 (76%)	10 (48%)		53 (78%)	7 (54%)	
IB-IC	27 (24%)	11 (52%)		15 (22%)	6 (46%)	
Lympho-vascular invasion			0.595[f]			0.282[f]
No	103 (94%)	14 (88%)		66 (99%)	11 (92%)	
Yes	6 (6%)	2 (12%)		1 (1%)	1 (8%)	
N/A	*74*	*28*		*10*	*6*	
AH/EIN and/or EEC			-			0.744[f]
No	67 (65%)	0 (-)		44 (64%)	7 (70%)	
Yes	36 (54%)	0 (-)		25 (36%)	3 (30%)	
N/A	*80*	*44*		*8*	*8*	
Radicality of surgery			0.353[f]			0.337[f]
R0	95 (98%)	14 (93%)		52 (98%)	11 (92%)	
R1	2 (2%)	1 (7%)		1 (2%)	1 (8%)	
N/A	*86*	*29*		*24*	*6*	
Adjuvant therapy			0.053[p]			0.072[f]
No	80 (82%)	23 (66%)		56 (84%)	9 (60%)	
Yes	18 (18%)	12 (34%)		11 (16%)	6 (40%)	
N/A	*85*	*9*		*10*	*3*	
Follow-up time (months)			**<0.001[m]**			**<0.001[m]**
mean (SD)	62 (60.6)	197 (105.3)		52 (48.4)	137 (58.5)	
median (range)	45 (1-252)	183 (13-432)		40 (1-181)	150 (35-252)	
Disease status at last control			-			-
NED	85 (83%)	19 (48%)		62 (95%)	7 (41%)	
AWD	6 (6%)	14 (35%)		1 (2%)	5 (29%)	
DOD	4 (4%)	5 (12%)		0 (0%)	4 (24%)	
DOC	8 (8%)	2 (5%)		2 (3%)	1 (6%)	
N/A	*80*	*4*		*12*	*1*	

p- values are based on Pearson chi-square test ([p]) or Fisher Exact test ([f]) for categorical variables and Mann-Whitney U-test ([m]) for continuous variables; **statistically significant *p*-values are indicated in bold.**

Percentages are counted only from available data and are rounded up/down.

AGCT – adult granulosa cell tumors, SD – standard deviation, N/A – data not available, AH – endometrial atypical hyperplasia, EIN – endometrial intraepithelial neoplasia, EEC – endometroid endometrial cancer, NED – no evidence of disease, AWD – alive with disease, DOD – death of disease, DOC – death of other cause

Table 2: Selected molecular characteristics of 227 AGCT patients stratified by tissue origin (Primary vs. Recurrence) and 95 primary AGCT patients stratified by known recurrence status (P non-recurrent vs. P recurrent)

Variables	Primary (n = 183)	Recurrence (n = 44)	*p*-value	P non-recurrent (n = 77)	P recurrent (n = 18)	*p*-value
FOXL2 status			> 0.995[m]			> 0.995[f]
Wild type	2 (1%)	0 (0%)		2 (3%)	0 (0%)	
C134W	181 (99%)	44 (100%)		75 (97%)	18 (100%)	
TERT promoter status*			**0.007[p]**			0.393[p]
Wild-type	112 (61%)	17 (39%)		47 (61%)	9 (50%)	
Mutated	71 (39%)	27 (61%)		30 (39%)	9 (50%)	
C228T	64 (90%)	20 (74%)		29 (97%)	7 (78%)	
C250T	7 (10%)	7 (26%)		1 (3%)	2 (22%)	
KMT2D status			0.406[f]			0.394[f]
Wild-type	166 (91%)	38 (86%)		70 (91%)	15 (83%)	
Mutated (truncating)	17 (9%)	6 (14%)		7 (9%)	3 (7%)	
FOXO1 status			0.314[f]			> 0.995[f]
Wild-type	169 (92%)	43 (98%)		71 (92%)	16 (89%)	
Mutated	14 (8%)	1 (2%)		6 (8%)	2 (11%)	
CHEK2 status			0.469[f]			> 0.995[f]
Wild-type	172 (94%)	43 (98%)		71 (92%)	17 (94%)	
Mutated	11 (6%)	1 (2%)		6 (8%)	1 (6%)	
TP53 status			0.481[f]			> 0.995[f]
Wild-type	177 (97%)	42 (95%)		75 (97%)	18 (100%)	
Mutated	6 (3%)	2 (5%)		2 (3%)	0 (0%)	
TMB status			0.599[f]			> 0.995[f]
TMB-Low (< 10 mut/Mb)	179 (98%)	42 (95%)		76 (99%)	18 (100%)	
TMB-High (≥ 10 mut/Mb)	4 (2%)	2 (5%)		1 (1%)	0 (0%)	
MSI status			> 0.995[f]			> 0.995[f]
MSS	182 (99%)	44 (100%)		76 (99%)	18 (100%)	
MSI-High	1 (1%)	0 (0%)		1 (1%)	0 (0%)	

p- values are based on Pearson chi-square test ([p]) or Fisher Exact test ([f]) for categorical variables and Mann-Whitney U-test ([m]) for continuous variables; **statistically significant *p*-values are indicated in bold.**

Percentage is counted only from available data and are rounded up/down.

* - *p*-value is based only on status wild-type vs. mutated.

AGCT – adult granulosa cell tumors, SD – standard deviation, MSS – microsatellite stable, MSI – microsatellite instable.

Table 3: Selected clinico-pathological features and mutational status of primaries and their matched recurrences in 9 AGCT patients.

Sample ID	Origin	Age	Survival status	FIGO	MSI status	TMB (mut/MB)	FOXL2 status	TERTp status	KMT2D status	FOXO1 status	CHEK2 status	TP53 status	PIK3CA status	SMARCA4 status	NAV3 status	PASK status	SPOP status	MSH5 status
P_8	Primary	59	AWD	IIIC	MSS	6	**C134W**	**C228T**	wt	**H134fs**	wt	wt	wt	wt	wt	wt	wt	wt
R_8	Matched Recurrence				MSS	7	**C134W**	**C228T**	wt	**H134fs**	wt	wt	wt	**E700fs**	**R272***	wt	wt	wt
P_62	Primary	46	NED	IA	MSS	4	**C134W**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R_62	Matched Recurrence				MSS	3	**C134W**	wt	**F3672fs**	wt	wt	wt	wt	wt	wt	wt	wt	wt
P_74	Primary	55	N/A	IB	MSS	5	**C134W**	**C228T**	wt	wt	wt	wt	wt	wt	wt	wt	wt	**R181***
R_74	Matched Recurrence				MSS	5	**C134W**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	**R181***
P_86	Primary	53	NED	IC1	MSS	6	**C134W**	wt	wt	wt	wt	wt	wt	wt	wt	**splice**	wt	wt
R_86	Matched Recurrence				MSS	6	**C134W**	wt	wt	wt	wt	wt	wt	wt	wt	**splice**	wt	wt
P_95	Primary	55	AWD	IC1	MSS	7	**C134W**	**C250T**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R_95	Matched Recurrence				MSS	5	**C134W**	**C250T**	wt	wt	wt	wt	**H1047R**	wt	wt	wt	wt	wt
P_112	Primary	60	N/A	IA	MSS	5	**C134W**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R_112	Matched Recurrence				MSS	5	**C134W**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
P_123	Primary	33	N/A	IC1	MSS	4	**C134W**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R_123	Matched Recurrence				MSS	4	**C134W**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
P_173	Primary	69	AWD	IC1	MSS	6	**C134W**	wt	**L3428fs**	wt	wt	wt	wt	wt	wt	wt	**Y87C**	wt
R_173	Matched Recurrence				MSS	6	**C134W**	**C228T**	**L3428fs**	wt	wt	wt	wt	wt	wt	wt	**Y87C**	wt
P_176	Primary	66	DOD	IC1	MSS	3	**C134W**	**C228T**	**H3000fs**	wt	wt	wt	wt	wt	wt	wt	wt	wt
R_176	Matched Recurrence				MSS	5	**C134W**	**C228T**	**H3000fs**	wt	wt	wt	wt	wt	wt	wt	wt	wt

AGCT – adult granulosa cell tumors, AWD – alive with disease, NED – no evidence of disease, DOD – death of disease, N/A – data not available, TERTp – TERT promoter, wt – wild-type, **mutated cases are indicated in bold**.

Table 4: Results of minimal adequate models of multivariate Cox Proportional Hazard regression models for overall survival and recurrence-free survival

Predictor	β	SE	HR (95%CI)	*p*-value
Overall survival				
FOXO1 (wild-type vs. mutated)	1.817	0.776	6.15 (1.34-28.23)	**0.019**
age (continuous)	0.076	0.022	1.08 (1.03-1.13)	**< 0.001**
Recurrence-free survival				
FOXO1 (wild-type vs. mutated)	1.622	0.765	5.1 (1.13-22.72)	**0.034**
FIGO stage (I vs. II-III)	1.342	0.394	3.83 (1.76-8.28)	**< 0.001**

β – regression coefficient, SE – standard errors, HR – hazard ratio, CI – confidence intervals, *p*-values are based on log-rank test, **statistically significant *p*-values are indicated in bold**.

Full models included age, FIGO stage and adjuvant therapy as covariates.