

**Molecularly Defined Adult Granulosa Cell Tumor of the Ovary: the clinical phenotype**

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## Abstract

The histopathologic features of Adult Granulosa Cell Tumors (AGCTs) are relatively non-specific, resulting in misdiagnosis of other cancers as AGCT, a problem that has not been well characterized. *FOXL2* mutation testing was used to stratify 336 AGCTs from three European centers into three categories: 1) *FOXL2* mutant molecularly defined AGCT (MD-AGCT)(n=256 of 336), 2) *FOXL2* wild-type AGCT (n=17 of 336), 3) misdiagnosed other tumor types (n=63 of 336). All statistical tests were two-sided. The overall and disease-specific survival of the misdiagnosed cases was lower than in the MD-AGCTs ( $P<0.001$ ). The misdiagnosed cases accounted for 71.9% of disease-specific deaths within five years. In the population-based cohort, overall survival of MD-AGCT patients was not different from age-matched population-based controls. Even though 35.2% of all the MD-AGCT patients, in our study, experienced a relapse, AGCT is usually an indolent disease. The historical, pre-molecular data underpinning our clinical understanding of AGCT was likely skewed by inclusion of misdiagnosed cases, and future management strategies should reflect the potential for surgical cure and long survival even after relapse.

## Main text

Adult Granulosa Cell Tumor (AGCT) accounts for 3-5% of all ovarian cancers (1, 2) and is characterized by an unpredictable disease course with reported recurrence rates between 6-50% (3-6). AGCTs can show histomorphological patterns similar to a variety of unrelated tumors, and diagnosis can be challenging. In historical series false positive diagnosis rates of up to 36% have been recorded (7, 8), hampering our ability to understand the clinical behavior of AGCT.

We identified a single somatic point mutation in the forkhead transcription factor *FOXL2* (402C>G) C134W in 97% of centrally reviewed AGCTs (9). This mutation is a pathognomonic defining feature of AGCT, and is not seen in other tumors in particular other ovarian cancers (10-15). Analysis of the *FOXL2* mutation has proven useful in the differential diagnosis of AGCT, and its incorporation into diagnostic algorithms has been proposed (16-18), yet the clinical impact of using this diagnostic tool has not been determined. Our objective was to analyze the C134W *FOXL2* mutation status and clinical outcomes of three European cohorts of AGCT patients to determine, for the first time, the clinical behavior of true AGCTs when diagnosis is based on a robust molecular marker.

A cohort of 369 ovarian AGCTs were identified in pathology records of three European centers: Helsinki University Hospital, Finland (248 cases), the Center for Gynecologic Oncology Amsterdam (CGOA), The Netherlands (79 cases), and Tuebingen University Hospital, Germany (42 cases). The Helsinki and Tuebingen University Hospitals both serve populations, whereas the CGOA consists of 3 referral hospitals. Clinical and follow-up data were retrospectively collected, as previously described (19, 20). The ethics committees of Helsinki University Central Hospital and the National Supervisory Authority for Welfare and Health approved this study. The study material was strictly handled after anonymization of the data according to national ethical guidelines of 'Code for Proper Secondary Use of Human Tissue' developed by Federation of Medical Societies (FMWV) in the Netherlands. Therefore the need for obtaining informed consent was waived by the three-referral centers. The Independent Ethics Committee (IEC) of the University of Tuebingen approved this study.

We were able to perform *FOXL2* (C>G) C134W mutation analysis in 336 out of 369 cases with the allelic discrimination assay (9, 16). After *FOXL2* mutation testing, tumors were stratified as mutation positive molecularly defined AGCT (MD-AGCT) or negative (*FOXL2* wild-type), and all *FOXL2* wild-type tumors were subjected to histopathological review, and additional immunohistochemical (IHC) analysis (11) (**Supplementary Materials**) was performed to further refine diagnoses and cases assigned to one of three final categories; 1) MD-AGCT (n=256/336, 76.2%), 2) AGCT *FOXL2* wild-type (AGCT-WT) (i.e. typical AGCT morphology/immunophenotype but *FOXL2* mutation negative) (n=17/336, 5.1%), 3) Misdiagnosed other tumor types (n=63/336, 18.8%). The misdiagnosis rate in each of the three cohorts was: Helsinki; 18.8%, Amsterdam; 19.5%, Tuebingen; 17.1%, and the revised diagnoses included other sex cord-stromal tumors (49.2%), epithelial malignancies (44.4%) and miscellaneous tumors (6.3%) many of which have their own distinct molecular features and treatment strategies (**Supplementary Table 1**). If we disregard the misdiagnosed other tumor types, 256/273 (93.8%) AGCTs harbor the *FOXL2* mutation.

Clinical characteristics of patients with *FOXL2* MD-AGCTs, AGCT-WT and misdiagnosed other tumor types are described in **Table 1**. All calculated P-values are two-sided and statistical significance was assessed at the 0.05 level. Univariable associations were examined using Fisher's exact test for categorical variables and t-tests for continuous ones (**Supplementary Materials**). As this was a multicenter study, we used a stratified log-rank test to account for cohort. The overall survival (OS) (**Figure 1A**) and disease specific survival (DSS) (**Figure 1B**) were clearly distinct ( $P < 0.001$ ) between MD-AGCTs and the misdiagnosed other tumor types. Molecularly defined diagnoses remains statistically significant when cohort, stage and age at diagnosis were included as covariates in a Cox proportional hazard model; however, we note that due to the long term follow-up in one cohort, proportional hazard assumptions are not met (**Supplementary Table 2**). Whilst the number of AGCT-WT cases was small (n=17), these tumors were associated with a similar OS compared to the MD-AGCT cases (**Figure 1A**). Although 35.2% of all MD-AGCT recurred, we found that 71.9% (23/32) of all disease-specific deaths in the first five years after diagnosis were within the subset of misdiagnosed other types of tumors. We

determined the OS of patients after their first disease relapse and found that all patients with recurrent misdiagnosed other tumor types (primarily carcinomas) died within 5-years of the first relapse (**Figure 1C**). In contrast, 76.4% of the patients with relapsed MD-ACGT survive to five years, and 53.6% survive to ten years after their first relapse.

As MD-AGCTs generally show an indolent disease course, we assessed whether the life expectancies of Finnish MD-AGCT patients differ from an age and gender-matched Finnish population. The OS of MD-AGCT patients with and without recurrence were similar to the population for the first 10 years (**Figure 1D**). The expected survival curves were estimated using the Hakulinen method (21) from life tables. Data with diagnoses prior to 2010 were used to compare to expected population data in order to match to available survivals dates.

This is the first large retrospective clinical outcomes study of AGCTs where unrelated tumors that mimic the histomorphological features of AGCT have been excluded. We confirmed the presence of the *FOXL2* mutation in 94.1% of AGCTs from three European centers with all showing a similar misdiagnosis rate (17-20%). AGCT-WT cases are morphologically typical, are rare but do exist and merit further study; they appear to have similar clinical features to MD-AGCTs. The role of other mutations in modifying the behavior of MD-AGCT is unknown. The conflicting data from previous studies on AGCT prognosis (22, 23) is attributable to the inclusion of non-AGCT cases. The majority (71.8%) of disease-specific deaths were caused by misdiagnosed tumors. Conversely, only 6 (2.2%) patients with MD-AGCTs died of disease within the first five years, and 56.8% of all MD-AGCT patients who experienced a recurrence were alive five years thereafter. Most recurrences and deaths in historical molecularly unconfirmed cohorts of AGCT likely occur in patients with misdiagnosed other tumor types. We propose that in all cases with diagnostic uncertainty where the differential diagnosis includes AGCT, *FOXL2* mutation testing should be performed to allow for accurate diagnosis and appropriate treatment and clinical follow-up (24, 25) (**Figure 1E**). Incorporating *FOXL2* mutation testing into routine pathological assessment will aid in moving towards reproducible diagnosis of AGCT; allowing for future studies to accurately study the clinical course of true ovarian AGCTs creating uncontaminated cohorts for clinical

trials. The *FOXL2* mutation frequency in any future AGCT series should be over 90% or the accuracy of primary diagnoses is questionable.

The median time to recurrence of MD-AGCT, for those who recurred, in the Finnish population based cohort was 7.2 years, suggesting that both the clinical standard follow-up time of five years, as recommended by the NCCN (26), SGO (27) and ESMO (28) is illogical and of unproven benefit.

Our study cohort originates from three different European centers, which results in limitations typical of multicenter studies. The Helsinki and Tuebingen centers provide service to a general population, however the Amsterdam center consists of 3 tertiary hospitals, making the Dutch cohort a selected population. This has likely resulted in a selection bias, resulting in an overestimation of disease recurrences in the Amsterdam cohort. The cohort from Germany is also limited by the shorter follow-up time. Differences in stage distributions between the cohorts may be explained by differences in staging methods and procedures over the 60 year ascertainment period of this study (19).. Due to these potential limitations, we have presented analyses for both the individual and combined cohorts. It was not possible to properly generalize conclusions to the combined cohorts for some specific clinicopathological features and outcome analysis, such as stage and relapse-free survival. However, the distribution and prognosis of MD-AGCTs were comparable within each cohort and we therefore have presented survival curves based on the combined cohorts. Moreover, since cancer survival is known to vary greatly between countries (29), these data from the three combined cohorts will putatively more accurately depict the clinical behavior of MD-AGCT.

This study provides an explicit view of the clinical behavior of MD-AGCT, in particular the long latency to relapse and overall favorable outcomes, highlighting the critical importance of accurate diagnosis.

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## **Notes**

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**Table 1: Clinical characteristics of patients with molecularly defined AGCT**

	Total	Finland	Netherlands	Germany	P-value <sup>†</sup>
<b>Age in Years (at last birthday)</b>					
Median (IQR)	54 (45-61)	54 (44-62)	52 (45-58)	57 (47-70)	0.28
<b>Stage (FIGO 2009)</b>					
Stage I	218 (85.2%)	148 (90.2%)	46 (76.7%)	24 (75.0%)	0.03
I	11 (4.3%)	6 (3.7%)	5 (8.3%)	0 (0%)	0.002
Ia	130 (50.8%)	88 (53.7%)	21 (35.0%)	21 (65.6%)	
Ib	4 (1.6%)	1 (0.6%)	2 (3.3%)	1 (3.1%)	
Ic	73 (28.5%)	53 (32.3%)	18 (30.0%)	2 (6.2%)	
Stage II	23 (9.0%)	10 (6.1%)	8 (13.3%)	5 (15.6%)	
Stage III	10 (3.9%)	3 (1.8%)	4 (6.7%)	3 (9.4%)	
Unknown	5 (2.0%)	3 (1.8%)	2 (3.3%)	0 (0%)	
<b>Type of Surgery</b>					
HYS+BSO	157 (61.3%)	112 (68.3%)	26 (43.3%)	19 (59.4%)	<0.001
USO	66 (25.8%)	31 (18.9%)	28 (46.7%)	7 (21.9%)	
BSO	26 (10.2%)	14 (8.5%)	6 (10.0%)	6 (18.6%)	
Other/Unknown	5 (2.0%)	5 (3.0%)	0 (0.0%)	0 (0%)	
<b>Any Adjuvant Treatment*</b>					
Yes	52 (20.3%)	40 (24.4%)	8 (13.3%)	4 (12.5%)	0.15
No	197 (77.0%)	123 (75.0%)	52 (86.7%)	22 (68.8%)	
Unknown	7 (2.7%)	1 (0.6%)	0 (0.0%)	6 (18.8%)	
<b>Follow-up Time</b>					
Reverse KM	10.49	14.32	8.96	5.34	
<b>OS % (95%CI)</b>					
5-year	93.3 (91.5-98.4)	94.9 (91.5-98.4)	92.6 (85.8-99.9)	85.2 (72.7-99.8)	
10-year	84.4 (79.5-89.5)	89.8 (81.4-92.5)	80.5 (69.7-91.3)	79.5 (64.6-98.0)	
15-year	72.1 (65.6-79.2)	77.0 (70.0-84.7)	59.4 (44.7-79.1)	39.8 (9.8-100)	
<b>Deaths</b>					
Yes	92 (35.9%)	65 (39.6%)	6 (18.8%)	21 (35.0%)	0.07
No	164 (64.1%)	99 (60.4%)	26 (81.2%)	39 (65.0%)	
<b>DSS % (95%CI)</b>					
5-year	97.4 (95.3-99.5)	98.6 (96.8-100)	94.6 (88.9-100)	95.5 (87.1-100)	
10-year	91.8 (88.0-95.8)	93.2 (88.9-97.6)	87.4 (78.3-97.5)	95.5 (87.1-100)	
15-year	85.4 (80.0-91.2)	89.0 (83.5-95.0)	71.9 (57.6-89.7)	95.5 (87.1-100)	
<b>Deaths of Disease</b>					
Yes	42 (16.4%)	25 (15.2%)	1 (3.1%)	16 (26.7%)	0.01
No	213 (83.2%)	138 (84.1%)	31 (96.9%)	44 (73.3%)	
Unknown	1 (0.4%)	1 (0.6%)	0 (0.0%)	0 (0.0%)	
<b>Number of Patients with Recurrent Disease</b>					
Yes	90 (35.2%)	46 (28.0%)	38 (63.3%)	6 (18.8%)	< 0.001
No	143 (55.9%)	110 (67.1%)	20 (33.3%)	13 (40.6%)	
Unknown	23 (9.0%)	8 (4.9%)	2 (3.3%)	13 (40.6%)	
<b>Time to Event for Patients who Recurred (Median)</b>					
OS	13.4	16.5	3.2	11.6	
DSS	12.7	14.7	3.3	12.5	

RFS	13.9	7.2	4.2	5.3
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\*Includes chemotherapy and radiation, IQR = Interquartile Range, HYS = Hysterectomy, USO = Unilateral Salpingo-Oophorectomy, BSO = Bilateral Salpingo-Oophorectomy, CI = Confidence Interval, OS= Overall Survival, DSS= Disease Specific Survival, RFS=Relapse Free Survival. The percentages shown are reflected in the column groupings.

†All P-values are two-sided and calculated using the fisher’s exact test to assess associations with categorical variables, and t-tests were used for continuous ones

## Figure Legend

**Figure 1. Survival curves of molecularly defined AGCT patients (MD-AGCT), AGCT *FOXL2* WT (wild-type) and misdiagnosed other tumor types.**

**Panel A.** Overall survival (OS) of the combined three AGCT (adult granulosa cell tumor) cohorts from three European centers. All outcome analyses were performed using Kaplan-Meier methods with log rank (LR) testing and stratified log rank (SLR) testing where cohorts were combined. All statistical tests were two-tailed. **Panel B.** Disease-specific survival (DSS) of the combined three AGCT cohorts from three European centers. **Panel C.** Overall survival after the first recurrence of MD-AGCT (molecularly defined-adult granulosa cell tumor) patients compared to patients diagnosed with misdiagnosed other types of cancers. Shown is only the patient cohort from Helsinki, Finland. Survival data is only after a patient’s first recurrence in the MD-AGCT group and the misdiagnosed other cancer types group. **Panel D.** Overall survival for MD-AGCT from the Finnish population for patients that recurred and did not recur compared to an age, gender and calendar-year matched Finnish population acquired as one-year intervals from the human mortality database ([www.mortality.org](http://www.mortality.org)). The green solid lines indicate MD-AGCTs with no observed survival event, the orange solid lines indicate MD-AGCTs with an observed survival event, and the red solid line indicates the expected survival of the general population. 95% Confidence bands are indicated by broken lines. Overlapping confidence bands indicates that there is no statistically significant difference in survival between those patients that recurred or did not recur compared to the survival of the matched population. **Panel E.** Recommended diagnostic algorithm for

accurate diagnosis of AGCT. Morphological assessment, FOXL2 immunohistochemistry, and *FOXL2* mutation testing are applied in a step-wise fashion, as indicated, to identify MD-AGCTs, morphologically typical AGCT-WT (*FOXL2* mutation negative), and other tumors (e.g. sex cord stromal tumors, carcinomas, metastasis).