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FOXL2 and *TERT* promoter mutation detection in circulating tumor DNA of adult granulosa cell tumors as biomarker for disease monitoring

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HIGHLIGHTS

- CtDNA harboring a *FOXL2* mutation was identified in plasma of 79% of patients with an adult granulosa cell tumor (aGCT).
- CtDNA containing a *TERT* promoter mutation was detected in a smaller subset of included aGCT patients.
- Both *FOXL2* mutant ctDNA and *TERT* mutant ctDNA levels correlated with clinical disease activity in the majority of patients.
- These findings suggest the clinical value of ctDNA detection as a biomarker for disease monitoring in aGCT.

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ABSTRACT

Objective. Adult granulosa cell tumors (aGCTs) represent a rare, hormonally active subtype of ovarian cancer that has a tendency to relapse late and repeatedly. Current serum hormone markers are inaccurate in reflecting tumor burden in a subset of aGCT patients, indicating the need for a novel biomarker. We investigated the presence of circulating tumor DNA (ctDNA) harboring a *FOXL2* or *TERT* promoter mutation in serial plasma samples of aGCT patients to determine its clinical value for monitoring disease.

Methods. In a national multicenter study, plasma samples ($n = 110$) were prospectively collected from 21 patients with primary ($n = 3$) or recurrent ($n = 18$) aGCT harboring a *FOXL2* 402C > G and/or *TERT* (C228T or C250T) promoter mutation. Circulating cell-free DNA was extracted and assessed for ctDNA containing one of either mutations using droplet digital PCR (ddPCR). Fractional abundance of *FOXL2* mutant and *TERT* mutant ctDNA was correlated with clinical parameters.

Results. FOXL2 mutant ctDNA was found in plasma of 11 out of 14 patients (78.6%) with aGCT with a confirmed *FOXL2* mutation. *TERT* C228T or *TERT* C250T mutant ctDNA was detected in plasma of 4 of 10 (40%) and 1 of 2 patients, respectively. Both *FOXL2* mutant ctDNA and *TERT* promoter mutant ctDNA levels correlated with disease progression and treatment response in the majority of patients.

Conclusions. FOXL2 mutant ctDNA was present in the majority of aGCT patients and *TERT* promoter mutant ctDNA has been identified in a smaller subset of patients. Both *FOXL2* and *TERT* mutant ctDNA detection may have clinical value in disease monitoring.

1. Introduction

Adult granulosa cell tumors (aGCTs) belong to the subgroup of sex cord-stromal cell tumors of the ovary, accounting for 3–5% of ovarian cancers. Although a granulosa cell tumor can be suspected in patients presenting with abnormal vaginal bleeding or abdominal pain and an

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ovarian mass, the majority of aGCTs are diagnosed after surgical removal of the tumor [1,2]. Most aGCTs are found at an early stage with a 5-year survival rate of >90%, and characterized as indolent tumors. However, recurrent disease is seen in at least one third of aGCT patients, typically occurring late with a time to relapse of 4–8 years [3,4]. Surgery remains the cornerstone of treatment for both primary and recurrent aGCT, as alternative therapeutic options such as chemotherapy and anti-hormonal therapy have shown limited response [5–7]. Relapsed aGCTs tend to recur multiple times, eventually leading to death in 50–80% of patients.

The hormonal activity of granulosa cells permits the use of several serum hormones as biomarkers in the follow-up of aGCTs. Inhibin is a hormone produced by granulosa cells during follicular development and a member of the TGF-β family of growth factors. It consists of an alpha and beta (β) subunit, the latter being either a βA subunit forming inhibin A or a βB subunit forming inhibin B. Inhibin levels fluctuate during the menstrual cycle, and decrease to undetectable or very low levels after menopause [8,9]. Inhibin B is superior to inhibin A as a marker of disease activity in aGCT, with reported sensitivities of 89–93% for inhibin B and 67% for inhibin A [10,11]. Anti-Müllerian hormone (AMH) is another member of the TGF-β growth factor family and is secreted by granulosa cells of developing follicles. Serum AMH has also been validated as a biomarker for aGCT and its accuracy was found to be similar to inhibin B, with a described sensitivity of 92% [11,12]. Although estrogen is a well-known hormone produced by granulosa cells and frequently secreted by aGCTs, no consistent correlation between serum estradiol levels and tumor activity has been reported [12,13]. Despite the high sensitivity of inhibin B and AMH, the use of serum hormone markers for aGCT has its limitations. Hormone levels are physiologically elevated and fluctuating over time in premenopausal women. In addition, up to 15% of aGCTs do not produce inhibin B, and normal or fluctuating levels of the described markers can be found in patients with recurrent disease. Also, elevated levels of hormone markers without evidence of disease are sometimes seen [10,11]. Therefore, currently utilized serum hormone markers do not always correlate with disease activity, hampering their use as a biomarker. This highlights the need for a novel method to monitor aGCT relapse and treatment response.

The identification of circulating tumor DNA (ctDNA) as a fraction of cell-free DNA (cfDNA) in plasma has emerged as a non-invasive "liquid biopsy" in a variety of cancers [14,15]. Small ctDNA fragments are released from tumor cells into the bloodstream following processes such as necrosis or apoptosis. As a consequence, ctDNA harbors tumor-specific genetic alterations [16]. With the detection of these tumor-specific mutations in patients' plasma samples, ctDNA has been investigated as a genomic biomarker for disease monitoring and the assessment of treatment response in many cancer types including non-small cell lung cancer, breast cancer, colorectal cancer, gastric cancer, bladder cancer and ovarian cancer [17–22]. Techniques used for the detection of ctDNA include polymerase chain reaction (PCR) to identify specific mutations in single genes, and targeted next-generation sequencing (NGS) to assess alterations in multiple genes at once.

The most defining molecular feature of aGCT is a mutation in the transcription factor *FOXL2*. This 402C > G (C134W) *FOXL2* mutation is present in approximately 95% of tumors [23]. One study has described the development of a specific digital droplet PCR (ddPCR) assay to analyze the *FOXL2* 402C > G mutation in plasma ctDNA of aGCT patients. *FOXL2* mutant ctDNA was identified in 36% of subjects and the authors suggested the use of this assay for the diagnosis and monitoring of aGCT [24]. Recent studies have reported alterations in cancer-related genes in a subset of aGCTs, including *TERT, TP53, PIK3CA, KMT2D* and *CTNNB1* [25–29]. Most notably, the C228T *TERT* promoter mutation was found in 24–40% of aGCTs, with a higher prevalence in recurrent tumors as compared to primary tumors [26,27,29]. In addition, two studies also revealed the presence of the C250T *TERT* promoter mutation in a smaller percentage of aGCTs [28,29]. Both *TERT* promoter mutations have previously been detected in ctDNA of melanoma and hepatocellular carcinoma patients [30–32].

In the current study, we evaluate the clinical potential of *FOXL2* mutant ctDNA as biomarker for monitoring disease activity and treatment response in a prospective cohort of aGCT patients in different disease stages. In addition, our investigation is the first to identify ctDNA harboring a *TERT* C228T or C250T mutation in plasma of aGCT patients by ddPCR and correlate these findings with disease status and *FOXL2* mutant ctDNA detection.

2. Methods

2.1. Patients and samples

A national multicenter prospective study was conducted, with approval of our institutional review board (UMCU METC 17–868) and the review boards of the participating centers. Patients diagnosed with a primary or recurrent granulosa cell tumor were included in the study during a hospital visit between 2018 and 2020. All patients provided written informed consent. Tumor tissue was obtained at the time of surgery as previously described [29]. In addition, blood samples were collected prospectively from participating aGCT patients: at diagnosis of primary or recurrent disease, prior to surgery or other treatment, during systemic treatment and at regular follow-up visits. Clinical data including patient characteristics, treatments, serum marker levels, histopathology and radiology reports and follow-up information were collected. Upon blood withdrawal for study purposes, 2 × 10 ml of venous whole blood was collected into PAXgene® tubes (BD Biosciences, Eysins, Switzerland). Within 7 days after collection, samples were centrifuged for 10 min at 1900 *xg* (3000 rpm) at 4 °C. The supernatant plasma was then transferred to 15 ml centrifuge tubes and centrifuged for 10 min at 16,000 xg (in fixed-angle rotor) to remove additional cellular nucleic acids attached to cell debris. The supernatant was divided in 1 ml aliquots and stored at −80 °C until further analysis.

2.2. Selection and preparation of plasma samples

We confirmed the presence of the *FOXL2* 402C > G mutation using whole genome sequencing (WGS) on DNA isolated from tumor tissue of all aGCT patients whose plasma samples were used for analysis of ctDNA harboring the *FOXL2* mutation [29]. In addition, we used the plasma samples of patients with a *TERT* promoter mutation in their tumor, identified by previous WGS analyses, for detection of ctDNA with the *TERT* C228T or *TERT* C250T mutation. In order to extend this number of samples, additional tumor specimens of patients with active disease and at least one plasma sample available were tested for the *TERT* C228T or *TERT* C250T mutation by PCR amplification followed by Sanger sequencing using the primers 5′-AGCACCTCGCGGTAGTG-3′ and 5′-GGGCTCCCAGTGGATTC-3′. The thermal cycling conditions were 95 °C for 5 min, followed by 32 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 10 min and infinite hold at 4 °C.

Plasma aliquots were thawed and cfDNA was isolated from 3 ml plasma per sample using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated DNA samples were eluted in 30–40 μl elution buffer. The quantity of cfDNA was measured using a Qubit fluorometer with the dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Quality control of DNA was performed using the Agilent TapeStation system with D5000 ScreenTape assay (Agilent, Santa Clara, California, USA).

2.3. Digital droplet PCR (ddPCR)

CfDNA extracted from plasma samples was analyzed for *FOXL2* or *TERT* mutations by ddPCR. For *FOXL2* mutation detection, validated ddPCR Mutation Assays for *FOXL2* wild-type (WT) and *FOXL2* c.402C >

Table 1
Patient characteristics.

Patient	Age (years)	Primary/ recurrence	Age at diagnosis	Stage at diagnosis	Recurrence number	Previous treatment	Tumor load	Size of largest tumor	Inhibin B level	FOXL2 mutation status	TERT mutation status	Disease status
P1	64	Recurrence	53	IC	5th	Surgery, CT, HT	Multifocal: IP, liver, lung	3 cm	185	402C > G	C228T	Progressive disease
P2	76	Recurrence	50	IC	5th	Surgery	Multifocal: IP, liver, lymph nodes	4 cm	128	402C > G	C250T	Progressive disease
P3	74	Recurrence	68	IIB	3rd	Surgery	Multifocal, IP	3 cm	168	402C > G	WT	Progressive disease
P4	44	Recurrence	35	IC	3rd	Surgery, CT	Multifocal, IP	2 cm	84	402C > G	WT	Stable disease
P5	74	Recurrence	57	IA	4th	Surgery, CT	Multifocal: IP, liver	7 cm	1783	402C > G	WT	Died of disease
P6	63	Recurrence	61	IC	1st	Surgery	Unilocal: IP	5 cm	43	WT	C228T	No evidence of disease
P7	48	Primary	48	IA	n/a	n/a	Left ovary	6 cm	329	402C > G	WT	No evidence of disease
P8	50	Recurrence	48	I	2nd	Surgery	Multifocal: IP	4 cm	84	402C > G	C228T	No evidence of disease
P9	42	Recurrence	30	IA	5th	Surgery	Liver	2 cm	334	402C > G	C228T	Stable disease
P10	59	Recurrence	52	IC	1st	Surgery	Unilocal: IP	1.5 cm	54	402C > G	C228T	No evidence of disease
P11	49	Recurrence	43	IC	3rd	Surgery, CT	Multifocal: IP	5 cm	772	402C > G	C228T	Died of disease
P12	49	Primary	49	IA	n/a	Surgery	n/a	n/a	<10	402C > G	WT	No evidence of disease
P13	56	Recurrence	50	IA/C	2nd	Surgery	Unifocal: IP	3 cm	31	402C > G	WT	Progressive disease
P14	57	Recurrence	53	IA	3rd	Surgery, CT, HT	Multifocal: IP, lymph nodes	5 cm	2615	402C > G	WT	Died of disease
P15	68	Recurrence	52	IIB	5th	Surgery, CT, HT	Multifocal: IP	7 cm	<10	402C > G	WT	No evidence of disease
P16	47	Recurrence	40	IA	3rd	Surgery, HT	Multifocal: IP	7 cm	122	402C > G	WT	Stable disease
P17	74	Recurrence	71	IC	1st	Surgery	Multifocal: IP	4 cm	39	Unknown	C228T	No evidence of disease
P18	44	Primary	44	IA	n/a	n/a	Right ovary	13 cm	4932	Unknown	C250T	No evidence of disease
P19	84	Recurrence	60	I	1st	Surgery	Multifocal: IP, lung	23 cm	1813	Unknown	C228T	No evidence of disease
P20	68	Recurrence	52	I	5th	Surgery, RT	Multifocal: IP, liver	7 cm	295	Unknown	C228T	Progressive disease
P21	64	Recurrence	49	IC	3rd	Surgery	IP, liver, spleen, abdominal wall	5 cm	376	402C > G	C228T	No evidence of disease

Recurrence number, previous treatment, tumor load, size of largest tumor and inhibin B level (ng/L) at collection of first blood sample are shown. Most recent disease status is shown. CT = chemotherapy, HT = anti-hormonal therapy, RT = radiotherapy, IP = intraperitoneal, n/a = not applicable, WT = wild-type.

G p.C134W were used (Bio-Rad Laboratories, Hercules, California, USA). For *TERT* promoter mutation detection by ddPCR, the *TERT* C228T_88 Expert Design Assay and the *TERT* C250T_88 Expert Design Assay (Bio-Rad Laboratories) were utilized as previously described [30]. Reaction volumes of 22 μl per well of a 96-well plate were prepared. Each reaction for *FOXL2* testing contained 11 μl supermix for probes (no dUTP) (Bio-Rad Laboratories), 1 μl primer-probe mix for both mutant (labeled with FAM) and WT (labeled with HEX) *FOXL2*, 4–8 μl cfDNA from patient plasma, and purified water to a total of 22 μl. Each reaction for *TERT* testing consisted of 11 μl supermix for probes (no dUTP), 1 μl primer-probe mix of either *TERT* C228T or C250T assays (mutation specific probe labeled with FAM and WT specific probe labeled with HEX), 2 μl of 5 M betaine (Sigma-Aldrich, St. Louis, Missouri, USA), 1 μl of 20 mM EDTA (Thermo Fisher Scientific), 4–6 μl cfDNA from patient plasma, and purified water to a total of 22 μl. Reactions were subjected to ddPCR analysis using the QX200 system according to the manufacturer's protocol (Bio-Rad Laboratories). Each ddPCR experiment was optimized by performing a temperature gradient experiment to establish the best amplification temperature for separation of positive and negative droplets. The final thermal cycling conditions for the *FOXL2* assay were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 55 °C for 1 min, followed by 98 °C for 10 min and infinite hold at 12 °C. For the *TERT* C228T assay, the PCR conditions were 95 °C for 10 min, followed by 50 cycles of 96 °C for 30 s and 60 °C for 1 min, followed by 98 °C for 10 min and infinite hold at 12 °C. For the *TERT* C250T assay, the PCR cycling conditions were 95 °C for 10 min, followed by 50 cycles of 96 °C for 30 s and 62 °C for 1 min, followed by 98 °C for 10 min and infinite hold at 12 °C. Positive and negative controls, consisting of aGCT tumor DNA samples with and without the *FOXL2* or *TERT* promoter mutation, as well as no-template controls were included in every run. Each cfDNA sample was analyzed at least in duplicate wells in each run, and in two separate ddPCR runs.

2.4. Data analysis

Quantasoft software (Bio-Rad Laboratories) was utilized for analysis of the ddPCR data. Only wells with total droplet counts greater than 10,000 droplets were analyzed. For each ddPCR assay, thresholds separating positive and negative droplets were set manually based on the droplet distribution in the positive and negative control samples. Double positive droplets were excluded. Allowing for not more than two false-positive droplets in negative controls, samples with ≥3 mutation positive droplets per well were considered true positive. A relative quantification of ctDNA in each plasma sample was done by calculating the fractional abundance as a percentage of mutant to total (mutant + WT) copies.

Table 2
Presence of *FOXL2* mutant ctDNA and *TERT* promoter mutant ctDNA in aGCT plasma samples.

Patient	Samples (*n*)	*FOXL2* mutant ctDNA detected	Fractional abundance, range (%)	*TERT* mutation in tumor	*TERT* mutant ctDNA detected	Fractional abundance, range (%)
P1	8	Yes	0.07–1.75	C228T	Yes	0.00–0.24
P2	9	Yes	0.00–2.03	C250T	Yes	0.00–0.59
P3	9	Yes	0.00–18.07	WT	n/a	
P4	7	Yes	0.00–1.86	WT	n/a	
P5	11	Yes	0.09–48.56	WT	n/a	
P6	3	WT		C228T	No	
P7	2	No		WT	n/a	
P8	6	NT		C228T	No	
P9	9	Yes	0.00–7.97	C228T	Yes	0.00–1.68
P10	6	No		C228T	No	
P11	10	Yes	0.00–1.25	C228T	Yes	0.00–0.29
P12	5	Yes	0.00–0.49	WT	n/a	
P13	3	Yes	0.00–0.82	WT	n/a	
P14	11	Yes	0.00–18.61	WT	n/a	
P15	2	No		WT	n/a	
P16	2	Yes	0.22–1.43	WT	n/a	
P17	3	NT		C228T	No	
P18	1	NT		C250T	No	
P19	1	NT		C228T	Yes	4.64
P20	1	NT		C228T	No	
P21	1	NT		C228T	No	

WT = wild-type, NT = not tested, n/a = not applicable.

3. Results

3.1. Patient cohort

Twenty-one patients with histologically confirmed aGCT and active disease on CT or MRI imaging or elevated serum markers were included in the current study. A total of 110 serial blood samples were prospectively obtained. Patient characteristics are shown in Table 1. Three of the included patients had a primary aGCT, while all other patients had recurrent disease. Plasma samples ($n = 94$) of 14 patients were used for ddPCR analysis of the *FOXL2* mutation. For *TERT* promoter mutation testing, 46 plasma samples of 12 patients were used; 36 samples from ten patients harboring a *TERT* C228T mutation and ten samples from two patients harboring a *TERT* C250T mutation, as confirmed by WGS or Sanger sequencing of corresponding tumor tissue.

3.2. Prevalence of FOXL2 mutant ctDNA in plasma of aGCT patients

The isolated cfDNA from plasma of aGCT patients was analyzed for presence of the *FOXL2* 402C > G mutation by ddPCR. *FOXL2* mutation positive ctDNA was found in cfDNA of 11 out of 14 patients (78.6%). Fractional abundance, defined as the relative fraction of mutation positive ctDNA in a background of WT cfDNA, varied between patients and ranged from 0.49% to 48.56% (Table 2). In one of three patients (Patient (P) 7) without detectable plasma *FOXL2* mutant ctDNA, blood samples from before and after surgery for a primary aGCT (stage IA) were analyzed. The other two patients (P10 and P15) who were negative for *FOXL2* mutant ctDNA had their first and fifth recurrence of aGCT with unifocal and multifocal intraperitoneal disease, respectively, both requiring cytoreductive surgery. Contrary to the patients with active disease but undetectable ctDNA, in one patient (P12) *FOXL2* mutant ctDNA was detected in plasma during follow-up after a surgically treated primary aGCT, although there was no evidence of disease. Two of her five plasma samples showed low fractions of ctDNA (0.33% and 0.49%, four and seven months after surgery) whereas no detectable ctDNA fraction was found in the two subsequent follow-up samples, with inhibin A and AMH levels also slightly elevated followed by normal values and inhibin B remaining at normal levels. The remaining ten patients whose plasma contained *FOXL2* mutant ctDNA had active, measurable recurrent disease at the time of ctDNA detection.

3.3. Detection of TERT mutant ctDNA in plasma of aGCT patients

After optimizing the ddPCR assays for *TERT* mutation testing in tumor DNA of aGCT patients, cfDNA from patients with a confirmed *TERT* mutant tumor was analyzed for the presence of the *TERT* C228T or *TERT* C250T mutation by ddPCR. In plasma of four out of ten patients (40%) the *TERT* C228T mutation was found, with mutant ctDNA fractions ranging from 0.24% to 4.64% (Table 2, Supplementary Fig. 1). In addition, ctDNA with the *TERT* C250T mutation was detected in one of two studied patients with a fractional abundance of 0.59% (Table 2, Supplementary Fig. 1). Examples of ddPCR positive and negative control tissue samples for both *TERT* mutations are shown in Supplementary Fig. 2.

3.4. FOXL2 mutant ctDNA testing for disease monitoring

From eight patients (P1–5, P9, P11, P14) with recurrent aGCT whose plasma harbored *FOXL2* mutant ctDNA, 7–11 plasma samples per patient were collected at multiple time points during follow-up and treatment. These serial samples allowed for the evaluation of ctDNA levels related to disease progression and treatment response. Fig. 1 depicts the changes in *FOXL2* mutant ctDNA fractions during the course of each patient's disease, with inhibin B levels shown as a comparison. In six of eight patients (75%), changes in ctDNA levels correlated with disease activity and response to treatment.

3.5. Case descriptions

Patient P1 was included in the study when she was being treated with carboplatin and paclitaxel for a fifth aGCT recurrence, followed by incomplete cytoreductive surgery. She developed progression of liver metastases within three months after surgery, for which she underwent radiofrequency ablation (RFA). After a period of stable disease she again developed progressive disease and was treated with letrozole. In plasma samples collected during this course, *FOXL2* mutant ctDNA was found in low amounts. A peak at 1.8% was seen when her liver metastases increased in size, then a *FOXL2* mutant ctDNA decrease was observed after RFA treatment. No rise in *FOXL2* mutant ctDNA was seen alongside the subsequent disease progression. Patient P2 was monitored after a complete cytoreductive surgery for a fourth aGCT recurrence when serial plasma collections were initiated. Inhibin and *FOXL2* mutant ctDNA levels increased (ctDNA peak at 2.03%) and CT scans confirmed a multifocal fifth recurrence. The patient was treated with six cycles of carboplatin and paclitaxel, leading to a partial response on CT scan, decreased inhibin B and absence of *FOXL2* mutant ctDNA. The first blood sample of patient P3 was collected prior to a complete cytoreductive surgery for a third recurrence. *FOXL2* mutant ctDNA was highly present (18.1%), which subsequently declined to 0.7% six weeks after surgery. Another relapse was confirmed four months later by CT imaging and was accompanied by increased ctDNA and inhibin B levels. This recurrence was treated with carboplatin and paclitaxel, with a partial response and a sharp decline of ctDNA to undetectable levels. Despite anti-hormonal therapy that was initiated after completion of chemotherapy, the disease progressed as was also shown by a rise of *FOXL2* mutant ctDNA fractions. Patient P4 was initially found to harbor low fractions of *FOXL2* mutant ctDNA during chemotherapeutic treatment (carboplatin and paclitaxel) for a third aGCT recurrence, resulting in a partial response with absent *FOXL2* mutant ctDNA after treatment. Complete cytoreductive surgery was performed, which was followed by a rise in *FOXL2* mutant ctDNA and suspicion of limited recurrent or residual disease on CT scan, then stable disease during the following year. Similar to patient P3, *FOXL2* mutant ctDNA levels in plasma of patient P5 with a fourth aGCT recurrence were high (up to 48.6%) and a clinical response to treatment with chemotherapy and cytoreductive

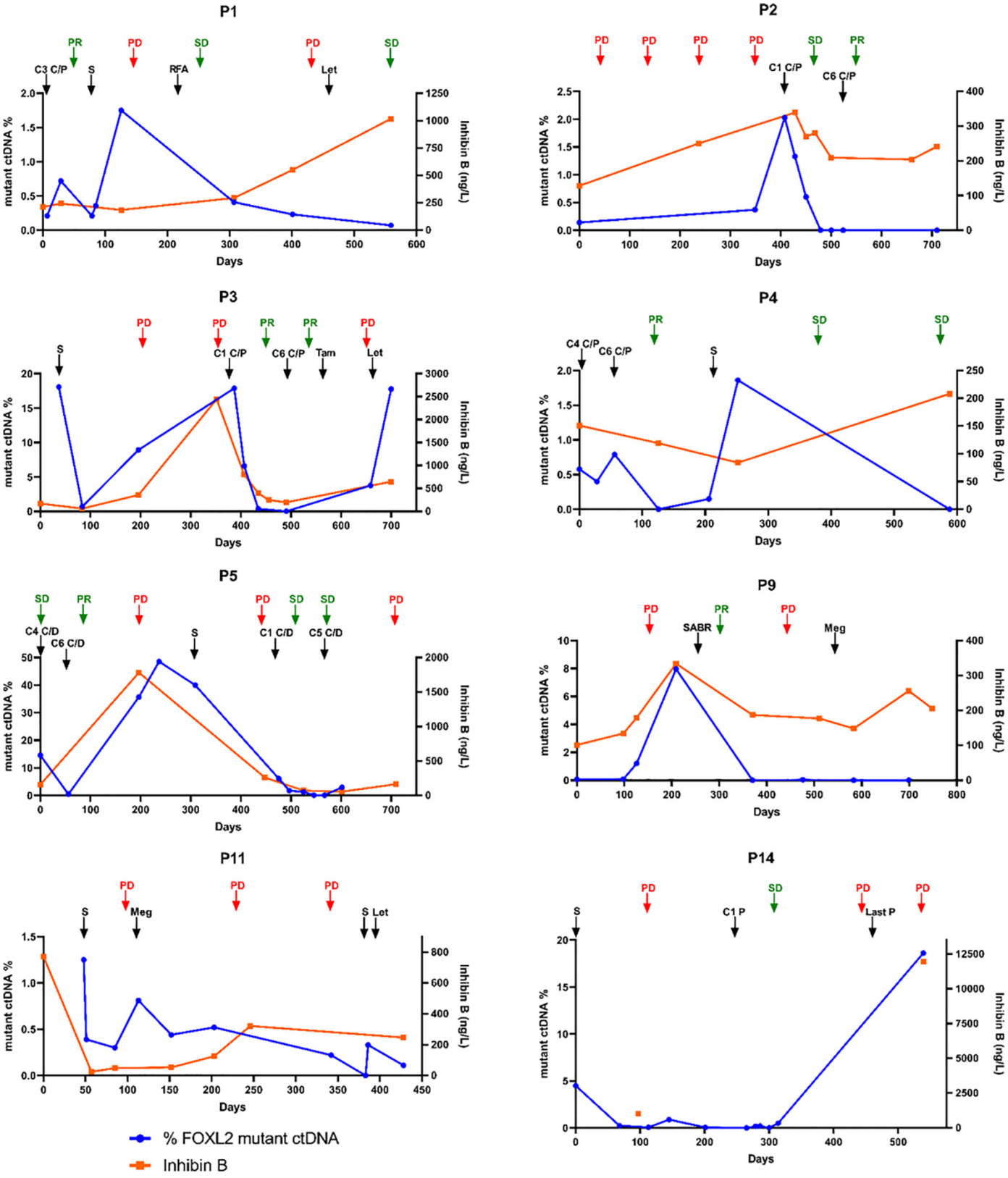

Fig. 1. Clinical course of aGCT patients and their changes in *FOXL2* mutant ctDNA and inhibin B.
Graphs depicting the changes in *FOXL2* mutant ctDNA over time of all patients whose serial plasma samples were analyzed, with inhibin B levels shown as a comparison. Detailed information on the clinical course of each patient and the corresponding ctDNA levels can be found in the main text. Black arrows mark the start of a treatment. Green and red arrows indicate the disease status, based on CT or MRI imaging findings. Blue line = *FOXL2* mutant ctDNA, orange line = inhibin B. PR = partial response, PD = progressive disease, SD = stable disease, S = surgery, C = cycle, C/P = carboplatin/paclitaxel, RFA = radiofrequency ablation, Let = letrozole, Tam = tamoxifen, C/D = carboplatin/docetaxel, SABR = stereotactic ablative radiotherapy, Meg = megestrol, P = paclitaxel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

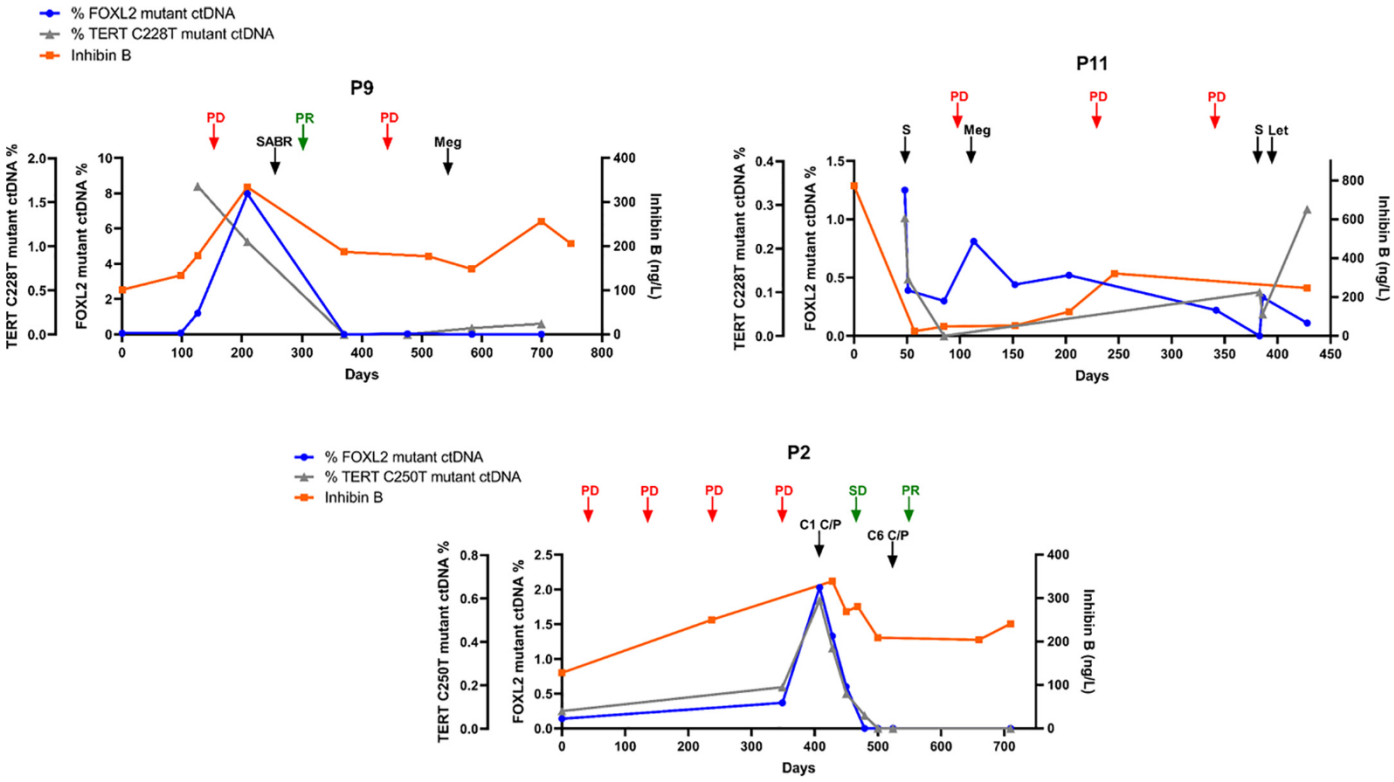

Fig. 2. Clinical course of aGCT patients and their changes in *TERT* mutant ctDNA, *FOXL2* mutant ctDNA and inhibin B.
Graphs depicting the changes in *TERT* mutant ctDNA over time of three patients whose serial plasma samples were analyzed, with *FOXL2* mutant ctDNA and inhibin B levels shown as a comparison. Detailed information on the clinical course of each patient and the corresponding ctDNA levels can be found in the main text. Black arrows mark the start of a treatment. Green and red arrows indicate the disease status, based on CT or MRI imaging findings. Grey line = *TERT* mutant ctDNA, blue line = *FOXL2* mutant ctDNA, orange line = inhibin B. PR = partial response, PD = progressive disease, SD = stable disease, S = surgery, C = cycle, C/P = carboplatin/paclitaxel, SABR = stereotactic ablative radiotherapy, Meg = megestrol, Let = letrozole. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surgery was accompanied by a decrease in *FOXL2* mutant ctDNA levels. Patient P9 had a fifth recurrence located in the liver with rising *FOXL2* mutant ctDNA and inhibin B. She was treated with stereotactic ablative radiotherapy with a partial response which was confirmed by decreasing ctDNA and inhibin B levels. Subsequently, disease progression occurred which was treated with megestrol leading to stable disease. Patient P11 harbored low amounts of *FOXL2* mutant ctDNA, with the highest fraction (1.2%) found just prior to cytoreductive surgery for a third aGCT recurrence and lower fractions thereafter, despite progressive disease requiring another cytoreductive surgery and antihormonal therapy. Finally, patient P14 was also included just prior to surgical treatment for a third aGCT recurrence. The first measurement showed a *FOXL2* mutant ctDNA level of 4.5%, which declined to 0.2% after surgery. This *FOXL2* mutant ctDNA fraction mildly increased upon disease progression, then was undetectable during treatment with paclitaxel and stable disease, and then strongly increased to 18.6% when disease progressed rapidly leading to the patient succumbing to her disease.

3.6. *TERT* promoter mutant ctDNA levels during disease monitoring

Similar to studying *FOXL2* mutant ctDNA as a biomarker in aGCT, we analyzed *TERT* promoter mutant ctDNA fractions during the course of the disease in three patients (P2, P9, P11) from whom serial plasma samples were available (Fig. 2). In all three cases, *TERT* mutant ctDNA fractions were found to correlate with disease progression or response to treatment, resembling the *FOXL2* mutant ctDNA changes in the same patients. Patient P2 was found to harbor *TERT* C250T mutant ctDNA and although fractions were small, a clear increase was seen during progression of disease and a decline accompanying the clinical response to treatment with carboplatin and paclitaxel. In plasma samples of patient P9, an initial *TERT* C228T mutant ctDNA fraction of 1.7% during disease progression decreased to undetectable levels as a result of treatment with a partial response. Moreover, patient P11 had a low *TERT* C228T mutant ctDNA fraction that decreased to zero following surgery, and subsequently increased with progression of disease.

4. Discussion

In the current study, *FOXL2* mutant ctDNA was detected in 79% of analyzed patients with an aGCT harboring a *FOXL2* 402C > G mutation. We are the first to report the presence of *TERT* promoter mutant ctDNA in a subset of patients with aGCT harboring a C228T or a C250T *TERT* promoter mutation. In addition, ctDNA analysis of longitudinal samples showed a correlation between *FOXL2* mutant or *TERT* mutant ctDNA levels and disease activity in the majority of patients.

Current biomarkers in aGCT, most notably inhibin B and AMH, sufficiently measure disease activity in most aGCT patients. However, they can be inadequate in premenopausal patients due to hormonal fluctuations related to the menstrual cycle, or show false negative or false positive results for unexplained reasons. The course of relapsed aGCT commonly includes multiple recurrences requiring repeated surgery and other therapeutic strategies, underscoring the importance of monitoring disease reliably to determine the timing and effectiveness of treatment. For all patients included in the current study, inhibin B was used to monitor disease activity. The majority (86%) of our cohort had recurrent disease and a postmenopausal status because of previous bilateral salpingo-oophorectomy, this includes all patients whose serial samples were studied. In these patients, inhibin B was mostly accurate in reflecting tumor burden. When comparing changes in ctDNA

fractions with inhibin B levels at different time points during the course of disease progression or treatment, ctDNA performed largely similar to inhibin B in monitoring disease activity.

The potential application of ctDNA as a genomic biomarker for disease monitoring has been a main focus of investigation in a variety of cancers [15,33]. The presence of plasma ctDNA was found to reflect minimal residual disease and serve as a prognostic biomarker in several solid tumors including breast, colorectal, lung and pancreatic cancer [15,34]. In addition, other studies have described the use of ctDNA for the assessment of treatment response. In breast cancer, a clear suppression of plasma *PIK3CA* mutant ctDNA within four weeks of treatment with paclitaxel with or without the AKT inhibitor capivasertib was seen in a subset of patients, in some cases followed by a rise in ctDNA that marked the development of resistance [35]. Several studies have described the use of *KRAS* mutant ctDNA as a marker of response to chemotherapy and disease progression in pancreatic cancer, with changes in ctDNA during treatment being more rapid and pronounced than changes in protein-based tumor markers [36,37]. A recent study in metastatic colorectal cancer showed similar results, comparing ctDNA with carcinoembryonic antigen (CEA) for the assessment of chemotherapy response and finding ctDNA to be a more sensitive and responsive marker of tumor burden than CEA [19]. In line with these studies, we observed a correlation between ctDNA levels and response to treatment or progressive disease in the majority of aGCT patients, with chemotherapy leading to a more pronounced decrease in ctDNA as compared to inhibin B.

In aGCT, plasma ctDNA containing the highly prevalent 402C > G (C134W) *FOXL2* mutation has been previously studied by other investigators, who detected *FOXL2* mutated ctDNA in 35% of patients with primary disease and in 19% of patients with recurrent disease [24]. A sensitivity of their ddPCR assay of 23% was described, which the authors attributed at least in part to the use of limited amounts of plasma (1 ml on average) resulting in small quantities of cfDNA being analyzed. Serial plasma samples of six aGCT patients showed limited correlation with clinical disease activity. In our study of mostly recurrent aGCT patients, larger volumes of plasma and consequently larger amounts of cfDNA were used for ddPCR analyses, showing a substantially higher frequency of *FOXL2* mutant ctDNA detection and a better reflection of the clinical course. These findings suggest *FOXL2* mutant ctDNA may be a useful marker in the management of patients with aGCT. In addition, recent studies have provided further insight into the mechanisms by which mutant *FOXL2* drives adult granulosa cell tumorigenesis thereby identifying potential therapeutic targets [38,39]. If treatment strategies targeting the function of mutant *FOXL2* in aGCT become available, the use of *FOXL2* mutant ctDNA may be particularly helpful for monitoring treatment response.

Detection of ctDNA with the *TERT* promoter mutation has not yet been reported in aGCT, although previous studies have identified the C228T or C250T *TERT* promoter mutation in 24–49% of tumor specimens of aGCT patients [26–29]. A *TERT* promoter mutation was found more frequently in recurrent aGCT as compared to primary tumors, and was associated with a worse prognosis [26,28,29]. Others have detected *TERT* C228T mutant plasma ctDNA in 51% of patients with advanced hepatocellular carcinoma and *TERT* C228T or C250T mutant ctDNA in 78% of melanoma patients [30,32]. In our small pilot cohort of plasma samples from ten patients with aGCT harboring a C228T *TERT* promoter mutation and two aGCT patients harboring a C250T *TERT* promoter mutation, we detected *TERT* mutant ctDNA in four of ten (40%) and in one of two (50%) patients, respectively.

Consistent with available evidence on the detection of ctDNA in other malignancies, the observed fractions of plasma ctDNA in our cohort are highly variable and range from 0.24% to 48.56% of total plasma cfDNA [33]. The presence of cfDNA, including ctDNA, is thought to be the result of its release from cells through apoptosis, necrosis and possibly active secretion [16,40]. The largest proportion of plasma cfDNA is released following hematopoietic cell death, and shedding of cfDNA may vary depending on processes such as infection or intense exercise, or body mass index. In addition, the half-life of cfDNA is very short, estimated to be shorter than 2.5 h, which is why ctDNA assessment is considered a 'real-time' measure of tumor burden [14]. These characteristics of cfDNA lead to the possibility of intrapatient and interpatient variability in plasma ctDNA levels. Also, the relatively low amounts of ctDNA in plasma samples can reduce the sensitivity of ctDNA detection assays. Low ctDNA fractions in small quantities of cfDNA can be difficult to assess and lead to false negative results. Despite recent advances in the development of technologies such as ddPCR or targeted sequencing for the detection of plasma ctDNA, it remains a challenge to quantify very low ctDNA fractions. We observed the best correlation of ctDNA levels with clinical disease status in patients whose plasma contained the highest ctDNA fractions, suggesting that changes in ctDNA are measured more accurately when its abundance is higher.

Limitations of our study include the small sample size and the relatively short follow-up for this disease with a prolonged time to recurrence and relapsed aGCT often being managed over the course of several years. As aGCT comprises a rare malignancy, studies including a sufficient number of patients are a challenge and require international collaboration. Due to the small size of our cohort, we were unable to make a comparison between primary and recurrent aGCT or evaluate the value of ctDNA detection as a diagnostic tool. In addition, the prognostic relevance of the presence and abundance of *FOXL2* mutant or *TERT* mutant ctDNA could not be established and remains to be investigated. Finally, further research is needed to determine the value of ctDNA as an alternative to the currently used hormone markers when serum hormones insufficiently reflect tumor burden, in cases such as premenopausal patients.

In conclusion, *FOXL2* mutant ctDNA is present in the majority of aGCT patients and *TERT* promoter mutant ctDNA has been identified in a smaller subset of patients. Both *FOXL2* and *TERT* mutant ctDNA levels were found to correlate with disease progression and response to treatment in most patients, suggesting the clinical potential of ctDNA as a biomarker in aGCT. Larger collaborative studies are warranted to validate our findings and establish the clinical value of ctDNA testing in aGCT.

Author contributions

Study design: J.G., J.R., G.v.H. and R.Z.. Patient enrollment and sample collection: J.G., J.R., A.B., S.P., H.N., H.v.M., L.v.L., J.P., C.L., G.M. and R.Z.. Experiments, data collection and data analysis: J.G., J.R., E.P., F.S. and A.B.. Manuscript writing: J.G., J.R., G.M. and R.Z.. All authors revised the manuscript and approved its final version.

Conflict of interest statement

H.N. reports grants from Aduro, DCPrime and the Dutch Cancer Society, and non-financial support from Merck, ViciniVax and BioNTech, outside the submitted work. The other authors report no conflict of interest.

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Appendix A. Supplementary data

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