Virchows Archiv
https://doi.org/10.1007/s00428-024-03854-0

ORIGINAL ARTICLE

An extensive immunohistochemical analysis of 290 ovarian adult granulosa cell tumors with 29 markers

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Received: 13 May 2024 / Revised: 10 June 2024 / Accepted: 13 June 2024

Abstract

The current knowledge about the immunohistochemical features of adult granulosa cell tumor (AGCT) is mostly limited to the "traditional" immunohistochemical markers of sex cord differentiation, such as inhibin, calretinin, FOXL2, SF1, and CD99. Knowledge about the immunohistochemical markers possibly used for predictive purpose is limited. In our study, we focused on the immunohistochemical examination of 290 cases of AGCT classified based on strict diagnostic criteria, including molecular testing. The antibodies used included 12 of the "diagnostic" antibodies already examined in previous studies, 10 antibodies whose expression has not yet been examined in AGCT, and 7 antibodies with possible predictive significance, including the expression of HER2, PD-L1, CTLA4, and 4 mismatch repair (MMR) proteins. The results of our study showed expression of FOXL2, SF1, CD99, inhibin A, calretinin, ER, PR, AR, CKAE1/3, and CAIX in 98%, 100%, 90%, 78%, 45%, 41%, 94%, 82%, 26%, and 9% of AGCT, respectively. GATA3, SATB2, napsin A, MUC4, TTF1, and CD44 were all negative. PTEN showed a loss of expression in 71% of cases and DPC4 in 4% of cases. The aberrant staining pattern (overexpression) of p53 was found in 1% (3/268) of cases, 2 primary tumors, and 1 recurrent case. Concerning the predictive markers, the results of our study showed that AGCT is microsatellite stable, do not express PD-L1, and are HER2 negative. The CTLA4 expression was found in almost 70% of AGCT tumor cells.

Keywords Ovarian tumors · Sex cord-stromal tumors · Granulosa cell tumors · Immunohistochemistry

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Published online: 21 June 2024

Introduction

Adult granulosa cell tumors (AGCTs) comprise approximately 2–5% of all ovarian malignant tumors and 95% of malignant sex cord-stromal tumors [1, 2]. These tumors mostly occur in perimenopausal patients, usually 50–55 years old at diagnosis. A proportion of the tumors can present with hormonal secretion, particularly estrogen production [3]. The diagnosis of AGCT is usually straightforward and can be based on morphological and immunohistochemical features. Nevertheless, there are tumors with ambiguous features and a morphological and immunohistochemical overlap with other sex cord-stromal tumors, especially juvenile granulosa cell tumor, Sertoli-Leydig cell tumor, and thecoma. Distinguishing AGCT from tumors of other histogenesis (including epithelial tumors) can also be challenging in some cases. Molecular testing can be helpful in tumors with equivocal features, as about 95% of AGCTs harbor the missense *FOXL2* mutation (c.402C > G, p.(Cys134Trp)) [4–6]. However, the *FOXL2* mutation is not entirely specific and was also described in a subset of Sertoli-Leydig cell tumors [7]. The current knowledge about the immunohistochemical features of AGCT concerning diagnostic markers is mostly limited to the "traditional" immunohistochemical markers of sex cord differentiation, which have a limited value in the differential diagnosis with other sex cord-stromal tumors. Knowledge about immunohistochemical markers which can be used for predictive purposes is rather poor, as only a few studies have focused on this issue so far.

In our study, we focused on the immunohistochemical examination of a large cohort of AGCT including 290 cases classified based on strict diagnostic criteria, including molecular testing. The antibodies used included 12 of the "diagnostic" antibodies already examined in previously published studies (but sometimes on a low number of cases), 10 antibodies whose expression has not yet been examined in AGCT, and 7 antibodies with possible predictive significance, including the expression of HER2, PD-L1, CTLA4, and 4 mismatch repair (MMR) proteins.

Methods

Samples

The total of 319 cases diagnosed as AGCT were retrieved from the archives of the co-operating institutions. All tumors were reviewed by two pathologists with expertise in gynecopathology (KN and PD). The morphological features assessed in all tumors included the determination of the predominant growth pattern, presence of necrosis, lymphovascular invasion (LVSI), mitotic rate, and presence of nuclear atypia (no atypia, mild and moderate atypia, versus high-grade atypia) on whole-tissue sections of each tumor.

After a central review of the cases supplied for the study, 29 cases were excluded from further analysis, 22 cases were reclassified as other tumors, 4 were duplicate tissue blocks of already included cases, and in 3 cases, there was not enough material for the necessary analyses. The final sample set included 290 cases, with 241 primary tumors and 49 non-matched recurrences (primary tumor tissue was not available for these cases). Molecular testing using the NGS approach was possible in 225 cases and showed the *FOXL2* mutation in all but 2 cases. The results of the molecular analysis of these cases are not presented in the current study and will be described in detail in a separate upcoming study. The clinico-pathological and survival characteristics of the 290 patients are summarized in Table 1.

Immunohistochemical analysis

The immunohistochemical (IHC) analysis was performed using 4 µm thick sections of formalin-fixed and paraffin-embedded (FFPE) tissue using tissue microarrays (TMAs). The eligible areas of each tumor were selected and two tissue cores (each 2 mm in diameter) were taken from the donor block using the tissue microarray instrument TMA Master (3DHISTECH Ltd., Budapest, Hungary). The antibodies used included the "diagnostic" markers (FOXL2, SF1, CD99, inhibin A, calretinin, Ki67, ER, PR, AR, p53, p16, and CKAE1/3), new markers which have not yet been analyzed in AGCT (PTEN, CAIX (carbonic anhydrase IX), DPC4, CD44, GATA3, napsin A, ARID1A, SATB2, MUC4, and TTF1), and selected predictive markers (CTLA4, PD-L1, HER2, MLH1, PMS2, MSH2, and MSH6). The list of their manufacturers, clones, and dilutions is provided in Supplementary table S1.

The expression of all markers was double-blindly evaluated by two pathologists (KN, AŠ). Cases were classified based on the overall percentage of positive tumor cells as negative (entirely negative or < 5% of positive tumor cells) or positive (≥ 5% of positive tumor cells) with the exception of p53, p16, Ki67, HER2, and PD-L1. The p53 protein expression was assessed as either the "wild-type" or "aberrant type." The "aberrant type" of staining was defined as diffuse intense nuclear positivity of > 80% of tumor cells, cytoplasmic p53 positivity, or the complete absence of staining with positive internal control (the so-called null pattern) [8]. The expression of p16 was regarded as block positive (diffuse staining of tumor cells in the nuclei and/or cytoplasm), or negative (focal/patchy or absent staining). Ki67 was assessed as a continuous variable based on the proportion of positive tumor cells (0–100%). It was counted

Table 1 The clinico-pathological, morphological, and survival characteristics of 290 patients with adult granulosa cell tumors

Variables	Primary ($N=241$)	Recurrence ($N=49$)
Age at diagnosis (years)		
Mean (SD)	56 (14.1)	50 (13.6)
Median (range)	58 (17–83)	47.5 (27–77)
FIGO		
I	127 (91%)	22 (45%)
II	6 (4%)	4 (8%)
III	6 (4%)	2 (4%)
IV	1 (1%)	0 (0%)
N/A	*101*	*21*
Lymphovascular invasion		
No	39 (85%)	15 (94%)
Yes	7 (15%)	1 (6%)
N/A	*195*	*33*
Recurrence		
No	92 (90%)	0 (0%)
Yes	23 (10%)	43 (100%)
Single site*	13 (57%)	23 (53%)
Multiple site*	10 (43%)	20 (47%)
N/A	*126*	*6*
Association with AH/EIN and/or EEC		
No	78 (64%)	13 (93%)
Yes	43 (36%)	1 (7%)
N/A	*120*	*35*
HG nuclear atypia		
No	230 (96%)	46 (94%)
Yes	10 (4%)	3 (6%)
N/A	*1*	*0*
Mitosis/10 HPF		
<5 mitoses/10 HPF	203 (84%)	41 (84%)
≥ 5 mitoses/10 HPF	38 (16%)	8 (16%)
Necrosis		
No	194 (80%)	32 (65%)
Yes	47 (20%)	17 (35%)
Disease status at last control		
NED	100 (82%)	23 (53%)
AWD	8 (7%)	13 (30%)
DOD	5 (4%)	5 (12%)
DOC	9 (7%)	2 (5%)
N/A	*119*	*6*

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

*Percentages are counted only from the cases with local recurrence

SD, standard deviation; *N/A*, data not available; *AH*, endometrial atypical hyperplasia; *EIN*, endometrial intraepithelial neoplasia; *EEC*, endometroid endometrial carcinoma; *HPF*, high-power field; *NED*, no evidence of disease; *AWD*, alive with disease; *DOD*, death of disease; *DOC*, death of other cause (or unknown cause)

manually in 200 tumor cells in the hot-spots, or in randomly selected fields in cases of homogenous expression. For ARID1A, MMR, PTEN, and DPC4, the loss of expression in tumor cells with retained staining in stromal cells was evaluated (negativity was defined as less than 5% of tumor cells). HER2 scoring was performed in accordance with the 2018 ASCO Guidelines for breast carcinoma, as there is currently no established scoring system for ovarian tumors [9]. PD-L1 expression was evaluated as the percentage of positive tumor cells (tumor proportion score; TPS). Only

occasional rare lymphocytes were present in the stroma of a few cases, so neither CTLA4 expression in immune cells nor PD-L1 combined positive score (CPS) could be assessed.

Statistical analyses

Standard descriptive statistics were employed to summarize and characterize the entire dataset: categorical variables were described using absolute and relative frequencies (%), while continuous variables were characterized by both the mean with standard deviation and the median with range.

The correlation between the selected clinico-pathological/morphological variables was assessed using the chi-squared test for categorical markers (negative vs. positive, as described above), and the Mann–Whitney *U*-test for continuous markers.

Survival analyses were not conducted due to the limited number of cases with the event of interest in particular groups. Instead, survival status was tested solely as a categorical variable (living vs. deceased) in relation to the expression levels of selected markers.

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

Results

The morphological characteristics are presented in Table 1. Most cases (92%) displayed a mixed architectural pattern. The most common pattern was diffuse, seen in 56% (161/290), followed by nested/insular/trabecular in 27% (79/290), microfollicular in 6% (18/290), and the remaining 11% of cases were made up of other, less common patterns (30/290).

The results of the immunohistochemical analyses are summarized in Table 2 (see also Fig. 1). Briefly, the traditional diagnostic markers; FOXL2, SF1, CD99, inhibin A, calretinin, ER, PR, AR, and CKAE1/3 showed expression in 98%, 100%, 90%, 78%, 45%, 41%, 94%, 82%, and 26%, respectively. CAIX showed expression in 9% (25/285) of cases. GATA3, SATB2, napsin A, MUC4, TTF1, and CD44 were all negative. PTEN showed loss of expression in 71% (185/261) of cases. DPC4 showed loss of expression in 4% (10/257) of cases. The aberrant staining pattern (overexpression) of p53 was found in 1% (3/268) of cases; 2 primary tumors and 1 non-matched recurrent case. One primary case developed a subsequent recurrence, but the follow-up for the other primary tumor was not available. All three p53-aberrant tumors showed high-grade nuclear atypia, 3–7 mitoses/10HPF, and Ki67 up to 18%. P16 was diffusely positive in 1% (3/285) of cases. Ki67 showed a median value of 3 (range 0–40), and mean value of 4 (SD 5.1). Concerning the predictive markers, all tumors were HER2 negative and PD-L1 negative (TPS < 1%) and showed a retained expression of MMR proteins. CTLA4 showed weak to moderate expression in 69% (194/284) of cases.

The correlation between expression of hormonal receptors and selected clinico-pathological/morphological variables, as described in Table 3, revealed that ER-negative cases were more frequently observed in association with endometrial atypical hyperplasia (AH)/endometrial intraepithelial neoplasia (EIN), and/or endometrial endometroid cancer (EEC) ($p = 0.005$). High-grade nuclear atypia was associated with AR negativity ($p = 0.007$), while necrosis was more frequently found in AR positive cases ($p = 0.043$). We found a higher expression of ER ($p < 0.001$) and CAIX ($p = 0.034$) in the recurrent cases (Supplementary Table 2), which were not matched with primary samples.

The association between survival status and expression levels did not reveal any significant differences in either hormone receptor expression or Ki67.

Discussion

AGCTs are regarded as low-grade malignant tumors mostly diagnosed in the early stages, when they can be successfully treated surgically. However, approximately 30% of AGCTs have the propensity for late recurrence and metastasis, which can even occur after more than 10 years.

Therapeutic options for recurrent AGCT are limited, and about 50% of patients with recurrent or metastatic AGCT die of the disease; therefore, knowledge about possible new predictive markers in these tumors is important [1, 2, 10, 11]. Nevertheless, in AGCT, this knowledge is still limited. Immune check point inhibitors in cancer immunotherapy play a significant role in tumor treatment, and their significance is increasing [12, 13]. In our study, we focused on selected predictive markers, including MMR proteins, HER2, CTLA4, and PD-L1 expression. The expression of CTLA4 in tumor cells themselves is currently not yet a clinically established predictive marker, but its expression has been described in various tumors including hematological malignancies, breast cancer, and lung cancer [14–17]. In female genital tract tumors, CTLA4 positivity was found in ovarian, uterine, and cervical cancer cell lines, as well as in cervical tumor cells in one study [18, 19]. The expression of CTLA4 in AGCTs has not yet been examined. In our study, we found CTLA4 expression in 69% of AGCTs, mostly of weak to medium intensity.

Concerning CTLA4's prognostic significance, the results of published studies are equivocal. Some studies found a relationship between higher expression and adverse outcome, others reported inverse correlation, while the

Table 2 Overview of the overall positivity and ratio of positive/negative cases for the examined immunomarkers in the AGCT cohort

Marker		Marker		Marker	
FOXL2		p53*		CD44	
Median (range)	90 (0–100)	Median (range)	N/A	Median (range)	0
Mean (SD)	77 (14.2)	Mean (SD)	N/A	Mean (SD)	0
No. of positive cases	277 (98%)	No. of positive cases	3 (1%)	No. of positive cases	0 (0%)
No. of negative cases	7 (2%)	No. of negative cases	265 (99%)	No. of negative cases	278 (100%)
SF1		p16*		CTLA4	
Median (range)	98 (0–100)	Median (range)	N/A	Median (range)	100 (0–100)
Mean (SD)	89 (21.5)	Mean (SD)	N/A	Mean (SD)	67 (45.6)
No. of positive cases	285 (100%)	No. of positive cases	3 (1%)	No. of positive cases	195 (69%)
No. of negative cases	1 (0%)	No. of negative cases	282 (99%)	No. of negative cases	89 (31%)
CD99		GATA3		PTEN	
Median (range)	80 (0–100)	Median (range)	0	Median (range)	0 (0–100)
Mean (SD)	62 (34.9)	Mean (SD)	0	Mean (SD)	12 (26.2)
No. of positive cases	257 (90%)	No. of positive cases	0 (0%)	No. of positive cases	76 (29%)
No. of negative cases	29 (10%)	No. of negative cases	283 (100%)	No. of negative cases	185 (71%)
Inhibin A		ARID1A		HER2	
Median (range)	40 (0–100)	Median (range)	100 (6–100)	Median (range)	0
Mean (SD)	46 (36.8)	Mean (SD)	97 (8.1)	Mean (SD)	0
No. of positive cases	223 (78%)	No. of positive cases	283 (100%)	No. of positive cases	0 (0%)
No. of negative cases	64 (22%)	No. of negative cases	0 (0%)	No. of negative cases	286 (100%)
Calretinin		Napsin A		PD-L1	
Median (range)	2 (0–100)	Median (range)	0	Median (range)	0
Mean (SD)	22 (32.8)	Mean (SD)	0	Mean (SD)	0
No. of positive cases	129 (45%)	No. of positive cases	0 (0%)	No. of positive cases	0 (0%)
No. of negative cases	158 (55%)	No. of negative cases	285 (100%)	No. of negative cases	285 (100%)
ER		SATB2		MLH1	
Median (range)	1 (0–100)	Median (range)	0	Median (range)	95 (5–100)
Mean (SD)	15 (25.3)	Mean (SD)	0	Mean (SD)	87 (22.6)
No. of positive cases	118 (41%)	No. of positive cases	0 (0%)	No. of positive cases	279 (100%)
No. of negative cases	167 (59%)	No. of negative cases	286 (100%)	No. of negative cases	0 (0%)
PR		MUC4		PMS2	
Median (range)	55 (0–100)	Median (range)	0	Median (range)	99 (8–100)
Mean (SD)	53 (32.8)	Mean (SD)	0	Mean (SD)	91 (17.8)
No. of positive cases	268 (94%)	No. of positive cases	0 (0%)	No. of positive cases	282 (100%)
No. of negative cases	17 (6%)	No. of negative cases	286 (100%)	No. of negative cases	0 (0%)
AR		TTF1		MSH2	
Median (range)	24 (0–98)	Median (range)	0	Median (range)	100 (80–100)
Mean (SD)	38 (33.4)	Mean (SD)	0	Mean (SD)	99 (2.8)
No. of positive cases	234 (82%)	No. of positive cases	0 (0%)	No. of positive cases	287 (100%)
No. of negative cases	51 (18%)	No. of negative cases	286 (100%)	No. of negative cases	0 (0%)
CKAE1/3		DPC4		MSH6	
Median (range)	0 (0–100)	Median (range)	85 (1–100)	Median (range)	100 (11–100)
Mean (SD)	11 (24.0)	Mean (SD)	70 (33.4)	Mean (SD)	98 (8.6)
No. of positive cases	72 (26%)	No. of positive cases	247 (96%)	No. of positive cases	287 (100%)
No. of negative cases	210 (74%)	No. of negative cases	10 (4%)	No. of negative cases	0 (0%)
Ki67		CAIX			
Median (range)	3 (0–40)	Median (range)	0 (0–80)		
Mean (SD)	4 (5.1)	Mean (SD)	2 (8.8)		
No. of positive cases	N/A	No. of positive cases	25 (9%)		
No. of negative cases	N/A	No. of negative cases	260 (91%)		

IHC, immunohistochemical; *SD*, standard deviation; *N/A*, not available

Cutoff for positive/negative case is 5% (Methods section)

*In case of p53, aberrant cases are marked as positive; wild-type cases are marked as negative

*In case of p16, negative and focal cases are marked as negative; diffusely positive cases are marked as positive

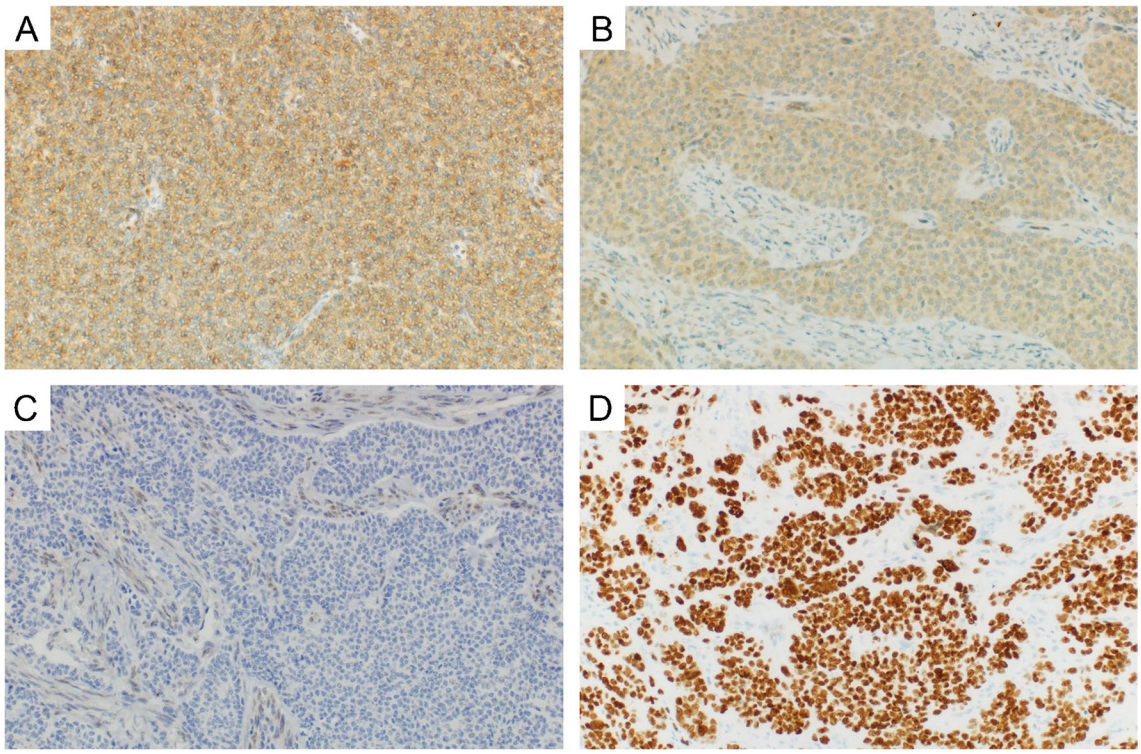

Fig. 1 **A** CTLA4 moderate expression 200×, **B** CTLA4 weak expression 200×, **C** PTEN loss of expression 200×, **D** p53 aberrant expression 200×

remaining studies (including ours) found no association between CTLA4 expression and prognosis [15, 19–21].

Regarding PD-L1, one study investigated the PD-L1 expression in 29 AGCTs [13]. The authors used a different antibody clone (SP142), but their results echoed ours with no detected PD-L1 positivity in the tumor cells.

HER2 expression in AGCTs was also investigated in only a few studies. The works of five groups, including ours, reported all examined cases to be HER2 negative [22–25]. However, there are two studies which did report some HER2-positive AGCT cases. The first one found HER2 positivity in 98% of cases of 80 primary AGCT [26]. The second one found HER2 positivity in 2 matched recurrent cases of 81 granulosa cell tumors (GCT) [2]. Both of the studies used the same antibody and similar methodology as ours. It is not clear why the first study found such a high percentage of positive cases when compared to other works, including ours. However, despite the reported high immunohistochemical expression of HER2 (in 23% of primary AGCT), the detected levels of HER2 amplification did not correspond with these results, as only 8 cases showed 3–6 copies of HER2 and remaining showed no amplification [26].

MMR proteins play an essential role in DNA repair, and their deficiency is linked with an increased risk of the development of cancer. However, MMR deficiency and/or high microsatellite instability are predictors of a favorable response to immune checkpoint inhibitor therapy in solid tumors [27]. So far only one study focused on the expression of MMR proteins in AGCT [28]. Their results were similar to ours, as none of their 40 cases examined showed MMR protein expression deficiency.

We have also examined the expression of some markers which have not yet been studied in AGCT, including PTEN, CAIX, DPC4, CD44, GATA3, napsin A, ARID1A, SATB2, MUC4, and TTF1. Two studies have explored the impaired functions of the *PTEN* pathways which lead to the development of AGCT from granulosa cells in animal models and a few samples of granulosa cell tumors, but no works have explored PTEN expression in a cohort of AGCTs [29, 30]. We have found loss of PTEN expression in 71% (185/261) of AGCTs. PTEN function can be influenced by a variety of genetic and epigenetic mechanisms, and post-transcriptional or post-translational modifications. The role of PTEN as a potential prognostic and/or predictive biomarker is not yet clear, although there are studies in which either the loss of expression as detected by immunohistochemistry, or *PTEN* mutation are considered predictive biomarkers [31, 32]. Currently, there are no uniform scoring criteria for PTEN immunohistochemistry or validating test predicting the lack of PTEN function [31].

Table 3 Correlation between the expression of hormone receptors (categorical scoring, positive vs. negative) and selected clinico-pathological/morphological variables

Characteristics	ER positive	ER negative	*p*-value	PR positive	PR negative	*p*-value	AR positive	AR negative	*p*-value
FIGO			0.242[p]			1.000[f]			0.701[f]
Low (I)	60 (86%)	86 (91%)		142 (89%)	6 (100%)		128 (88%)	19 (95%)	
High (II–IV)	10 (14%)	8 (9%)		18 (11%)	0 (0%)		17 (12%)	1 (5%)	
LVSI			0.264[p]			0.430[f]			1.000[f]
No	27 (95%)	27 (81%)		51 (86%)	4 (100%)		43 (86%)	10 (91%)	
Yes	2 (5%)	6 (19%)		8 (14%)	0 (0%)		7 (14%)	1 (9%)	
Recurrence			**0.001**[p]			0.203[f]			0.608[p]
No	34 (45%)	55 (71%)		86 (57%)	5 (83%)		80 (59%)	10 (53%)	
Yes	42 (55%)	23 (29%)		64 (43%)	1 (17%)		56 (41%)	9 (47%)	
AH/EIN and/or EEC			**0.005**[p]			1.000[f]			0.904[p]
No	43 (81%)	45 (58%)		85 (67%)	5 (71%)		78 (67%)	11 (69%)	
Yes	10 (19%)	33 (42%)		41 (33%)	2 (29%)		38 (33%)	5 (31%)	
HG nuclear atypia			0.817[p]			1.000[f]			**0.007**[p]
No	113 (96%)	158 (95%)		254 (95%)	17 (100%)		226 (97%)	45 (88%)	
Yes	5 (4%)	8 (5%)		13 (5%)	0 (0%)		7 (3%)	6 (12%)	
Mitoses/10 HPFs			0.755[p]			0.325[f]			0.747[p]
<5 mitoses/10 HPF	98 (83%)	141 (84%)		223 (83%)	16 (94%)		197 (84%)	42 (82%)	
≥ 5 mitoses/10 HPF	20 (17%)	26 (16%)		45 (17%)	1 (6%)		37 (16%)	9 (18%)	
Necrosis			0.887[p]			0.377[f]			**0.043**[p]
No	91 (77%)	130 (78%)		206 (77%)	15 (88%)		176 (75%)	45 (88%)	
Yes	27 (23%)	37 (22%)		62 (23%)	2 (12%)		58 (25%)	6 (12%)	
Survival status			0.756[p]			0.221[f]			0.109[p]
Live	66 (88%)	76 (86%)		138 (88%)	5 (71%)		126 (89%)	16 (76%)	
Deceased	9 (12%)	12 (14%)		19 (12%)	2 (29%)		16 (11%)	5 (24%)	

AH, endometrial atypical hyperplasia; *EIN*, endometrial intraepithelial neoplasia; *EEC*, endometrial endometroid carcinoma; *HPF*, high-power field; *LVSI*, lymphovascular invasion; *p*-value is based on Pearson-squared test (*p*) or Fisher Exact test (*f*); significant *p*-values are indicated in bold

The differential diagnosis of AGCT includes mainly other types of ovarian sex cord-stromal tumors, such as thecoma and Sertoli-Leydig cell tumor. Tumors of other histogenesis such as primary or metastatic endometrial stromal sarcoma, undifferentiated carcinoma, hypercalcemic type of small cell carcinoma, and endometrioid carcinoma can enter the differential diagnosis as well. Generally, AGCT expresses "traditional" sex cord markers, most commonly calretinin and inhibin, but also some other markers such as CD56, and WT1, which were not included in our analysis. According to the literature, inhibin and calretinin are positive in a rather broad range of tumors (inhibin 44–100% and calretinin 38–100%) [2, 3, 33–45]. In our study, 78% of tumors expressed inhibin A and 45% of tumors showed the expression of calretinin. In some instances, both of these markers can be negative which can be problematic, especially in cases without typical morphological features. In our study, complete negativity of both those markers was present in 46 cases. In these cases, additional markers including FOXL2 and SF1 are needed. Knowledge about the expression of these markers on a large sample set of AGCT is, however, missing. Previously published studies found the expression of FOXL2 in 70–100% (in 5, 10, 17, 30, 42, and 46 cases) of AGCT, and SF1 in 100% (in 20, 32, and 80 cases) of AGCT [33, 34, 39, 43, 45–49]. The sensitivity of these markers seems to be higher compared to inhibin A and calretinin. This is in concordance with our results, showing the expression of FOXL2 in 98% of cases and SF1 in 100% of cases. The sensitivity of these markers seems to be high, but their specificity and possible use in differential diagnosis with other sex cord-stromal tumors are limited. In this context, molecular testing seems to be beneficial, as there are distinct molecular aberrations such as *FOXL2* and *DICER1* mutation, which can be helpful in the differential diagnosis. Immunohistochemical testing can still be useful in distinguishing tumors of other histogenesis. The relevant diagnostic algorithms are described in detail in other studies [43, 50].

Other markers which can be expressed in AGCT include cytokeratins, such as CKAE1/3. In our study, we found mostly dot-like cytoplasmic positivity of CKAE1/3 in 26% of cases. This number is lower than in the three previously

published studies which examined the expression of this marker. These studies found CKAE1/3 expression in the range of 30–58% of cases [37, 39, 51, 52]. However, the number of cases examined in those was lower (17–47 cases), and only one work mentioned the cutoff used for positivity [37, 39, 51, 52]. The lower number of positive cases in the current study might also be related to the TMA approach since the positivity of this marker is often only focal.

Other markers which can be of both diagnostic and therapeutic significance are hormonal receptors. We found expression of ER in 41%, PR in 94%, and AR in 82% of cases. Those results are in accordance with previous studies, which reported positivity of ER in 16–66%, PR in 5–100%, and AR in 59–100% of cases, with the cutoff ranging from 1 to 10%, using variable antibody clones [3, 11, 13, 39, 53–56]. In our study, we noted higher ER expression in the recurrent cases, but the comparison between primary and recurrent cases was not possible as the samples were not matched. Interestingly, the ER-negative cases were more commonly associated with AH/EIN and/or ECC ($p=0.005$). Most studies (including ours) did not find any correlation between the hormonal receptor expression and prognosis, with the exception of one study in which the authors found PR expression to act as a predictor of recurrence free survival and overall survival [3, 11, 13, 56].

The Ki67 proliferation index is regarded as a prognostic marker in some tumors, but it is a well-known fact that Ki67 is difficult to compare due to a lack of consensus about the scoring methods and cutoff values, and thus a potential lack of reproducibility [57]. In general, AGCT mostly show a lower proliferation index, and only a small proportion of the cases reach higher values [23, 34, 44, 58]. The median proliferation index found in our study was 3 (range 0–40), and mean 4 (SD 5.1), with a positive association of Ki67 with the mitotic rate. We have noticed a higher proliferative index in the recurrent cases, possibly reflecting more biologically aggressive behavior as was found in one previous study [44]. Another study reported a positive correlation between higher Ki67 index and tumor stage, but most of the published works did not find a significant correlation between Ki67 and prognosis [3, 58–62].

The aberrant expression of p53 can be seen in many malignant tumors, but only a few studies have focused on p53 expression in AGCT [1, 23, 38, 39, 49, 53, 59, 60, 63, 64]. Two used the same methodology as us but included a smaller number of cases. The first study found p53 overexpression in 1 of 5 AGCTs [49]. The second study evaluated only 4 AGCT cases, which showed high-grade transformation [1]. They found p53 overexpression in the high-grade areas of 3 of the 4 cases, while the low-grade areas showed wild-type expression [1]. The other works which describe p53 expression are all more than 20 years old and used various cutoffs for positivity which both influence the comparability [23, 38, 39, 49, 53, 59, 60, 63, 64]. Our analysis revealed only 3 cases with aberrant expression (2 primary and 1 recurrent case), all with high-grade nuclear atypia. One of the primary AGCT developed a subsequent recurrence, but for the second case the follow-up was not available.

The expression of p16 in AGCT has so far been examined only in the one aforementioned study on 4 AGCT cases with high-grade transformation. Diffuse block-type p16 staining was found only in the high-grade component in one case [1]. Our results showed diffuse block-type 16 positivity in 1% of AGCT.

The expression of CAIX, GATA3, SATB2, napsin A, MUC4, TTF1, CD44, DPC4, and ARID1A has not been investigated in AGCTs to date. CAIX expression was found in 9% of AGCT, with higher expression in the recurrent cases, suggesting adverse biological behavior. This is in accordance with other studies since CAIX expression has been associated with a worse prognosis in several carcinomas, including breast cancer, gastric cancer, and some others [65, 66]. None of our cases showed positive staining with GATA3, SATB2, napsin A, MUC4, TTF1, or CD44 antibodies, which can be significant with respect to differential diagnosis.

Conclusion

We have immunohistochemically characterized the so far largest, well-defined cohort of 290 AGCTs using 29 markers, including markers which have not yet been examined in AGCT, or were examined only in a few studies and on small sample sets. Our results can be of diagnostic significance, especially in diagnostically challenging cases. Concerning predictive markers, the results of our study showed that AGCT is microsatellite stable, do not express PD-L1, and are HER2 negative. However, we have found the expression of CTLA4 in almost 70% of AGCT, but further studies examining the precise role of this marker are warranted. We have also found the loss of PTEN expression in a significant proportion of AGCT, which can be of therapeutic significance, as there are new treatment strategies targeting the PTEN or mTOR pathways.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00428-024-03854-0.

Acknowledgements The authors wish to extend their gratitude to Mgr. Zachary H. K. Kendall, B.A. (Institute for History of Medicine and Foreign Languages, First Faculty of Medicine, Charles University in Prague) for the English language editing.

Author contribution KN and PD drew up the study concept and design. All authors participated on material preparation, data collection, and/or analyses. The first draft of the manuscript was written by KN. All authors commented on previous versions of the manuscript and approved the final manuscript.

Funding This work was supported by the Ministry of Health, Czech Republic (MH CZ DRO-VFN 64165 and AZV NU21-03–00238), by Charles University (Project UNCE24/MED/018, SVV 260631), and by the European Regional Development Fund (EF16_013/0001674 and BBMRI_CZ LM2023033).

Data availability All data generated or analyzed during this study is included in this published article (and its Supplementary information files).

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.

Competing interests The authors declare no competing interests.

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