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COMPLEX IMMUNOHISTOCHEMICAL AND MOLECULAR STUDY ON 5 CASES OF OVARIAN JUVENILE GRANULOSA CELL TUMORS REVEALS A CONSISTENT ALTERATION IN THE PI3K/AKT/MTOR SIGNALING PATHWAY.

--Manuscript Draft--

Manuscript Number:	
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Article Type:	Original Article
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Funding Information:	Ministerstvo Zdravotnictví Ceské Republiky (MH CZ DRO-VFN 64165 and AZV NU21-03-00238) — Assoc. Prof. Kristýna Němejcová
Abstract:	Juvenile granulosa cell tumor (JGCT) of the ovary is a rare malignant tumor with distinct clinicopathological and hormonal features primarily affecting young women and children. We conducted a complex clinicopathological analysis of five cases of JGCT. The immunohistochemical examination was performed with 33 markers, including markers that have not been previously investigated. Moreover, DNA next-generation sequencing (NGS) and PTEN methylation analysis was performered. We found the expression of calretinin, inhibin A, SF1, FOXL2, CD99, CKAE1/3, ER, PR, AR in all cases. WT-1 was expressed in 20% of cases. Conversely, the expression of p16, OCT3/4, SALL4, GATA3, Napsin A, SATB2, MUC4, TTF1, and CAIX was completely negative. All tumors showed the wild-type pattern of p53 expression. Regarding predictive markers, all tumors were HER2 negative, did not express PD-L1, and were CTLA4 positive. Mismatch repair proteins (MMR) showed no loss or restriction of expression, similarly to ARID1A, DPC4, BRG1, and INI1. The molecular analysis revealed AKT1 internal tandem duplication in 40% of tumors. Two other cases exhibited mutations in TERT and EP400, respectively, and both developed recurrence.

	All AKT1-wild type tumors exhibited immunohistochemical loss of PTEN expression. However, no mutations, deletions (as assessed by CNV analysis), or promoter hypermethylation in the PTEN gene were detected. The results of our study further support the hypothesis that the pathogenesis of JGCT may be driven by activation of the PIK3/AKT/mTOR pathway. These findings could potentially have future therapeutic implications, as treatment strategies targeting the PTEN/mTOR pathways are currently under investigation.
Suggested Reviewers:	
Opposed Reviewers:	

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ABSTRACT

Juvenile granulosa cell tumor (JGCT) of the ovary is a rare malignant tumor with distinct clinicopathological and hormonal features primarily affecting young women and children. We conducted a complex clinicopathological analysis of five cases of JGCT. The immunohistochemical examination was performed with 33 markers, including markers that have not been previously investigated. Moreover, DNA next-generation sequencing (NGS) and *PTEN* methylation analysis was performered. We found the expression of calretinin, inhibin A, SF1, FOXL2, CD99, CKAE1/3, ER, PR, AR in all cases. WT-1 was expressed in 20% of cases. Conversely, the expression of p16, OCT3/4, SALL4, GATA3, Napsin A, SATB2, MUC4, TTF1, and CAIX was completely negative. All tumors showed the wild-type pattern of p53 expression. Regarding predictive markers, all tumors were HER2 negative, did not express PD-L1, and were CTLA4 positive. Mismatch repair proteins (MMR) showed no loss or restriction of expression, similarly to ARID1A, DPC4, BRG1, and INI1. The molecular analysis revealed *AKT1* internal tandem duplication in 40% of tumors. Two other cases exhibited mutations in *TERT* and *EP400*, respectively, and both developed recurrence. All *AKT1*-wild type tumors exhibited immunohistochemical loss of *PTEN* expression. However, no mutations, deletions (as assessed by CNV analysis), or promoter hypermethylation in the *PTEN* gene were detected. The results of our study further support the hypothesis that the pathogenesis of JGCT may be driven by activation of the *PIK3/AKT/mTOR* pathway. These findings could potentially have future therapeutic implications, as treatment strategies targeting the PTEN/mTOR pathways are currently under investigation.

Key words: juvenile granulosa cell tumor, immunohistochemistry, NGS, sex cord-stromal tumors, ovary

STATEMENTS AND DECLARATIONS:

Competing Interests: The authors declare no competing financial and/or non-financial interests relevant to the content of this article.

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).

Institutional Review Board Statement - 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.

INTRODUCTION

Granulosa cell tumors (GCT) of the ovary are malignant tumors belonging to the group of pure sex cord-stromal tumors and represent 3-5% of all ovarian malignancies. GCT have distinct hormonal features and are divided into two groups based on their clinical and pathological attributes: adult granulosa cell tumors (AGCT) and juvenile granulosa cell tumors (JGCT) [1]. Adult granulosa cell tumors are significantly more common (95%), typically arise in perimenopausal women in the age between 55-60 years, and are known for their late recurrences [1-2]. Conversely, JGCT (5%) predominantly occur in younger patients with a median age of 13 years; however, instances of occurrence have also been recorded in infants as well as in patients up to 67 years old [3-4]. Typical clinical symptoms include abdominal pain, menstrual irregularities, amenorhea, and precocious pseudopuberty, most of which are attributed to the tumor's capacity to secrete hormones, most commonly estrogen. However, some JGCT can produce androgens, prolactin, or cause hypercalcaemia [3,5-7]. The majority of JGCT are diagnosed in the early stages, resulting in a favorable prognosis and minimal risk of recurrence. The primary treatment for JGCT involves surgical resection, typically without the necessity for adjuvant chemotherapy. In contrast, the prognosis for higher-stage JGCT is significantly worse, with reduced survival rates and inconclusive treatment modalities. Unlike AGCT, recurrences of JGCT typically occur within the first 3 years following diagnosis [3, 8-10]. The diagnosis of JGCT typically relies on a combination of morphology, immunohistochemistry, and clinical features. Diagnosing JGCT can be challenging and potentially misleading in some cases due to the overlap with other sex-cord stromal tumors or malignancies of epithelial origin.

Studies on the molecular background of JGCT have identified the *AKT1* mutation as a potential driver in the pathogenesis of these tumors. Additionally, *GNAS* mutations, somatic *DICER1* mutations, and *FOXL2* mutations have also been found in some JGCT, although all of these mutations are present only in a subset of cases in contrast to AGCT, where 98% of cases harbor the previously mentioned missense FOXL2 mutation (c.402C > G,p.Cys134Trp) [11-15]. Rare cases of JGCT have been associated with Ollier disease, Maffucci syndrome, or tuberous sclerosis, and the literature also describes these tumors with germline mutations in *TP53*, *PTEN*, and *DICER1* [16-20].

In this study, we present a comprehensive analysis of 5 primary JGCT cases and 1 associated recurrence encompassing the clinicopathological features, extensive immunohistochemistry (including predictive markers not previously described in the literature), DNA next-generation sequencing (NGS), and methylation analysis with the aim of expanding current knowledge on these extremely rare entities.

MATERIALS AND METHODS

Samples

A total of 10 cases previously diagnosed as JGCT were retrieved from the archives of our department, supplemented by contributions from collaborating institutions. All tumors were examined and reviewed by two experts in gynecologic pathology (KN and PD). The morphologic analysis included the determination of the predominant growth pattern, presence of necrosis and hemorrhage, lymphovascular space invasion (LVSI), and mitotic rate per 10 high-power fields (HPFs). These assessments were conducted on whole tissue sections from each tumor. After a central review of all selected cases, the diagnoses of 4 cases were reclassified to other entities within the sex-cord stromal tumors group (2 Sertoli-Leydig cell tumors (SLCT), 2 sex cord-stromal tumors NOS), and 1 case was identified as a duplicate tissue sample (primary tumor of a recurrent case). Ultimately, our subset consists of 5 primary tumors and 1 associated recurrence. All samples underwent comprehensive immunohistochemical evaluation along with targeted-capture DNA next-generation sequencing (NGS) and *PTEN* methylation analysis.

Patient clinical characteristics

Clinical data on patient and tumor characteristics at the time of diagnosis, along with survival information, were obtained retrospectively from the medical records. However, follow-up data could not be obtained for one case (#2). Detailed clinical characteristics of all cases are summarized in Table 1.

Immunohistochemical analysis

Immunohistochemistry was conducted on tissue microarrays (TMAs) utilizing 4 μm thick sections of formalin-fixed, paraffin-embedded (FFPE) tissue. To construct the TMAs, suitable areas of each tumor were identified, and two tissue cores (each 2.0 mm in diameter) were extracted from the donor block using the TMA Master tissue microarray instrument (3DHISTECH Ltd., Budapest, Hungary). In this study, we employed markers analogous to those utilized in our previous research on AGCT [21]. The antibodies utilized included the "diagnostic" markers (FOXL2, SF1, CD99, inhibin A, calretinin, Ki67, ER, PR, AR, p53, p16, CKAE1/3) and other markers which were incorporated because of the important role they play in the differential diagnosis, such as BRG1, INI1, SALL4, OCT3/4, and WT1. We have also evaluated the expression of markers not previously analyzed in JGCT (CAIX (carbonic anhydrase IX), DPC4, PTEN, ARID1A, GATA3, MUC4, napsin A, TTF1, and SATB2), and selected predictive markers (PD-L1, HER2, CTLA4, MLH1, PMS2, MSH2, and MSH6).

Details on their manufacturers, clones, and dilutions are provided in Supplementary Table S1. The expression of all markers was evaluated independently by two pathologists (AŠ, KN) in a double-blind manner. Tumor cases were classified based on the overall percentage of positive cells as either negative (entirely negative or < 5% positive tumor cells) or positive (≥ 5% positive tumor cells), with the exceptions of Ki67, p16, p53, HER2, and PD-L1. The expression of the p53 protein was categorized as either "wild-type" or "aberrant type." The "aberrant type" staining was characterized by one of the following: diffuse intense nuclear positivity in more than 80% of tumor cells, cytoplasmic positivity for p53, or a complete absence of staining in the presence of a positive internal control (referred to as the null pattern) [22]. Ki67 expression was evaluated as a continuous variable, quantified by the percentage of positive tumor cells, ranging from 0 to 100% and analyzed manually in 200 tumor cells in the hot-spot, or in randomly chosen fields for cases exhibiting homogeneous expression. The expression of p16 was classified as either block positive (diffuse staining of tumor cell nuclei and/or cytoplasm) or negative (focal, patchy, or absent staining). The evaluation of PTEN, DPC4, ARID1A, BRG1, INI1, and mismatch repair (MMR) proteins concentrated on the loss of expression in tumor cells with preserved staining in stromal cells. Tumor cells were classified as negative if less than 5% showed expression. PD-L1 expression was evaluated as the percentage of positive tumor cells (tumor proportion score; TPS). The presence of stromal lymphocytes in cases of JGCT is sparse, rendering the evaluation of CTLA4 expression in immune cells and the PD-L1 combined positive score (CPS) unfeasible. HER2 scoring was conducted according to the 2018 ASCO Guidelines for breast carcinoma, due to the absence of an established scoring system for ovarian tumors [23].

Next generation sequencing (NGS)

Genomic DNA were isolated from FFPE tissue from the tumor using the Magcore Genomic DNA FFPE One step kit (RBC Bioscience). Sequence capture NGS analysis of DNA was performed using the KAPA HyperPlus kit according to KAPA HyperCap Workflow v3.0 (Roche) and a panel of hybridization probes against multiple targets of cancer relevant genes (Supplementary Table S2, 788 genes or gene parts; 2440 kbp of target sequence including 1992 kbp of coding regions; Roche). The prepared sample libraries were pair-end sequenced by the NextSeq 500 instrument (Illumina) using the NextSeq 500/550 High Output Kit v2.5 (Illumina). The biostatistical evaluation was performed using the CLC Genomics Workbench software (CLC GW; Qiagen, Venlo, The Netherlands). The interpretation of the DNA variants and calculation of tumor mutation burden (TMB) was performed as previously described [24]. Due to inconsistent DNA quality from FFPE tissues, which limited extensive CNV analysis, we specifically examined *PTEN* CNV based on the observed loss of PTEN expression at the immunohistochemical level.

PTEN methylation analysis

The methylation-specific qPCR to analyze the methylation status of the *PTEN* gene was conducted using primers designed by Garcia et al. and the 5X HOT FIREpol® PROBE qPCR mix (Solis BioDyne) [25]. The experiments included non-methylated DNA and universally methylated DNA controls (Zymo Research).

RESULTS

Clinical and morphological features

The ages of patients in our cohort ranged from 6 to 43 years, with a mean age of 19 years and a median age of 16 years. The follow-up period for these patients ranged from 20 to 79 months, with a mean follow-up duration of 41 months. Interestingly, none of our cases manifested with hormonal irregularities. However, the clinical data of case #2 was missing. All cases with available data presented with abdominal pain. Notably, case #4 exhibited an acute abdomen due to torsion, a condition infrequently observed in ovarian tumors, including JGCT [26]. In all three cases of recurrence within our cohort, the recurrence manifested within the first year following the initial diagnosis. The most frequently administered chemotherapy for recurrent disease was platinum-based, with taxane-based chemotherapy (specifically paclitaxel) being used less commonly. In case #1, biological treatment with bevacizumab was also administered, resulting in a limited therapeutic response.

Regarding macroscopy, all tumors had multicystic appearance and their size varied between 8 to 18 centimeters with mean size of 12.6 and a median of 12. the morphological atributes are summarized in Fig. 1. The histopathology of JGCTs predominantly exhibited a characteristic microfollicular pattern with a variable proportion of solid component, and is documented in Fig. 2. The recurrence of case #3 exhibited morphology comparable to that of the primary tumor.

Immunohistochemistry

Detailed immunohistochemical results of each case are highlighted in Fig. 1. In summary, immunohistochemical analysis revealed positive expression of SF1, inhibin A, calretinin, FOXL2, ER, PR, AR, CKAE1/3, and CD99 in 100% (5/5) of cases. Conversely, there was no expression of GATA3, Napsin A, SATB2, MUC4, CA IX, OCT 3/4, SALL-4, and TTF1. WT1 expression was observed in 20% (1/5) of cases. All tumors exhibited the "wild type" p53 expression pattern. The expression of p16 was negative in all cases. Notably, 60% (3/5) of cases showed loss of PTEN expression, while BRG1, INI1, ARID1A, and DPC4 expression was fully retained. Overall, Ki67 showed a median value of 30 (range 4-47) and mean value of 26,8.

Concerning the possible predictive markers, all tumors were HER2 negative, and PD-L1 negative (TPS < 1%). CTLA4 was weakly expressed in the tumor cells of all five cases. Analysis of mismatch repair (MMR) proteins indicated retained expression across all cases.

Molecular findings

Targeted NGS DNA analysis was successfully performed in all cases. Pathogenic or likely pathogenic mutations were detected in 80% of cases. The *AKT1* gene mutation was identified in 40% of cases. Additionally, one of these tumors also exhibited a mutation in the *CHEK2* gene. A *TERT* promoter mutation (c.-124C>T) was detected in one case, while an *EP400* gene mutation was identified in another case. None of the tumours harbored the missense *FOXL2* mutation (c.402C > G, p.Cys134Trp). The detailed mutation status of each tumor is summarized in Fig. 1. The recurrence of case #3 exhibited an identical mutational profile to the primary tumor. Tumor mutation burden was low in all 5 cases (average 5 Mutation/Megabase).

We did not detect promoter hypermethylation or the deletion of the *PTEN* gene in any of the tumors.

DISCUSSION

The diagnosis of JGCT relies primarily on morphology and clinical features in combination with immunohistochemistry. The majority of patients are under 30 years of age, with the tumors typically exhibiting a histomorphology characterized by lobular growth pattern, microfollicular spaces lined with atypical juvenile granulosa cells, and varying amounts of solid components. These observations are consistent with the findings in our cohort and are demonstrated in Fig. 2. However, distinguishing these tumors from other sex cord-stromal tumors based on morphology alone can be challenging, as there are potentially cases with overlap, particularly regarding AGCT or SLCT. Immunohistochemistry is frequently limited in its utility for differentiating between entities within the sex cord-stromal tumor group. This limitation is due to the fact that the majority of tumors in this category exhibit similar immunohistochemical profiles when analyzed using common markers of sex cord differentiation. This similarity in expression has been consistently documented across numerous studies [27-37]. Notably, inhibin A was first identified as a helpfull diagnostic tool in differential diagnosis of sex cord-stromal tumors, including JGCT, compared to tumors of other histogenesis [34]. Subsequent studies have analyzed the expression of inhibin A and provided similar results, with positivity reported in 95% to 100% of cases [27-33, 35]. Similar attributes apply to calretinin, which has been described as more specific but less sensitive than inhibin for identifying sex cord differentiation [32]. Studies have reported variable expression of calretinin in

JGCT, with 85% to 100% of cases showing positive results [27, 29, 32-33]. Both of these markers (inhibin A, calretinin) were positive in all cases in our cohort. Another useful auxiliary marker used in confirming sex-cord differentiation is SF1. Although the number of studies examining SF1 expression in JGCT is limited, the available data supports its reliability as a diagnostic tool as it has higher sensitivity than inhibin and calretinin, and was expressed in 100% cases of JGCT, consistent with our findings [27, 37]. Regarding expression of FOXL2, all tumors in our cohort were positive. Previous studies have reported FOXL2 expression ranging from 74% to 100% [38-40]. Notably, DeAngelo et al. found that increased FOXL2 expression in JGCT cases was associated with worse prognostic outcome [38]. In contrast, Kalfa et al. described a reduction or lack of FOXL2 expression in aggressive JGCT cases using the treshold of <70% as reduced expression [39]. Due to our limited dataset and these conflicting findings, our study cannot assess the prognostic significance of FOXL2 immunohistochemical expression andfurther research is necessary to clarify this hypothesis. At the molecular level, two studies have reported FOXL2 missense mutation in JGCT with a frequency of 10% and 12% of cases, respectively [13-14]. However, the sample sizes in these studies were also limited, consisting of 10 and 8 cases. Moreover, several recent studies with larger datasets provided entirely negative results and are consistent with the findings in our study [27, 38, 41]. Another marker commonly expressed in sex cord-stromal tumors is CD99 and its expression has already been previously examined in JGCT with 100% of positive cases [42-43]. Results in our cohort were similar as all tumors expressed CD99.

Epithelial markers such as cytokeratins can also be expressed in JGCT, though with variable frequency. Previous studies have reported cytokeratin expression rates of 15%, 60%, and 80% [27, 44-45]. In contrast, all of our cases were cytokeratin positive.

The aberrant expression of p53 is a common finding in many malignant tumors and has a proven diagnostic utility. However, only a limited number of studies have specifically examined p53 expression in JGCT [27, 30, 46]. Furthermore, none of the referenced studies distinguished between the wild-type and aberrant p53 expression in their analyses, making direct comparison with our findings challenging. In our cohort, all JGCT cases exhibited wild-type p53 expression and, based on the reported findings from previous studies, it can be inferred that they likely observed the wild-type expression as well.

Regarding hormonal receptors, we found the expression of ER, PR, and AR in 100% of cases. Only ER had been previously studied in a cohort of JGCT with mostly positive results of 81% and 85% [27, 47]. A study of Staibano et al. found a correlation of ER expression loss with worse prognostic outcome [47]. However, they used a different antibody clone and analysed only 11 cases of JGCT. We did not provide any statistical analysis

in our study due to the limited dataset.

The Ki67 proliferation index is a recognized prognostic marker in certain tumors, but its use is hindered by inconsistent scoring methods and cutoff values, leading to poor reproducibility [48]. The expression of Ki-67 in our cohort varied with a range from 4% to 47% (median 30, mean 27). These results were similar in other studies evaluating proliferation index which ranged from <5% to 61% [27, 30, 46, 49]. Generally, the mitotic activity of JGCT is typically brisk, but lacks prognostic significance. In contrast, AGCT exhibit a lower proliferation index and mitotic count, yet are associated with worse prognosis [2, 21].

Other important markers in the differential diagnosis of JGCT are WT1 and germ cell markers such as OCT3/4 and especially SALL4. Yolk sac tumor is an extremely variable tumor histologically, and can exhibit similar morphological features to JGCT in cases of reticular growth pattern. Both SALL4 and OCT3/4 were completely negative in our cohort, similar to other studies that also examined its expression [27, 37]. WT1 is commonly expressed in malignant tumors of various localizations including ovarian cancer [50]. Data concerning WT1 expression in JGCT are limited to only one study with 43% of positive cases [27]. We found the expression of WT1 in 20% (1/5) of our cases.

Lastly, BRG1 and INI1 are crucial markers in distinguishing JGCT from the hypercalcemic type of small cell carcinoma of the ovary (SCCOHT), which usually affects younger women and children as well. SCCOHT can have a similar morphology to JGCT but are characterized by much worse prognosis [51]. They belong to the group of SMARCA4 deficient tumors and the diagnosis is verified by examining INI1 and BRG1 through immunohistochemistry, as their loss of expression is a diagnostic feature [52]. All cases of JGCT in our cohort showed retained BRG1 and INI1 expression.

Knowledge of predictive markers in JGCT is limited, with only two studies having analyzed HER2 status. These studies, conducted by Sakr et al. and Leibl et al., provided results consistent with ours by confirming that JGCT do not express HER2, although both studies were based on small sample sizes of 2 and 10 cases, respectively [53-54]. A recent study of Němejcová et al. evaluating HER2 status in 290 AGCT also reported completely negative findings [21]. This suggests that JGCT likely shares similar HER2-negative attributes with AGCT. The novelty of our study was the analysis of other predictive markers such as MMR and PD-L1. The results showed that JGCT is PD-L1 negative and MMR proficient. Another potentially predictive marker is CTLA4, the expression of which has been described in several malignant tumors including those of female genital system [55-56]. In our study we found weak cytoplasmatic expression in all 5 cases (Fig. 3). Positive expression of CTLA4 in tumor cells was also recently described in AGCT [21].

We also investigated the expression of additional markers including GATA3, Napsin A, SATB2, MUC4, TTF1, and CAIX, which had not been previously described in the context of JGCT. None of these markers exhibited positive expression. Similarly, the expression of DPC4 and ARID1A, which had not been previously studied in this setting, showed no loss or restriction of expression.

Another aim of our study was to analyze the molecular background of our JGCT cases. Bessiere et al. found an internal tandem duplication (ITD) of the *AKT1* gene in 60% of a cohort consisting of 16 JGCTs and these mutations were restricted to patients under 15 years of age [11]. Our findings are consistent with these observations as we detected an ITD of the *AKT1* gene in 40% of cases (2/5). One of the *AKT1*-mutated tumors also harbored a likely pathogenic mutation (class 4) in the *CHEK2* gene, while the second *AKT1*-mutated tumor did not have any additional mutations detected. Both of the *AKT1* mutated tumors in our cohort exhibited decreased expression of Ki-67 compared to *AKT1* wild-type tumors. *AKT1* ITD activate the oncogene *AKT1* and subsequently the *PI3K–AKT* pathway, and probably act as driver events in the pathogenesis of JGCT [11]. In our cohort, three cases lacked *AKT1* mutations. Among these, one tumor exhibited a *TERT* promoter mutation which has also been previously described in the study of Vougiouklakis et al. in 3% of JGCT (1/33) [41]. The second case presented with a mutation in *EP400* which is involved in chromatin remodeling and regulation of gene expression. A *TERT* promoter mutation was detected in the primary tumor as well as in the subsequent recurrence. Interestingly, we observed the immunohistochemical loss of PTEN expression in 60% (3/5) cases of JGCT (Fig. 3). All cases with the loss of PTEN expression were *AKT1* wild-type JGCT tumors, but we did not detect mutations, deletions (as determined by CNV analysis), or promoter hypermethylation in the *PTEN* gene. This suggests that the loss of PTEN expression may result from alternative mechanisms, such as miRNA interference or post-translational modifications. The loss of *PTEN* function is a common event in many cancers and could lead to the activation of the *PI3K/AKT/mTOR* signaling pathway but to our knowledge has not been previously examined in JGCT [57]. Recent studies on animal models suggest that the impaired function of *PI3K/AKT* pathway by PTEN loss has a possible effect on the growth of granulosa cell tumors [58-59]. Our results further supplement the theory that the majority of JGCTs are driven by the activation of the *PI3K/AKT/mTOR* signaling pathway. However, this hypothesis needs further investigation in a larger cohort of JGCTs. Ultimately, our study found no somatic *DICER1* mutations, which stands in contrast to the findings of Onder et al., who reported *DICER1* mutations in 25% (2/8) of JGCT [40].

CONCLUSION

We provide a complex analysis of 5 cases of JGCT including morphological assesment, 33 immunohistochemical markers, and detailed molecular biological analysis using NGS and *PTEN* methylation analysis. Our results further expand knowledge of the immunohistochemical markers in the differential diagnosis and prediction. Regarding the predictive markers, we found that JGCT does not express PD-L1, is HER2 negative, microsatellite stable, and expressed CTLA4 in 100% cases (5/5). We also found the loss of PTEN in 60% (3/5) cases in corelation with the *AKT1* wild-type status, which further supports the theory that the pathogenesis of JGCT is possibly driven by the activation of the PIK3/AKT/mTOR pathway. Nonetheless, this hypothesis warrants further exploration in a larger cohort of JGCTs. Two other cases exhibited mutations in *TERT* and *EP400*, and both developed recurrence. Our findings may hold future therapeutic significance as treatment strategies targeting the PTEN/mTOR pathways are currently being investigated.

Author contribution: Kristýna Němejcová and Pavel Dundr conceptualized and designed the study. All authors contributed to the material preparation, data collection, and/or analysis. The molecular biological analysis was provided by Nikola Hájková. Adam Šafanda wrote the first draft of the manuscript. All authors provided feedback on previous drafts and approved the final version of the manuscript.

Informed Consent Statement: Not applicable.

Acknowledgments: 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.

Data availability

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

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Figure legends:

Fig. 1: Summary of the morphological, immunohistochemical, and molecular features of ovarian juvenile granulosa cell tumor cohort.

Fig. 2: Histomorphology of JGCT (H&E stain): **A** Case #1 (100x), **B** Case #2 (100x), **C** Case #3 (100x), **D** Case #3 recurrence (100x), **E** Case #4 (100x), **F** Case #5 (100x)

Fig. 3: **A** PTEN loss of expression case #4 (200x), **B** PTEN loss of expression case #5 (200x), **C** SF1 expression (200x), **D** CTLA4 expression (200x)

Tables:

Table 1: Clinical features of ovarian juvenile granulosa cell tumors.

Supplementary files:

Table S1: Overview of the immunostains used and their main characterstics.

Table S2: List of genes or gene parts included in the NGS DNA targeted panel

Table 1

Table 1. Clinical features of ovarian Juvenile granulosa cell tumors (n=5).

Sample ID	Age at diagnosis	Primary site - ovary	Clinical presentation	Hormonal features	Stage (FIGO)	Follow up (months)	Primary treatment	Recurrences	Positive ascites	Treatment of recurrences	Status
1	43	left	abdominal pain	no	IC	21	HYE + AE bilateral	yes	N/A	CHT, BT	DOD
2	6	left	N/A	N/A	IC	N/A	AE unilateral	N/A	N/A	N/A	N/A
3	16	right	abdominal pain	no	IIIB	79	AE unilateral, CHT	yes	yes	R, CHT	AWD
4	10	right	acute abdomen	no	IC	44	AE unilateral	yes	N/A	R, CHT	NED
5	21	right	abdominal pain	no	IC	20	AE unilateral	no	no	N/A	NED

HYE – hysterectomy, AE – adnexectomy, N/A – data not avalible, CHT – chemotherapy, BT – biological therapy, R- surgical resection, NED – no evidence of disease, AWD – alive with disease, DOD – death of disease.

Figure 1

Sample ID	Tumor size (cm)	Haemorrhage	Necrosis	LVSI	Mitoses (per 10 HPFs)	class 4-5 mutation
1	8	yes	yes	no	25	*EP400*: p.Ala2577fs
2	10	yes	no	no	6	*AKT1*: p.Arg76_Val83dup
3	15	yes	no	no	10	*TERT* promoter: c.-124C>T
4	18	yes	no	no	8	*AKT1*: p.Arg67_Leu78dup *CHEK2*: p.Arg145Trp
5	12	yes	no	no	20	none

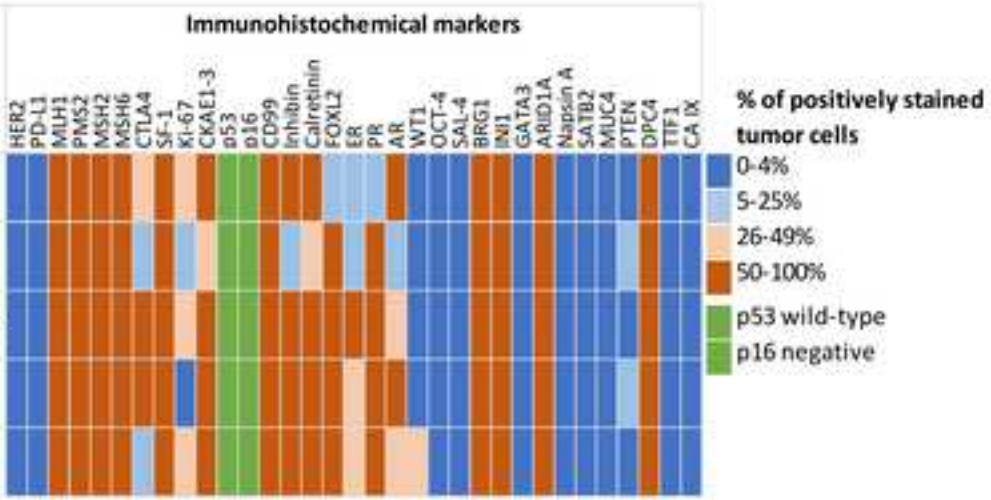

Figure 2

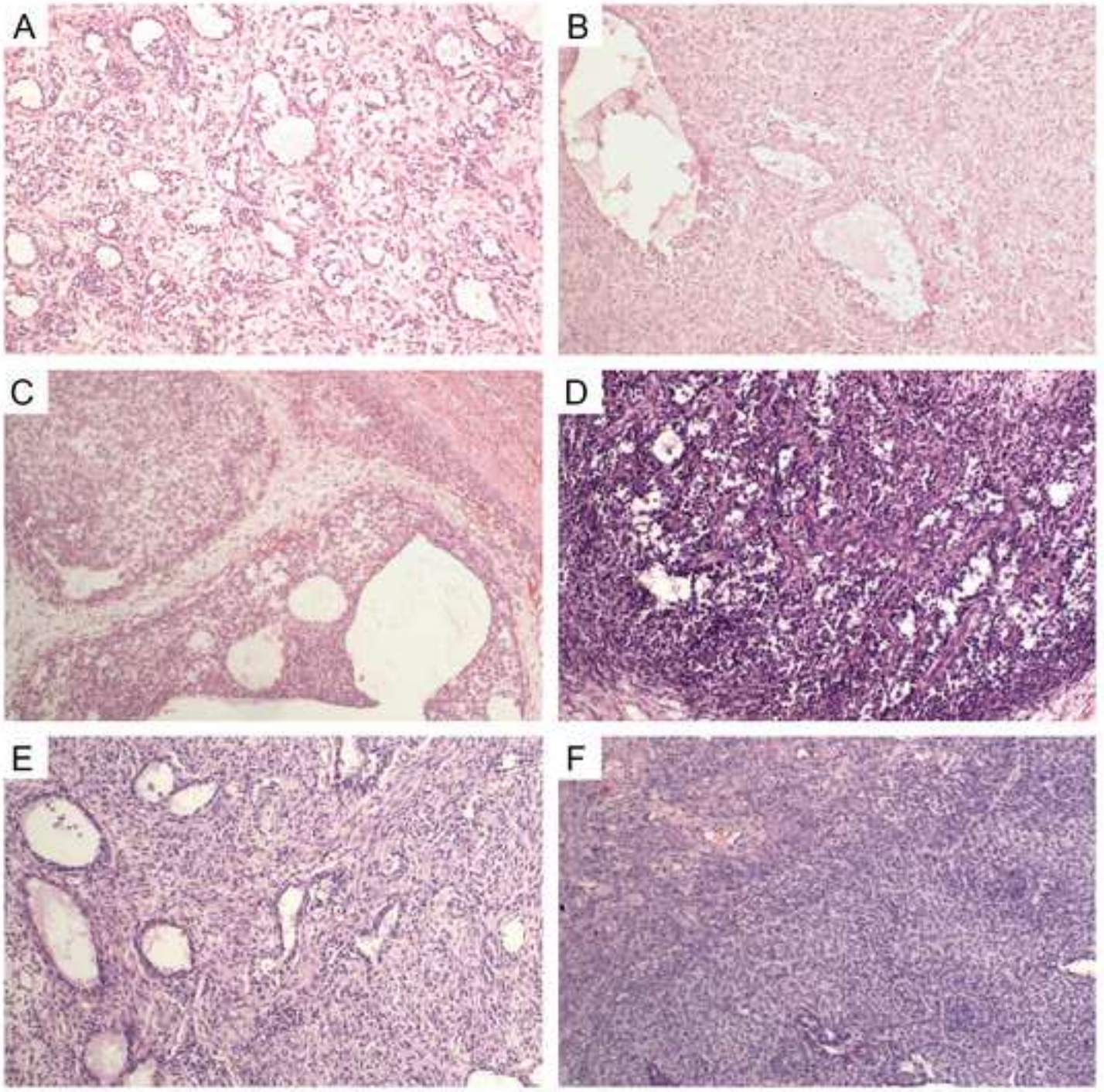

Figure 3