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Original contribution

PIK3CA hotspot mutations and cyclooxygenase-2 expression in ovarian clear cell carcinomas: a close association with stromal features☆

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Summary Ovarian clear cell carcinomas (CCCs) have 2 distinct stromas: a hyalinized/mucoid stroma and a plasma cell–rich inflammatory stroma. Clinically, CCC is the most common ovarian cancer associated with thromboembolism. Recent studies suggested a potential role of *PIK3CA* mutation in the cyclooxygenase (COX) pathway, which mediates inflammation or hemostasis. In the present study, 54 ovarian CCCs and 3 CCC cell lines were analyzed for *PIK3CA* hotspot mutation and COX-2 expression with special reference to stromal features. Among the 54 CCCs, 20 (37.0%) and 8 (14.8%) were classified as CCCs with a hyalinized/mucoid stroma and an inflammatory stroma, respectively. *PIK3CA* mutation was identified in 11 (55%) of the 20 CCCs with a hyalinized/mucoid stroma, but not in any of the 8 CCCs with an inflammatory stroma. In contrast, COX-2 expression was frequent in CCCs with an inflammatory stroma (1/20 [5%] versus 7/8 [87.5%], respectively). Such a relationship between the *PIK3CA* mutation, COX-2 expression, and stromal features was repeated in the 3 CCC cell lines. Thromboembolism was noted in 9 (16.7%) of the 54 CCC patients, and it was more frequent in CCCs with a hyalinized/mucoid stroma (7/20 [35%]) than in those with an inflammatory stroma (0/8 [0%]). In conclusion, there is a difference in *PIK3CA* mutation, COX-2 expression, and paraneoplastic thromboembolism between CCCs with a different stroma. It is suggested that a different stromal feature, either hyalinized/mucoid change or inflammation, represents a different molecular genetic background or hemostatic potential in ovarian CCCs.

1. Introduction

Ovarian clear cell carcinomas (CCCs) are characterized by both their epithelial component and stroma. The common epithelial types are clear and hobnail cells. In the stroma, CCCs often show hyalinization and/or mucoid change [1], and less frequently show plasma cell–rich inflammation [2] (Fig. 1). The hyalinized/mucoid stroma is caused by the deposition of

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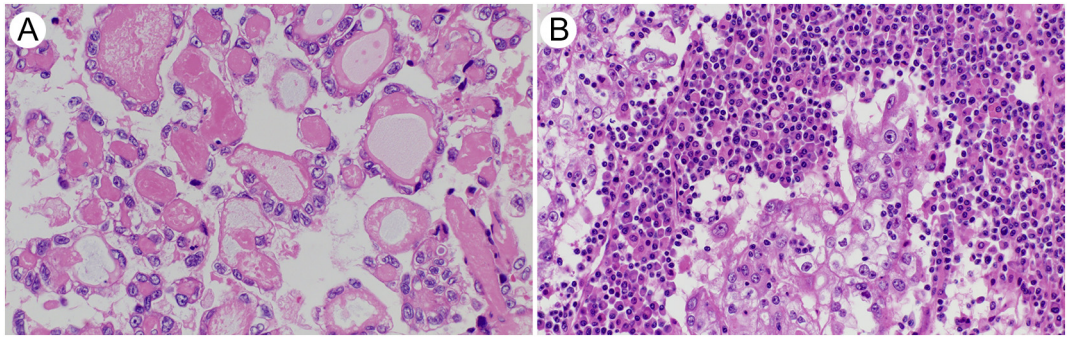

Fig. 1 Representative histology of ovarian CCC with a hyalinized/mucoid stroma (A) or an inflammatory stroma (B). In the hyalinized/mucoid stroma, hyalinization and mucoid change were found concomitantly. In the inflammatory stroma, the infiltrating cells were predominantly plasma cells. Hematoxylin and eosin, original magnification ×40.

different extracellular matrices produced by CCC cells [1], and the plasma cell–rich inflammatory stroma is also likely to be induced by CCC cells [2]. Both stromas are rarely concurrent.

In ovarian CCCs, one of the most common genetic alterations is *PIK3CA* mutation [3]. The *PIK3CA* gene encodes the catalytic subunit p110a of phosphatidylinositol-3 kinases (PI3Ks), and hotspot mutations of *PIK3CA* increase PI3K activity [4,5]. Recently, an interaction between the PI3K and cyclooxygenase (COX)-2 pathways was suggested in colorectal and breast cancers, attracting interest as a potential novel therapy [6,7]. We previously showed that COX-2 was expressed in some CCCs, especially those with an inflammatory stroma [2]. However, there have been no systemic studies on the relation between *PIK3CA* mutation and COX-2 expression in CCCs.

Clinically, CCC is the most common ovarian cancer associated with thromboembolism [8,9]. It would be of interest to investigate whether there are any histopathologic signs of CCC-related thromboembolism. Previous studies failed to identify any such features in the epithelial component of CCC. However, little attention has been paid to the stroma.

In the present study, 54 CCCs and 3 CCC cell lines were analyzed to clarify the *PIK3CA* hotspot mutation and COX-2 expression with special reference to stromal features. The 54 CCCs were also analyzed to assess the relation between stromal features and thromboembolism.

2. Materials and methods

2.1. Surgical specimens

We investigated 54 ovarian CCCs that had been surgically resected from 54 patients between 2000 and 2018. Of the 54, 39 were stage I, 5 were stage II, 9 were stage III, and 1 was stage IV, according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO) [10]. All of them had been confirmed to be immunoreactive for hepatocyte nuclear factor-1β. We used only specimens from patients who had not received any treatment before surgery. All specimens were fixed in 10% formalin and embedded in paraffin. This study was approved by the Institutional Ethics Committee (approval code: 2017-1151(= updated approval code)., Hirosaki University Graduate School of Medicine).

Hematoxylin and eosin–stained slides (5-25 slides) were reviewed by pathologists, and the stromal features were evaluated. Hyalinized/mucoid stroma was defined as stroma that is diffusely accumulated by hyalinized and/or mucoid materials greater than 20% of tumor areas. Inflammatory stroma was defined as stroma that is diffusely obliterated by marked inflammatory infiltrates in multiple foci, which is evident at low-power magnification (×4).

2.2. CCC cell lines

HAC-2 was a gift from Dr M. Nishida (Kasumigaura Medical Center, Tsuchiura, Japan), and SMOV-2 and JHOC-5 were obtained from Immunobiological Laboratory (Takasaki, Japan) and Riken Cell Bank (Tsukuba, Japan), respectively. HAC-2 and SMOV-2 show a mucoid or hyalinized stroma, whereas JHOC-5 shows a plasma cell–rich inflammatory stroma in xenografts of nude mice, confirmed by our previous study [2]. Each cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C. After the 3 cell lines were cultured to confluence, the cells were gently scraped and served for genetic and immunocytochemical analyses.

2.3. Mutation analysis of *PIK3CA*

For total DNA isolation from surgical specimens, areas of paraffin sections (10 μm) showing core histologic features by definition were manually dissected using a sterile scalpel, and their genomic DNA was extracted with the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). For total DNA isolation from cell lines, the QIAamp DNA Mini Kit (Qiagen) was used. Polymerase chain reaction (PCR) was performed with the KOD-Plus ver.2 (Toyobo, Osaka, Japan) on a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). The PCR primers used were 5′-cagagtaaca gactagctagagac-3′ (forward) or 5′-ccagaggggaaaaatatgac-3′

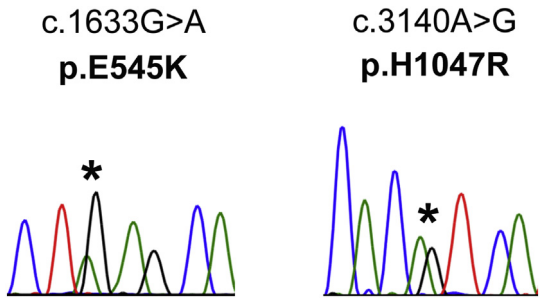

Fig. 2 Representative sequence electropherograms of the 2 *PIK3CA* hotspot mutations (E545K and H1047R) in ovarian CCCs analyzed in the present study.

(forward) and 5′-gcacttacctgtgactccatag-3′ (reverse) for exon 9, and 5′-ttgatgacattgcatacattcg-3′ (forward) and 5′-aattgtgtg-gaagatccaatcc-3′ (reverse) [11] for exon 20, which covered the hotspots of mutations.

The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), and sequencing reactions were performed on an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific) using the BigDye Terminator v.1.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and sequencing primer 5′-agactagctagagacaatgaat-3′ (exon 9) or 5′-tgcatacattcgaaagacccta-3′ (exon 20) [11]. Sequences were analyzed with the Applied Biosystems DNA Sequencing Analysis Software v.6.0 and Sequence Scanner Software v.2.0 (Thermo Fisher Scientific).

2.4. Immunohistochemistry and immunocytochemistry for COX-2

Paraffin sections (3 μm) of surgical specimens that showed core histologic features by definition and those of cell line blocks were autostained using a rabbit antibody against the carboxyl region (C295) of human COX-2 (Immunobiological Laboratory; dilution 1:50). Details of the immunoreaction have been described in our previous study [2].

Table *PIK3CA* hotspot mutation, COX-2 expression, and association with thromboembolism in ovarian CCCs showing a different stroma

Type of stroma	n	*PIK3CA* mutation	COX-2 expression	Thromboembolism
Hyalinized and/or mucoid stroma	20	11 (55%)	1 (5%)	7 (35%)
Plasma cell–rich inflammatory stroma	8	0 (0%)	7 (87.5%)	0 (0%)
Stroma, not specified	26	6 (23.1%)	2 (7.7%)	2 (7.7%)
Total	54	17 (31.5%)	10 (18.5%)	9 (16.7%)

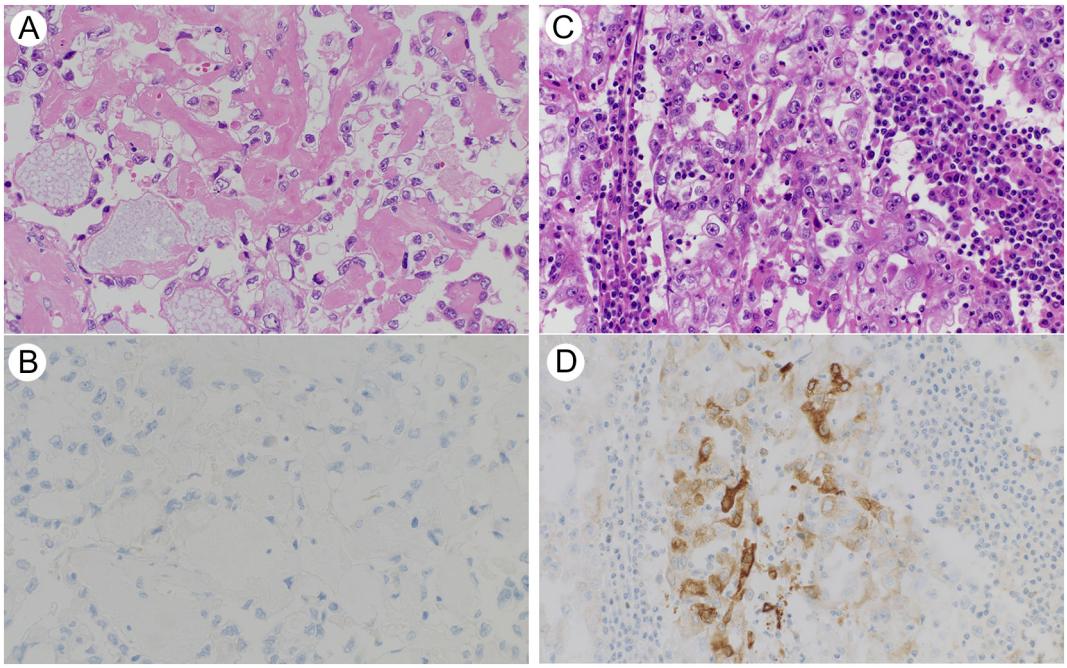

Fig. 3 Immunohistochemistry for COX-2 in ovarian CCCs with a hyalinized/mucoid stroma (A and B) or an inflammatory stroma (C and D). COX-2 was expressed by tumor cells that interfaced with the inflammatory stroma, but not by tumor cells adjacent to the hyalinized/mucoid stroma. A and C, Hematoxylin and eosin. B and D, COX-2 with hematoxylin counterstain. Original magnification ×40.

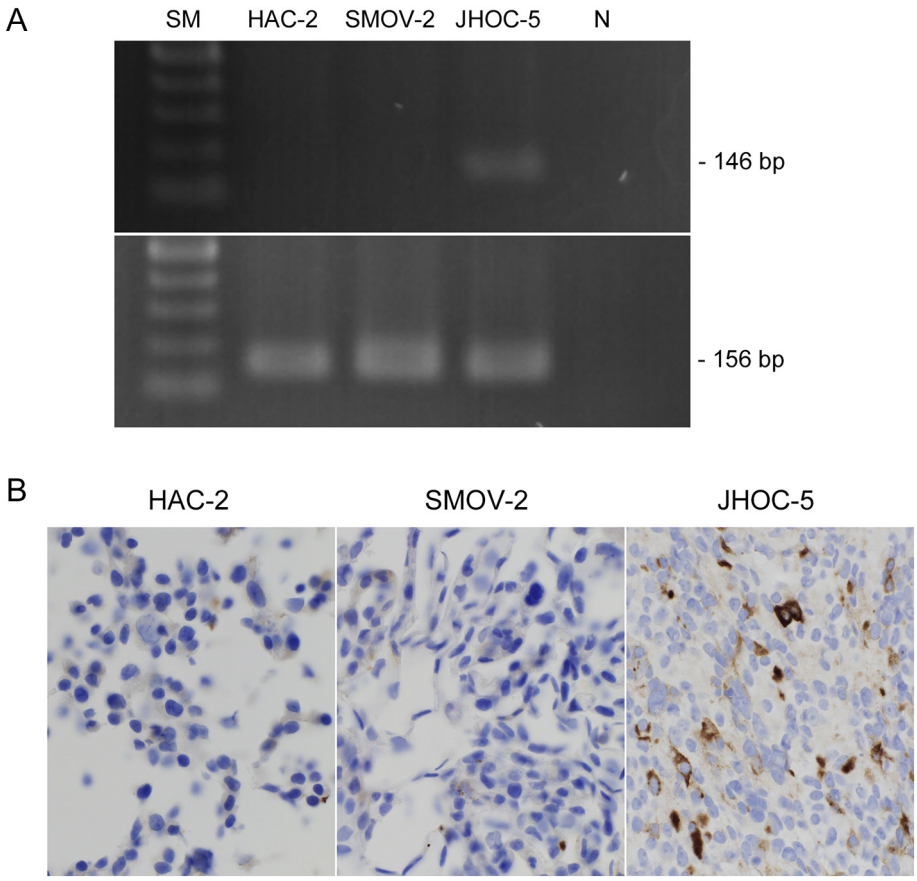

Fig. 4 A, Reverse-transcription PCR for COX-2 in ovarian CCC cell lines. JHOC-5 showed COX-2 mRNA (146 bp), whereas HAC-2 and SMOV-2 did not. β-Actin (156 bp) served as an internal control. SM, size marker. N, negative control (a water blank). B, Immunocytochemistry for COX-2 in CCC cell lines. JHOC-5 expressed COX-2 in the cytoplasm, whereas HAC-2 and SMOV-2 did not. COX-2 with hematoxylin counterstain, original magnification ×40.

2.5. Reverse-transcription PCR for COX-2

After the 3 cell lines were cultured to confluence, total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) and RNeasy Mini Kit (Qiagen). The RNA was then reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). PCR of cDNA was performed with the KOD FX Neo (Toyobo). The primers used were as follows: 5′-ggtggagaagtgggttttca-3′ (forward) and 5′-cgggaagaacttgcattgat-3′ (reverse) [12]. The PCR products were resolved by 2% agarose gel electrophoresis, followed by staining with Atlas ClearSight DNA Stain (Bioatlas, Tartu, Estonia).

3. Results

3.1. Pathological findings

Among the 54 CCCs, 20 (37.0%) and 8 (14.8%) were classified as CCCs with a hyalinized/mucoid stroma and an inflammatory stroma, respectively. Both stromas were mutually exclusive. The predominant growth pattern was papillary in the former and solid in the latter. On the other hand, 26 CCCs with nonspecified stroma often showed a tubular or tubulocystic pattern, and 5 had an adenocarcinofibromatous component, although not more than 20% of tumor areas.

3.2. *PIK3CA* mutation status

In total, 17 (31.5%) of the 54 CCCs showed *PIK3CA* hotspot mutations, either at exon 9 (10 cases) or at exon 20 (7 cases). The breakdown of the mutations was as follows: E542K (2 cases), E545K (4 cases), H1047R (6 cases), and E545A, E545Q, Q546P, Q546R, and G1049R (1 each case; Fig. 2). Among the 20 CCCs with a hyalinized/mucoid stroma, 11 (55%) had a *PIK3CA* mutation. On the other hand, *PIK3CA* mutations were absent in 8 CCCs with an inflammatory stroma, and less frequent in CCCs with nonspecified stroma (6/26 [23.1%]; $P = .026$; Table).

3.3. COX-2 expression

In total, 10 (18.5%) of the 54 CCCs contained COX-2–positive tumor cells, which were distributed either in aggregation (9 cases) or as scattered single cells (1 cases). Seven (87.5%) of the 8 CCCs with an inflammatory stroma were positive for

COX-2 (6 aggregated, 1 scattered). COX-2–positive tumor cells were distributed adjacent to the stroma. On the other hand, COX-2–positive tumor cells were rarely found in CCCs with a hyalinized/mucoid stroma (1/20 [5%]; $P < .001$) or nonspecified stroma (2/26 [7.7%]; $P < .001$; Table, Fig. 3).

3.4. Correlation between *PIK3CA* mutation and COX-2 expression

None of the 20 CCCs with a hyalinized/mucoid stroma or 8 CCCs with an inflammatory stroma showed concurrent *PIK3CA* mutation and COX-2 expression. Among the 26 CCCs with nonspecified stroma, only 2 showed a *PIK3CA* mutation (H1047R and Q546P, 1 each) and COX-2 expression, concurrently. These 2 tumors were devoid of hyalinized/mucoid or inflammatory stromas, or adenocarcinofibromatous areas.

3.5. Cell line study

HAC-2 and SMOV-2 were *PIK3CA* mutants. They had an H1047R mutation and an H1047L mutation, respectively. On the other hand, JHOC-5 was of the *PIK3CA* wild-type.

HAC-2 and SMOV-2 did not express COX-2, neither the mRNA nor protein, whereas JHOC-5 expressed both (Fig. 4).

3.6. Thromboembolism

In total, 9 (18.5%) of the 54 CCC patients showed thromboembolism, including pulmonary thromboembolism (5 cases) and/or arterial thromboembolism (cerebral, splenic, or renal infarctions; 6 cases). Four (44.4%) of the 9 patients died of thromboembolism. Eight (88.9%) of the 9 patients were in FIGO stages II to IV.

Thromboembolism was noted in 7 (35%) of the 20 patients with CCCs with a hyalinized/mucoid stroma, but not in any of the 8 patients with CCCs with an inflammatory stroma (Table). The presence of hyalinized/mucoid stroma was more frequent in CCCs with thromboembolism (7/9 [77.8%]) than in those without thromboembolism (13/45 [28.9%]; $P = .009$).

4. Discussion

In the present study, *PIK3CA* hotspot mutations were identified in 17 (31.5%) of the 54 CCCs, being concordant with previous studies [3,13,14]. Interestingly, the presence of *PIK3CA* mutations was different between CCCs with a different stroma: *PIK3CA* hotspot mutations were more frequent in CCCs with a hyalinized/mucoid stroma than in CCCs with an inflammatory stroma or non-specified stroma. Such a relation between *PIK3CA* mutations and stromal features was repeated in the 3 CCC cell lines. A previous study also noted an association between *PIK3CA* mutations and the presence of hyalinized and mucoid stroma [13]. Taken together, it is strongly suggested that a hyalinized/mucoid stroma is a histologic indicator of *PIK3CA* hotspot mutations in ovarian CCCs. In many cancers, *PIK3CA* mutations increase PI3K activity and activate the downstream AKT signaling pathway and subsequently increase cell growth, survival, and motility [4,15]. In ovarian CCCs, the role of *PIK3CA* mutations remains unclear. The hyalinized/mucoid stroma of CCC has excessive extracellular matrix with few intervening blood vessels [1], which should affect tumor cell growth or survival. According to previous studies, *PIK3CA* mutation is an early genetic event in the development of CCCs [11,13]. It is speculated that an excessive hyalinized/mucoid stroma is sustained by underlying *PIK3CA* mutation. Experimental study is necessary to elucidate the relationship between *PIK3CA* mutation and hyalinized/mucoid stroma in CCCs.

Recently, *PIK3CA* mutation attracted attention regarding some cancers, including colorectal and breast cancers, because *PIK3CA* mutation is a potential predictive biomarker for adjuvant aspirin therapy [6,7,16]. Aspirin is the most common inhibitor of COX-2 [17]. In colorectal cancers, the adjuvant use of aspirin was associated with a better prognosis in *PIK3CA*-mutated cancer patients than in *PIK3CA* wild-type cancer patients [6,16]. In breast cancers, *PIK3CA*-mutant cell lines showed a higher level of COX-2 expression than did *PIK3CA* wild-type, and aspirin suppressed tumor growth of *PIK3CA*-mutant breast cancer in vivo [7]. We previously showed that COX-2 was expressed in some CCCs, especially those with an inflammatory stroma [2]. In the present study, however, *PIK3CA* mutation inversely correlated with COX-2 expression in both surgical specimens and cell lines, and so it is less likely that *PIK3CA* mutation predicts the benefit of aspirin for CCC patients. CCC with an inflammatory stroma now attracts another interest in a potential targeted immunotherapy, particularly in the setting of mismatch repair (MMR) defects. A recent study showed that diffuse intratumoral stromal inflammation correlated with MMR defects in CCCs [18]. In the present study, loss of MMR proteins was observed in 2 CCCs with an inflammatory stroma (data not shown), although molecular genetic testing was not performed. More attention to stromal features might shed light on the novel therapy for CCC, which is resistant to conventional chemotherapy.

In CCC patients, thromboembolism, such as pulmonary thromboembolism or cerebral infarction, is a serious paraneoplastic complication [8,9]. In the current series, 9 (16.7%) of the 54 CCC patients were associated with thromboembolism, and the 4 died of thromboembolism. Little is known about the clinicopathological signs of CCC-related thromboembolism, except for the advanced FIGO stage [8,9]. In the present study, CCCs with a hyalinized/mucoid stroma were more likely to be associated with thromboembolism. Although the number of cases was limited, there is a possibility that hyalinized/mucoid stroma indicates the risk of thromboembolism. Contrary to our expectation, there was no association between the presence of an inflammatory stroma and thromboembolism. As noted above, CCCs with an inflammatory stroma commonly express COX-2. COX-2, as well as its isoform,

COX-1, is an important mediator of platelet aggregation through catalyzing the conversion of arachidonic acid to prostagrandin H_2 [19]. In a previous experimental study, the COX-2 gene was markedly upregulated in a mouse model of paraneoplastic thromboembolism [20]. From the present data, however, COX-2 is less likely to be involved in CCC-related thromboembolism. To date, various factors, including tissue factor, mucin, and interleukin-6, have been suggested to be responsible for cancer-related thromboembolism [21]. In ovarian CCCs, the extracellular matrix making up the hyalinized/mucoid stroma may cooperate with these factors and facilitate hemostasis.

In conclusion, the status of *PIK3CA* mutation and COX-2 expression is different between CCCs with a different stroma: frequent *PIK3CA* mutation and rare COX-2 expression in CCCs with a hyalinized/mucoid stroma, but the reverse in CCCs with an inflammatory stroma. CCCs with a hyalinized/mucoid stroma are more likely to be associated with thromboembolism. A different stromal feature, either hyalinized/mucoid change or inflammation, may represent a different molecular genetic background or hemostatic potential in ovarian CCCs.

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