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TITLE: A Deep Learning Strategy to Integrate Karyometric Features with Underlying Molecular Pathways in Ovarian Cancer Initiation

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CONTRACTING ORGANIZATION: University of California Los Angeles

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6. AUTHOR(S) Sandra Orsulic and Arkadiusz Gertych E-Mail: sorsulic@mednet.ucla.edu & arkadiusz.gertych@cshs.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
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14. ABSTRACT High-grade serous ovarian carcinoma is an aggressive cancer type that primarily affects postmenopausal women and usually originates in the fallopian tube cells. However, the exact process of how these cells transform into cancer isn't fully understood. The goal of the proposed project is to provide comprehensive biological information about the early steps in ovarian carcinogenesis that could be used as targets for ovarian cancer prevention. The biological information is gathered from tissue images, omics-based analyses, and processed by machine learning tools to be linked with outcomes data for in-depth biological and clinical interpretation and functional analyses in experimental models. The major project outcomes at this stage include single-cell karyotyping data from the fallopian tube epithelium and tools for distilling the data from histologic images.					
15. SUBJECT TERMS machine learning, deep learning, artificial intelligence, ovarian cancer initiation, BRCA1, karyometric features, histomorphometric features, computational imaging, fallopian tube					
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1. INTRODUCTION:

The proposed research will lead to a better understanding of the interplay between predisposing genetic mutations and microenvironmental changes that precede cancer initiation in BRCA1 mutation carriers. We expect that our AI-assisted multiplatform data integration will identify discrete image features that can be applied for diagnosing early cancerous lesions in fallopian tubes. In the long term, we hope that our functional analyses will reveal molecular pathways that contribute to cancer initiation and could be used as novel targets for prevention. We envision that some of these genes will be suitable for the development of vaccines, similar to the vaccines currently used to prevent cervical cancer.

2. KEYWORDS:

machine learning, deep learning, artificial intelligence, ovarian cancer initiation, BRCA1, karyometric features, histomorphometric features, computational imaging, fallopian tube

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goal of the proposed project is to provide comprehensive biological information about the early steps in ovarian carcinogenesis that could be used as targets for ovarian cancer prevention.

The percent completions listed below relate to year 1 of the total 4-year project duration.

<i>Aim 1. Identification of karyometric and topologic features associated with a precancer niche</i>	Timeline (months)	Site 1	Site 2
Major Task 1: Extraction of karyometric features			
Subtask 1: Obtain approval for existing IRB modification specific to this project and select fallopian tube specimens with p53 signatures and STICs. Note that normal fallopian tubes and HGSOc have been collected and sequenced.	Upon grant approval	Orsulic 100%	Gertych 100%
Subtask 2: Cut blocks, stain slides with H&E, scan, de-stain, double stain with the secretory and ciliated cell markers PAX8 and FOXJ1, and re-scan slides. Scan H&E slides.	1-2	Orsulic 100%	
Subtask 3: Annotate regions of interest (ROIs).	3-4	Orsulic 50%	
Subtask 4: Segment the nuclei in all ROIs (Mask-RCNN), co-register H&E with PAX8 and FOXJ1 staining, and annotate ciliated and secretory cell nuclei. Conduct Q/C.	5-7		Gertych 50%
Subtask 5: Optimize machine learning tools and image analysis algorithms for automated feature extraction, pattern recognition/classification in cells.	8-11		Gertych 100%
Subtask 6: Extract quantitative karyometric features from each group and exclude features that are invariant or highly correlated. Apply Deep Feature Synthesis algorithm for feature engineering based on extracted features.	12-14		Gertych 50%
Major Task 2: Topology analyses			
Subtask 1: Annotate ROIs on scanned slides of fallopian tubes and STIC lesions as in Aim 1. Segment nuclei. Use	1-6	Orsulic 70%	

StrataQuest software (QuPath software for some applications) to conduct spatial analyses of secretory and ciliated cells.			
Subtask 2: Conduct Q/C and preliminary data analyses. Modify app setting in the StrataQuest software as needed.	7-11	Orsulic 30%	
Major Task 3: Correlation of karyometric and topologic features with clinical parameters, BRCA1 status, and spatial organization of secretory and ciliated cells			
Subtask 1: Scale all of the data to a uniform range using z-scores, Box-Cox, Tanh estimation, or binning.	15-16		Gertych 0%
Subtask 2: Analyze the data and examine the associations between different measured parameters and study groups. Use a Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) plot to visualize subjects represented by multidimensional feature vectors from the deep analysis in two-dimensional space. Use clustering to identify differential expression of some features with the possibility of separating cases using machine learning.	17-22		Gertych 0%
Subtask 3: Correlate karyometric features to the topography of secretory and ciliated cells (i.e. secretory cell runs and proximity of ciliated cells).	23-24	Orsulic 0%	Gertych 0%
Subtask 4: Correlate karyometric and topologic data with BRCA1 status and clinical parameters (normal fallopian tube, p53 signature, STIC, HGSOc) in postmenopausal women. Test for confounding factors (age, BMI, contraception, HRT).	25-26	Orsulic 0%	
Milestone(s) Achieved: Demonstrated the correlation between karyometric features, topology, and clinical data in fallopian tubes (normal, p53 signatures, STICs) and HGSOc.	26		
<i>Aim 2. Identification of molecular pathways associated with karyometric and topologic features</i>			
Major Task 1: Digital Spatial Profiling			
Subtask 1: Conduct NanoString GeoMx Digital Spatial Profiler (DSP) analysis (UCLA Genomics Core).	1-4	Orsulic 100%	
Subtask 2: Analyze DSP data (UCLA Genomics Core).	5-10	Orsulic 70%	
Major Task 2: Molecular and spatial data integration			
Subtask 1: Use clustering to lower the dimensionality of data to reduce overfitting. Use DESeq2 to identify differentially expressed genes associated with karyometric features and clinical/demographic data and use gene- and pathway-level association within a mixed-model framework.	16-26	Orsulic 0%	Gertych 0%
Subtask 2: Review candidate genes in public databases and research literature to prioritize genes as potential targets for ovarian cancer prevention.	27-30	Orsulic 0%	
Milestone(s) Achieved: Identified and prioritized genes and molecular pathways associated with karyometric and topologic features.	30		
<i>Aim 3. Validation and functional characterization of prioritized genes</i>			
Major Task 1: Validation by immunohistochemistry (IHC) and/or in situ hybridization (ISH)			
Subtask 1: Select specimens for validation.	31-32	Orsulic	

		50%	
Subtask 2: Purchase and optimize probes for IHC and ISH. Conduct IHC and ISH. Quantitate and analyze the data.	31-35	Orsulic 0%	
Subtask 3: Identify karyometric features as in Aim 1 and correlate with features identified in Aim 1.	36-38		Gertych 0%
Subtask 4: Identify topologic features as in Aim 1 and correlate with features identified in Aim 1.	36-38	Orsulic 0%	Gertych 0%
Major Task 2: Functional characterization in cell culture			
Subtask 1: Purchase cell culture reagents, shRNAs and plasmids. Inhibit or overexpress the genes of interest in 2D cell lines and in iPSC-derived fallopian tube organoids.	31-37	Orsulic 10%	
Subtask 2: Conduct 2D and 3D proliferation, invasion, and migration assays. Quantitate morphologic differences.	38-44	Orsulic 0%	
Subtask 3: Identify karyometric features as in Aim 1 and correlate with features identified in Aim 1.	45-46		Gertych 0%
Major Task 3: Functional characterization in mice			
Subtask 1: Amend approved IACUC protocol and submit ACURO protocol for the use of human cell lines in mice.	25-30	Orsulic 100%	
Subtask 2: Purchase nude mice and surgically implant iPSC-derived fallopian tube organoids. Monitor tumor growth.	31-40	Orsulic 0%	
Subtask 3: Analyze mouse tumors by IHC and ISH.	41-42	Orsulic 0%	
Subtask 4: Identify karyometric features as in Aim 1 and correlate with features identified in Aim 1.	43-44	Orsulic 0%	Gertych 0%
Subtask 8: - Consider all data and repeat experiments if necessary. - Run final statistical analyses and validation. - Generate a comprehensive integrated spatiotemporal map of morphologic and molecular changes in the fallopian tube epithelia and microenvironment that precede malignant transformation. - Upload all datasets (image features and molecular data) into appropriate public repositories. - Prepare manuscript(s) for publication. - Consider translational implications and initiate collaborations and MTAs with other institutions to collect samples for validation analyses or provide collaborators with selected image features/genes for validation in their laboratories.	45-48	Orsulic 0%	Gertych 0%
Milestone(s) Achieved: Generated a comprehensive integrated spatiotemporal map of morphologic and molecular changes in the fallopian tube epithelia and microenvironment that precede malignant transformation. Validated and functionally analyzed genes that may play a role in the formation of a pre-cancer niche and could be used as potential targets for ovarian cancer prevention.	48		

What was accomplished under these goals?

1) Major activities:

- a) Obtaining local and federal approvals for work with mice and human tissues, and re-obtaining the approvals for work with mice due to the triennial renewal (Orsulic, Site 1, mice and human tissues; Gertych, Site 2, human tissues).
- b) Cutting blocks, staining slides with H&E, scanning, de-staining, double staining with the secretory and ciliated cell markers PAX8 and FOXJ, and re-scanning slides (Orsulic, Site 1).
- c) Development of: a pipeline to delineate and distinguish epithelial cell nuclei from other nuclei for digital karyotyping, a feature panel for nuclear karyotyping, a karyotyping feature space reduction tool, and karyotyping approach testing to predict outcomes in own or external datasets (Gertych, Site 2)
- d) Digital spatial profiling (Orsulic, Site 1)
- e) Preliminary functional analysis of an identified candidate target for ovarian cancer prevention (Orsulic, Site 1)
- f) Writing 3 manuscripts related to this project

2) Specific objectives:

The specific objective of the proposed project is to provide comprehensive biological information about the early steps in ovarian carcinogenesis that could be used as targets for ovarian cancer prevention.

Aim 1: Identification of karyometric and topologic features associated with a precancer niche

Major Task 1: Extraction of karyometric features.

Major Task 2: Topology analyses.

Major Task 3: Correlation of karyometric and topologic features with clinical parameters, BRCA1 status, and spatial organization of secretory and ciliated cells.

Aim 2: Identification of molecular pathways associated with karyometric and topologic features

Major Task 1: Digital Spatial Profiling.

Major Task 2: Molecular and spatial data integration.

Aim 3: Validation and functional characterization of prioritized genes

Major Task 1: Validation by immunohistochemistry (IHC) and/or in situ hybridization (ISH)

Major Task 2: Functional characterization in cell culture.

Major Task 3: Functional characterization in mice.

3) Significant results:

Aim 1: Identification of karyometric and topologic features associated with a precancer niche

a) (Gertych, Site 2) When tested on high-resolution images of histologic preparations from ovarian and other human cancer tissues, the deep learning model (HoVerNet) identified and delineated cell nuclei with an accuracy of 88.7% and 85.8%, respectively. To achieve more robust karyotyping of epithelial cells, we trained this model so that it can exclude lymphocytes and neutrophils. The model was trained and validated using immunohistochemical staining.

b) (Gertych, Site 2) The feature panel for nuclear karyotyping includes 6 size & shape, 7 staining hematoxylin and eosin intensity, 54 staining texture and 2 nuclear proximity features. The feature panel is applied to digitally unmixed images to separately quantify hematoxylin and eosin staining characteristics. In total, 125 features per nucleus are quantified.

c) (Gertych, Site 2) For downstream karyotyping, features that are invariant or mutually associated with other features are removed. This feature reduction step involves a parametric Gaussian copula feature normalization that provides robust rank-based statistics to handle multidimensional, continuous as well as discrete variables

(regardless their distributions). This approach is more general and robust than commonly used variance- and correlation-based feature reduction approaches.

d) (Gertych, Site 2) Prior applying to images of fallopian tube epithelia, the feature panel was tested on images with pancreatic ductal adenocarcinoma with the goal of predict clinical outcomes (i.e., death). A classifier trained with nuclear features extracted from the institutional cohort (71 cases) predicted death in the TCGA cohort (33 cases) with an accuracy of 79% (76% sensitivity and 83% specificity) thereby providing evidence that nuclear features bear prognostic potential. A manuscript reporting these results is currently under review.

e) (Orsulic, Site 1 & Gertych, Site 2) Our computational image analyses identified differences in the tumor microenvironment between the primary tumors and synchronous and/or metachronous metastases in: %s of fibroblasts, epithelial cells, and %s of COL11A1+ fibroblasts. The results were reported in our manuscript (*Gertych et al., 2022, funding acknowledged*)

f) (Orsulic, Site 1 & Gertych, Site 2) Recognizing the absence of any existing publications on computational pathology imaging in ovarian cancer, Drs. Orsulic and Gertych took the initiative to release the pioneering review encapsulating the present landscape of computational pathology in ovarian cancer (*Orsulic et al., 2022, funding acknowledged*).

Aim 2: Identification of molecular pathways associated with karyometric and topologic features

a) (Orsulic, Site 1) In our recent study (Wu et al., 2022), we observed a decline in the occurrence of fallopian tube ciliated cells associated with aging and postmenopausal conditions. We expanded this analysis to benign and early ovarian cancer lesions. Our focus was directed at deciphering the significance of the ciliated cell markers FOXJ1 and CAPS, along with the secretory cell marker PAX8. The findings from this study are described in our recent publication (*Richardson et al., 2022, funding acknowledged*).

b) (Orsulic, Site 1) During our preliminary digital spatial profile analysis utilizing GeoMx technology, we came to the realization that this method is less than optimal for scrutinizing fallopian tube epithelial cells, primarily due to their unique thin and elongated spatial organization, as illustrated in Figure 1. The GeoMx ROI dimensions span from a minimum of 10 microns to a maximum of 785 microns. To achieve efficient library preparation, it is imperative to capture at least 100-200 cells within each ROI. However, the majority of the ROIs within the fallopian tubes exceeded the 785-micron limit. Attempting to divide these ROIs into segments of 785 microns was unfeasible, as it prevented us from attaining the required minimum cell count for each ROI. Fortunately, over the past year, a breakthrough emerged with the introduction of CosMx technology. This innovative tool has offered us a solution aligned with our initial vision. CosMx facilitates comprehensive single-cell spatial analysis across an entire tissue, enabling the subcellular mapping of each RNA molecule to its precise spatial location within the tissue. Notably, the UCLA Genomics Core has recently acquired the CosMx instrument. At present, we are collaboratively engaged in testing this transformative technology. Our strategy involves executing the 1000 NanoString gene panel across six slides, each encompassing multiple areas of interest. An inherent advantage of this technique lies in its capacity not only to assess the phenotype and genotype of epithelial cells but also to encompass the entire microenvironment of the fallopian tube. This will allow us to identify specific

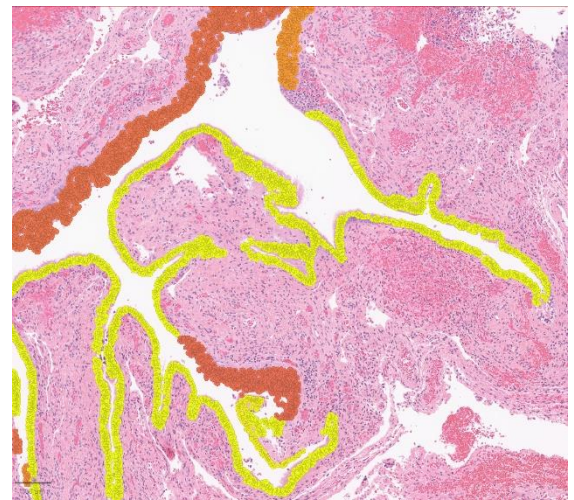


Fig. 1. The complex morphology of STIC lesions (depicted in orange) and the intricate structure of normal fallopian tube epithelium (highlighted in yellow) pose challenges for GeoMx digital spatial analysis.

patterns associated with pre-cancer lesions. For instance, we can define the spatial arrangement of distinct immune cell subtypes, nerves, and blood vessels.

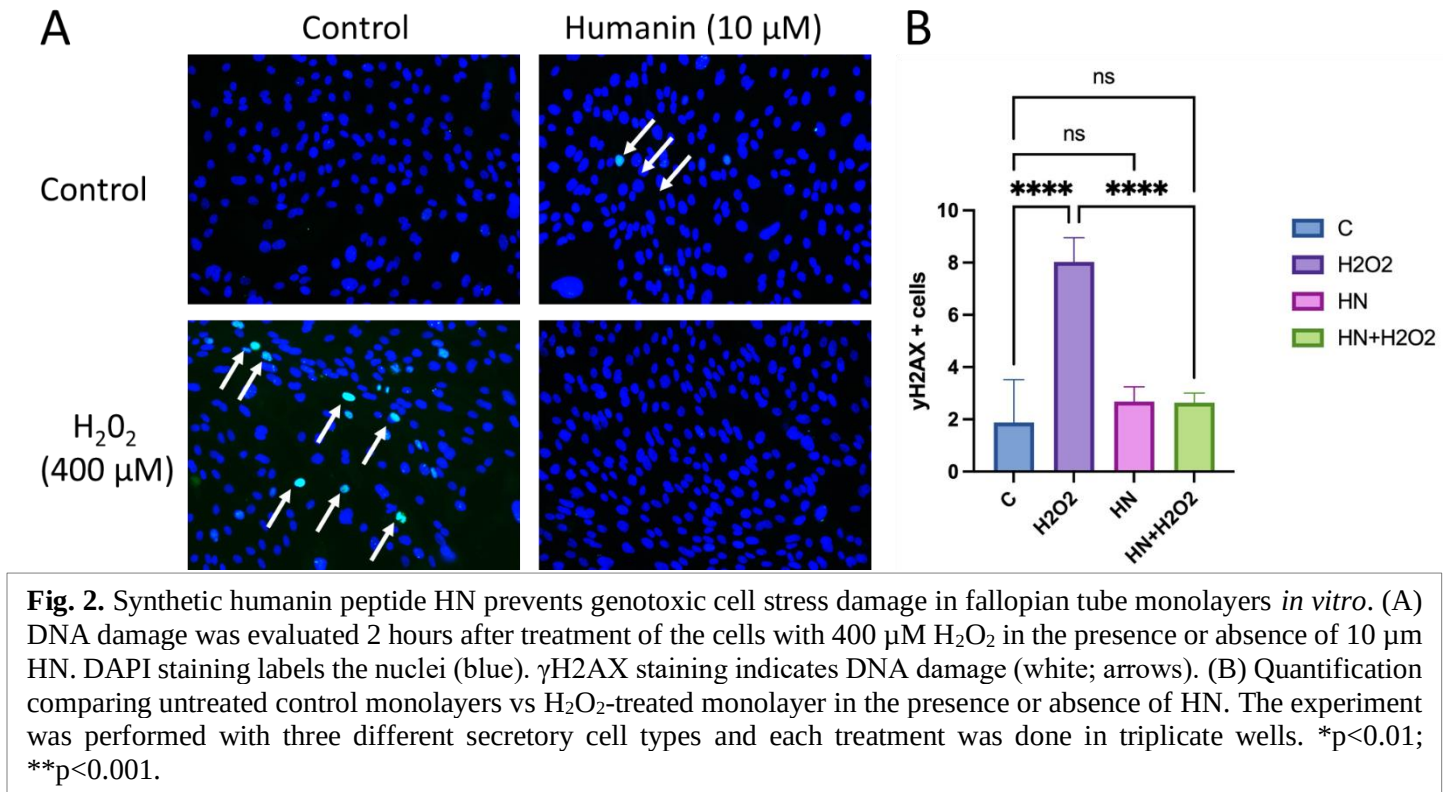
In the interim, we undertook a comprehensive comparison involving the entire segments of fallopian tube fimbria derived from premenopausal (n=5) and postmenopausal (n=7) women. Our rationale was that postmenopausal fallopian tubes could potentially manifest a decline in ciliated cell transcripts while concurrently exhibiting elevated levels of transcripts linked to the pre-cancerous alteration of secretory cells. Using established gene signatures for identification of individual cell types, we identified several immune cell types enriched in postmenopausal fallopian tubes, including macrophages, myeloid-derived suppressor cells (MDSCs), B cells, and plasma cells. Ciliated cell frequency was reduced in postmenopausal fallopian tubes confirming our previous histopathology observations (Wu et al, 2022). Interestingly, a new subpopulation of secretory cells appeared to emerge in postmenopausal fallopian tubes. We hypothesized that these transcriptionally distinct secretory cells might represent senescent cells or cells in the early stages of transformation. This subset of secretory cells enriched specifically in postmenopausal tubes had an elevated expression of the ovarian/fallopian tube epithelial cell marker KRT18. Another gene that was identified as predominantly expressed in secretory cells in postmenopausal fallopian tubes was stanniocalcin-1 (STC1). STC1 has previously been implicated in the promotion of early steps of ovarian cancer metastasis and as a potential diagnostic marker of ovarian cancer. Studies in other cancer types suggest that STC1 is a marker of multipotent mesenchymal stem cells in myeloid leukemia, plays a role in augmenting stem-like traits in glioblastoma, and is involved in tumor dormancy in breast cancer. Consistent with the stem-cell-like phenotype, we observed an increased expression of several ovarian cancer stem cell markers, such as ALDH1A3. We expect to validate these data and uncover additional spatial details using CosMx.

Aim 3: Validation and functional characterization of prioritized genes

a) (Orsulic, Site 1) One promising candidate identified in Aim 2 to be significantly reduced in postmenopausal fallopian tubes was the humanin-like peptide, MTRNR2L1, a nuclear genome-encoded derivative of the mitochondrial genome-encoded MTRNR2 (humanin). The nuclear- and mitochondrial-encoded humanin is thought to have the same functions. Although these data are preliminary, we have initiated the functional analysis of humanin because of its high translational potential for ovarian cancer prevention. Humanin levels decline with age in most organisms, except for the naked mole rat, which exhibits naturally reduced senescence, decreased cancer incidence, and an extended lifespan of up to 30 years. Overexpression of humanin in genetically engineered *C. elegans* and mice increases lifespan and healthspan, respectively. In middle-aged mice, biweekly intraperitoneal injection of the synthetic humanin peptide decreased visceral fat, improved healthspan, and provided protection against toxic insults. Age-associated decline in humanin levels in most organisms, including mice, monkeys, and humans, suggests that depletion of humanin may be associated with the onset of various age-related diseases and that its restoration may alleviate some of the effects of biological aging. Indeed, in various animal models, supplementation of humanin with synthetic peptides seems to protect from age-related diseases, such as macular degeneration, Alzheimer's disease, atherosclerosis, intracerebral hemorrhage, and cardiac ischemia. Mechanistically, it has been shown in various cell types that both nuclear- and mitochondrial-encoded humanin peptides have the ability to safeguard against oxidative stress and reduce aberrant cell death by enhancing mitochondrial function and biogenesis, activating STAT3, and inhibiting caspase-3 activation by interfering with the activation of the pro-apoptotic protein BAX. Given the multipotent effects of humanin in protecting against inflammation, mitochondrial oxidative stress, and oxidative stress-induced cell senescence, humanin and its synthetic analogs could prove to be a valuable approach for ovarian cancer prevention.

We hypothesize that humanin can reduce or even prevent the harmful effects of genotoxic stress and cancer formation in the fallopian tube. To assess the efficacy of humanin restoration in ameliorating genotoxic stress in vitro, fallopian tube cells were grown as monolayers in the presence or absence of the genotoxic stress inducer hydrogen peroxide (H₂O₂) and the presence or absence of 10 µM of synthetic humanin (HN). The fallopian tube

cells treated with HN sustained less DNA damage as evidenced by lower accumulation of the DNA damage marker γ H2AX (Fig. 2).



4) Other achievements:

Nothing to report.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Results were discussed and disseminated through publications and presentations to scientists, medical faculty, students and staff interested in relevant technologies at the home institution.

What do you plan to do during the next reporting period to accomplish the goals?

Planned activities:

- Q/C of digital slides with ovarian tumors stained with H&E and IHC biomarkers
- spatial analyses of secretory and ciliated cells using available software
- aggregation of karyomeric and topologic features, their normalization and statistical analyses
- correlative analysis of karyometric features to the topography of secretory and ciliated cells (i.e., secretory cell runs and proximity of ciliated cells)
- aggregation of features with clinical data and identification of associations using UMAP and statistical and machine learning models
- repeating the digital spatial GeoMx experiment with CosMx technology
- molecular and spatial data integration through a mixed model network to seek biological feature explanations and identify biomarker candidates

- assessing the efficacy of synthetic humanin in preventing genotoxic stress in organoid fallopian tube cultures
- reviewing other candidate genes in public databases and researching literature to prioritize genes as potential targets for ovarian cancer prevention

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

(Active grant participants are highlighted)

1. **Gertych A, Walts AE**, Cheng K, Liu M, John J, Lester J, **Karlan BY**, and **Orsulic S**. Dynamic changes in the extracellular matrix in primary, metastatic, and recurrent ovarian cancers. *Cells* 11(23), 3769, 2022. PMID: 36497028, PMCID: PMC9736731, *Status: Published, Acknowledgement of federal support: Yes*
2. **Orsulic S**, John J, **Walts AE**, **Gertych A**. Computational Pathology in Ovarian Cancer. *Frontiers in Oncology* 12:924945, 2022. PMID: 35965569, PMCID: PMC9372445. *Status: Published, Acknowledgement of federal support: Yes*
3. Richardson M*, **Recouvreux MS***, **Karlan BY**, **Walts AE**, **Orsulic S**. Ciliated cells in ovarian cancer decrease with increasing tumor grade and disease progression. *Cells*, 2022 Dec 11;11(24):4009. PMID: 36552773, PMCID: PMC9776429, *equal contribution. *Status: Published, Acknowledgement of federal support: Yes*

- **Books or other non-periodical, one-time publications**

4. Malpale U, **Orsulic S**. Editorial: Molecular pathology and computational image analyses in gynecologic malignancies. *Frontiers in Oncology* 2022 Nov 21;12:1082220. PMID: 36479087, PMCID: PMC9720375. *Status: Published, Acknowledgement of federal support: Yes*

- **Other publications, conference papers and presentations.**

The collaborative work of the four senior investigators (highlighted below) that provided preliminary data for the current project has now been published:

Wu J, Raz Y, Hu Y, Recouvreux MS, **Diniz MA**, Lester J, **Karlan BY**, **Walts AE**, **Gertych A**, **Orsulic S**. Focal serous tubal intra-epithelial carcinoma (STIC) lesions are associated with global changes in the fallopian tube epithelia and stroma. *Frontiers in Oncology* 2022; Mar 21;12:853755. PMID: 35387127 PMCID: PMC8977528

Invited presentations (*funding acknowledged*):

Orsulic, S. The translational potential for computational pathology for biomarker development in cancer. 2nd Annual Marie Skłodowska-Curie Symposium on Cancer Research and Care. Roswell Park Comprehensive Cancer Center, Buffalo, NY. September 10, 2022. Virtual.

Orsulic, S. Uterine Fibroids: Molecular Differences between African American and Caucasian Women. Mini-symposium on Research in Disparities in OB/GYN Care. Department of OB/GYN. David Geffen School of Medicine, UCLA. Los Angeles, CA. October 21, 2022. In person. *Dr. Orsulic was the organizer of this mini-symposium.*

Orsulic, S. Understanding the Earliest Stages of Cancer Cell Attachment to the Peritoneal Mesothelium. 2023 Director's Lectureship Seminar Series at The University of New Mexico Comprehensive Cancer Center, Albuquerque, NM. April 2, 2023. In person.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Site 1

Name:	Sandra Orsulic
Project Role:	Initiating PI
Researcher Identifier (ORCID ID):	SORSULIC
Nearest person month worked:	1.2
Contribution to Project:	Dr. Orsulic oversaw all projects at UCLA (Site 1) and coordinated progress with the Partnering PI (Dr. Gertych)
Funding Support:	Department of Veterans Administration BX006020 (PI)
	Department of Veterans Administration BX004974 (PI)
	Department of Defense W81XWH2210354 (Co-PI; PI Rowat)
	NIH/NCI R01 CA149385 (Co-I; PI Tanaka)
	NIH/NCI R21 CA245667 (Co-I; PI Rowat)
	Mary Kay Foundation (PI)

Name:	Beth Y. Karlan
Project Role:	Co-I
Researcher Identifier (ORCID ID):	KARLANB
Nearest person month worked:	0.12
Contribution to Project:	Dr. Karlan participated in the selection of clinically-relevant samples and advised the team on the translational aspects of the research and potential clinical applications of the research findings
Funding Support:	Mary Kay Foundation (co-PI; PI Orsulic)
	UCLA Women's Cancer Family History Cohort (Boutros, Karlan, Geschwind)
	Feldman Family Foundation (PI)
	Sherwood Family Foundation (PI)

Name:	Paul Boutros
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Project Role:	Co-I
Researcher Identifier (ORCID ID):	PBOUTROS
Nearest person month worked:	0.6
Contribution to Project:	Dr. Boutros used his expertise and resources to advise the team on an open-platform multidimensional fallopian tube database for the identification of novel clinically-relevant biomarkers and therapeutic targets
Funding Support:	NCI ITCR U24CA248265 (Guinney & Boutros MPI)
	NCI 1R01CA244729-01A1 (Soragni & Boutros MPI)
	The University of Melbourne – Movember Foundation (PI)
	Movember Foundation Global Action Plan 6 (PI)
	UCLA Women’s Cancer Family History Cohort (Boutros, Karlan, Geschwind)

Name:	Shinya Kusumoto
Project Role:	Postdoctoral Fellow
Researcher Identifier (ORCID ID):	-
Nearest person month worked:	3.48
Contribution to Project:	Dr. Kusumoto conducted GeoMx digital spatial analyses and transcriptomic data analyses
Funding Support:	Orsulic Lab

Name:	Maria Sol Recouvreux
Project Role:	Postdoctoral Fellow
Researcher Identifier (ORCID ID):	-
Nearest person month worked:	3.48
Contribution to Project:	Dr. Recouvreux conducted in vitro validation analyses
Funding Support:	Tina’s Wish Foundation (PI), Orsulic Lab

Site 2

Name: Arkadiusz Gertych
 Project Role: Partnering PI
 Researcher Identifier (ORCID ID): 0000-0002-3107-602X
 Nearest person month worked: 3.0

Contribution to Project: Dr. Gertych has designed computational pipelines for: identification and delineation of nuclei, assembled feature libraries for nuclear karyotyping, devised, coded, and tested nuclear feature reduction approaches, contributed to the automation of H&E slide annotations, collected raw data, and contributed to feature analysis and initially validated the karyotyping. He has oversaw the project at Cedars-Sinai and coordinated progress with the initiating PI (Dr. Orsulic).

Funding Support:

Source of Funding: VA Greater Los Angeles Healthcare System
 Title: Precancer Niche Formation in the Fallopian Tube
 Project number: VA-ORD BX004974
 Role: co-I, (S.Orsulic PI)

Source of Funding: The Helmsley Foundation

Title: Integrating Genomic and Imaging data with scRNAseq to better understand the causes of, and heterogeneity in, Crohn's Disease

Role: co-PI, D. McGovern (leading PI)

Source of Funding: NIH/NCI

Title: Fully Quantitative Low-Dose, Motion-Resolved Dynamic Contrast-Enhanced MRI in Pancreatic Adenocarcinoma

Project number: 1 R01 EB032801-01

Role: co-I, (A.Christodoulou, D.Li co-PIs)

Source of Funding: Department of Defense/CDMRP

Title: RIPK2 as a Therapeutic Target in Advanced Prostate Cancers

Project number: GRANT13433214

Role: co-I, (W.Yang, PI)

Name: Natalia Zurek
Project Role: staff, Research Bioinformatician I
Researcher Identifier (ORCID ID): 0009-0008-5080-242X
Nearest person month worked: 4.0

Contribution to Project: Mrs. Zurek trained using in house generated data and implemented HoVerNet model for an automated identification and delineation of nuclei epithelial and exclusion of immune cells, collected raw data from digital slides for karyotyping, and statistically analyzed nuclear features.

Name: Ann E. Walts
Project Role: Consultant
Researcher Identifier (Scopus author ID): 7003766897
Nearest person month worked: 1.0

Contribution to Project: Dr. Walts has provided pathology expertise in all aspects of the project, contributed to digital slide annotations, manuscript writing and IRB protocols preparations.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Sandra Orsulic has closed three grants:

R01 CA208753 (Orsulic)	09/02/16 – 07/31/22
National Cancer Institute, National Institutes of Health	\$228,750
Cancer-Associated Fibroblasts Alter the Composition of B Cells in Solid Malignancies	
Role: PI (20%)	

R21 CA245667 (Rowat)	02/01/21 – 08/31/23
National Cancer Institute, National Institutes of Health	\$35,063
Re-purposing beta-blockers to improve chemotherapy response	
Role: Co-Investigator (3%)	

Cancer Research Project (Orsulic)	07/01/21 – 06/30/23
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The Mary Kay Foundation
Implantable "Tumor Trap" for Metastatic Cancer Cells
Role: PI (3%) \$50,000

Dr. Sandra Orsulic has received two new grants:

VA-ORD BX006020 (Orsulic) 04/01/23 – 03/31/27
Department of Veterans Administration \$300,000
BCCMA: Overcoming chemoresistance in ovarian cancer: Identification and validation of biomarkers and targetable drivers of platinum resistance
Role: PI (32.5%)
There is no overlap with the current grant in effort or funding.

W81XWH2210354 (Rowat) 05/01/22 – 04/30/24
Department of Defense/CDMRP \$55,575
NUDT5 as a Novel Target for Ovarian Cancer
Role: Co-PI (7%)
There is no overlap with the current grant in effort or funding.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ebrap.org/eBRAP/public/index.htm> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil/Pages/Resources.aspx>) should be updated and submitted with attachments.*

9. APPENDICES:

Four Publication Reprints

Article

Dynamic Changes in the Extracellular Matrix in Primary, Metastatic, and Recurrent Ovarian Cancers

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Abstract: Cancer-associated fibroblasts (CAFs) and their extracellular matrix are active participants in cancer progression. While it is known that functionally different subpopulations of CAFs co-exist in ovarian cancer, it is unclear whether certain CAF subsets are enriched during metastatic progression and/or chemotherapy. Using computational image analyses of patient-matched primary high-grade serous ovarian carcinomas, synchronous pre-chemotherapy metastases, and metachronous post-chemotherapy metastases from 42 patients, we documented the dynamic spatiotemporal changes in the extracellular matrix, fibroblasts, epithelial cells, immune cells, and CAF subsets expressing different extracellular matrix components. Among the different CAF subsets, COL11A1⁺ CAFs were associated with linearized collagen fibers and exhibited the greatest enrichment in pre- and post-chemotherapy metastases compared to matched primary tumors. Although pre- and post-chemotherapy metastases were associated with increased CD8⁺ T cell infiltration, the infiltrate was not always evenly distributed between the stroma and cancer cells, leading to an increased frequency of the immune-excluded phenotype where the majority of CD8⁺ T cells are present in the tumor stroma but absent from the tumor parenchyma. Overall, most of the differences in the tumor microenvironment were observed between primary tumors and metastases, while fewer differences were observed between pre- and post-treatment metastases. These data suggest that the tumor microenvironment is largely determined by the primary vs. metastatic location of the tumor while chemotherapy does not have a significant impact on the host microenvironment.

Keywords: computational imaging; COL11A1; ECM; CAF; collagen; extracellular matrix; carcinoma-associated fibroblasts; high-grade serous ovarian cancer; tumor microenvironment



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1. Introduction

Despite substantial progress in the molecular understanding of cancer, several cancers commonly associated with desmoplastic stroma, such as pancreatic, ovarian, colorectal, breast, and lung cancers, remain difficult to treat with conventional therapies. Most therapeutic approaches have focused on epithelial tumor cells and their genetic alterations. However, tumor response to and/or the beneficial effects of personalized therapies targeting specific mutations in these cancers are typically temporary due to genomic instability and the rapid proliferation of resistant clones. Increasingly, normalization of the tumor

microenvironment is thought to explain the efficacy observed with recent immunotherapies and some targeted agents. Identification of additional molecular events and cellular factors whose disruption might undermine tumor progression could lead to more successful anti-cancer therapies.

Cancer-associated fibroblasts (CAFs) are common in solid malignancies and their presence is associated with poor clinical outcomes (reviewed in [1]). CAFs secrete a dense extracellular matrix (ECM) that traps immune cells thus physically restricting their access to tumor islets [2–4]. In mouse tumor models, it has been observed that T cells are enriched in the CAF-secreted ECM while fewer T cells are seen in contact with cancer cells [2–4]. Immunostaining and real-time imaging of T cell distribution in viable slices of human tumors have revealed that fiber alignment and the density of ECM control T-cell migration [5]. The mechanisms by which CAF-secreted ECM might limit T-cell access to tumor islets are not clear but could result from a steric barrier of dense fibers within the stroma and/or increased interstitial fluid pressure within the tumor.

Although CAFs and immune cells are viewed as promising therapeutic targets, significant challenges remain in developing adequate tools to modify the stromal environment in cancer. Studies to assign specific functions to distinct types of stromal cells have been confounded by a poor understanding of stromal cell heterogeneity and a lack of effective targeted approaches. Several therapeutic approaches targeting stromal cells, including endothelial cells, hematopoietic cells, and CAFs, have been used with variable success in ovarian cancer patients. Anti-angiogenic agents, such as bevacizumab, effectively prolong progression-free, but not overall, survival [6]. Immunotherapies with antagonists to CTLA-4 [7], PD-1 [8], and PD-L1 [9] have yielded remarkable results, including complete responses in chemoresistant cancers and long-term remissions in melanoma and lung cancer [10], but only a small subset of patients respond to immunotherapy [10]. A clinical trial of anti-PD-1 therapy in melanoma revealed that innately resistant tumors upregulate the stromal genes involved in epithelial-mesenchymal transition, extracellular matrix remodeling, and wound healing [11], suggesting a possible role for CAFs in the immune escape. However, methods to effectively target CAFs without subjecting normal fibroblasts and stem cells to toxicity are still in the early stages of development.

High-grade serous ovarian carcinoma (HGSOC) is the most common and most lethal subtype of ovarian cancer. Standard treatment for HGSOC combines surgical cytoreduction with platinum-based chemotherapy. Typically, the treatment initially achieves remission; however, cancer recurs in the vast majority of women. Patients with the recurrent disease might continue to respond to additional cycles of platinum-based chemotherapy but most will ultimately develop platinum resistance. An analysis of human ovarian cancer samples demonstrated that the balance between CAFs and CD8⁺ T cells profoundly affects chemotherapeutic sensitivity [12]. While immune cell distribution has been extensively studied in primary tumors (reviewed [13]), less is known about the distribution of immune cell subsets during tumor progression and chemotherapy resistance. To elucidate changes in the tumor microenvironment during ovarian cancer progression, we investigated ECM structure, percentages of fibroblasts, epithelial cancer cells, and immune cells as well as the enrichment of cancer- and stroma-associated CD8⁺ T cells in longitudinally collected HGSOC samples.

2. Materials and Methods

2.1. Tissue Microarray (TMA)

Studies involving human tissue samples were approved by the Cedars-Sinai Institutional Review Board (IRB). We generated a TMA comprising triplicate cores of matched primary HGSOC, synchronous metastasis, and metachronous/recurrent metastasis samples from 42 patients. Primary and synchronous metastatic tumors were acquired during primary debulking surgery (pre-chemotherapy) while metachronous metastatic/recurrent tumors were acquired during second-look surgery (post-chemotherapy). Primary tumors were collected from the ovary ($n = 38$), fallopian tube ($n = 2$), and peritoneum ($n = 2$).

Synchronous metastases and metachronous recurrent metastases were collected from various intraperitoneal sites, including the omentum, gastrointestinal organs, abdominal wall, liver, spleen, and lymph nodes. After a review of diagnostic H&E slides by a pathologist (AEW), the matched primary, synchronous metastatic, and metachronous recurrent HGSOc samples from 42 patients were assembled into a formalin-fixed paraffin-embedded (FFPE) TMA. Each primary, metastatic, and recurrent sample was represented by three cores (1 mm in diameter) that were punched at different locations in the tumor block (Figure 1). The 378 cores were distributed on two slides with tumor samples from 21 patients represented on each slide. The high correlation in the percentage of CD8⁺ T cells in whole slide sections and the corresponding TMA cores confirmed that the TMA cores were representative of the whole tumor section (Figure S1).

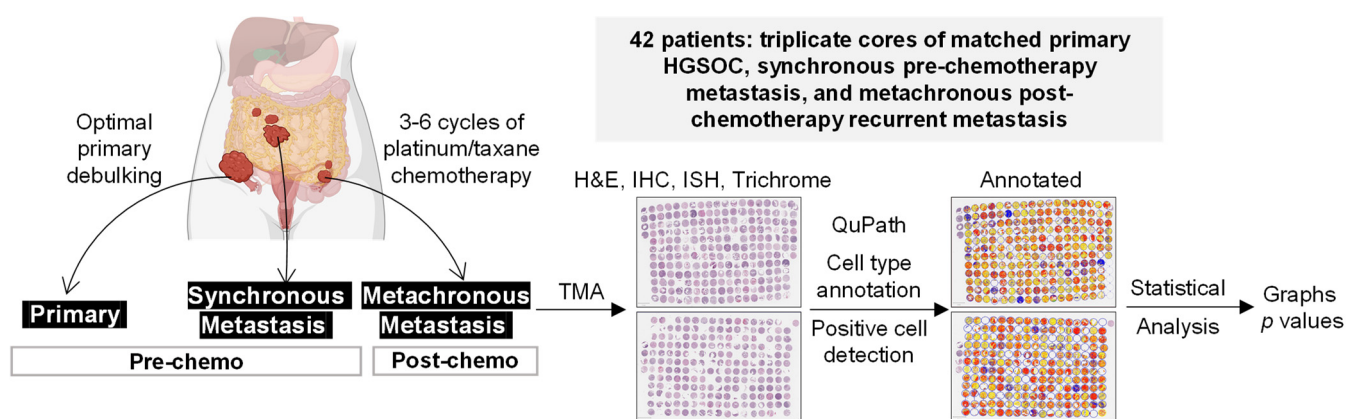


Figure 1. Workflow diagram of TMA generation, cell type annotation/positive cell detection, and statistical analysis. HGSOc, high-grade serous ovarian cancer; TMA, tissue microarray; H&E, hematoxylin, and eosin; IHC, immunohistochemistry; ISH, in situ hybridization.

2.2. Immunohistochemistry (IHC), In Situ Hybridization (ISH), Masson's Trichrome Staining, and Hematoxylin and Eosin (H&E) Staining

IHC with antibodies against alpha-smooth muscle actin (α -SMA) (α -am-1, Leica Biosystems, Wetzlar, Germany), podoplanin (PDPN) (D2-40 Cell Marque, Rocklin, CA, USA), and cluster of differentiation 8 (CD8) (JCB117, Ventana, Dallas, TX, USA) was performed by the Cedars-Sinai Medical Center Biobank and Translational Research Core using 3,3'-Diaminobenzidine (DAB) chromogen and standard protocols for automated immunostaining. The RNA hybridization kit (RNAscope 2.0 FFPE Assay) and probes for *COL11A1*, the bacterial gene *dapB* (negative control), and the housekeeping gene *HPRT* (positive control) were acquired from Advanced Cell Diagnostics, Inc. The ISH was performed according to the protocol provided by the manufacturer. Masson's trichrome staining (hematoxylin, Biebrich scarlet/acid fuchsin, and aniline blue, Dako, Carpinteria, CA, USA) and H&E staining were performed by the Cedars-Sinai Medical Center Biobank and Translational Research Core using the Ventana Discovery XT autostainer (Roche Diagnostics, Basel, Switzerland) and standard pathology protocols.

2.3. Cell Type Annotation by Computational Image Analysis

H&E- and IHC-stained TMA slides were scanned at 40 $\times$ magnification using the Aperio AT Turbo whole slide scanner. The image analysis was performed using QuPath software for TMA analysis (TMA DeArrayer) [14]. H&E images were used to identify and quantify fibroblasts, epithelial cancer cells, and immune cells. IHC and ISH images were used to identify and quantify subsets of fibroblasts positive for *COL11A1*, α -SMA, and PDPN and classify CD8⁺ cells into stroma- and cancer cell-associated CD8⁺ T cells. The workflow consisted of tissue selection (excluding folded, necrotic, and non-cancer tissues), cell/nucleus detection, annotation of regions containing three major cell types (fibroblasts, epithelial cancer cells, and immune cells), creation of the cell detection classifier

using Random Trees (RTrees), application of the classifier to the TMA cores, and statistical analysis of the data (Figure 1). Cores with <200 epithelial cancer cells were excluded from statistical analyses. For multiple cores representing the same tumor, the median value was used for comparative analyses.

2.4. Masson's Trichrome Image Feature Extraction and Analysis

Masson's trichrome-stained TMA slides were scanned at 20× magnification using the Aperio AT Turbo whole slide scanner. Manual sampling was done by extracting a 200-by-200 pixels image tile of stroma adjacent to tumor from each core. Cores in which the stromal area could not be captured within the 200-by-200 pixels image tile without including cancer cells were excluded from the analysis. Image tiles were analyzed for differences in texture patterns using the blue and red channels that were digitally separated from Masson's trichrome-stained image by a color-deconvolution algorithm [15]. Staining texture patterns were characterized by our previously applied feature panel [16,17] comprising: 1 average staining intensity, 45 segmentation-based fractal texture features (SFTA) [18], 30 Gabor mean-squared energy, and 30 Gabor mean amplitude features [19]. We chose these features because they are invariant to image rotation and can extract image intensities, stained area size, fractal dimension, and texture energy. As such, these features are applicable to many image texture quantification tasks, including computational pathology. Features were extracted separately from the blue and red stain images resulting in 212 features per tile. To characterize a tile with SFTA features, the tile undergoes a binary image decomposition into 15 binary masks and each mask is used to calculate fractal dimension, stained area, and mean intensity under the mask in the original image.

2.5. Identification and Validation of Computational Image Features Associated with COL11A1 Positivity

Paired digital slides with TMAs stained with Masson's trichrome and separately with COL11A1 ISH were split into discovery and validation sets. The discovery TMA with COL11A1 staining was manually annotated for regions of interest (ROIs) with COL11A1 positive (COL11A1⁺) and COL11A1 negative (COL11A1[−]) staining, and the ROI boundaries were transferred to the corresponding digital Masson's trichrome-stained TMA slide. Sampling was done by extracting a 200-by-200 pixels image tile from each ROI and labeled based on COL11A1 staining positivity. Image tiles (100 COL11A1⁺ and 99 COL11A1[−]) were analyzed for differences in texture patterns using the features extracted from images as described in Section 2.4. and statistical analysis as described in Section 2.6. Extraction of image tiles ($n = 151$) and features from the validation TMA stained with Masson's trichrome was performed identically to the training set except that the COL11A1 positivity for these tiles was unknown at the time of feature extraction. To determine whether COL11A1⁺ and COL11A1[−] regions can be predicted from Masson's trichrome-stained slides in the validation TMA, the full set of features from the discovery TMA tiles was used to train a Random Forest (RF) classifier. The RF-based predictions of COL11A1 were then compared with the COL11A1 positivity assessed under the microscope (10× objective) in a COL11A1 ISH-stained consecutive slide.

2.6. Statistical Analyses

The statistical analyses were performed with SAS 9.4. In the descriptive statistics, boxplots were used to show the overall results among the three groups (primary, metastatic, and recurrent tumors). Significant variables were then analyzed using the ANOVA test. A p -value threshold of 0.05 was considered statistically significant. For texture pattern comparisons between primary, metastatic, and recurrent tumors, as well as between COL11A1⁺ and COL11A1[−] regions, our null hypothesis for each analysis was that there was no difference between the groups. To test this hypothesis, the 212 features extracted from the tiles were z-scored and statistically analyzed using one-way ANOVA followed by the Tukey–Kramer test for multiple comparisons to identify differential texture features

between the groups. The significance level α in the ANOVA was Bonferroni-corrected ($\alpha = 0.05/212$). We rejected the null hypothesis, if at least one differentially expressed feature was found. Low-dimensional representation of the tiles characterized by differentially expressed features was visualized by the uniform manifold approximation and projection (UMAP) plot. The image analyses and visualizations were carried out in Matlab (ver. R2022a, Matlab, Natick, MA, USA). Populations of CAFs expressing COL11A1, α -SMA (ACTA2), and PDPN were visualized using t-distributed stochastic neighborhood embedding (t-SNE) plots generated by the SCoPe visualization tool for single-cell RNA-seq datasets (<https://scope.aertslab.org/>, accessed on 16 August 2022).

3. Results

3.1. Primary Tumors and Metastases Have Different Percentages of Fibroblasts, Epithelial Cancer Cells, and Immune Cells

To study potential changes in the microenvironment during HGSOc progression, we utilized our TMA from 42 patients comprising triplicate cores of matched primary HGSOc, synchronous metastasis, and metachronous recurrent metastasis samples (hereafter referred to as primary, metastatic, and recurrent, respectively) (Figure 1). Using QuPath, we trained the object classifier to annotate the three major cell types in HGSOc (fibroblasts, epithelial cancer cells, and immune cells, Figure 2A) and quantified their percentages in TMA cores of primary, synchronous metastatic, and metachronous recurrent metastatic tumors. Compared to primary HGSOc, synchronous metastatic and metachronous recurrent metastatic HGSOc had a higher percentage of immune cells and fibroblasts but a lower percentage of epithelial cells although most of the observed differences were not statistically significant (Figure 2B). Synchronous metastatic and metachronous recurrent metastatic tumors had similar percentages of fibroblasts, epithelial cancer cells, and immune cells, suggesting that post-tumor-debulking platinum-based chemotherapy does not have an effect on the percentage of these cell types.

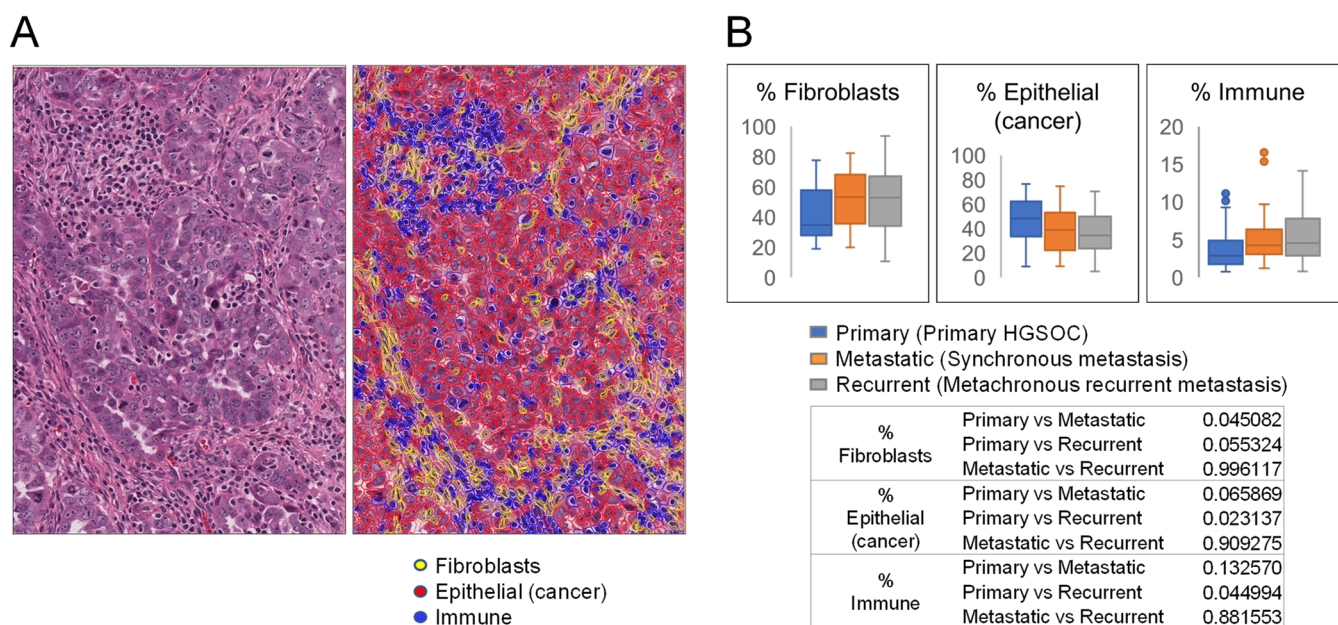


Figure 2. Percentages of fibroblasts, epithelial cancer cells, and immune cells in primary tumors and synchronous and metachronous metastases. (A) Example of H&E-stained HGSOc before and after annotation of fibroblasts, epithelial cancer cells, and immune cells by QuPath analysis. (B) Box and whisker plots show percentages of fibroblasts, epithelial cancer cells, and immune cells in primary HGSOc and synchronous and metachronous recurrent metastases. The Y-axis represents the percentage of a specific cell type among all detected cells. The table shows p values for comparisons between every two groups of samples within the three groups.

3.2. COL11A1⁺, α -SMA⁺, and PDPN⁺ CAF Subsets Show Differential Distribution during HGSOC Progression

Studies in different types of cancer have demonstrated the presence of multiple CAF subtypes that might have different and even opposing roles in cancer progression [1]. In particular, COL11A1, α -SMA, and PDPN might be associated with functionally distinct subpopulations of CAFs. We have previously shown that COL11A1 is a specific marker of activated CAFs that are associated with poor survival and enriched in metastatic HGSOC [20,21]. Although PDPN⁺ CAFs have not been studied in HGSOC, they have been associated with an immunosuppressive microenvironment and/or poor prognosis in multiple cancers, including breast, lung, esophageal, and pancreatic cancers (reviewed in [22]). A t-SNE plot of the ovarian cancer single-cell RNA sequencing profile of the tumor microenvironment [23] shows that COL11A1, ACTA2 (gene encoding for α -SMA), and PDPN are expressed in different, partially overlapping, ovarian CAF subsets, with COL11A1 exhibiting the most restricted expression (Figure 3A).

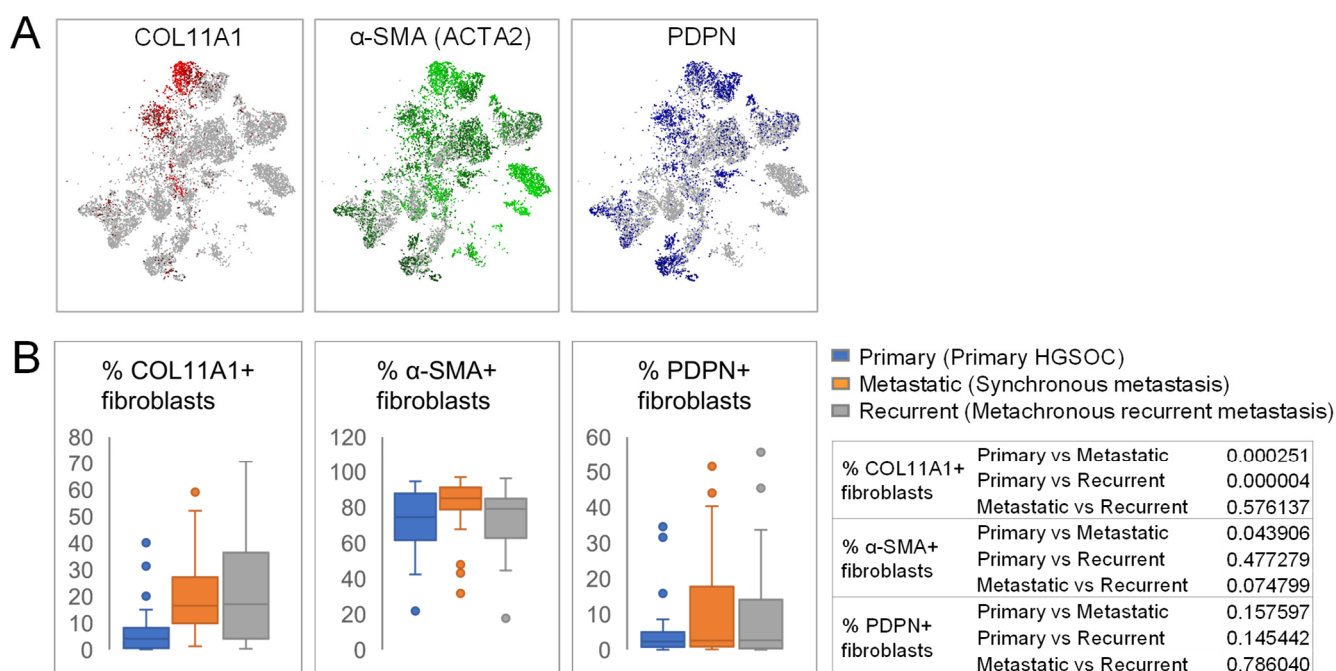


Figure 3. Distribution of three CAF subsets during HGSOC progression. **(A)** t-SNE plots of COL11A1, ACTA2 (encoding for α -SMA), and PDPN mRNA expression in ovarian CAFs. The images were generated using SScope software and public single-cell RNA sequencing data deposited into the ArrayExpress database at EMBL-EBI under accession numbers E-MTAB-8107. **(B)** Box and whisker plots show percentages of COL11A1⁺, α -SMA⁺, and PDPN⁺ CAFs in primary tumors, synchronous metastases, and metachronous recurrent metastases. The Y-axis represents the percentage of the CAF subtype among all morphologically defined CAFs. The table shows *p* values for comparisons between every two groups of samples within the three groups.

We compared the percentages of COL11A1⁺, α -SMA⁺, and PDPN⁺ CAF subsets in primary tumors, synchronous metastases, and metachronous recurrent metastases. QuPath [14] was used to identify COL11A1⁺, α -SMA⁺, and PDPN⁺ CAFs by setting a threshold for the average DAB staining intensity for each cell type. The results were displayed as percentages of CAF subsets (COL11A1⁺, α -SMA⁺, or PDPN⁺) among all CAFs (defined by morphology, Figure 2A) present in a TMA core. The percentage of each CAF subtype was increased in metastases; however, the difference was most significant for COL11A1 (Figure 3B).

3.3. Extracellular Matrix Texture and Pattern Differ in Primary HGSOE and Metastases

To test for possible changes in ECM texture during HGSOE progression, representative stroma image tiles were obtained from each core in Masson's trichrome-stained TMA slides (Figure 4A). The tiles (92 primary, 105 metastatic, 95 recurrent) were used for computational extraction of 212 image features (106 features from the blue channel and 106 features from the red channel). Statistical analysis of the 212 features revealed 8 differentially expressed features between the stroma in primary HGSOE and metastases (synchronous and metachronous combined into one group) (Figure 4A). Among these 8 features, 2 were mean blue intensity and 6 were texture features (1 fractal dimension, 1 stained area, and 4 Gabor) (Figure 4B). Seven of these 8 features were differentially expressed between primary HGSOE and synchronous metastases while 2 of the 8 features were differentially expressed between primary HGSOE and metachronous metastases (Figure 4A). None of the 212 features showed significant differential expression between synchronous metastases and matched metachronous recurrent metastases. These results suggest that collagen fibers in the ECM are different in metastases than in the primary tumors but are similar in pre- and post-treatment metastases. The identification of differentially expressed texture features (Figure 4B) also suggests that the arrangement of collagen fibers differs between primary HGSOE and metastases.

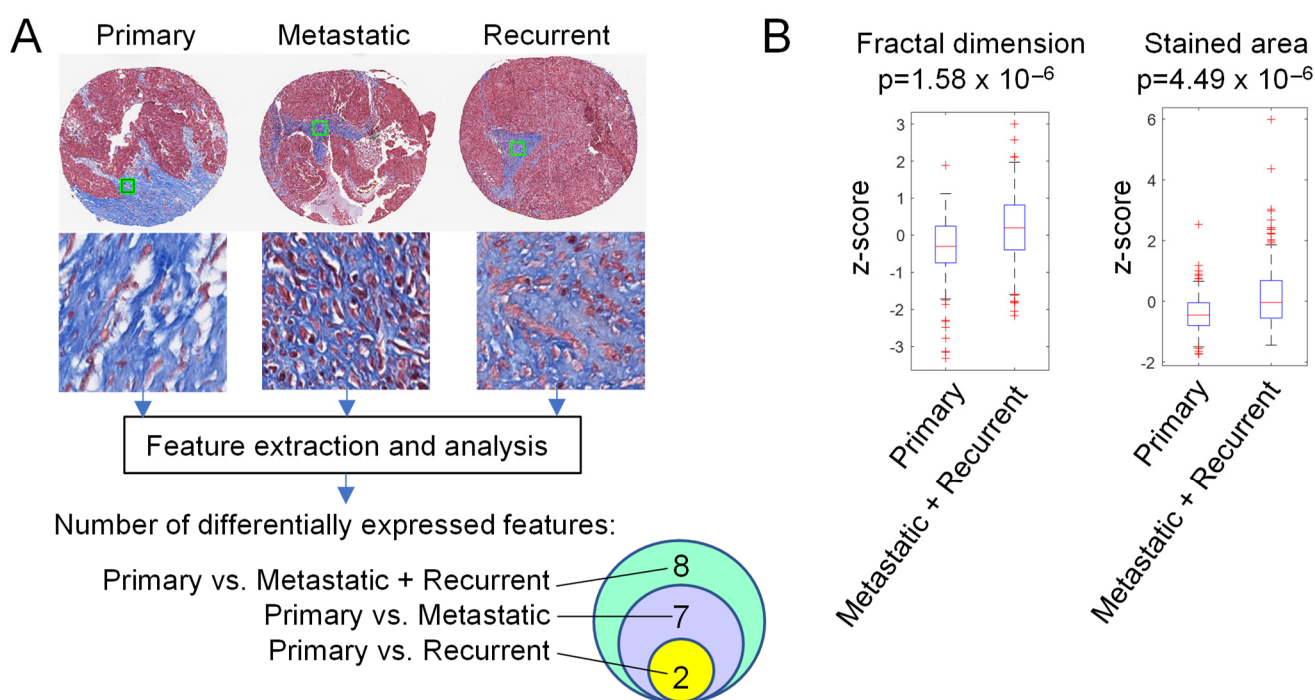


Figure 4. Metastatic and recurrent tumors have a different ECM texture and pattern than primary HGSOE. (A) Workflow diagram of tile selection for feature extraction and analysis. Shown is a randomly selected (not necessarily representative) set of TMA cores from primary, metastatic, and recurrent tumors and the corresponding manually selected stroma tiles (green squares in cores shown at higher magnification below). The number of differentially expressed features identified through statistical analysis is shown in the Venn diagram. (B) Examples of expression boxplots for two image features differentially expressed between Primary vs. Metastasis + Recurrence groups of image tiles.

3.4. ECM Texture and Pattern Are Altered in Tumor Areas Positive for COL11A1

Collagen type XI is primarily expressed in fetal and adult cartilage, where it assembles with collagen type II in a heterotrimeric molecule composed of $\alpha 1(XI)$, $\alpha 2(XI)$ and $\alpha 1(II)$ chains, which are encoded by COL11A1, COL11A2, and COL2A1 genes, respectively [24]. Unlike most collagens, which are processed upon incorporation into the fibril structure, COL11A1 retains its N-terminal extension as a functional component throughout

the lifespan of the fibril [25]. This has important implications for collagen structure and fibril diameter because the bulky N-terminal domains sterically hinder the deposition of additional collagens onto the fibril thereby preventing further increase in diameter and resulting in thin linear fibers, as in cartilage where type XI collagen helps maintain the spacing and diameter of type II collagen fibrils [26–31]. We show that this feature of thin, linear collagen fibers in the presence of COL11A1 is preserved in the tumor microenvironment. A comparison of COL11A1 ISH and Masson's trichrome-stained TMAs revealed that COL11A1⁺ ECM frequently exhibited thin linear fibers, most of which were stained with aniline blue and Biebrich scarlet/acid fuchsin as is characteristic of newly remodeled ECM [32]. The COL11A1[−] ECM exhibited thick curly fibers and stained primarily with aniline blue as is characteristic of mature ECM [32] (compare rectangles labeled [1] and [2] in Figure 5). Thin linear fibers have been associated with increased invasion in breast cancer [33] and increased stiffness of activated myofibroblasts in fibrosis [34].

To test in an unbiased manner if COL11A1⁺ and COL11A1[−] tumor regions have distinct ECM patterns, we conducted computational image feature extraction and statistical analyses. Tiles from Masson's trichrome-stained slides were divided into discovery and validation sets. Statistical analyses of 212 features extracted from the discovery tiles revealed 50 features that were differentially expressed between COL11A1⁺ and COL11A1[−] tiles: 41 blue-channel features and 9 red-channel features ($p < 1.66 \times 10^{-4}$). Of the 41 blue-channel features, one was the mean blue staining intensity in the entire tile and 40 were SFTA features, which included 8 fractal features, 13 mean intensity, 11 area under the mask related features, and 8 Gabor texture features. The UMAP plot showed that COL11A1⁺ and COL11A1[−] tiles represented by all 50 features form separate clusters (Figure 5D). Differential expressions of two of the 50 features are shown as examples in Figure 5E,F. Using the 212 features from the discovery tiles, we trained the RF classifier and applied it to classify features from the validation tiles to test whether COL11A1 status can be predicted in the validation set of Masson's trichrome images, which were selected without prior knowledge of the COL11A1 status. Of note, during training, the RF classifier assigned a high importance score to the majority of the 50 significantly different features. Once the COL11A1 positivity of the Masson's trichrome-stained validation tiles had been predicted by the RF classifier (54 predicted COL11A1⁺ and 97 predicted COL11A1[−]), the prediction (positive and negative) was compared to the pathology score of the entire corresponding TMA core in a consecutive COL11A1 ISH-stained slide. The pathology score of each core was determined manually using a 10× microscope objective. The COL11A1⁺ score was assigned when any COL11A1⁺ cells were observed, otherwise the TMA core was scored as COL11A1[−]. The RF's classification performance was evaluated using a confusion matrix with juxtaposed results of the computationally predicted COL11A1 positivity in the Masson's trichrome validation tiles and the manually determined scores from the TMA cores on the COL11A1 ISH slide (Figure 5G). The RF's accuracy was 70.86%, and the sensitivity and specificity were 70.37% and 71.13%, respectively. Since differentially expressed features were identified between COL11A1⁺ and COL11A1[−] regions, we rejected the null hypothesis (no difference in ECM patterns between COL11A1⁺ and COL11A1[−] regions). This observation was supported by the accurate image classification results in the validation set.

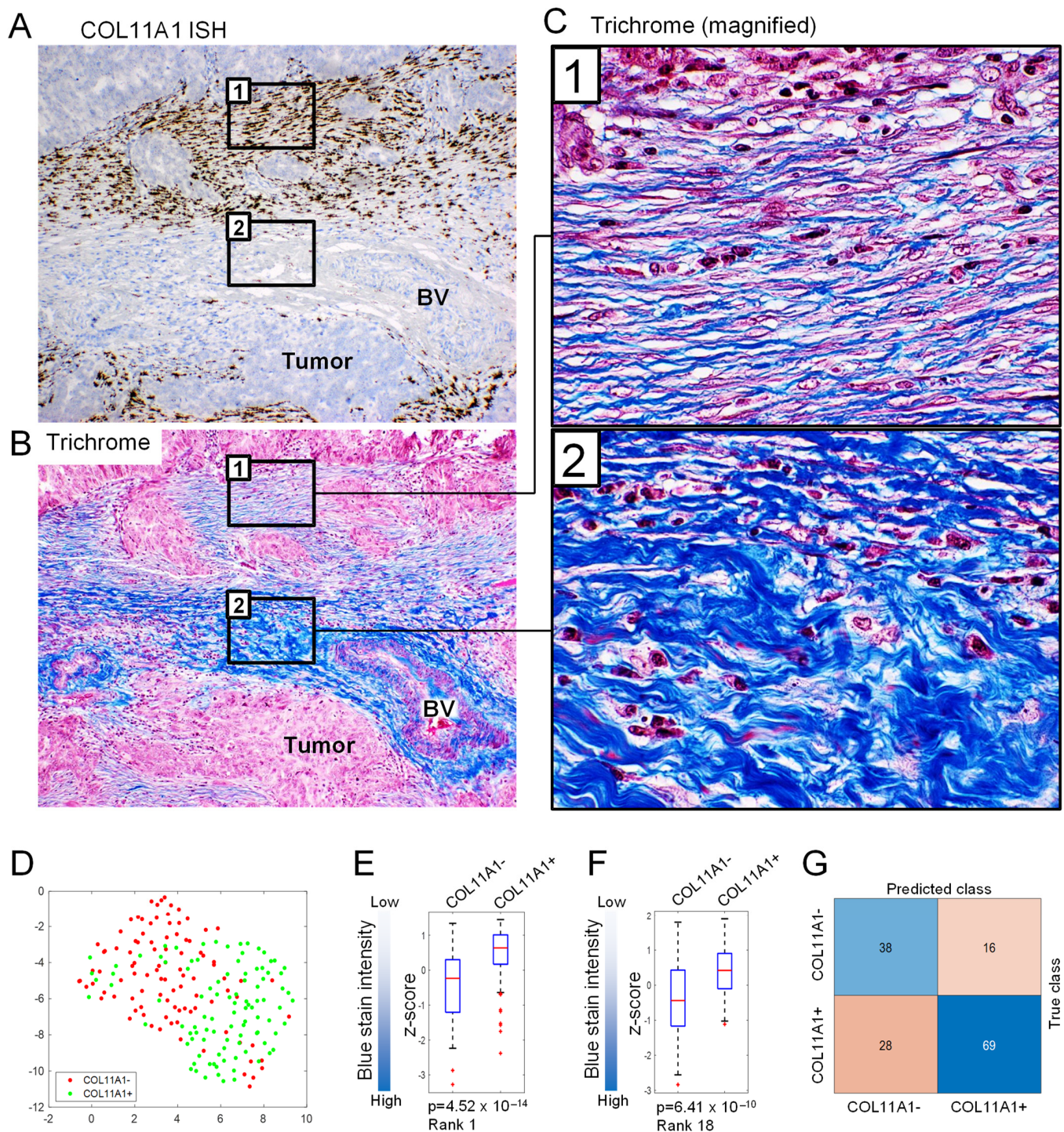


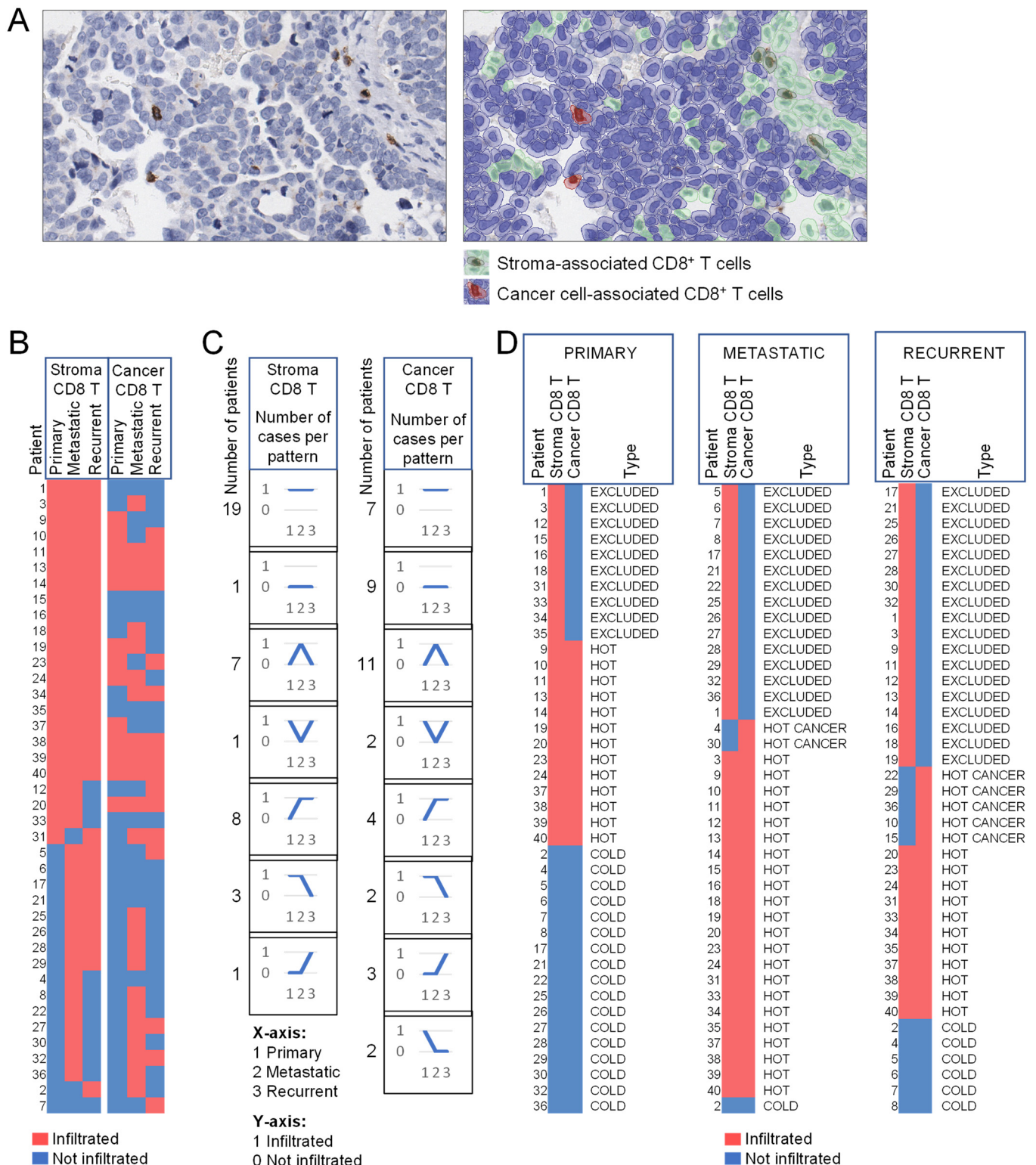
Figure 5. COL11A1⁺ ECM exhibits altered texture and organization. Tumor sections were processed for (A) COL11A1 ISH and (B) Masson's trichrome staining. (C) COL11A1⁺ ECM (rectangles labeled [1] in (A,B)) contains thin linear collagen fibers while COL11A1⁻ ECM (rectangles labeled [2] in (A,B)) contains thick curly collagen fibers. ISH, in situ hybridization; BV, blood vessel. (D) UMAP plot of images characterized by the 50 differentially expressed features identified by statistical analysis of the discovery set of Masson's trichrome tiles with known COL11A1 status. (E,F) Examples of expression boxplots for two mean blue intensity features quantitated in (E) image areas with strong staining (an SFTA feature) and (F) the entire image. (G) Confusion matrix showing predicted COL11A1 positivity in the validation set of Masson's trichrome images. The classifier correctly predicted COL11A1⁺ and COL11A1⁻ scores in 107/151 images (70.86% accuracy).

3.5. HGSOC Metastases Have an Increased CD8⁺ T Cell Infiltration, However, Not All CD8⁺ T Cells Are Reaching the Tumor Parenchyma

T-lymphocyte infiltration has been linked to improved survival in multiple ovarian cancer studies [12,35–45]. However, most of the studies were only done on primary tumors and did not distinguish between stroma- and cancer-cell-associated CD8⁺ T cells. We annotated stroma- and cancer cell-associated CD8⁺ T cells using QuPath (Figure 6A) in a cohort of 40 patients represented by TMA cores from matched primary, metastatic, and recurrent tumors (two of the 42 patients were excluded from the analysis due to an insufficient quantity of fibroblasts or epithelial cancer cells in the cores). The median percentage of stroma- and cancer cell-associated CD8⁺ T cells in the cores from primary, metastatic, and recurrent tumors from each patient are shown in Supplementary Table S1. For simplification, an arbitrary threshold of 2% was used to assign ‘infiltrated’ (>2% CD8⁺ T cells) or ‘not infiltrated’ (<2% CD8⁺ T cells) status. The tumor stroma was infiltrated in the majority of patients, with primary tumors being less frequently infiltrated than metastases and recurrent tumors (Figure 6B). Cancer cell-associated CD8⁺ T cells in the same cores showed a more complex distribution and did not necessarily follow the same pattern of infiltration as the stroma-associated CD8⁺ T cells (Figure 6B). We quantified different patterns of CD8⁺ T cell infiltration in matched primary, metastatic, and recurrent tumors. The ‘infiltrated’ phenotype across primary, metastatic, and recurrent tumors was present in 19 of 40 patients in the stromal region but only in 7 of 40 patients in the cancer cell region (Figure 6C). In contrast, the ‘not infiltrated’ phenotype across primary, metastatic, and recurrent tumors was present in 1 of 40 patients in the stromal region and in 9 of 40 patients in the cancer cell region (Figure 6C).

Based on the distribution of immune infiltrates in the stroma and cancer cells, solid malignancies can be divided into hot (inflamed), cold (desert) and excluded (immune cells excluded from the vicinity of cancer cells) [46,47]. While hot and cold tumors are characterized by the ubiquitous presence and absence of immune cells, respectively, excluded tumors are characterized by immune cells ‘trapped’ in the stroma without access to cancer cells [46,47]. In general, patients with hot tumors have better survival than patients with cold tumors. Patients with excluded tumors also have poor survival despite the presence of immune cells in the stroma [48], suggesting that stroma-sequestered immune cells are deficient in providing immune protection against cancer cells.

Using the same definition of ‘infiltrated’ and ‘not infiltrated’ phenotype as in Figure 6C, we classified the patients into hot, cold, and excluded tumors. While all primary tumors could be assigned into relatively evenly distributed hot, cold, and excluded tumors, the synchronous and metachronous metastases were primarily excluded or hot (Figure 6C). A new category, which we designated as hot cancer (not infiltrated stroma but infiltrated cancer), appeared in synchronous and metachronous metastases (Figure 6C). Together, these data show that metastases are typically better infiltrated with CD8⁺ T cells than primary tumors but many of these infiltrates are excluded from the tumor parenchyma.



and ‘not infiltrated’ phenotype in the stroma and cancer cell area across patient-matched primary, metastatic, and recurrent tumors. The patterns of immune infiltration are represented by diagrams. The number of patients exhibiting a specific pattern is shown on the left of each corresponding diagram. (D) Classification of the same patients into hot, cold, and excluded tumor phenotypes. EXCLUDED = infiltrated in stroma but not cancer, HOT = infiltrated in stroma and cancer, HOT CANCER = infiltrated in cancer but not stroma, COLD = not infiltrated.

4. Discussion

Despite technological advances in the molecular characterization of primary ovarian cancer, the evolution of the tumor microenvironment during metastatic progression and recurrence after chemotherapy remains poorly understood. Several bulk RNA-seq studies have analyzed differences in the tumor microenvironment of longitudinally collected primary, metastatic, and/or recurrent HGSOC samples. Recent RNA-seq expression analysis of primary, metastatic, and recurrent ovarian cancer from 32 patients by Pietila et al. [49] provided detailed gene signatures of the ECM transcriptome (1027 matrisome genes defined by Naba et al. [50]). Similar to our observation that primary HGSOC are different from synchronous pre-treatment metastases and metachronous recurrent metastases in cell type composition (fibroblasts, epithelial cancer cells, and immune cells) and specific ECM proteins, Pietila et al. found the largest differences in transcriptomes between primary ovarian tumors and the matched metastases [49]. Although notable transcriptomic differences were observed between pre- and post-chemotherapy samples, the individual differentially expressed genes varied greatly depending on the anatomic location of sample collection [49] making it difficult to attribute specific changes in the matrisome to chemotherapy alone. Using RNA-seq analysis, Kreuzinger et al. [51] compared patient-matched primary and recurrent fresh-frozen tissue samples from 66 HGSOC patients. In their study, tumor microenvironment was the most significant contributor to the differentially expressed genes, which included multiple ECM genes [51]. Similar to our findings, Kreuzinger et al. found that most of the changes in the tumor microenvironment already existed in pre-treatment metastases [51]. Using the NanoString gene expression profile and IHC analyses of FFPE samples, Westergaard et al. investigated the molecular features of matched primary and recurrent HGSOC from nine patients with specific emphasis on the immune infiltrates [52]. Although the site of tumor collection was not defined in their study, based on the observed high expression of the adipocyte marker CD36 in recurrent samples, it is likely that primary tumors were obtained from the ovary/fallopian tube/adnexa while recurrent tumors were from the omentum or soft tissue in the peritoneal cavity. In the study by Westergaard et al., gene signatures of fibroblasts and immune cells were generally expressed at higher levels in recurrent tumors than in primary tumors [52]. Two additional transcriptomic studies reported increased transcriptomic signatures of the overall population of immune cells [53] and extensive ECM changes [54] in HGSOC metastases when compared to primary tumors. Thus, our computational cell morphology and IHC analyses in primary HGSOC, pre-treatment metastases, and post-treatment recurrent metastases concur with the observed differential expression of gene signatures in published transcriptomic studies.

Transcriptomic studies have shown that primary ovarian carcinomas can be classified into 4 main molecular subtypes: mesenchymal (C1), immunoreactive (C2), proliferative (C4), and differentiated (C5) [55–57]. Abdominal/omental metastases, however, are typically classified as the mesenchymal (C1) subtype irrespective of the subtype of patient-matched primary ovarian tumor [56,58]. The mesenchymal subtype is characterized by desmoplasia and enrichment in myofibroblasts expressing COL11A1, α -SMA, and PDPN. The mesenchymal phenotype of metastases is likely a reflection of the tumor location (ovary/adnexa in the pelvis vs. omentum/peritoneum in the upper abdomen) rather than differences between primary tumors and metastases because the majority of primary peritoneal carcinomas (PPC) are classified as the mesenchymal subtype [59] and there

is evidence that mesenchymal primary tumors may actually be PPC metastases to the ovary [60] or secondary metastases to the ovary [61,62]. Together, these results suggest that the mesenchymal phenotype is determined by the metastatic microenvironment rather than by the intrinsic molecular subtype of epithelial cancer cells.

Notably, bulk RNA-seq analyses lack the spatial information necessary to attribute the differentially expressed genes to specific cell types and ECM organization. Our quantitation of the percentage of CAFs expressing extracellular matrix components COL11A1, α -SMA, and PDPN revealed that COL11A1⁺ fibroblasts were increased in both synchronous and metachronous recurrent metastases compared to matched primary HGSOC. We and others have shown that COL11A1 expression in primary HGSOC is associated with adverse clinical outcomes, including late-stage diagnosis, residual disease after primary cytoreduction, chemotherapy resistance, higher rate of metastasis and recurrence, and poor overall survival [20,21,63–67]. While it remains unclear how COL11A1 might contribute to the poor outcomes in ovarian cancer, we observed that areas enriched for COL11A1 are associated with altered ECM texture and pattern, including fiber linearization. In breast cancer, collagen fibers aligned into linear tracks at the cancer-stroma interface has been associated with an increased incidence of metastasis [33,68–71], suggesting that such changes in collagen organization might facilitate chemotaxis of cancer cells along the linear collagen tracks. Although evidence is currently lacking, it is also possible that COL11A1-rich ECM is less penetrable to chemotherapy and immunotherapy.

Collagen structure in ovarian cancers has been analyzed by two-photon excited fluorescence (TPF) and second harmonic generation (SHG) imaging [72]. A comparison of normal peritoneal biopsies and matched peritoneal metastases revealed that collagen fibers in metastases were thinner than their normal tissue counterparts [73]. Through the analysis of tissue images, we found that COL11A1 expression is associated with distinct ECM architecture in Masson's trichrome-stained slides (thin, linear collagen fibers with reduced aniline blue staining). By applying a trained classifier to random tiles extracted from TMA cores of Masson's trichrome-stained slides, we were able to predict which TMA cores were COL11A1⁺. Although the classifier is not sufficiently accurate to use in a clinical setting, it provides evidence that the expression of a gene associated with poor outcomes in ovarian cancer can be predicted based on ECM architecture.

5. Conclusions

Our computational image analyses of primary HGSOC and patient-matched pre-treatment and post-treatment peritoneal metastases show differences between primary HGSOC and synchronous and/or metachronous metastases in: (1) percentages of fibroblasts, epithelial cells, and immune cells, (2) percentages of COL11A1⁺ fibroblasts, (3) ECM architecture, and (4) percentages of stroma- and cancer-associated CD8⁺ T cells. Pre-treatment synchronous metastases and post-treatment metachronous metastases did not differ significantly in their ECM composition and architecture, suggesting that the observed molecular and structural differences between primary and recurrent HGSOC can be largely attributed to the tumor location rather than the effects of treatment on the host microenvironment that subsequently contributes to tumor recurrence.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells11233769/s1>, Figure S1: Correlation between % of CD8-positive immune cells in whole slides vs. TMA cores; Table S1: Forty patients with high-grade serous ovarian cancer (HGSOC) were analyzed for the median percentage of stroma- and cancer cell-associated CD8-positive cells using QuPath.

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Data Availability Statement: The raw and processed data presented in this study are available upon request from the corresponding author.

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Computational pathology in ovarian cancer

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Histopathologic evaluations of tissue sections are key to diagnosing and managing ovarian cancer. Pathologists empirically assess and integrate visual information, such as cellular density, nuclear atypia, mitotic figures, architectural growth patterns, and higher-order patterns, to determine the tumor type and grade, which guides oncologists in selecting appropriate treatment options. Latent data embedded in pathology slides can be extracted using computational imaging. Computers can analyze digital slide images to simultaneously quantify thousands of features, some of which are visible with a manual microscope, such as nuclear size and shape, while others, such as entropy, eccentricity, and fractal dimensions, are quantitatively beyond the grasp of the human mind. Applications of artificial intelligence and machine learning tools to interpret digital image data provide new opportunities to explore and quantify the spatial organization of tissues, cells, and subcellular structures. In comparison to genomic, epigenomic, transcriptomic, and proteomic patterns, morphologic and spatial patterns are expected to be more informative as quantitative biomarkers of complex and dynamic tumor biology. As computational pathology is not limited to visual data, nuanced subvisual alterations that occur in the seemingly “normal” pre-cancer microenvironment could facilitate research in early cancer detection and prevention. Currently, efforts to maximize the utility of computational pathology are focused on integrating image data with other -omics platforms that lack spatial information, thereby providing a new way to relate the molecular, spatial, and microenvironmental characteristics of cancer. Despite a dire need for improvements in ovarian cancer prevention, early detection, and treatment, the ovarian cancer field has lagged behind other cancers in the application of computational pathology. The intent of this review is to encourage ovarian cancer research teams to apply existing and/or develop additional tools in computational pathology for ovarian cancer and actively contribute to advancing this important field.

KEYWORDS

artificial intelligence, computational pathology, convolutional neural network (CNN), deep learning, artificial neural network, digital pathology, machine learning, ovarian cancer

Computational imaging as a tool to study cancer from a novel perspective

Opinions about the role of computational imaging in the future of pathology range from the belief that computers will entirely replace pathologists to the conviction that computers will never achieve the competency of a well-trained pathologist. Similar to advanced chess-playing software, which is now practically unbeatable by humans, it is likely that computers will be faster and more accurate than pathologists in performing specific tasks, but the main purpose of computational pathology is not to outperform pathologists in routine analyses but to provide them with a completely new and complementary set of tools, some of which are highlighted below:

Large-scale searchable integrative quantitation

Ovarian cancer is the fifth most deadly cancer in women, accounting for more than 200,000 deaths per year worldwide (1). Pathologic diagnosis is primarily confirmed by histopathologic evaluation and interpretation of H&E stained sections of tumor obtained during surgical debulking and/or biopsies obtained during treatment. Years of training with meticulous attention to histopathologic details in numerous examples of normal, altered but benign, and cancerous tissues enable pathologists to extract and empirically integrate an array of visual information from H&E slides into discrete features, such as tumor type, grade, mitotic count, and lymphovascular invasion. Using these features and additional pathological parameters allows the pathologist to categorize each tumor within a universally accepted classification, such as the World Health Organization (WHO) classification of tumors, which ultimately guides clinicians in patient management. Whereas each pathologist is variably limited by a composite of experience, memory, ability to recall visual detail, and case-correlated clinical outcome information locally available, computer algorithms take advantage of a searchable database with quantitative image features linked to clinical outcomes from thousands of patients. By applying computational image analyses, a new patient's tumor can be compared to tumors in the database to find similar tumor phenotypes and help predict the most likely clinical outcome for the new patient.

Subvisual features

The invention of microscopes enabled the visualization of subcellular details, including nuclear enlargement, atypia, nucleolar prominence, hyperchromatism, and mitotic activity,

which are usually important in establishing cancer diagnoses. However, a computer can extract and quantify hundreds, if not thousands, of additional visual and subvisual features (Figure 1), which can be configured into an algorithm to predict specific tumor behavior (i.e. likelihood of sensitivity to immunotherapy) or assess the efficacy of chemotherapy in tumor biopsies obtained at different time points during treatment. Extracting and analyzing information that is beyond human visual perception might also help identify new tissue, cellular, or subcellular structures, features, and/or spatial relationships that are difficult to characterize in H&E slides, such as liquid flow and vacuolarity, and intratumoral areas with increased tension, pH, metabolic activity, and/or genotoxic stress.

Complex spatial information

In most solid malignancies, including ovarian cancer, cancer cells exist within a complex microenvironment that supports tumor growth. In general, the presence of immune cells is associated with better prognosis while the presence of cancer-associated fibroblasts (CAFs) is associated with worse prognosis. In addition to phenotypic diversity, it is becoming apparent that spatial relationships between cancer cells, immune cells, and CAFs play key roles in tumor aggressiveness and response to therapy. However, our knowledge and understanding about the composition of, and the spatial organization within, the tumor microenvironment is still in its infancy. Higher-order spatial patterns, such as cell-cell interactions, tissue interfaces, collagen alignment, and extent of tumor heterogeneity (Figure 2), are important readouts of the complexities of tumor pathophysiology and response to treatment yet remain largely unexplored. While the quantitation of individual cell types and the algorithms that are currently applied to summarize the extent and intensity of tumor staining in a biomarker panel are sufficient for patient stratification into broad groups, i.e. responders and non-responders, comprehensive spatial and morphometric readouts will be necessary to effectively tailor medical treatment to the individual characteristics of each patient (2, 3).

Unbiased discovery

Knowing where and what to look for when examining a specimen or a histologic slide is crucial. Information that is not considered clinically relevant is more likely to be only cursorily examined or implicitly overlooked. For example, precursor lesions in the fallopian tube became obvious to pathologists only at the beginning of the 21st century after decades of searching for these lesions in the ovary, which at the time seemed to be the most logical place for ovarian cancer initiation. Computers can record information extracted from hundreds of scanned slide images in a

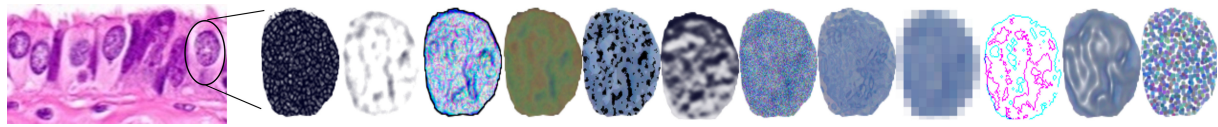


FIGURE 1
Artistic rendering of how a computer may “see” quantifiable nuclear features.

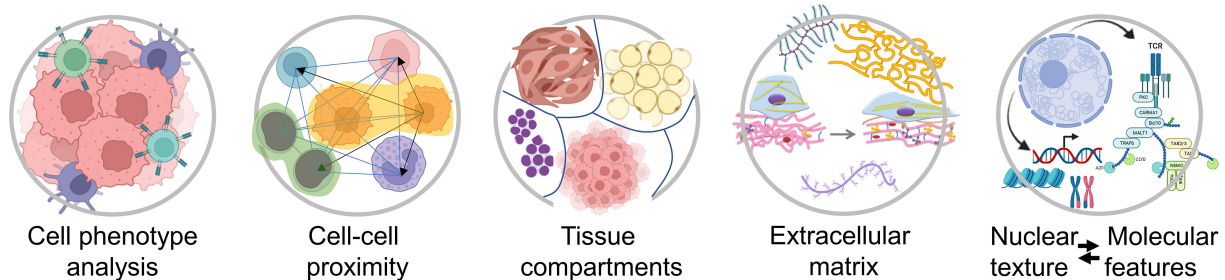
matter of hours. Using unbiased systematic processing of the recorded data, new and/or non-intuitive patterns can emerge (4). For example, although precursor lesions, such as serous tubal intraepithelial carcinoma (STIC) are identifiable in serially sectioned fallopian tubes processed with standard histopathology methods (5), the morphologic events that precede STIC formation are still unknown. Indeed, it is likely that STIC lesions are preceded by subtle morphologic changes that are either not visible to the human eye or are too nuanced to detect without prior knowledge of where and what to look for. A recent analysis of global stromal features in fallopian tubes from postmenopausal women showed that fallopian tubes with STIC lesions exhibit subvisual global changes in the stroma that might precede STIC formation and/or provide a permissible microenvironment for the neoplastic transformation of tubal epithelial cells (6). The ability to detect and understand what constitutes a “permissible” microenvironment for cancerous transformation provides new opportunities for the early detection of cancer and for the identification of rate-limiting events in the early stages of cancer development.

Artificial intelligence, machine learning, and neural networks

Artificial Intelligence (AI) comprises a set of algorithms that mimic human intelligence, enabling machines to perform complex tasks, such as cognitive perception, decision-making,

and communication. Machine learning is a subcategory of AI in which a machine is initially provided with large amounts of training data needed to build models to accurately analyze and interpret new data (7, 8). Machine learning models can automatically learn, improve their performance, and solve problems. Support vector machines, clustering algorithms, k-nearest neighbor classifiers, and logistic regression models (7, 8) are examples of commonly used models. However, because they can learn only a handful of features, they are being replaced by models that use deep learning. Deep learning is a type of machine learning that uses a neural network consisting of three or more functional layers, which include an input layer, multiple hidden layers, and an output layer (9, 10). The hidden layers promote learning and refinement of information “seen” by the input layers to increase the accuracy of predictions of the model’s output. The layers are connected to each other, and the strengths of these connections (termed “weights”) are learned from the training data.

A convolutional neural network (CNN) is a type of a deep learning model used to build advanced decision-making workflows in digital image analysis, ranging from image processing, classification and segmentation of tissue images to the prediction of patient outcomes. CNN often employs more than three convolutional sets of learnable filters (kernels) to extract features from the images. Kernel filters provide low, high and selective filtering (smoothing and sharpening, respectively). The filters distill various basic features such as edges, shapes, color, intensity and location of objects in the image. By flattening



an image, removing or reducing the dimensions, convolutional kernels conduct pre-processing steps that enable selection and organization of the most informative learned features and recognition of these features in previously unseen images. In deeper layers of the CNN, the features are transformed into feature maps that summarize the presence of detected features in the input image (11). Another model that could prove useful in pathology is the fully convolutional network (FCN), characterized by a hierarchy of convolutional layers that are not fully connected. Unlike CNN, which learns from repetitive features that occur throughout the entire image, FCN learns from every pixel thereby allowing detection of objects or features that might be only sporadically present in an entire image (12), for example cancer stem cells which comprise a minute fraction of the tumor. CNN and FCN models are well suited for tumor/object detection, classification, and segmentation tasks (13–16). A recurrent neural network (RNN) model is characterized by dynamic behavior, meaning that it can memorize inputs over different time points or time intervals and learn from them in a sequential manner. RNN could be useful to analyze serial biopsies from a patient who is under surveillance for disease development, progression, or emergence of therapy resistance (17). Machine learning can be applied in a supervised or unsupervised manner. Supervised machine learning uses labeled data sets to train algorithms that classify data or predict outcomes. As input data are fed into the model, the significance of the individual data is adjusted until the model has been appropriately fitted to infer a function that maps input images to a designated outcome label or an object. In contrast, unsupervised machine learning uses unlabeled images to infer a function and detect patterns in the data, clustering them by any distinguishing characteristic(s). Both supervised and unsupervised learning can be useful to reveal new non-intuitive patterns that correlate with specific clinical outcomes.

Computational pathology as a window into the molecular composition of cancer

Oncologists in consultation with pathologists increasingly rely on molecular assays to guide patient-tailored cancer therapy. Such assays can be costly and time-consuming in comparison to routine H&E slides, which can be digitized and analyzed on site. If the application of AI to H&E stained slides could provide molecular information, i.e. predict mutation status or expression levels of actionable cancer drivers in a patient's tumor, several of the currently used technologies, such as immunohistochemistry, *in situ* hybridization, and next generation sequencing, might become superfluous. Recent efforts to integrate digitized H&E image data with molecular data indicate that determining molecular information from digitized H&E slides is not only feasible (18–

21) but might achieve greater accuracy than other techniques that are subject to human error-prone steps, such as specialized specimen preparation and complex laboratory tasks.

Since tissue and cellular morphologies are known to reflect their physiological and pathological conditions, it would be reasonable to expect that higher-order features extracted from histopathology slides are associated with distinct molecular profiles, such as specific gene and protein expression, metabolism, and sensitivity to specific drugs. For example, a higher-order chromatin structure was shown to be the main determinant of genomic instability and mutation frequency in cancer cells (22–24) as well as a strong prognostic indicator in a pan-cancer study (25). Relying only on tumor histomorphology in H&E slides, recent reports indicate that it was possible to predict microsatellite instability in gastrointestinal cancers (26–28), determine molecular expression of ER, PR, and HER2 in breast cancer (29), and detect mutations in prognostically and therapeutically relevant driver genes in different cancer types, including BRAF in melanoma (30), APC, KRAS, PIK3CA, SMAD4, and TP53 in colorectal cancer (31), EGFR, KRAS, TP53, STK11, FAT1, and SETBP1 in lung cancer (32), TP53, CTNNB, FMN2, and ZFX4 in hepatocellular carcinoma (33), FGFR3 in bladder cancer (34), and IDH1 in glioma (35).

Biomarker discovery

With an increasing number of available cancer therapies, it is crucial to develop companion diagnostic tests that identify and stratify subpopulations of patients by their likelihood to benefit from specific therapies. For example, in ovarian cancer, immunotherapy can be associated with serious side effects but only shows efficacy in about 10% of ovarian cancer patients (36). Another example is the need to identify patients who could benefit from PARP inhibitors. In the absence of better biomarkers, detection of a gene mutation (i.e. BRCA1 mutation) or elevated protein expression (i.e. PD-1 and PD-L1) of the intended therapeutic target are frequently used as proxies for treatment response. However, most of the current treatment response biomarkers perform poorly. While it is logical to expect that BRCA mutation carriers would be more sensitive to PARP inhibitors, clinical trials have shown that many non-carriers are responsive to PARP inhibitors although the rationale for this response is still unclear (37). Similarly, immunohistochemical detection of tumor-infiltrating lymphocytes and PD-L1 is a poor proxy for sensitivity to immunotherapy in most cancer types, including ovarian cancer (38). In fact, microsatellite instability, a less specific biomarker, seems to be a better predictor of sensitivity to immunotherapy in various solid cancers (39), possibly because microsatellite instability encompasses multiple proteins and pathways that are involved in the immune response. Using the same reasoning, it is likely that morphometric tumor features that reflect complex pathophysiological states will prove to be

more clinically useful biomarkers even if they are initially non-intuitive or currently non-explainable.

Finding a biomarker in an unexpected place is exemplified in the tumor microenvironment. While cancer research has mostly focused on cancer cells, the unbiased application of deep imaging to digitized H&E slides combined with software that identifies areas of the image that influence algorithm-based classification has frequently revealed stromal features as better biomarkers of clinical outcomes than features of cancer cells (40). In ovarian cancer, a high ratio of stromal CAFs to cancer cells is an independent biomarker of poor survival and chemotherapy resistance (41–44). Indeed, stromal features were identified as the most effective biomarkers in colon cancer (45–47), mesothelioma (48), breast cancer (49, 50), lung adenocarcinoma (51) and early-stage non-small cell lung cancer (52). The discovery of stromal features as the most relevant predictors of clinical outcomes is not surprising as multiple molecular studies of expression profiles and proteomic signatures have also shown that an increased stroma/cancer ratio and increased expression of gene signatures associated with stromal remodeling were the strongest predictors of clinical outcomes (53–62). Studies in several cancer types have also shown that the signature of TGF β -mediated extracellular matrix remodeling is the best predictor of therapy failure (63). The tumor stroma could contribute to poor survival and therapeutic resistance through multiple mechanisms, including promotion of tumor growth, angiogenesis, invasion and metastasis, provision of protective niches for cancer stem cells, creation of an immunosuppressive microenvironment that excludes immune cells from proximity to cancer cells, and/or generation of physical barriers that block access of chemotherapies and immunotherapies to cancer cells (64–68). Thus, it is unlikely that a single molecular pathway will be sufficient as a biomarker of the complex biology that dictates clinical outcomes. A recent computational image analysis of H&E slides of non-small cell lung and gynecologic cancers has shown that the spatial architecture and the interaction of cancer cells and tumor-infiltrating lymphocytes can predict clinical benefit in patients receiving immune checkpoint inhibitors. Importantly, the computational image classifier was associated with clinical outcome independent of clinical factors and PD-L1 expression levels (69). In addition to identifying new biomarkers by highlighting specific structures and regions, computational tissue imaging could assist with the identification of new therapeutic targets. For example, targeting CAFs with various TGF β inhibitors was effective in improving immunotherapeutic efficacy in several preclinical cancer models (66–68).

Future implementation of computational pathology in clinical practice

Potential applications of computational pathology in cancer include diagnosis, phenotyping, subtype classification, early

detection, prognostication, assessment of sensitivity to chemotherapy and immunotherapy, and identification of suitable targeted therapies (20). For example, open-source software, QuPath (70), was capable of classifying serous borderline ovarian tumors and high-grade serous ovarian cancer with >90% accuracy by examining only a small number of tiles extracted from whole H&E slide images (71). While this approach does not reach the accuracy achieved by pathologists who examine multiple sections from different areas of each tumor and have access to surgical records and other clinical information, this example suggests that improvements in the software recognition pattern and computational capacity to analyze pathology slides could achieve clinical-grade tumor classification (72).

Several examples report the utility of computational imaging to automate cancer diagnoses without compromising accuracy. For example, a CNN trained to classify images as malignant melanoma or benign nevi demonstrated superior performance compared to manual scoring by histopathologists (73, 74). Another study that assessed the ability of deep learning algorithms to accurately detect breast cancer metastases in H&E slides of lymph node sections reported that the algorithms were superior in detecting micrometastases and equivalent to the best performing pathologists when under time constraints in detecting macrometastases (75). Similarly, an AI system has reached a clinically acceptable level of cancer detection accuracy in prostate needle biopsies (76). A study of deep CNN in Gleason scoring of prostatectomy specimens reported that the deep learning-based Gleason classification achieved higher sensitivity and specificity than 9 out of 10 pathologists (77). Computational analyses of H&E images have been applied for the prediction of patient survival and recurrence. Deep learning-based algorithms have also been reported to predict prostate cancer recurrence comparably to a genomic companion diagnostic test (78). Other studies utilizing deep learning-based classification involve determining histologic subtype in ovarian cancer (79), predicting platinum resistance in ovarian cancer (41, 80), predicting survival outcome in patients with mesothelioma (48), predicting metastatic recurrence and death in patients with primary melanoma (81) and colon cancer (46), predicting lung cancer recurrence after surgical resection to identify patients who should receive additional adjuvant therapy (52, 82), and predicting response to ipilimumab immunotherapy in patients with malignant melanoma (83). A computational quantitative characterization of the architecture of tumor-infiltrating lymphocytes and their interplay with cancer cells from H&E slides of three different gynecologic cancer types (ovarian, cervical, and endometrial) and across three different treatment approaches (platinum, radiation and immunotherapy) showed that the geospatial profile was prognostic of disease progression and survival irrespective of the treatment modality (84). This computationally-derived profile even outperformed the stage variable, which is the

current standard for gynecologic oncology (84). Upon closer inspection, features related to the increased density of lymphocytes in the epithelium and invasive tumor front were associated with better survival compared with features related to the increased density of lymphocytes in the stromal compartment (84). Although these patterns can be observed by conventional pathology examination, the value of the computational geospatial profile is in the inherently quantitative output rather than the descriptive output from conventional pathology.

Computational pathology has already become an integral component of cancer research and is increasingly being used in anatomic pathology to automate tasks, reduce subjectivity, and improve accuracy and reproducibility (17, 85, 86). The development of high-resolution whole slide image scanners, which are specialized microscopes fitted with high resolution cameras, high magnification objectives, and software for the identification and automated correction of out-of-focus points, constitutes a major advance in computational pathology. Two of these high-quality scanners, the Leica Aperio AT2 DX System and the Philips IntelliSite Pathology Solution, now have FDA approval for clinical review and expert interpretation of pathology slides in routine diagnoses (87). To facilitate the adoption and implementation of this technology into pathology workflows, the Digital Pathology Association has published several practical guides to whole slide imaging (88–91). Digitization of pathology slides enables simultaneous review of slides by multiple pathologists at different institutions and the creation and growth of centralized cloud-based image repositories and databases that are accessible for remote high-throughput studies and/or the development of multiple freely-available machine learning-based tools that can recognize and quantify positive immunostaining or basic structures, such as epithelial-lined ducts, blood vessels, and mitotic figures in H&E slides. Proof-of-concept studies show that automated analyses of simple morphologic patterns can assist pathologists and researchers, allowing them to focus on more complex tasks (2, 92).

Academic pathology departments are increasingly interested in adopting AI in routine practice to optimize operations, reduce costs, and improve patient care. A survey of physician perspectives on the integration of AI into diagnostic pathology revealed that 75% of pathologists across more than fifty countries are interested in using AI as a diagnostic tool in cancer care (93). With the continuous increase in the aged population and the growing number of available tests for personalized medicine, pathologists are overburdened by tasks, such as counting mitotic figures or scoring Ki67 staining, that could be easily performed by machines. Hence, automated computational imaging platforms for the detection and quantitation of stained markers in defined cell subpopulations are the first candidates to be integrated into pathology workflows as these analyses could provide accurate, unbiased, reproducible, and standardized results that can be

viewed and verified (72, 89, 94–96). In contrast, results of artificial neural network analyses that cannot be verified visually are more likely to be met with mistrust by the pathology community because the pathologist needs to be confident in the result before signing out a report (97). This problem might be solved by the further development of tools that introduce transparency for non-linear machine learning methods, such as gradient-weighted class activation mapping (grad-CAM) that overlays images and heatmaps to better visualize the cell type or region in which the informative features were expressed (98).

Although research results utilizing computational imaging in pathology are promising, significant improvements need to be made to achieve the safety and reliability required for endorsement by medical specialty societies, legal approvals, and implementation of this technology into daily clinical pathological diagnostics (99–101). One of the first steps will involve the standardization of slide scanners, display devices, image formats, platforms for image processing and analysis as well as the standardization of the interfaces with clinical information systems and reimbursement mechanisms. Among the technical challenges that must be overcome are normalization methods for histological data preparation at different institutions. This is a pressing issue given that histologic slides are stained either manually or with auto-stainers using site-specific protocols and then digitized on scanners from various manufacturers. Tissue handling and processing can also introduce artefacts, such as air bubbles, fingerprints, excessive glue, blurry/out of focus tissue image, unstained or overstained tissue, and folded or cauterized tissue and digitization (102). While the human eye might not be sensitive to slight differences in tissue fixation, sectioning, staining, and coverslip mounting, all of these factors indiscriminately impact machine learning, and each can have drastic implications on the final results (102). For these reasons, the computational pathology community strongly promotes rigorous algorithm development and testing schemes. First, innovative methods must be used to address pixel data normalization, color standardization, and recognition of the areas on the slide that are out of focus, blurred or contain folded or damaged tissue (103–109). Second, algorithm generalizability on unseen digitized specimens collected at different institutions or deposited in public repositories, such as The Cancer Genome Atlas (TCGA) (110) and The Cancer Imaging Archive (TCIA) (111), will need to be tested and standardized (7, 112, 113).

Many commercial and freely available viewers and image analysis software packages support different whole slide image formats for Windows, Linux, and MacOS. Commercial slide viewers include ImageScope (Aperio/Leica), CaseViewer (3DHistech), and ObjectiveView. The development of web-based viewing interfaces and custom computational pathology pipelines is supported by OpenSlide and SlideIO software libraries. For users with little to no programming skills, the most popular free software packages for general tissue image analysis include ImageJ (114), Fiji (115), Icy (116), Orbit (117), ilastik (118), Cell Profiler (119), QuPath (70), SlicerScope (120),

and PathML (121). These platforms have been embraced in the research arena because they can be easily adapted to different types of image analyses by introducing new codes or incorporating plugins from other programs. They are also flexible in processing different types of image files. Additionally, a plethora of fit-for-purpose packages with custom-made computational pathology pipelines can be found on GitHub (<https://github.com/>). Commercial digital pathology solutions, such as PerkinElmer (USA), Tissue Gnostics (Austria), Halo (United States), Visophram (Denmark) and Definiens (Germany), are often preferred by pathology departments because these platforms offer built-in databases of analytical engines and available technical support. Recognizing the importance of standardization in clinical practice, the freely-accessible Pathology Analytic Imaging Standards (PAIS) was created to support the standardization of image analysis algorithms and image features (122, 123). Resources of web-based interfaces and online tools, such as The Cancer Digital Slide Archive (CDSA, <http://cancer.digitalslidearchive.net/>), for visualization and analysis of pathology image data are also becoming more readily available (124).

Conclusion

Computational image analysis holds great promise in advancing ovarian cancer research, including a better understanding of the events that provide a permissible microenvironment for early cancerous transformation in the fallopian tube, the role of the stroma in therapeutic resistance, and biomarkers for the selection of patients most likely to benefit from different therapeutic approaches. Although computational pathology in the ovarian cancer research field is still too rudimentary to use in a clinical setting, we anticipate that it will become one of the main tools for cancer research and precision medicine in the next decade, surpassing current methods for the selection of patient-tailored therapy, such as immunostaining, mutation sequencing, and expression profiling. Among the key factors that will propel computational pathology to the forefront of medical research are the accessibility of histopathology slides, the

low-cost of image analysis and storage, seamless integration with other computational imaging platforms, and the potential for rapid, accurate, and reliable prediction of patient outcomes.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Ciliated Cells in Ovarian Cancer Decrease with Increasing Tumor Grade and Disease Progression

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Abstract: Ciliated cell markers expressed in epithelial ovarian cancers (EOC) are associated with improved survival. We examined the distribution of cells expressing ciliated cell markers in various EOC histologies and stages. Immunohistochemistry and/or multiplex immunofluorescence were used to determine the expression of FOXJ1 and/or CAPS (ciliated cell markers) in tissue microarrays including 4 normal fallopian tubes, 6 normal endometria, 16 cystadenomas, 25 borderline tumors, 21 low-grade carcinomas, and 118 high-grade carcinomas (HGSOC) (46 serous, 29 endometrioid, 30 clear cell, 13 mucinous). CAPS+ cells were observed in normal fallopian tubes and endometria and in ~85% of serous benign and borderline tumors and low-grade carcinomas but only in <40% of HGSOC. mRNA data from an independent cohort showed higher FOXJ1 and CAPS expression in serous borderline tumors and low-grade carcinomas compared to HGSOC. In HGSOC, ciliated cell-positive markers were observed in 52% primary tumors compared to 26% of patient-matched synchronous metastases, and 24% metachronous metastases ($p = 0.009$). mRNA data from an independent HGSOC cohort showed lower levels of CAPS in metastases than in primary tumors ($p = 0.03$). Overall, the study revealed that ciliated cells were less common in mucinous EOC, the percentage of ciliated cell marker-positive cases decreased with increasing grade, and the percentage of ciliated cells decreased in HGSOC metastases compared to patient-matched primary tumors.

Keywords: fallopian tube; ciliated cells; secretory cells; ovarian cancer; serous; mucinous; endometrioid; clear cell; CAPS; FOXJ1



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1. Introduction

In the United States, nearly 20,000 women are diagnosed with ovarian cancer annually and 12,000 succumb to their disease [1]. Epithelial ovarian cancer (EOC) is a heterogeneous disease that includes serous, endometrioid, mucinous and clear cell histologic subtypes. These histologic subtypes are further divided into high-grade, low-grade, and borderline (previously termed low-malignant potential or LMP) tumors [2]. High-grade serous ovarian cancer (HGSOC) which accounts for more than two-thirds of ovarian cancer deaths [3] was initially thought to originate from the ovarian surface epithelium or peritoneum [4,5]. However, more recent studies indicate tumor origin from the secretory cells at the fimbriated end of the fallopian tube where precursor lesions, including serous tubal intraepithelial carcinoma (STIC) and a TP53 mutation signature, are typically identified [3,6,7].

The fallopian tube epithelium is composed primarily of secretory cells and ciliated cells. Whereas the secretory cells produce the liquid film which overlays the tubal epithelium and are more numerous in all segments of the tube [8–10], ciliated cells help

transport the ovum through the tube and are more numerous in the fimbriated end of the tube, decreasing in number toward the uterus [11]. Ciliated cells are also thought to aid in follicular fluid clearance, thereby possibly reducing the genotoxic stress that follows ovulation [4]. Ciliated cells are also present in the endometrium (both eutopic endometrium and endometriosis) [12] and in some ovarian cortical inclusion cysts (CIC) [13–15].

Relatively little is known about the role of ciliated cells in the development of EOC. Ciliated cell dysfunction has been proposed to enable DNA damage and carcinogenesis [16,17], although the mechanism is unknown. The presence of ciliated cell markers has also been shown to be associated with improved survival in EOC [18].

We sought to better understand the relationships between ciliated cells and the biologic behavior of various benign and malignant epithelial ovarian tumors and histologies. We utilized patient tumor tissue microarrays (TMAs), immunohistochemistry (IHC) for ciliated cell marker-positive (FOXJ1 and/or CAPS) cells, multiplex immunofluorescence (mIF) for ciliated cell marker-positive (CAPS, TUBB4) cells and secretory cell marker (PAX8), and mRNA expression profiles to characterize the distribution of ciliated and secretory cell markers in normal fallopian tubes and endometria, benign and borderline ovarian tumors, and various histologic subtypes and grades of EOC. We also utilized a TMA of patient-matched tumors to compare the presence of ciliated cell markers in primary, synchronous metastatic, and metachronous metastatic/recurrent HGSOC.

2. Materials and Methods

2.1. TMAs

Our study was approved by the UCLA and Cedars-Sinai Medical Center Institutional Review Boards. We utilized commercial and in-house generated TMAs with de-identified formalin-fixed, paraffin-embedded (FFPE) patient tissue samples and associated clinico-pathologic data (age, histologic subtype, malignant potential, grade, and stage). Four TMAs with non-overlapping patient tumors were used: TMA1 (OV991 Ovarian Tumor Series; US Biomax, Inc, Derwood, MD, USA; a single 1.2 mm core/case), TMA2 (CJ3 Ovary Cancer; Super Bio Chips; a single 2 mm core/case), TMA3 (CHTN OvCa2 Ovarian Carcinoma Survey; four 0.6 mm cores/case), and TMA4 (Ovarian Cancer Histologic Subtypes; in-house generated TMA; two 1.5 mm cores/case). Table S1 shows all cases that were analyzed after excluding cases with missing cores or insufficient tissue and cases irrelevant to this study.

We also generated a “HGSOC Progression” TMA comprising triplicate 1 mm cores of HGSOC from 42 patients. In this TMA, each patient was represented by 3 tumors: primary tumor, concurrent (synchronous) metastasis, and recurrent (metachronous) metastasis. The primary tumors and concurrent/synchronous metastases were chemotherapy-naïve while recurrent/metachronous metastases were obtained following treatment (usually 6 cycles of platinum/taxane combination chemotherapy).

2.2. Multiplex Immunofluorescence (mIF)

Opal mIF staining was performed by the UCLA Translational Pathology Core Laboratory based on tyramide signal amplification (TSA). The Opal Polaris 7-Color Automation IHC Kit (Cat#NEL871001KT, Akoya Biosciences, Marlborough, MA, USA) was used with ER2 30 min retrieval and 1 h room temperature incubation. Staining was performed consecutively using each of the following antibodies: Paired Box 8 (PAX8, prediluted rabbit polyclonal antibody 363A-18, Cell Marque, Rocklin, CA, USA), calcyphosine (CAPS, Atlas Antibodies Cat#HPA043520, RRID:AB 10964138, 1:1000 dilution, Sigma-Aldrich, St. Louis, MO, USA), and tubulin beta 4 (TUBB4, Sigma-Aldrich, St. Louis, MO, USA), monoclonal anti-acetylated tubulin T7451, 1:500 dilution), and 4',6-diamidino-2-phenylindole (DAPI) (Cat# SKU FP1490, Akoya Biosciences, Marlborough, MA, USA). The slides were scanned at 20X with the Vectra Polaris scanner (Akoya Biosciences, Marlborough, MA, USA) and data from the multispectral camera were analyzed using the imaging InForm automated image analysis software (Akoya Biosciences, Marlborough, MA, USA). Images were scored at 10X

magnification using Phenochart whole slide viewer (Akoya Biosciences, Marlborough, MA, USA).

2.3. Immunohistochemistry (IHC)

IHC was performed by the UCLA Translational Pathology Core Laboratory using the Leica Bond RX processor based on Protocol F using Bond Polymer Refine Detection kit (Cat#DS9800, Leica Biosystems, Nussloch, Germany), ER2 buffer (BOND Epitope Retrieval Solution 2, Leica Biosystems, Cat#AR9640), DakoCytomation Envision System Labelled Polymer HRP anti rabbit (Agilent, Santa Clara, CA, USA, K4003) and antibodies against FOXJ1 (Sigma-Aldrich, St. Louis, MO, USA, Atlas Antibodies Cat#HPA005714, RRID:AB_1078902, 1:100 dilution) and CAPS (Sigma-Aldrich, Atlas Antibodies Cat#HPA043520, RRID:AB_10964138, 1:1000). Hematoxylin was used as the counterstain. The slides were scanned at 40X with the Aperio ScanScope AT (Aperio, Vista, CA, USA).

2.4. Scoring and Interpretation

The percentage of CAPS+ cells in different stages of ovarian cancer progression represented in TMAs 1–4 was evaluated by mIF. CAPS, TUBB4, and DAPI channels were used for scoring while the PAX8 channel was turned off to facilitate scoring of CAPS+ cells. Missing cores and cores containing <50 epithelial cells were excluded from analysis. For TMA3 (four adjacent cores per case) and TMA4 (two adjacent cores per case), all cores/case were scored as one contiguous sample. Percentage of CAPS+ cells in all epithelial cells per case was estimated by eye in increments of 10 in 10X Phenochart viewer images. Cases containing >5 CAPS+ cells but <1% of CAPS+ cells per case were scored as 1%. Cases without CAPS+ cells or with <5 CAPS+ cells per case were scored as 0%.

Since HGSOV cases typically contained no or only a few ciliated cell marker-positive cells, which could be missed by either FOXJ1 or CAPS staining, three non-consecutive “HGSOV Progression” TMA sections were stained using IHC for FOXJ1, IHC for CAPS, and mIF for CAPS. A case was considered positive when >5 cells in one or more cores stained positive by at least one of the three staining methods. The three methods yielded similar scores. Re-review of the cores with discrepant results revealed that positive cells had been undercounted by one or two of the three methods because the tissue containing positive cells was folded or damaged in individual TMA sections.

2.5. Transcriptome Dataset

The Ovarian Cancer Database of Cancer Science Institute Singapore, CSIOVDB (<http://csiovdb.mc.ntu.edu.tw/CSIOVDB.html>, accessed on 22 May 2022) is a transcriptomics database of 3431 human ovarian neoplasms, including serous, endometrioid, mucinous, and clear cell carcinomas, and serous-LMP (borderline) tumors, and mucinous-LMP (borderline) tumors [19]. Expression levels of FOXJ1 and CAPS (ciliated cell markers) and PAX8 (a secretory cell marker) were plotted using the CSIOVDB online tool, comparing ovarian tumor histology and malignant potential.

2.6. Statistical Analyses

mRNA expression levels, measured in mean and quantiles, were compared using the Mann–Whitney Test. CAPS+ expression between histology was determined with Chi-squared testing. A *p*-value of 0.05 was considered significant.

3. Results

3.1. Ciliated Cells Are Frequently Present in Ovarian Cystadenomas, Borderline Tumors, and Low-Grade Carcinomas

To validate FOXJ1 and CAPS antibodies as markers of ciliated cells, we assessed their localization by IHC (FOXJ1) and mIF (CAPS) in normal fallopian tubes and proliferative endometria. In ciliated cells from both sites, FOXJ1 was expressed in the nucleus

(Figure 1A,C), CAPS was present in all cellular compartments including at the base of cilia, and tubulin highlighted intracellular skeletal structures and cilia (Figure 1B,D).

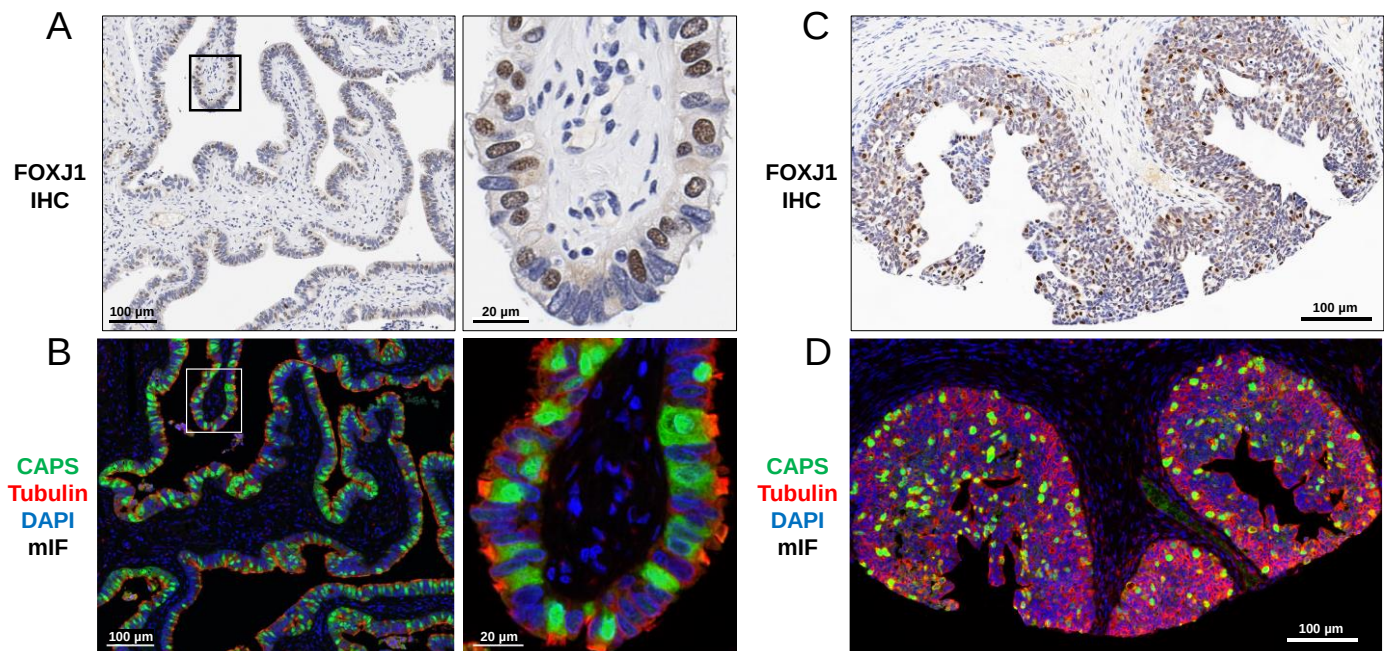


Figure 1. Ciliated cell markers FOXJ1 (IHC) and CAPS (mIF) in (A,B) normal fallopian tube and (C,D) proliferative endometrium. FOXJ1 shows nuclear staining (A,C) while CAPS shows diffuse intracellular staining (B,D). TUBB4 stains the cytoskeleton including base of cilia (B,D). Images (left) are shown at higher magnification (right). (A,C) Hematoxylin counterstain. (B,D) DAPI counterstain.

In ovarian cystadenomas, borderline tumors, and low-grade carcinomas, CAPS+ cells were typically associated with easily recognized cilia (Figure 2A–C) while cilia were rarely recognizable in high-grade carcinomas (Figure 2D–F). In ovarian high-grade carcinomas, even in the absence of distinct cilia, it was clear that CAPS+ cells did not show nuclear staining for PAX8 and thus were immunophenotypically distinct from the PAX8+ carcinoma cells (Figure 3).

While CAPS+ cells were detected in approximately 85% of serous cystadenomas, borderline tumors, and low-grade carcinomas, they were detected in <40% of HGSOCs. The average percentage of CAPS+ cells decreased with increasing tumor grade (40% in benign serous cystadenomas, 31.54% in serous borderline tumors, 18.86% in low-grade serous carcinomas, and 2.7% in HGSOCs (Figure 2A–D and Table 1). Among 39 mucinous tumors (9 benign, 12 borderline, 5 low-grade carcinomas, and 13 high-grade carcinomas), only one benign cystadenoma (11.11%) and one high-grade carcinoma (7.69%) had CAPS+ cells. In both cases, CAPS+ cells comprised approximately 20% of total epithelial cells (Table S1). In endometrioid carcinomas, CAPS+ cells were present in 8 of 9 (88.88%) low-grade and 9 of 29 (31.03%) high-grade carcinomas. The average percentage of CAPS+ cells for all cases was markedly different between low-grade and high-grade endometrioid carcinomas (43.33% vs. 4.31%, Table 1). Regarding clear cell tumors, only high-grade carcinomas were available. CAPS+ cells were observed in 9 of 30 (30%) of cases and the average percentage of CAPS+ cells per case was 3.2% (Table 1). Overall, CAPS+ cells were observed in 45% of the serous, 45% of the endometrioid, and 30% of clear cell compared to only 6% of mucinous carcinomas ($p = 0.01$).

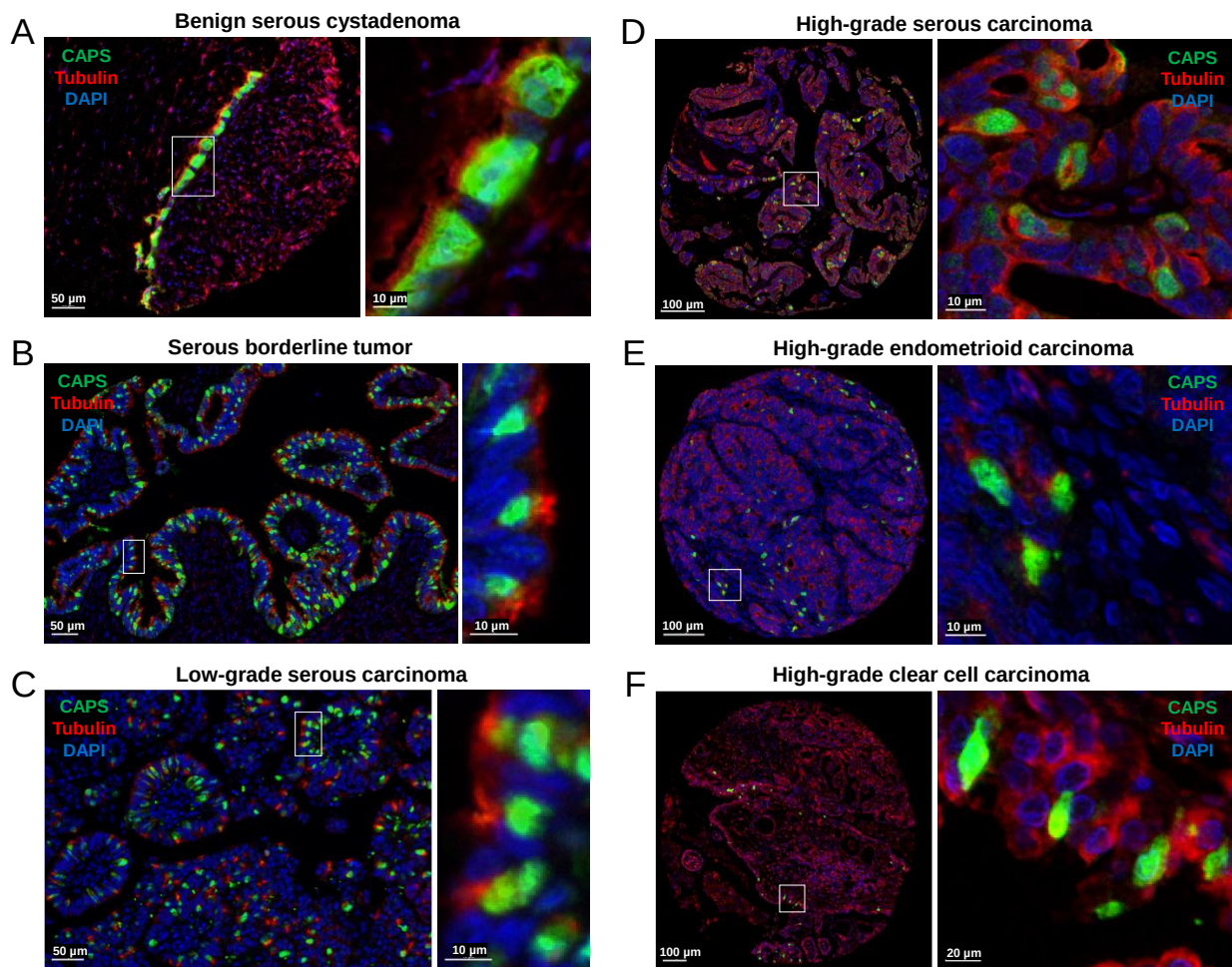


Figure 2. Ciliated cell markers CAPS and TUBB4 (mIF) in (A) benign serous cystadenoma, (B) serous borderline tumor, (C) serous low-grade carcinoma, (D) HGSOc, (E) high-grade endometrioid carcinoma, and (F) high-grade clear cell carcinoma. Images (left) are shown at higher magnification (right).

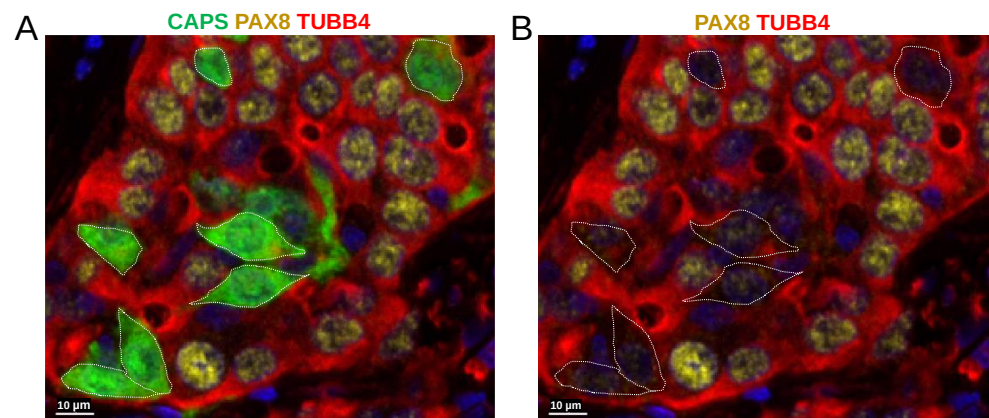


Figure 3. The nuclei of CAPS+ cells in HGSOc do not express PAX8. (A) CAPS, PAX8, and TUBB4 mIF, DAPI counterstain. (B) Same image as in (A) but without the CAPS channel (CAPS+ cells are outlined with a dotted line) for better visualization of the nuclear PAX8 staining.

Table 1. Summary of CAPS immunostaining results in ovarian tumors.

SEROUS	Ratio of	% CAPS+	Average % CAPS+
	CAPS+ Cases	Cases	Cells in All Cases
Benign cystadenoma	6/7	85.71	40.00
Borderline tumor	11/13	84.61	31.54
Low-grade carcinoma	6/7	85.71	18.86
High-grade carcinoma	18/46	39.13	2.70
MUCINOUS	Ratio of	% CAPS+	Average % CAPS+
	CAPS+ Cases	Cases	Cells in All Cases
Benign cystadenoma	1/9	11.11	2.22
Borderline tumor	0/12	0.00	0.00
Low-grade carcinoma	0/5	0.00	0.00
High-grade carcinoma	1/13	7.69	1.53
ENDOMETRIOID	Ratio of	% CAPS+	Average % CAPS+
	CAPS+ Cases	Cases	Cells in All Cases
Low-grade carcinoma	8/9	88.88	43.33
High-grade carcinoma	9/29	31.03	4.31
CLEAR CELL	Ratio of	% CAPS+	Average % CAPS+
	CAPS+ Cases	Cases	Cells in All Cases
High-grade carcinoma	9/30	30.00	3.20

3.2. mRNA Expression of Ciliated Cell Markers Is Higher in Borderline Tumors and Low-Grade Carcinomas than in High-Grade Carcinomas

We determined the mRNA expression levels of ciliated cell markers (FOXJ1 and CAPS) and a secretory cell marker (PAX8) in the CSIOVDB database, which includes 2433 serous, 185 endometrioid, 146 clear cell, and 78 mucinous ovarian carcinomas, 106 serous borderline (LMP) tumors, and 11 mucinous borderline (LMP) tumors. Both FOXJ1 and CAPS expression levels differed markedly between serous borderline tumors and serous carcinomas. The mean FOXJ1 expression in borderline serous tumors was 7.568 compared to 6.867 in serous carcinomas ($p = 2.35 \times 10^{-16}$) (Figure 4A). Similarly, the mean CAPS expression in serous borderline serous tumors was 9.508 compared to 8.207 in serous carcinomas ($p = 3.09 \times 10^{-27}$). In contrast, the mean PAX8 expression levels were similar in serous borderline tumors (9.978) and serous carcinomas (9.989; $p = 7.03 \times 10^{-1}$). Of note, mucinous tumors expressed low levels of both ciliated cell (FOXJ1, CAPS) and secretory cell (PAX8) cell markers, possibly suggesting a different cell of origin for this histologic subtype. FOXJ1 and CAPS expression levels also decreased with increasing tumor grade while PAX8 levels increased with tumor grade (Figure 4B). Thus, the mRNA expression results are highly concordant with the CAPS immunostaining results in TMAs 1–4 (Tables 1 and S1).

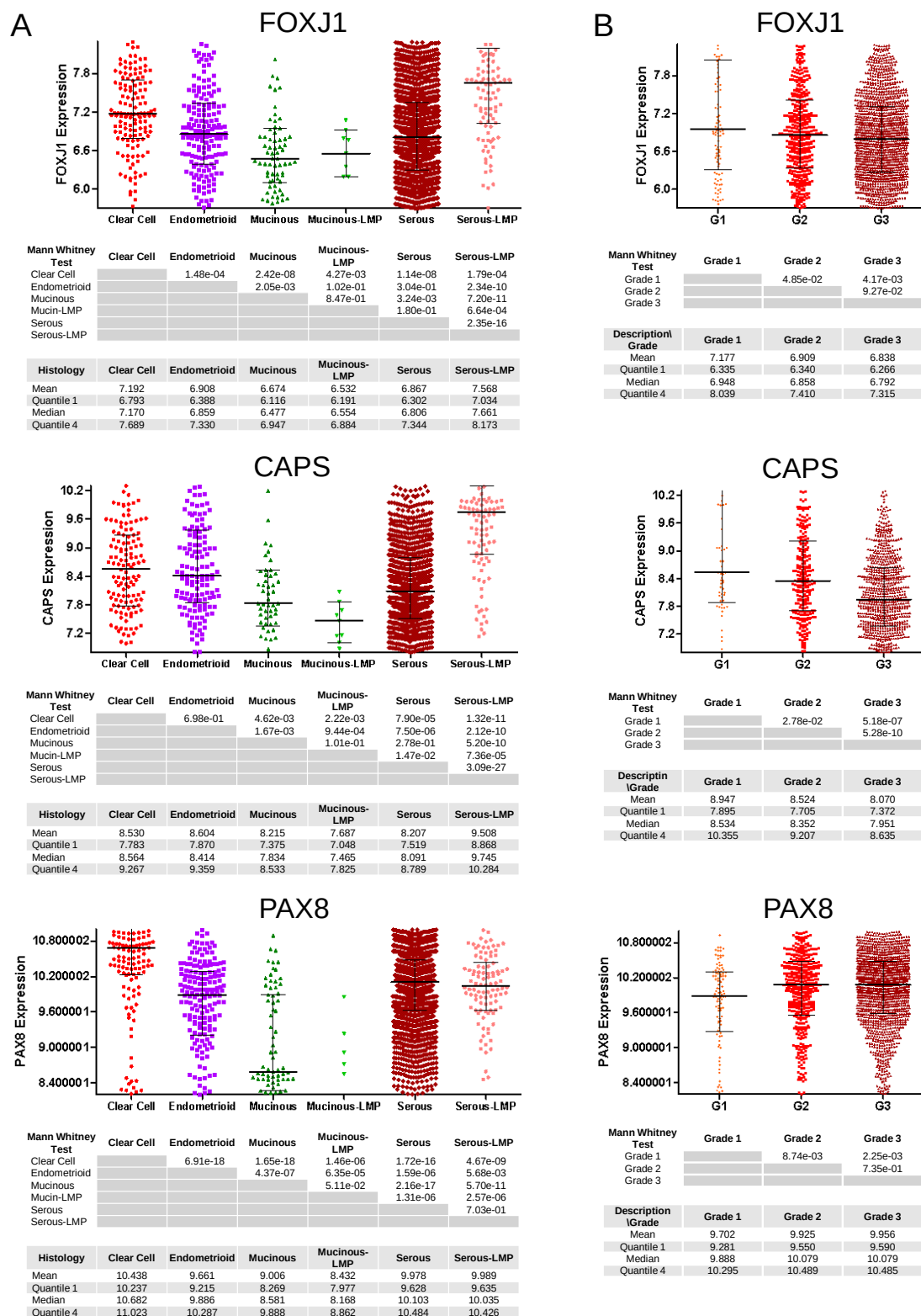
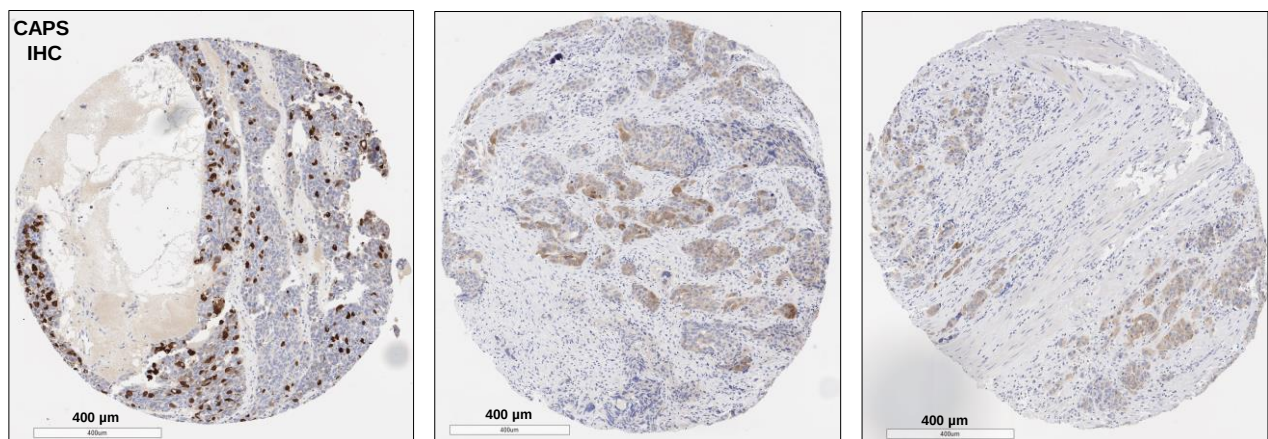


Figure 4. mRNA expression of ciliated cell markers FOXJ1 and CAPS and a secretory cell marker PAX8 in ovarian carcinomas (clear cell, endometrioid, mucinous, and serous) and borderline (LMP) tumors (mucinous, serous). (A) histologic subtypes and (B) cancer grade for all histologic subtypes combined. Y-axes represent relative mRNA levels.

3.3. Cells Expressing Ciliated Cell Markers Are Present in a Higher Percentage of Primary Tumors Than in Patient-Matched Metastases

Using the “HGSOC Progression” TMA, we compared the percentage of 42 patient-matched primary, synchronous metastatic, and metachronous/recurrent metastatic tumors for the presence of FOXJ1+ and/or CAPS+ cells. In this TMA, the primary and synchronous metastatic tumors were treatment naïve while the metachronous/recurrent metastases were obtained post-chemotherapy. FOXJ1+ and/or CAPS+ positive cells were identified in 22 of 42 (52%) primary tumors, 11 of 42 (26%) synchronous metastases, and 10 of 42 (24%) metachronous metastases. These percentages are consistent with 39.13% CAPS+ HGSOC in TMAs 1–4 (Table S1), considering that HGSOC samples in TMAs 1–4 were not necessarily primary tumors. The number of positive cells per core varied in the “HGSOC Progression” TMA, but in most cases where metastases contained FOXJ1+ and/or CAPS+ cells [8 of 11 (73%) synchronous and 8 of 10 (80%) metachronous], fewer positive cells were seen in the metastases than in the patient-matched primary tumor (Figure 5). Only 2 of 24 (8%) cases had FOXJ1+ and/or CAPS+ cells present in synchronous metastases but not in primary tumors. In one of those cases, both synchronous and metachronous metastases contained FOXJ1+ and/or CAPS+ cells.

A Patient 1



B Patient 2

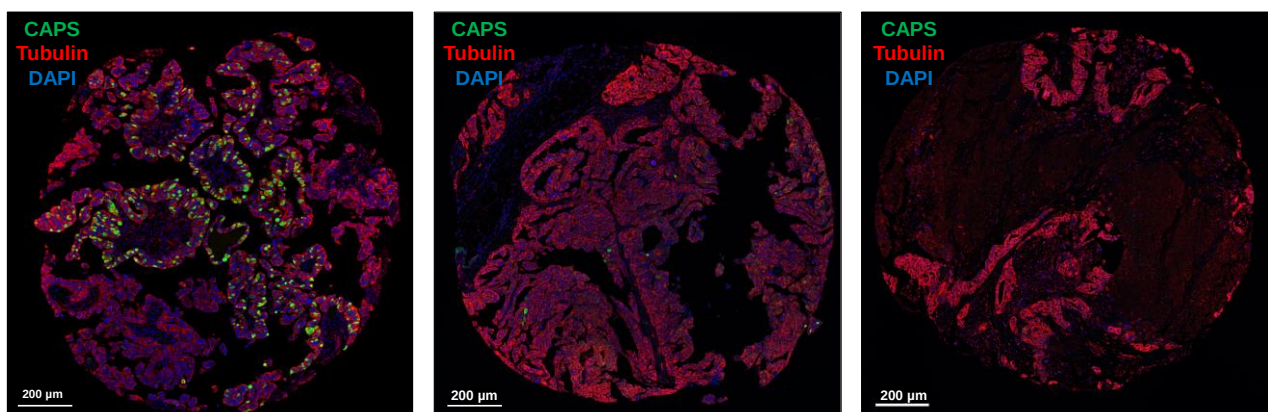


Figure 5. Detection of CAPS+ cells in primary, synchronous metastatic, and metachronous/recurrent metastatic HGSOC. Shown are two examples of HGSOC in which primary tumors exhibited high expression of CAPS by (A) IHC and (B) mIF.

4. Discussion

The role of ciliated cells in ovarian epithelial carcinogenesis is poorly understood. Recent evidence suggests that ciliated cells might help clear the oxidative and genotoxic

stress within the fallopian tube that is related to ovulation and the production of follicular fluid [4]. As terminally differentiated cells with low proliferative potential, ciliated cells themselves might be less prone to the accumulation of DNA damage [17]. The loss of ciliated cells is also associated with menopause and aging, which are known risk factors for EOC [20,21].

The presence of ciliated cells may underlie EOC development. In contrast to the well-described model of high-grade serous ovarian carcinogenesis-with progression of TP53 mutation to STIC lesions [3,6,7], the origin of other EOC histologic subtypes is less understood. Although both clear cell and endometrioid OC might arise from endometrial tissue via endometriosis, they can be of ciliated and secretory lineage, respectively [3,6,7,12,22]. Alternatively, an EOC could originate from a CIC, an invagination of ovarian surface mesothelium or displaced fallopian tube epithelium into the ovarian stroma and subsequent metaplasia of the cyst lining into Mullerian-type epithelium that can be a precursor for various carcinoma histologies [13,23]. The origin of serous borderline/LMP and serous low-grade carcinoma also remains relatively poorly understood. Kurman et al. proposed that mutation burden within CIC could determine low vs. high-grade disease [23] and Laury et al. suggested that serous borderline/LMP tumors might be linked to fallopian tube secretory cell outgrowths (SCOUTs) [24]. This sequence could also originate with ovarian cystadenoma as a principal precursor, and serous borderline/LMP tumor and low-grade carcinoma as eventual pathogenic variants [25]. Confirming the presence of ciliated cell markers in serous, endometrioid, and clear cell EOC supports the hypothesis that these histologic subtypes originate from precursor tissues containing ciliated cells, such as the fallopian tube, endometrium, and/or CIC.

We found that ciliated cells were rarely present in mucinous tumors, irrespective of lesion grade or malignant potential. Most mucinous tumors expressed relatively low mRNA levels of ciliated cell markers FOXJ1 and CAPS and there was a clear dichotomy in PAX8 mRNA expression when mucinous EOC were compared to serous, endometrioid, and clear cell EOC. The origin of mucinous EOC has been difficult to determine and is often confounded by possible confusion with metastases from other organs [26–28]. There may be a difference between subtypes of mucinous cancer such as endocervical or gastrointestinal which contribute to these findings, although these data were not available for our cases [29,30]. Our study adds to the scant literature on the relationship between ciliated cells and the origin of mucinous EOC in contrast to the origin of other EOC histologic subtypes [31].

Our results are consistent with a recent study that reported more numerous CAPS+ cells and higher levels of CAPS mRNA expression in low-grade serous EOC compared to HGSOC [32]. Additionally, we found that the presence of CAPS+ cells is not limited to low-grade serous EOC as we detected CAPS+ cells in approximately 85% of serous cystadenomas, borderline tumors, and low-grade carcinomas compared to <40% of HGSOC. Interestingly, the average percentage of CAPS+ cells for all cases decreased with increasing grade aggressiveness of serous lesions (~40% in cystadenomas, ~30% in borderline tumors, ~20% in low-grade carcinomas, and <3% in HGSOCs. This finding is consistent with previous associations between the loss of ciliated cell marker FOXJ1 and worsened prognosis [18]. Endometrioid EOC exhibited a similar phenomenon; CAPS+ cells were observed in 89% of low-grade endometrioid carcinomas compared to 31% of high-grade endometrioid carcinomas and the average percentage of CAPS+ in all cases was 10-times higher in low-grade compared to high-grade endometrioid carcinomas.

In HGSOC, we found an additional decline in the presence of ciliated cell marker-positive cells when primary tumors were compared to patient-matched metastases. Ciliated cell markers were identified in 52% of primary HGSOC compared to approximately 25% of patient-matched synchronous and metachronous/recurrent metastases. Little is known regarding either the mechanism(s) by which ciliated cells are lost during ovarian cancer progression or the possible active role ciliated cells play in preventing cancer initiation and progression. In a premenopausal fallopian tube, ciliated cells comprise up to 50%

of the tubal epithelial cells and are typically in direct contact with, or in close proximity to, secretory cells, which are the presumptive precursor cell type for ovarian cancer. The mechanism by which ciliated cells might protect secretory cells from genotoxic stress is unclear. One possibility is that the beating cilia provide physical protection like an umbrella over the secretory cells. Another possibility is that the juxtacrine and paracrine signaling between secretory and ciliated cells is needed to maintain homeostasis of the healthy cells and/or eliminate damaged and transformed cells from the epithelial monolayer through cell competition and extrusion. The reduction in the number of ciliated cells in menopause might alter this homeostasis and lead to the accumulation of damaged secretory cells that are vulnerable to tumorigenic transformation. However, it is less likely that ciliated cells have a protective role during the later stages of cancer progression as a sparse distribution of ciliated cells in tumors would dilute any physical or molecular means of protection. Based on the information currently available, fallopian tube and endometrial ciliated cells might initially be passively incorporated into the tumor mass. As terminally differentiated cells, ciliated cells cannot transdifferentiate or divide and are gradually outstripped by molecular (genetic, epigenetic, transcriptomic) conditions that result in a preferential proliferation of cancerous secretory-type cells during tumor progression. As such, ciliated cells are likely to be passive remnants of normal tissue engulfed by the tumor. Although ciliated cells in tumors might not have a protective role in tumor progression, their presence in a tumor might be a useful marker of reduced tumor aggressiveness, which is consistent with the observed positive correlation between the levels of ciliated cell markers and favorable clinical outcomes [18]. Our findings also raise the possibility that cellular mechanisms involved in tumor migration and/or adaptation to a new milieu (e.g., metastatic site) at least in part are responsible for, or contribute to, the decreased number of ciliated cells in HGSOC metastases compared to primary HGSOC.

Limitations to our study include the lack of demographic and clinical parameters, such as race, use of oral contraception, and use of hormone-replacement therapy, which might affect the number of ciliated cells. Our study was primarily histologic and did not link patient demographics to clinical outcomes. Our data do not clarify whether or not the presence or number of ciliated cells is determined by pre-existent patient or tumor characteristics or whether or not the number of ciliated cells present itself is prognostically significant. Additionally, although one of the largest studies to date, our number of TMAs and samples were relatively few and may be underpowered to detect differences. However, our study is strengthened by its utilization of a microarray validation dataset and by its unique focus on exploring ciliated cell markers in multiple stages of ovarian cancer progression.

In conclusion, we found that cells expressing ciliated cell markers were present in each of the five major histologic subtypes of EOC, and that ciliated cell marker-positive cells were more likely to be found in borderline and low-grade serous tumors compared to HGSOCs, as well as in primary HGSOCs compared to patient-matched metastases. Ciliated cell marker-positive cells were also less likely to be found in ovarian tumors of mucinous compared to other histologic subtypes. These findings support a model in which ciliated cell containing tissues, such as the fallopian tube, endometrium, and/or CIC, are the origin for the major histologic subtypes of EOC, with mucinous EOC being the exception. Future studies should focus on the mechanism(s) by which ciliated cells are lost during carcinogenesis, cancer progression, and metastatic spread. If ciliated cells provide protection from malignant transformation, understanding the mechanism of loss of ciliated cells may also lead to targets for prevention or treatment.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells11244009/s1>, Table S1. Detection and quantitation of CAPS+ cells in TMAs 1-4.

Author Contributions: M.T.R. reviewed the literature and wrote the manuscript. M.S.R. conducted the experiments. A.E.W. assisted in the design and analysis of tissue microarrays. B.Y.K. provided

patient samples for tissue microarrays and curated clinical data. S.O. designed the study and analyzed the data. All authors contributed to the writing of the manuscript. Conceptualization, S.O.; methodology, M.S.R. and S.O.; validation, S.O. and M.S.R.; formal analysis, S.O.; investigation, S.O. and M.T.R.; resources, B.Y.K. and A.E.W.; data curation, B.Y.K. and A.E.W.; writing—original draft preparation, S.O. and M.T.R.; writing—review and editing, S.O., M.S.R., M.T.R., B.Y.K. and A.E.W.; visualization, S.O.; supervision, S.O.; funding acquisition, S.O. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Written informed consent has been obtained from the patients at Cedars-Sinai Medical Center to use their archival specimens for research.

Data Availability Statement: The raw and processed data presented in this study are available upon request from the corresponding author.

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Editorial: Molecular pathology and computational image analyses in gynecologic malignancies

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Editorial on the Research Topic

Molecular pathology and computational image analyses in gynecologic malignancies

Gynecological malignancies represent an important public health problem due to high cancer-related mortality. Despite improvements in diagnosis and treatment, gynecological malignancies account for about 40% of all cancer incidence and more than 30% of all cancer-related mortality in women worldwide (1, 2). Recent advances in molecular pathology, digital pathology, and computational imaging are paving way for new approaches to the diagnosis and prediction of clinical outcomes in gynecological malignancies (Orsulic et al.; 3–7). The continuously increasing capacity to store and analyze digital data enables applications of new technologies, such as artificial intelligence- and deep learning-assisted image analyses to generate thousands of image features and other non-biased continuous quantifiable variables that can be readily integrated with other -omic platforms (8). Future clinical application of such features combined with improvements in molecular pathology may be an arrow in the quiver of pathologists in diagnosis and guiding patient treatment and management decisions.

In this Research Topic of Frontiers in Oncology, we attempt to address some major advances in molecular and computational pathology in gynecological malignancies.

Endometrial cancer is the most common gynecological cancer in developed countries. Ovarian preservation treatment (OPT) is an option for patients of child-bearing age and with early-stage endometrial cancer, however, the benefits need to be carefully assessed against the risk of cancer progression. In order to assist radiologists in assessing the depth of myometrial invasion and selecting eligible patients for OPT, Yan et al. developed and validated a radiomics nomogram based on multi-parametric magnetic resonance imaging

and the least absolute shrinkage and selection operator algorithm. Molecular analyses are a useful tool in the characterization and classification of recently identified malignancies, such as mesonephric-like adenocarcinoma (MLA), a rare and aggressive neoplasm that mostly arises in the uterine corpus. Ma et al. analyzed molecular alterations in four MLA cases and identified several immunohistochemical markers as well as recurrent mutations in PIK3CA, KRAS, and PTEN, which not only support the classification of these malignancies as Müllerian in origin with mesonephric differentiation but also provides important leads for targeted therapies.

Cervical cancer is the leading cause of gynecological tumor-related mortality worldwide and the second most common malignancy in women. Human papilloma virus (HPV) infection and integration within the human genome is the primary cause of cervical cancer. Integration can disrupt the function of nearby genes, including oncogenes and tumor suppressor genes, and cause genomic rearrangements and instability (9). However, the potential intra-tumoral heterogeneity in integration sites has not been explored. Using an optimized dual-color fluorescence *in situ* hybridization (FISH) method to detect HPV integration sites in formalin-fixed, paraffin-embedded cervical cancer samples, Xiong et al. showed that cervical cancer may comprise subpopulations of cells with distinct integration sites that are otherwise indistinguishable by cell and nuclear morphology. Beyond diagnosis, molecular testing may be adopted for prognostic and predictive purposes in cervical cancer patients. Ma et al. showed that the upregulation of *CD27*, *TNF*, *TNFRSF12A*, *TNFRSF13C*, and *TNFRSF9* and the downregulation of *EDA* mRNA expression levels may serve as prognostic biomarkers of cervical cancer because they are associated with the immunotherapy response of these patients. In addition to molecular tools, cervical cancer patients may benefit, in particular in the field of screening and early diagnosis, from artificial intelligence-based medical tools. As summarized by Hou et al., clinical application of artificial intelligence may reduce turn-around time and the need for professional and technical personnel as well as eliminate human bias in evaluating subjective variables.

Ovarian cancer is a complex gynecological malignancy with a high mortality rate. To shed light on the molecular heterogeneity of this disease, Chen et al. explored a novel molecular phenotyping method for ovarian cancer subtypes based on metabolic genes through a comprehensive analysis of genomic data. The authors identified three different molecular subtypes (C1, C2, and C3) of ovarian carcinomas, which improved our understanding of the molecular characteristics of human ovarian cancer and uncovered new potential therapeutic targets. Wang et al. reviewed the role of the glyoxalase system as a marker for diagnosis and a novel target for antitumor therapy in breast, ovarian, endometrial, and cervical cancers. Although research in the past two decades has revealed that the fallopian tube is the likely precursor tissue for most epithelial ovarian cancers, the cell-intrinsic and

microenvironmental conditions that lead to epithelial cell transformation into serous tubal intraepithelial carcinoma (STIC) are unknown. Wu et al. used computational image analyses to identify potential morphometric and topologic differences in fallopian tubes with and without STIC lesions. They showed that STIC lesions were accompanied by global stromal alterations and age-associated reorganization of tubal secretory and ciliated cells, which may provide a favorable microenvironment for cancer initiation. Another poorly understood aspect of ovarian cancer is metastasis outside of the peritoneal cavity. Brain metastases are rare in ovarian cancer, possibly because patients succumb to the peritoneal disease before the cancer has a chance to metastasize to other parts of the body. Using a spatially-oriented single-cell proteomics platform, Pejovic et al. identified cell populations that are shared between primary low-grade serous ovarian carcinomas and brain metastases, suggesting that cells predetermined for brain metastasis may exist in the early stages of cancer development. They also identified several brain metastasis-specific oncogenic and immunosuppressive pathways that could be used for targeted therapy.

Overall, this Research Topic has highlighted some major advances in molecular and computational pathology in gynecological malignancies. Ongoing research is warranted to improve the clinical outcome of these patients.

Author contributions

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Conflict of interest

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