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Ovarian Carcinoma Subtypes Are Different Diseases: Implications for Biomarker Studies

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Abbreviations: BCCA, British Columbia Cancer Agency; CI, confidence interval; DSS, disease-specific survival; RR, risk ratio; TMA, tissue microarray

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ABSTRACT

Background

Although it has long been appreciated that ovarian carcinoma subtypes (serous, clear cell, endometrioid, and mucinous) are associated with different natural histories, most ovarian carcinoma biomarker studies and current treatment protocols for women with this disease are not subtype specific. With the emergence of high-throughput molecular techniques, distinct pathogenetic pathways have been identified in these subtypes. We examined variation in biomarker expression rates between subtypes, and how this influences correlations between biomarker expression and stage at diagnosis or prognosis.

Methods and Findings

In this retrospective study we assessed the protein expression of 21 candidate tissue-based biomarkers (CA125, CRABP-II, EpCam, ER, F-Spondin, HE4, IGF2, K-Cadherin, Ki-67, KISS1, Matriptase, Mesothelin, MIF, MMP7, p21, p53, PAX8, PR, SLPI, TROP2, WT1) in a population-based cohort of 500 ovarian carcinomas that was collected over the period from 1984 to 2000. The expression of 20 of the 21 biomarkers differs significantly between subtypes, but does not vary across stage within each subtype. Survival analyses show that nine of the 21 biomarkers are prognostic indicators in the entire cohort but when analyzed by subtype only three remain prognostic indicators in the high-grade serous and none in the clear cell subtype. For example, tumor proliferation, as assessed by Ki-67 staining, varies markedly between different subtypes and is an unfavourable prognostic marker in the entire cohort (risk ratio [RR] 1.7, 95% confidence interval [CI] 1.2%–2.4%) but is not of prognostic significance within any subtype. Prognostic associations can even show an inverse correlation within the entire cohort, when compared to a specific subtype. For example, WT1 is more frequently expressed in high-grade serous carcinomas, an aggressive subtype, and is an unfavourable prognostic marker within the entire cohort of ovarian carcinomas (RR 1.7, 95% CI 1.2%–2.3%), but is a favourable prognostic marker within the high-grade serous subtype (RR 0.5, 95% CI 0.3%–0.8%).

Conclusions

The association of biomarker expression with survival varies substantially between subtypes, and can easily be overlooked in whole cohort analyses. To avoid this effect, each subtype within a cohort should be analyzed discretely. Ovarian carcinoma subtypes are different diseases, and these differences should be reflected in clinical research study design and ultimately in the management of ovarian carcinoma.

The Editors' Summary of this article follows the references.

Table 1. Study Population

Clinical Variable	Numerical Display	All	High-Grade Serous	Clear Cell	Endometrioid	Mucinous	Low-Grade Serous
Number of cases	*n*	500	200	132	125	31	12
Proportion	%	100	40.0	26.4	25.0	6.2	2.4
Age in years	Mean ± SE	58.1 ± 0.6	60.9 ± 0.8	56.2 ± 1.1	56.0 ± 1.2	55.4 ± 2.4	60.2 ± 4.1
Follow-up time in years	Mean ± SE	5.9 ± 0.2	5.4 ± 0.2	6.3 ± 0.4	6.4 ± 0.3	5.4 ± 0.7	5.8 ± 1.1
Death	*n* (%)	233 (46.6)	124 (62.0)	52 (39.4)	39 (31.2)	11 (35.5)	7 (58.3)
Death of disease	*n* (%)	164 (32.8)	92 (46.0)	40 (30.3)	19 (15.2)	8 (25.8)	5 (41.7)
10 YSR DSS	% ± SE	57.8 ± 2.9	38.9 ± 4.7	63.7 ± 5.2	83.9 ± 4.2	72.0 ± 10.0	48.0 ± 19.1
Stage I	*n* (%)	205 (41.0)	49 (24.5)	68 (51.5)	69 (55.2)	18 (58.1)	1 (8.3)
Stage II	*n* (%)	211 (42.2)	86 (43.0)	56 (42.4)	50 (40.0)	12 (38.7)	7 (58.3)
Stage III	*n* (%)	84 (16.8)	65 (32.5)	8 (6.1)	6 (4.8)	1 (3.2)	3 (33.3)
Grade 1	*n* (%)	105 (21.0)	0	0	82 (65.6)	11 (35.5)	12 (100)
Grade 2	*n* (%)	109 (21.8)	56 (28.0)	0	35 (28.0)	18 (58.1)	0
Grade 3	*n* (%)	286 (57.2)	144 (72.0)	132 (100)	8 (6.4)	2 (6.5)	0

YSR DSS, year disease-specific survival rate; SE, standard error of the mean.
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Introduction

Ovarian carcinoma is a heterogeneous disease. On the basis of histopathological examination, pathologists classify ovarian carcinoma into serous, clear cell, endometrioid, and mucinous subtypes. Each of theses subtypes is associated with different genetic risk factors and molecular events during oncogenesis [1,2], and characterized by distinct mRNA expression profiles [3,4]. These subtypes differ dramatically in frequency, when early stage carcinomas (where the majority are nonserous carcinomas [5]) and advanced stage carcinomas (which are predominantly of serous subtype [6]) are compared.

Oncologists have noted that subtypes respond differently to chemotherapy. The dismal response rate of clear cell carcinomas (15%) contrasts sharply with that of high-grade serous (80%), resulting in a lower 5-y survival for clear cell compared with high-grade serous carcinoma in patients with advanced stage tumors (20% versus 30%) [7,8]. Therefore, the National Cancer Institute (NCI) State of Science meeting recently singled out clear cell carcinoma as a candidate for clinical trials to identify more active therapy than what is currently available [9]. Although these data suggest substantial differences between subtypes, ovarian carcinoma is typically approached as a monolithic entity by researchers and clinicians. This practice impedes progress in understanding the biology or improving the management of the less common ovarian carcinoma subtypes.

We hypothesized that correlations between biomarker expression and stage at diagnosis or prognosis would reflect subtype variation in biomarker expression. To test this hypothesis we correlated protein expression rates of a panel of 21 candidate biomarkers with stage at diagnosis and disease-specific survival (DSS) in a large cohort of ovarian carcinomas and also analyzed these associations within ovarian carcinoma subtypes.

Methods

Study Population

The Cheryl Brown Ovarian Cancer Outcomes Unit is an ovarian cancer registry serving a population of approximately four million people in British Columbia. For the period 1984–2000, 2,555 patients with ovarian carcinoma were recorded in the registry. From these 834 patients were selected based on the criterion being free of macroscopic apparent residual disease after primary surgery and all histological slides underwent gynecopathological review. Subtypes were assigned according to refined World Health Organization (WHO) criteria [10] as recently described [5]. A further 91 patients diagnosed in stage 1a or 1b, grade 1 were excluded from the study because of excellent prognosis; only 3% of women in this group died of disease during the follow-up period. From the remaining patients 541 tissue blocks were available and used for tissue microarray (TMA) construction. A representative area of each tumor was selected and duplicate 0.6-mm tissue cores were punched to construct a TMA (Beecher Instruments). Review after TMA construction revealed that 23 cases were not adequately sampled. Of these 23 cases, 20 mixed carcinomas (>10% of tumor showing a second histological cell type) were excluded because their highest grade component was not sampled on the TMA; 18 cases were either of rare histological types (including seven undifferentiated, six transitional, and one squamous carcinoma) or could not be specified (five cases). This approach resulted in a study population of exactly 500 cases belonging to one of the four major cell types (serous, endometrioid, clear cell, and mucinous) (Table 1). The serous subtype was further subdivided into low- and high-grade [11]. Two cases of endometrioid carcinomas containing minor mucinous or low-grade serous components (>10%) are included in the study.

Adjuvant Therapy and Follow-up

All patients received standardized treatment according to the provincial treatment guidelines of the British Columbia Cancer Agency (BCCA) [12,13]; however, 3% of patients refused the advised adjuvant chemotherapy and were excluded from survival analysis. For 3% adjuvant therapy was not advised, hence 94% received platinum-based chemotherapy (with or without abdomino-pelvic radiotherapy) adjuvant treatments. Outcomes were tracked via the Cheryl Brown Ovarian Cancer Outcomes Unit at the BCCA and were available for all patients. Follow-up information was obtained

Table 2. Antibodies

Number	Biomarker	Supplier	Clone	Dilution	Full Name/Description
1	CA125	Cellmarque	OC125	1:100	Cancer antigen 125, cell surface glycoprotein
2	CRABP-II	Santa Cruz	Polyclonal	1:25	Cellular retinoic acid-binding protein II, transcriptional regulator of lipid metabolism
3	EpCam	R&D Systems	158206	1:25	Epithelial cell adhesion molecule, cell-cell adhesion
4	ER	Labvision	SP1	1:200	Estrogen receptor
5	F-Spondin	US Biological	Polyclonal	1:50	Neuronal development
6	HE4	Signet	Polyclonal	1:25	Human epididymis protein 4 is a member of 4-disulfide core protein with unknown function
7	IGF2	Abcam	Polyclonal	1:100	Insulin-like growth factor 2
8	K-Cadherin	Abcam	2B6	1:50	Cell-cell adhesion protein
9	Ki-67	Labvision	SP6	1:200	MKI, proliferation-associated antigen detected by Ki67
10	KISS1	Santa Cruz	Polyclonal	1:400	Kisspeptins, ligands of G-protein coupled receptor 54
11	Matriptase	Bethyl	Polyclonal	1:25	Type II transmembrane trypsin-like serine protease, degradation of extracellular matrix
12	Mesothelin	Novocastra	5B2	1:50	Cell surface glycoprotein
13	MIF	R&D Systems	Polyclonal	1:2500	Macophage inhibitory factor, modulator of chronic inflammation
14	MMP7	Chemicon	141–7B2	1:200	Matrix metalloproteinase 7, degradation of extracellular matrix
15	p21	Labvision	DCS-60.2	1:40	Cyclin-dependent kinase inhibitor 1A (Cip1)
16	p53	DAKO	DO-7	1:400	Tumor protein p53
17	PAX8	Donation[a]	Polyclonal	1:500	Thyroid specific transcription factor, Pax8/PPARgamma fusion gene in 50% of follicular thyroid carcinomas
18	PR	Labvision	SP2	1:400	Progesteron receptor
19	SLPI	Hycult	31	1:100	Secretory leukocyte protease inhibitor
20	TROP2	R&D Systems	Polyclonal	1:25	Tumor-associated calcium signal transducer 2
21	WT1	DAKO	6F-H2	1:100	Wilms tumor suppressor 1, zinc finger transcription factor

[a]The α-mPax8-bIII antibody was kindly provided by Roberto Di Lauro, Stazione Zoologica, Naples, Italy.
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through the electronic patient record of the BCCA or the patient's paper chart. Examples of documentation used to ascertain vital status include BCCA progress notes, death certificates, and correspondence indicating status from other care providers. Ovarian carcinoma specific death was defined where ovarian cancer was the primary or underlying cause of death. Death from concurrent disease (i.e., second malignancy) was coded as "died of other cause." Death resulting from toxicities relating to treatments for ovarian carcinoma was coded as "died of toxicities." Abstracted data were reviewed by an experienced medical oncologist (K.S.). Median follow-up time was 5.1 y. Approval for the study was obtained from the Research Ethics Board of the University of British Columbia.

Marker Selection and Immunohistochemistry

The goal of our marker selection was to use proteins that are consistently expressed in ovarian carcinomas and have been reported as prognosticators (p53, p21, Ki-67, PR, WT1) [14–19] or being developed as early detection markers in ovarian carcinomas [20]. This approach biased our results towards selection of markers mostly derived from and expressed in high-grade serous subtype. Serial 4-μm sections were cut for immunohistochemical (IHC) analysis and run through an automated protocol including heat antigen retrieval (Ventana System). The antibodies and suppliers are listed in Table 2. Specificity was determined by using appropriate positive controls, with omission of primary antibody as a negative control.

Evaluation of Immunohistochemistry

One or more pathologists (MK, DNI, or AR) scored these biomarkers after scanning with a BLISS scanner (Bacus Laboratories/Olympus America). Except KISS1 [21] and p53 [22] where recently published cut-off points were used, all markers were dichotomized into negative and positive cases (cut-off values for positive versus negative for all markers except Ki-67 are shown in Table S1). Ki-67 was assessed as a continuous variable as a percentage of positive tumor cells using automated image analysis software [23]. Prior to analysis a pathologist (MK) manually selected regions of interest so as to avoid noncancerous cellular areas. The median was used to dichotomize into low- and high-expressing groups for Ki-67.

Statistical Analysis

Contingency analysis and Pearson's Chi^2 statistic were used to test the change in the distribution of biomarker expression across stage and subtypes. The Kruskal-Wallis test was used to determine if Ki-67 was differentially expressed across stage and subtypes. Univariable DSS was illustrated by the generation of Kaplan-Meier curves and subgroup differences tested with a univariable Cox model. Multivariable DSS was tested using the Cox proportional hazards model. The Cox proportional hazards model was used to determine risk ratios (RRs) and *p*-values for all univariable and multivariable DSS analyses. Additionally, to assess significance in the presence of some small subgroups, permutation tests were performed and permutation *p*-values reported. Under the null hypothesis of no association of biomarker status with survival (for survival analyses) or stage/histology (for contingency table analyses), the biomarker outcomes are exchangeable across cases. For the survival analyses, permutations of biomarker outcomes were performed within stage/subtype subgroups, to preserve the observed distribution of biomarker frequencies within

subgroups. Permutation was performed by exchanging each case's entire biomarker panel at random without replacement among cases, to preserve correlation structure within case. A total of 10,000 permutation replications were performed. *p*-Values were obtained by finding the number of permutation sample estimates (Cox model parameter estimate for survival analyses, Pearson Chi^2 statistic for contingency table analyses) as extreme or more extreme than the observed value. $p < 0.05$ was considered statistically significant. Hence, any prognostic correlations for a single biomarker have to be interpreted with caution. Statistical analyses were performed using SPSS software (version 15.0; SPSS) and R (version 2.5.1; R Foundation for Statistical Computing).

Results

Biomarker Expression Profile Reflects Subtype

This cohort of 500 ovarian carcinomas was mainly selected based on the criterion of not having apparent residual tumor after primary surgery. Since successful surgery is typically achieved in lower stage, this case selection strategy can be anticipated to include more cases of tumors of histological subtypes that are commonly diagnosed at low stage, such as clear cell carcinoma (26.4%), endometrioid (25.0%), and mucinous (6.2%) carcinomas, although serous carcinomas were still the most common subtype (40.0% high-grade and 2.4% low-grade) in this cohort (Table 1).

Interpretable results of immunostains for the 21 candidate biomarkers (Figure 1) ranged from 363 to 493 (median 488, Table S2). The larger numbers of missing data for three biomarkers were caused by exhaustion of tumor material in the core. All immunostains with annotated clinical information are available online at http://www.gpecimage.ubc.ca (username: BCCA-VGH; password: OVCARE). The rate of positive cases for each biomarker ranged from 9% (KISS1) to 83% (EpCam) (detailed expression rates are listed in Table S2). Comparing biomarker expression in the entire cohort for tumors diagnosed at different stages revealed that ten biomarkers (CRABP-II, ER, F-Spondin, K-Cadherin, Ki-67, Matriptase, Mesothelin, p21, p53, and WT1) had significantly different expression levels between stages, suggesting differences between "early" and "late" stage disease (Figure 2, Table S2). However, comparing biomarker expression within one subtype across FIGO stages, no biomarker remained significantly differently expressed by stage (results for high-grade serous subtype are shown in Figure 3). This result was true for all four major subtypes (unpublished data for endometrioid, clear cell, and mucinous). In contrast, 20 of 21 biomarkers were significantly differentially expressed between the subtypes (Figure 4). Only, EpCam ($p = 0.23$) showed a consistent expression frequency across all subtypes. Additionally, *p*-values for biomarker expression rates in the entire cohort across subtypes were generally smaller than across stages (Table S2), indicating a stronger association with subtype than stage.

High-grade serous carcinoma showed positive staining in >75% of cases for WT1, Mesothelin, ER, and CA125 (Table S2). The biomarker expression pattern of low-grade serous carcinomas was similar to that of their high-grade counterparts. Three markers (PR, p53, K-Cadherin) showed a trend towards differential expression in low-grade versus high-grade serous subtypes. Only the median Ki-67 labelling index differed significantly between those groups, with median Ki-67 labelling index of 2.5% (95% confidence interval [CI] 0.5%–20.4%) in low-grade serous versus 22.4% (95% CI 3.6%–69.9%) in high-grade serous subtype (Figure 5). Endometrioid carcinomas coexpress high rates of hormone receptors ER and PR as well as CA125. Endometrioid and clear cell subtypes infrequently (<10%) expressed WT1 and p53. The median Ki-67 labelling index for endometrioid and clear cell carcinomas was similar (endometrioid 8.2%, 95% CI 0.8%–49.0%; clear cell 7.6%, 95% CI 0.5%–45.0%). Immunophenotypic characteristics of clear cell carcinomas included low levels of hormone receptors ER (10%) and PR (3%). The mucinous subtype displayed an intermediate proliferative capacity compared with the other subtypes (median Ki-67 labelling index 12.9%, 95% CI 2.1%–60.9%) and frequent expression of Matriptase (86%). Many of the markers expressed in other subtypes were either infrequently (<10%) expressed (p53, ER, PAX8, SLPI, K-Cadherin, and CA125), or completely absent (CRABP2, WT-1, and Mesothelin). Of note, EpCam was highly expressed across all subtypes included in this study.

Survival Analyses Can Be Confounded by Subtype Differences

To assess the biological importance of a biomarker, its expression is usually correlated with outcome. Survival analysis was restricted to the three major subtypes (high-grade serous, clear cell, and endometrioid) because of insufficient numbers of cases of mucinous or low-grade serous subtypes. The primary endpoint was defined as DSS and the rates after 10 y are shown for subtypes in Table 1. A multivariable Cox regression model including age, stage, and histological subtype showed significant differences across stage ($p < 0.0001$) and subtype ($p = 0.015$). Survival by stage showed little difference between stages I and II, with stage III showing poorer DSS (RR 3.0, 95% CI 1.87%–4.66% relative to stage I). Survival by subtype showed poorer DSS for clear cell (RR 2.31, 95% CI 1.29%–4.15%) and high-grade serous (RR 2.74, 95% CI 1.56%–4.81%) relative to endometrioid subtype. Age was not predictive in the model ($p = 0.211$) (Table S3).

Univariable Cox regression analysis for each biomarker was applied on the entire cohort as well as within the three largest subtypes (Figure S1, Table 3). RRs and *p*-values are presented in Table 3. Nine of 21 biomarkers show prognostic significance in the entire cohort. Of the nine biomarkers showing a significant association with DSS in the entire cohort, three remain prognostic indicators in the high-grade serous and one in the endometrioid subtype. As an extreme example, WT1 is an unfavourable prognostic biomarker in the entire cohort ($p = 0.0017$, Figure 6A) but is a favourable prognostic biomarker for high-grade serous carcinomas ($p = 0.0086$, Figure 6B). As WT1 is expressed in 80% of high-grade serous carcinomas but rarely in other subtypes, this negative prognostic significance in the entire cohort reflects subtype differences in expression, with WT1 most commonly expressed in the aggressive high-grade serous subtype. Four other biomarkers (KISS1, K-Cadherin, Mesothelin, Ki-67) that were significant in the entire cohort did not show significance in any subtype.

Ki-67 serves as an additional example, which is prognostic in the whole cohort but not when corrected for subtype. The median for Ki-67 labelling index in the entire cohort was

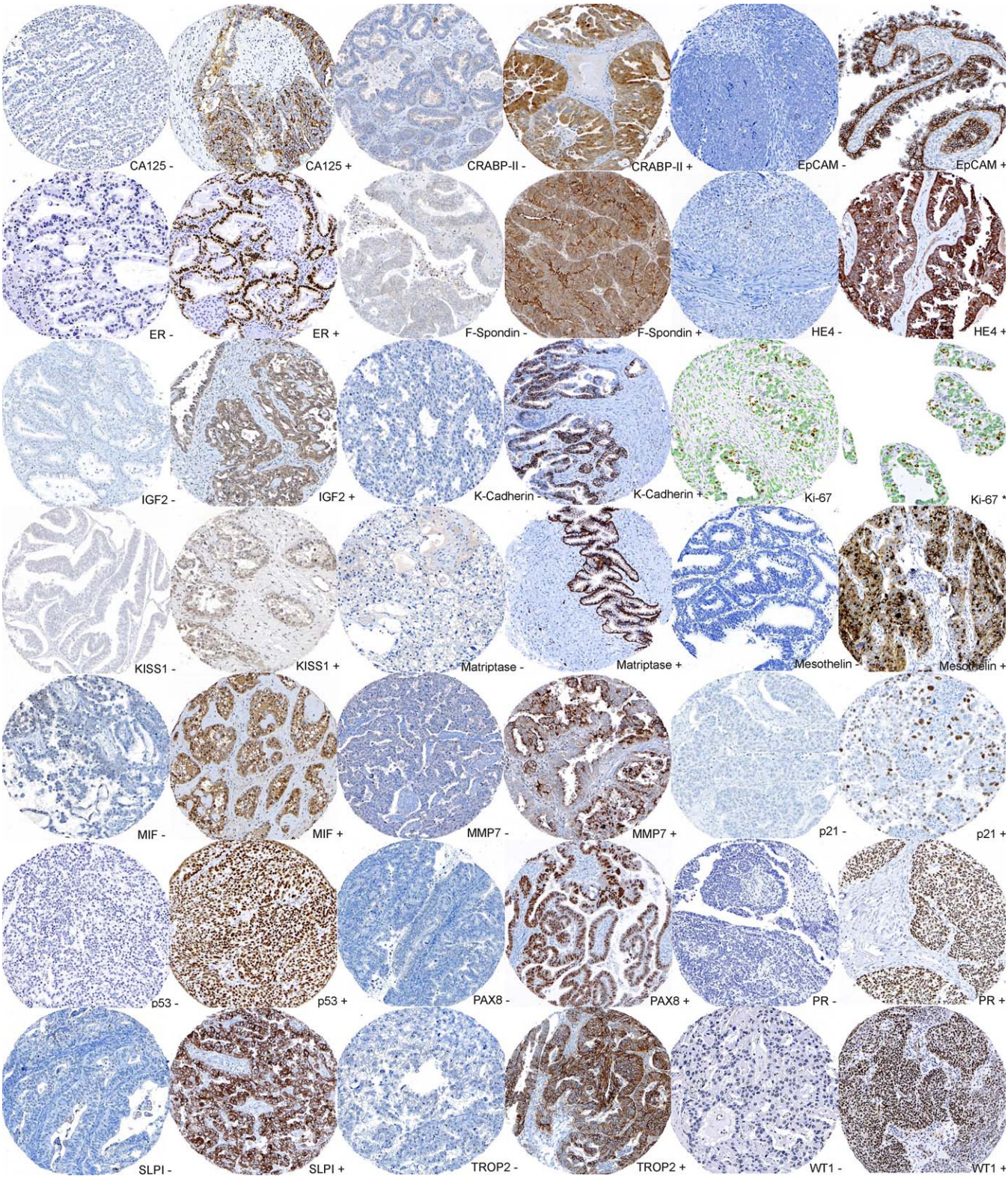

Figure 1. Representative Immunostains

Paired positive and negative examples for each biomarker.

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