A combination of the immunohistochemical markers CK7 and SATB2 is highly sensitive and specific for distinguishing primary ovarian mucinous tumors from colorectal and appendiceal metastases.

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Abstract

Primary ovarian mucinous tumors can be difficult to distinguish from metastatic gastrointestinal (GI) neoplasms by histology alone. The expected immunoprofile of a suspected metastatic lower GI tumor is $CK7^-/CK20^+/CDX2^+/PAX8^-$. This study assesses the addition of a novel marker SATB2, to improve the diagnostic algorithm. A test cohort included 155 ovarian mucinous tumors (105 carcinomas and 50 borderline tumors) and 230 primary lower gastrointestinal neoplasms (123 colorectal adenocarcinomas and 107 appendiceal neoplasms). All cases were assessed for SATB2, PAX8, CK7, CK20, and CDX2 expression on tissue microarrays. Expression was scored in a 3-tier system as absent, focal (1-50% of tumor cells) and diffuse (>50% of tumor cells) and further categorized into absent/present and nondiffuse/diffuse. SATB2 and PAX8 expression was further evaluated in ovarian tumors from an international cohort of 2876 patients (expansion cohort, including 159 mucinous carcinomas and 46 borderline mucinous tumors). The highest accuracy of an individual marker distinguishing lower GI from ovarian mucinous tumors was CK7 (91.7%, nondiffuse/diffuse cut-off) followed by SATB2 (88.8%, present/absent cut-off). The most effective combination was CK7 and SATB2 with accuracy of 95.3% using the 3-tier interpretation, absent/focal/diffuse. This combination outperformed the standard clinical set of CK7, CK20 and CDX2 (87.5%). Re-evaluation of outlier cases confirmed ovarian origin for the 6% of ovarian mucinous tumors with diffuse SATB2 expression. The accuracy of SATB2 was confirmed in the expansion cohort (91.5%). SATB2 expression was also detected in 15% of ovarian endometrioid carcinoma but less than 5% of other ovarian histotypes. A simple two marker combination of CK7 and SATB2 can distinguish lower GI from ovarian primary mucinous tumors with greater than 95% accuracy. PAX8 and CDX2 have value as

second-line markers. The utility of CK20 in this setting is low and this warrants replacement of this marker with SATB2 in clinical practice.

Introduction

Primary gastrointestinal (GI) neoplasms can present as metastatic ovarian masses and their potential to mimic an ovarian primary neoplasm, mostly mucinous type, is well recognized. (1-4) Ancillary immunohistochemistry is often applied with the standard panel including CK7, CK20 and CDX2. The expected immunoprofile of a GI tumor is CK7 negative, CK20 positive and CDX2 positive, with the reverse generally associated with a primary ovarian tumor. The clinical utility of this profile is hampered by reduced specificity due to focal and even diffuse positivity of CK20 and CDX2 in mucinous ovarian tumors.(4) This limitation warrants additional studies to validate more specific markers such as SATB2 and PAX8.(5-7) SATB2 (special AT-rich sequence-binding protein 2) is a transcriptional regulator (encoded on chromosome 2q32-33) that is involved in osteoblastic and cortical neuron differentiation and in skeletal development.(8) SATB2 is also expressed in epithelial cells of the lower GI tract including colon and appendix, therefore is expected to be present in lower GI tumors, but not primary ovarian neoplasms.(9) Another transcription factor, PAX8, is highly expressed in Müllerian epithelium (including approximately half of mucinous ovarian tumors), kidney and thyroid but not in lower GI tumors. (10)

Among the five main histotypes of ovarian carcinoma, mucinous carcinoma is the least common, accounting for only 3-4% of cases. This proportion is significantly lower than earlier estimations of approximately 12%.(11) The difference is likely due to improved recognition of metastatic adenocarcinomas to the ovary that mimic primary ovarian mucinous tumors. Despite these improvements, accurate diagnosis remains a challenge in clinical practice, with a lack of standardization in testing, and uncertainty over

optimum cut-offs with respect to focal and diffuse staining of immunohistochemical markers.(12) Due to its rarity, it is challenging to accumulate sufficient cases of mucinous carcinomas in a research setting to investigate this poorly understood histotype.(13) Previous studies of the Ovarian Tumor Tissue Association (OTTA) consortium included 6-7% of mucinous carcinomas.(14-17)

The primary aim of this study was to compare the sensitivity, specificity and accuracy of CK7, CK20, CDX2, SATB2 and PAX8 expression individually, and in combination to identify the most efficient panel to differentiate primary ovarian mucinous neoplasms (herein ovarian mucinous tumors refer to atypical proliferative/mucinous borderline tumors and mucinous carcinomas) from lower gastrointestinal primaries (colorectal adenocarcinomas and appendiceal neoplasms) in a well characterized test cohort. A second objective was to validate the specificity of SATB2 in a large expansion OTTA cohort including all the main ovarian carcinoma histotypes and explore survival associations of SATB2 and PAX8 in mucinous carcinomas.

Materials and Methods

Study population – test and expansion cohorts

Cases for the test cohort were ascertained as a subset of the Ovarian Tumor Tissue Analysis (OTTA) Consortium with paraffin-embedded tissue available for staining.(18) These cases were well characterized to ensure confidence that they are ovarian primary tumors, undergoing strict histopathology review before study entry to exclude cases deemed to be non-ovarian.(1) Matched clinical data were available (Supplementary Table S1), and all cases were stained and scored for all 5 immunohistochemical

markers. A cohort of 123 primary colorectal adenocarcinomas (all Stage II) was investigated for comparison, as well as 107 appendiceal neoplasms, which have previously been described.(19)

The expansion cohort (n=2876 cases of the major ovarian histotypes) was drawn from 14 centers participating in the OTTA consortium, with the initial diagnosis classified according to the original pathology report or following specialized central review(18) (Supplementary Table S2). These cases were all scored for SATB2 and PAX8.

Immunohistochemistry and scoring

All staining was performed in a central pathology laboratory. Samples were assembled in tissue microarrays, with duplicate or triplicate cores. Immunohistochemistry was performed on 4-micron sections from tissue microarrays on a DAKO Omnis platform. Immunohistochemical staining method details are provided in Supplementary Table S3. Two SATB2 antibodies were used for both the lower GI and ovarian cohorts, and the concordance between the two was assessed (Supplementary Table S4).

All markers were scored in a 3-tier system as absent if tumor cells showed no staining, focal if 1-50% of tumor cells exhibited unequivocal staining or as diffuse if >50% of tumor cells were stained in their respective subcellular compartment (nuclear for SATB2, PAX8, CDX2, cytoplasmic for CK7 and CK20), (Figure 1).

Reassessment of outlier cases in the test cohort

A focused reassessment of ovarian mucinous tumors with aberrant immunohistochemical staining by the most discriminatory markers potentially

suggesting an incorrect original diagnosis of a primary ovarian tumor underwent a morphological review by a single gynecological pathologist (author MK). Re-review was performed on two representative full H&E sections. The presence of features of metastatic lower gastrointestinal adenocarcinoma was recorded. These features were surface or hilar involvement by carcinoma, nodular pattern, destructive invasion, single cells, or signet ring cells.(1) Features suggestive of involvement by low-grade appendiceal neoplasms (LAMN) were sub-epithelial clefts, scalloped glands, pseudomyxoma ovarii, tall hypermucinous cells, fibrous hypocellular stroma and absence of mucin granulomas.(2)

Statistical analyses

The 3-tier scoring interpretation (absent/focal/diffuse) was categorized into 2 different binary datasets: the first cut-off was absent/present, with present including focal and diffuse staining, and the second cut-off was nondiffuse/diffuse with nondiffuse including absent and focal staining. Sensitivity, specificity, and balanced accuracy were calculated to assess test performance. Nominal logistic regression was used to rank the accuracy of different markers using the 3-tier scoring interpretation (absent/focal/diffuse) and binarized data.

All mucinous ovarian carcinomas from the OTTA consortium with available stage and survival data (n=214) were used to investigate associations between SATB2 and PAX8 expression (absent/present) and overall survival. Survival was estimated using the Kaplan Meier method, and Cox proportional hazards regression adjusted for age, stage of disease, and cohort (test/expansion). Due to differences in study entry within the

OTTA consortium, we applied left-truncation to account for observation time at risk versus date of primary diagnosis. Survival analyses were censored at 10 years. All data management and sensitivity analyses were performed using SAS version 9.4. Survival analyses were conducted using R Studio and nominal logistic regression model in JMPv14 (SAS).

Results

Performance of CK7, CK20, CDX2, SATB2 and PAX8 individually in the test cohort

A test cohort consisted of 155 ovarian primary mucinous neoplasms and 230 neoplasms of lower GI origin (Table 1, Supplementary Table S5). The ovarian primary mucinous neoplasms included 50 mucinous borderline tumors and 105 mucinous carcinomas. The GI primaries were comprised of 123 Stage II colorectal adenocarcinomas and 107 appendiceal neoplasms including 39 goblet cell carcinomas, 24 LAMN, 20 carcinoids, 12 high grade appendiceal mucinous neoplasms (HAMN) and 12 non-mucinous adenocarcinomas.

The frequencies of 3-tier marker expression are shown in Table 1. Expression of CK20, CDX2 and SATB2 was present in almost all GI primaries (87%, 98% 90% respectively), while CK7 expression was detectable in almost all (97%) and PAX8 in less than half (45%) of ovarian mucinous neoplasms. Interestingly, 6 (6%) of appendiceal cases (3 non-mucinous adenocarcinomas, 2 carcinoids and 1 goblet cell carcinoma) displayed diffuse PAX8 expression (Supplementary Table S5), but this was not seen in any of the 123 colorectal adenocarcinomas (Table 1, Supplementary Table S5). Differences in CDX2 and CK20 expression were noted between ovarian mucinous borderline tumors

and mucinous carcinomas, with carcinomas less likely to express CDX2. There was high concordance (94%) between the two SATB2 antibodies tested (Supplementary Table S4).

Sensitivity, specificity and accuracy of individual markers in distinguishing ovarian mucinous neoplasms from GI primaries are shown in Table 2. When the binary absence/presence cut-off is used, SATB2 shows the highest accuracy (88.9%) among all the markers. However, when a binary nondiffuse/diffuse cut-off was used, CK7 achieved the highest accuracy (91.7%). Notably, the nondiffuse/diffuse cut-off increased accuracy of CDX2 from 71% to 82%, while the absence/presence cut-off showed the higher accuracy for PAX8 (76% versus 63%). CK20 showed the lowest accuracy only reaching up to 65% with the nondiffuse/diffuse cut-off (Table 2).

Performance of marker combination in the test cohort

We then tested the marker combinations with different inputs (3-tier, binary absence/presence cut-off, nondiffuse/diffuse cut-off as well as different combinations of cut-offs) using nominal logistic regression modeling. Selected marker combinations arranged in descending order of accuracy are shown in Table 3. The standard clinical panel consisting of CK7, CK20, and CDX2 with a binary absent/present cut off shows the same accuracy as CK7 alone (Table 2 and 3: 87.5%). Once SATB2 and PAX8 are added to make a 5-marker panel, the accuracy increases to 95.3%. The effect was more pronounced for the distinction of appendiceal from ovarian (93.1% versus 86.6%) compared to colorectal primaries (94.6% versus 93.9%). Removal of CK20 or CK20 and

CDX2 only slightly affected AUC values for distinguishing GI from ovarian tumors (95.1%, 94.3% respectively).

Despite the fact that the binary nondiffuse/diffuse cut-off generally showed a higher accuracy compared to the absent/present cut-off for individual markers, this did not translate into higher accuracy for marker combinations. For example, the best two individual marker cut-offs (CK7 nondiffuse, SATB2 present) performed slightly worse than CK7 absent, SATB2 present. This is because SATB2 does not add information to CK7 nondiffuse alone. However, when the interpretation was left to 3-tier (absent, focal, diffuse), the two-marker combination of CK7 and SATB2 performed as well as a five-marker combination. A decision tree for the 3-tier interpretation of the CK7/SATB2 combination is shown in Figure 2.

Re-evaluation of ovarian outlier cases using CK7/SATB2 combination in the test cohort

We performed a focused clinical and morphological re-evaluation on the 11 primary ovarian mucinous carcinomas with aberrant CK7/SATB2 staining from Figure 1 (4 CK7 negative and 7 SATB2 diffusely positive ovarian mucinous tumors, supplementary table 6). During follow up, two of the eleven patients survived between 5 and 10 years, and 4 patients survived more than 10 years. The long survival time of these 6 patients is consistent with the classification of ovarian primaries. Four patients died of their disease within 2 years. Among these, two PAX8 negative cases had associated teratomas supporting primary ovarian origin. The other two cases were both PAX8 positive, also supporting an ovarian primary; one case showed anaplastic carcinoma within a mural nodule in a background of a mucinous borderline tumor and the other showed multifocal

destructive invasive mucinous carcinoma. The last patient was lost to follow up after 22 months but was alive at last contact. This tumor was CK7 focal, SATB2 diffuse, PAX8 absent, CDX2 diffuse, and CK20 diffuse. This was the only suspected misclassified case in the test cohort, which was originally diagnosed as an ovarian primary but on morphological review suggested a lower GI metastasis.

Frequency of SATB2 and PAX8 expression across five main ovarian carcinoma histotypes in the expansion cohort

Given the high performance of SATB2 (90% sensitivity, 87% specificity) in distinguishing primary ovarian mucinous neoplasms from lower GI primaries as an individual marker, we also investigated the frequency of SATB2 expression in an expansion set of tumors from the remaining OTTA cohort. This contained additional ovarian mucinous neoplasms (n=205, n=159 invasive and n=46 borderline) as well as 2,671 ovarian carcinomas of other histotypes. SATB2 expression frequencies for mucinous carcinomas were similar between the test and expansion sets (Table 4), however SATB2 was more frequently present in the borderline tumors of the test set, although not statistically significant (12% vs. 4%, p=0.3).

In the other ovarian carcinoma histotypes, we observed the highest frequency of positivity in endometrioid carcinomas with 13% showing focal and 2% diffuse SATB2 staining. 4% of high-grade serous and clear cell carcinomas also expressed SATB2. All these ovarian carcinoma histotypes showed a high frequency of PAX8 expression. Only very rare cases of endometrioid (3%), and high grade serous (1%) carcinoma showed an aberrant expression pattern of SATB2+/PAX8- (Table 4). Thus, the inclusion of

PAX8 should aid in the distinction of these ovarian tumors, especially for SATB2+ endometrioid ovarian carcinoma from GI metastases.

Table 2 also shows the validation of SATB2 and PAX8 in mucinous tumors from the expansion cohort, with an accuracy of 92% at an absent/present cut-off, and 95% when using a nondiffuse/diffuse cut-off. The low specificity of PAX8 absent to predict a GI tumor produced accuracy of only 71%.

Prognostic significance of SATB2 and PAX8 in all ovarian mucinous carcinomas from the OTTA consortium

As expected, the 5-year overall survival significantly differs between low (I/II) 80%, versus high stage mucinous carcinomas (III/IV) 17% ($p<0.0001$) (Figure 3a). Since a subset of ovarian mucinous carcinomas (13%) expressed SATB2 (Tables 1 and 4), we explored an association with survival and performed a Cox regression adjusted for patient age, disease stage, and cohort, and the proportional hazards assumption was not violated. We observed a significant association for SATB2 expression and poorer overall survival (Hazard ratio 2.49 (95% CI 1.22 – 5.09), $p=0.01$) (Figure 3b). Expression of PAX8 was not associated with survival ($p=0.3$, HR 0.76 (0.44-1.32)) (Figure 3c).

Discussion

Herein, we show that a combination of CK7 and SATB2 using a 3-tier interpretation is the most efficient ancillary test to distinguish primary ovarian mucinous neoplasms from metastatic lower GI primary tumors. This represents a refinement to previous recommendations for the use of different permutations of the five markers (CK7, CK20,

CDX2, SATB2 and PAX8) in routine clinical practice.(5-7) We also validated the specificity of SATB2 in the largest series of ovarian tumor tissue available to-date internationally.

It has long been known that CK7 is the best single discriminatory marker for lower GI primaries compared to ovarian mucinous neoplasms.(20) While CK7 is diffusely expressed in almost all ovarian primaries, it is largely absent in colorectal adenocarcinoma but can be expressed in BRAF-mutated mismatch repair proficient colorectal adenocarcinomas.(21) Its specificity towards appendiceal neoplasms is, however, limited. The combination of CK7 with CDX2 has been promoted particularly in a 3-tier staining distribution (absent/focal/diffuse).(12) In line with these suggestions, we show that increasing the cut-off for interpretation increases accuracy of CDX2 by more than 10%. Despite several publications questioning the specificity of CK20,(3) its use in routine clinical practice remains high. Based on our findings and those of previous publications,(3, 4, 12) we do not recommend the use of CK20 to distinguish lower GI from ovarian mucinous neoplasms. A balanced accuracy of 59% or 65% (depending on the cut-off) and sensitivity below 90% is suboptimal in terms of diagnostic accuracy and we propose that CK20 could be replaced by SATB2 to increase accuracy in a cost neutral way.

In publicly funded health care systems, finding the most efficient marker combinations to enable accurate tumor diagnosis is essential to deliver value-based care. Using a larger number of cases, we validated previous studies showing good performance for SATB2.(6) These results warrant adding SATB2 to the immunohistochemical arsenal. Other studies suggested that SATB2 is not optimally sensitive or specific when is used

as single marker. (6, 9) SATB2 has also been shown to have superior value in distinguishing certain lower from upper GI metastasis.(22, 23) Of note, upper GI metastasis can have the same immunohistochemical profile as ovarian mucinous carcinomas including some pancreatic adenocarcinoma showing PAX8 expression.(24) Herein, we found a higher sensitivity of SATB2 alone for colorectal adenocarcinomas (91.6%) and for LAMN (97.8%) compared to those reported by Moh et al. (71.3% and 80%, respectively).(6) The higher SATB2 expression frequency for ovarian mucinous neoplasms both in our testing and expansion cohorts (12%) compared to previously reported (5%) raises the possibility of an influx of misclassified lower GI primaries. Re-evaluation of outlier cases from the testing cohort revealed that some were teratoma-associated ovarian mucinous neoplasms while others were anaplastic carcinomas presenting as mural nodules in mucinous tumors. Only a single case was a likely misclassified metastatic GI primary. Ovarian mucinous tumors associated with teratomas, which account for approximately 5% of ovarian mucinous tumors, (12) have the same immunoprofile as those that originate in the GI tract, including SATB2 expression. The distinction in this scenario would rely on the identification of the teratoma and on clinico-pathological correlation. Although we could not re-evaluate outlier cases in the multi-institutional expansion cohort, we believe, that given the similar frequency of SATB2 expression in ovarian mucinous neoplasms in our extensively reviewed test and expansion cohorts, this provides a more realistic estimate of the SATB2 expression frequency in ovarian mucinous tumors (12%). Furthermore, the 5-year survival estimates of mucinous carcinomas in OTTA (84% for Stage I/II and 14% for Stage III/IV, Figure 3a) were similar to the SEER database (83% for localized, 69.5%

for regional spread and 14% for distant metastases).(25) Overall, this argues against a major component of misclassified metastatic lower GI tract neoplasms within the OTTA cohort and the relatively high proportion of primary mucinous carcinomas could be explained by study sites selectively enriching for rare tumor histotypes.

While CK7 and SATB2 together make the most efficient panel, CDX2 and PAX8 are reasonable second line markers. Particularly, the specificity of PAX8 helps to rule out a lower GI primary, despite the potential pitfalls: we found some appendiceal neoplasms exhibit diffuse PAX8 expression, and the frequency of PAX8 expression in ovarian mucinous tumors is much lower and often only focal when compared to the other ovarian carcinoma histotypes.(5)

We observed SATB2 expression in non-mucinous ovarian carcinoma histotypes: notably, in 14% of ovarian endometrioid carcinomas. Expression was commonly observed in squamous morules similar to CDX2 as reported previously,(26) although the significance of this finding is unclear. However, one should be aware of this possibility to avoid misdiagnosis of a metastatic lower GI neoplasm. Endometrioid carcinomas are PAX8 positive in a higher percentage compared to mucinous carcinomas and are also ER positive in almost 90% of cases.(27) Importantly, SATB2 is not entirely specific with regard to high-grade serous and clear cell carcinomas because almost 5% of these tumors did show at least focal expression. In this context, it is noteworthy that the osteosarcoma component of carcinosarcomas can also express SATB2.(28)

Our observation of an adverse survival association with SATB2 expression within mucinous carcinomas is intriguing. Despite our relatively large sample size, it is possible that this result could be still a false positive. It does raise the possibility that SATB2 expressing ovarian mucinous carcinomas such as teratoma associated or anaplastic carcinomas might be associated with a slightly worse outcome, although the current literature is very limited in this area.(29) The finding contrasts with previous reports of a survival benefit with SATB2 expression in colorectal cancer.(30) We did not observe differences in overall survival based on the PAX8 expression status arguing against a biological split of PAX8 positive versus negative mucinous carcinoma.

In future, molecular studies may assist in further refining classification. However, currently mutational profiles do not seem to achieve sensitivities and specificities of the biomarkers assessed here. For example, *TP53* or *KRAS* mutations are present in both ovarian mucinous and colorectal carcinomas and even though *APC* mutations are absent in ovarian mucinous carcinomas, and high in colorectal adenocarcinomas, this is limited to non-mucinous adenocarcinomas (88% vs 24% mucinous).(31) In addition, small numbers of mutations in *GNAS* have been reported mucinous ovarian tumors as well as in a subset of LAMN. (32-35)

Conclusion

The immunohistochemical profile of most “intestinal-type” primary ovarian mucinous primaries is distinct from lower gastrointestinal neoplasms. Our study provides strong evidence that SATB2 is a better marker than CK20 for the distinction of ovarian mucinous neoplasms from colorectal carcinomas and we recommend replacement of

CK20 by SATB2. In combination, CK7 and SATB2 efficiently help distinguish ovarian mucinous primaries from lower gastrointestinal metastasis, particularly if the distribution of staining in a 3-tier system is considered.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

Supplementary information is available at Modern Pathology's website.

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Figure legends

Figure 1

Immunohistochemical stains

First panel: typical staining pattern for an ovarian mucinous carcinoma: Hematoxylin and eosin (H&E) stain, CK7 diffuse, SATB2 absent, PAX8 nondiffuse, CDX2 nondiffuse, CK20 nondiffuse.

Second panel: typical staining pattern for low-grade appendiceal mucinous neoplasm: H&E stain, CK7 absent, SATB2 diffuse, PAX8 absent, CDX2 diffuse, CK20 diffuse

Third panel: typical staining pattern for colorectal carcinoma: H&E stain, CK7 absent, SATB2 diffuse, PAX8 absent, CDX2 diffuse, CK20 diffuse

Figure 2

Decision tree for the 3-tier interpretation of the CK7/SATB2 combination: If CK7 is absent, a lower gastrointestinal primary (red bar) is most likely regardless of the staining pattern of SATB2. If CK7 is nondiffuse and SATB2 is diffuse, a lower gastrointestinal primary should be favored. The category of CK7 nondiffuse and SATB2 absent or

nondiffuse suffers from low numbers and second line markers should be considered. If CK7 is diffuse and SATB2 is negative or focal, this represents an ovarian primary (blue bar) with 96% probability. However, if both CK7 and SATB2 are diffuse, this scenario more likely represents a lower gastrointestinal primary.

Figure 3 (a-c) Kaplan-Meier overall survival curves. **(a)** Overall survival in women diagnosed with mucinous carcinomas (n=214) by Stage (I/II vs. III/IV). **(b)** Overall survival in women diagnosed with mucinous carcinomas (n=214) by SATB2 expression (absent/present). **(c)** Overall survival in women diagnosed with mucinous carcinomas (n=214) by PAX8 expression (absent/present).

Table 1: Frequency of marker expression in ovarian and lower gastrointestinal tumors

		Lower gastrointestinal			Ovarian		
	Subtype	Total	CRC	Appendiceal	Total	MBOT	MC
	N	230	123	107	155	50	105
CK7	Absent (n, %)	186	110	76	4	3	1
		80.9	89.4	71.0	2.6	6.0	1.0
	Focal (n, %)	26	8	18	10	6	4
		11.3	6.5	16.8	6.5	12.0	3.8
	Diffuse (n, %)	18	5	13	141	41	100
		7.8	4.1	12.1	91.0	82.0	95.2
	Present (%)	19.1	10.6	29.0	97.4	94.0	99.0
CK20	Absent (n, %)	30	12	18	28	11	17
		13.0	9.8	16.8	18.1	22.0	16.2
	Focal (n, %)	38	26	12	60	16	44
		16.5	21.1	11.2	38.7	32.0	41.9
	Diffuse (n, %)	162	85	77	67	23	44
		70.4	69.1	72.0	43.2	46.0	41.9
	Present (%)	87.0	90.2	83.2	81.9	78.0	83.8
CDX2	Absent (n, %)	4	1	3	47	7	40
		1.7	0.8	2.8	30.3	14.0	38.1
	Focal (n, %)	18	12	6	60	20	40
		7.8	9.8	5.6	38.7	40.0	38.1
	Diffuse (n, %)	208	110	98	48	23	25
		90.4	89.4	91.6	31.0	46.0	23.8
	Present (%)	98.3	99.2	97.2	69.7	86.0	61.9
SATB2	Absent (n, %)	23	10	13	135	44	91
		10.0	8.1	12.1	87.1	88.0	86.7
	Focal (n, %)	23	19	4	11	3	8
		10.0	15.4	3.7	7.1	6.0	7.6
	Diffuse (n, %)	184	94	90	9	3	6
		80.0	76.4	84.1	5.8	6.0	5.7
	Present (%)	90.0	91.9	87.9	12.9	12.0	13.3
PAX8	Absent (n, %)	224	123	101	85	29	56
		97.4	100.0	94.4	54.8	58.0	53.3
	Focal (n, %)	0	0	0	52	14	38
		0.0	0.0	0.0	33.5	28.0	36.2
	Diffuse (n, %)	6	0	6	18	7	11
		2.6	0.0	5.6	11.6	14.0	10.5
	Present (%)	2.6	0.0	5.6	45.2	42.0	46.7

Table 2: Sensitivity, specificity and balanced accuracy of individual markers to predict a lower GI tumor in the test and expansion cohorts using different cut-offs

Test cohort

Binary criteria (absent vs. present)	Sensitivity	95% CI		Specificity	95% CI		Accuracy	95% CI	
SATB2 present	0.90	0.85	0.94	0.87	0.81	0.92	0.89	0.85	0.92
CK7 absent	0.81	0.75	0.86	0.97	0.94	0.99	0.88	0.84	0.91
PAX8 absent	0.97	0.94	0.99	0.45	0.37	0.53	0.76	0.72	0.81
CDX2 present	0.98	0.96	1.00	0.30	0.23	0.38	0.71	0.66	0.75
CK20 present	0.87	0.82	0.91	0.18	0.12	0.25	0.59	0.54	0.64
Binary criteria (diffuse vs. non-diffuse)									
CK7 nondiffuse	0.92	0.88	0.95	0.91	0.85	0.95	0.92	0.88	0.94
SATB2 diffuse	0.80	0.74	0.85	0.94	0.86	0.97	0.86	0.82	0.89
CDX2 diffuse	0.81	0.76	0.86	0.83	0.75	0.89	0.82	0.78	0.86
PAX8 nondiffuse	0.97	0.94	0.99	0.12	0.07	0.18	0.63	0.58	0.68
CK20 diffuse	0.71	0.64	0.77	0.56	0.48	0.64	0.65	0.60	0.70

Expansion cohort

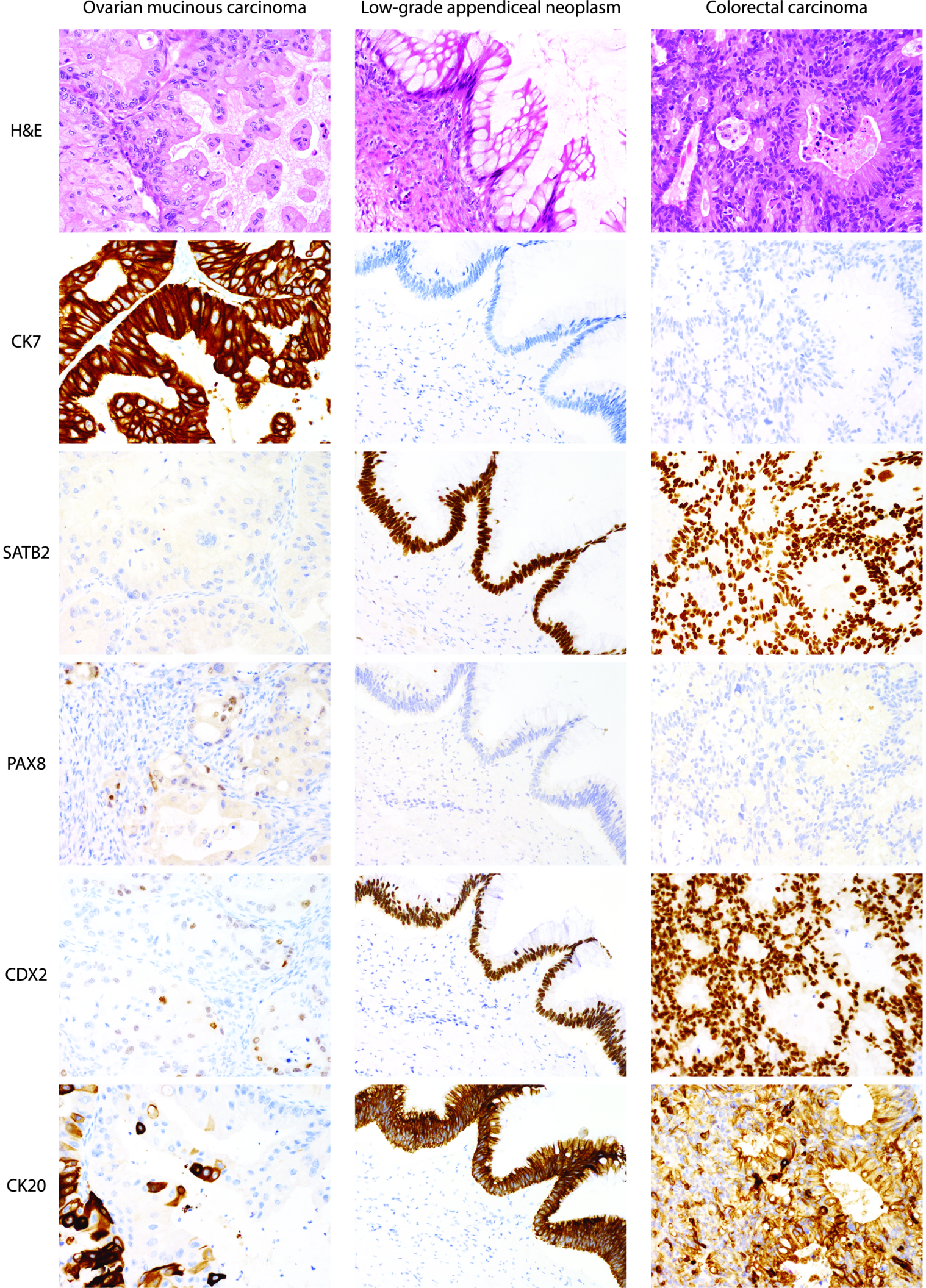
Binary criteria (absent vs. present)									
SATB2 present	0.95	0.91	0.97	0.88	0.83	0.92	0.92	0.88	0.94
PAX8 absent	0.97	0.94	0.99	0.41	0.35	0.49	0.71	0.67	0.75
Binary criteria (diffuse vs. non-diffuse)									
SATB2 diffuse	0.80	0.74	0.85	0.94	0.89	0.97	0.96	0.83	0.90
PAX8 nondiffuse	0.97	0.94	0.99	0.14	0.09	0.19	0.58	0.53	0.63

Table 3: Accuracy of markers in combination to predict a lower gastrointestinal primary								
			GI vs Ov		CRC vs Ov		App vs Ov	
Rank	Markers (N)	Marker combination assessed	**ROC**	**Accuracy**	ROC	Accuracy	ROC	Accuracy
1	5	CK7 3-tier, SATB2 3-tier, CDX2 3-tier, PAX8 3-tier, CK20 3-tier	**0.981**	**95.3**	0.988	96.0	0.976	94.7
2	4	CK7 3-tier, SATB2 3-tier, CDX2 3-tier, PAX8 3-tier	**0.978**	**95.8**	0.986	95.7	0.973	94.7
3	3	CK7 3-tier, SATB2 3-tier, PAX8 3-tier	**0.978**	**95.6**	0.986	95.7	0.969	93.9
4	3	CK7 3-tier, SATB2 3-tier, CDX2 3-tier	**0.976**	**95.6**	0.986	95.7	0.966	93.5
5	2	CK7 3-tier, SATB2 3-tier	**0.973**	**95.3**	0.984	95.3	0.957	93.5
6	5	SATB2 present, CK7 absent, PAX8 absent, CDX2 present, CK20 present	**0.972**	**95.3**	0.983	94.6	0.96	93.1
7	4	SATB2 present, CK7 absent, PAX8 absent, CDX2 present	**0.972**	**95.1**	0.982	96.0	0.959	92.0
8	3	SATB2 present, CK7 absent, PAX8 absent	**0.97**	**94.3**	0.981	95.0	0.957	91.6
9	3	CK7 3-tier, CDX2 3-tier, CK20 3-tier	**0.97**	**93.2**	0.98	95.0	0.962	91.6
10	2	SATB2 present, CK7 absent	**0.963**	**93.5**	0.979	93.9	0.945	90.5
11	2	CK7 nondiffuse, SATB2 present	**0.953**	**91.7**	0.963	93.2	0.942	89.7
12	3	CK7 absent, CDX2 present, CK20 present (clinical standard)	**0.922**	**87.5**	0.954	93.9	0.886	86.6

GI (gastrointestinal); Ov (ovarian); CRC (colorectal); App (appendiceal); ROC (Receiver Operating Characteristic)

Table 4: OTTA expansion cohort SATB2 and PAX8 expression by histotype											
Histotype		SATB2			PAX8			SATB2/PAX8			
	Total	absent n(%)	focal n(%)	diffuse n(%)	absent n(%)	focal n(%)	diffuse n(%)	SATB2-/PAX8+ n(%)	SATB2-/PAX8- n(%)	SATB2+/PAX8+ n(%)	SATB2+/PAX8- n(%)
MC	159	137 (86%)	11 (7%)	11 (7%)	97 (61%)	40 (25%)	22 (14%)	59 (37%)	78(49%)	3 (2%)	19 (12%)
MBOT	46	44 (96%)	0 (0%)	2 (4%)	23 (50%)	17 (37%)	6 (13%)	23 (50%)	21 (46%)	0 (0%)	2 (4%)
EC	515	439 (85%)	67 (13%)	9 (2%)	92 (18%)	148 (29%)	275 (53%)	364 (71%)	75 (15%)	59 (11%)	17 (3%)
CCC	386	371 (96%)	12 (3%)	3 (1%)	21 (5%)	29 (8%)	336 (87%)	351 (91%)	20 (5%)	14 (4%)	1 (0%)
HGSC	1698	1623 (96%)	69 (4%)	6 (0%)	80 (5%)	156 (9%)	1462 (86%)	1553 (91%)	70 (4%)	65 (4%)	10 (1%)
LGSC	72	67 (93%)	5 (7%)	0 (0%)	9 (13%)	4 (6%)	59 (82%)	58 (81%)	9 (13%)	5 (7%)	0 (0%)

Mucinous carcinoma (MC); Mucinous borderline ovarian tumor (MBOT); Endometrioid carcinoma (EC); Clear cell carcinoma (CCC); High grade serous carcinoma (HGSC); Low grade serous carcinoma (LGSC)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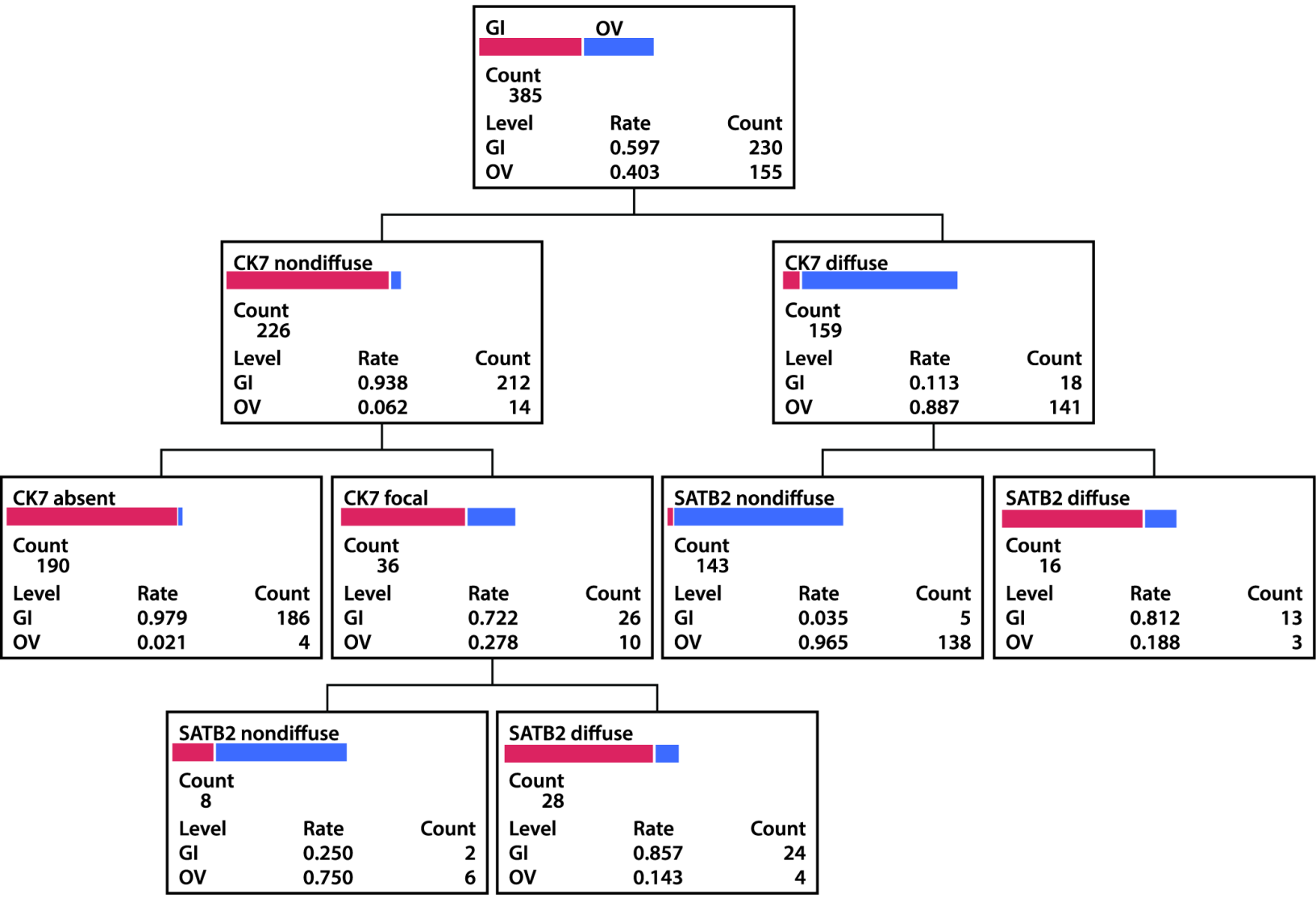
Low-grade appendiceal neoplasm
Colorectal carcinoma
H&E
CK7
SATB2
PAX8
CDX2
CK20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OV 0.403 155
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CK7 diffuse
Count 159
Level Rate Count
GI 0.113 18
OV 0.887 141
CK7 absent
Count 190
Level Rate Count
GI 0.979 186
OV 0.021 4
CK7 focal
Count 36
Level Rate Count
GI 0.722 26
OV 0.278 10
SATB2 nondiffuse
Count 143
Level Rate Count
GI 0.035 5
OV 0.965 138
SATB2 diffuse
Count 16
Level Rate Count
GI 0.812 13
OV 0.188 3
SATB2 nondiffuse
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Level Rate Count
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SATB2 diffuse
Count 28
Level Rate Count
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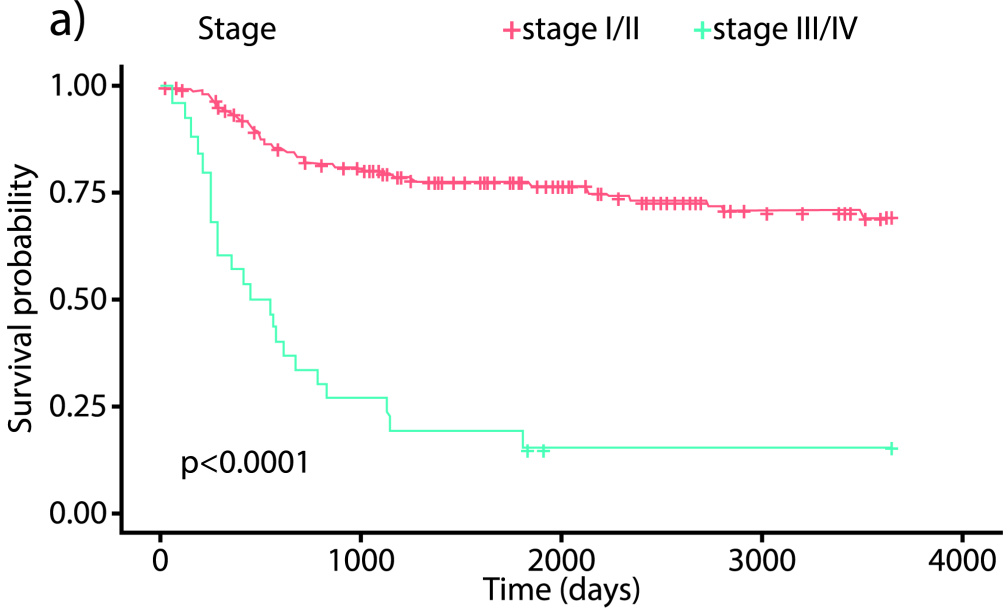
OV 0.143 4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Stage
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stage III/IV
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0
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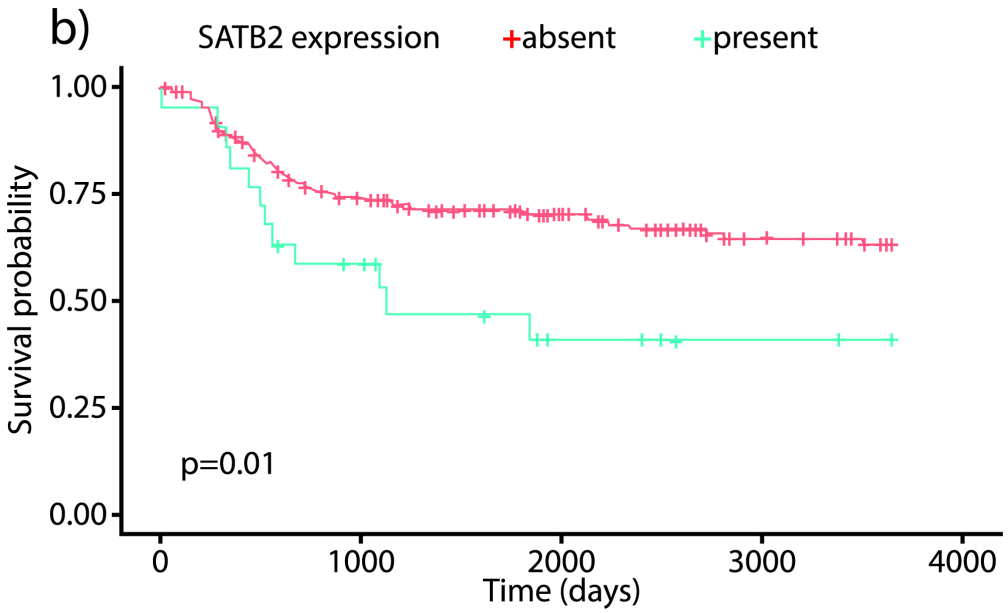
2000
3000
4000
Time (days)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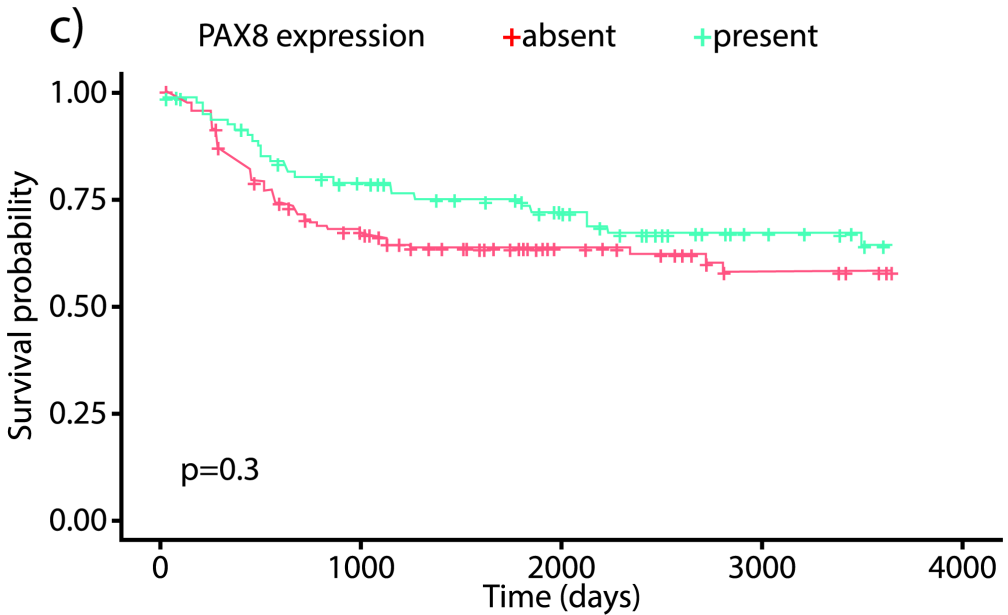
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4000
Time (days)