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Primary cilia discriminate benign nevi from thin melanoma

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Abstract of the Thesis

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Melanoma accounts for an overwhelming majority of mortality and morbidity from skin neoplasms. The aggressiveness of melanoma necessitates accurate and efficient diagnosis, which currently relies on analysis of histological samples by dermatopathologists as the gold standard. However, this gold standard allows for many uncertain diagnoses along with increased morbidity and even mortality. Recently, studies of primary cilia have shown their expression to be reduced in cutaneous malignancies, including melanoma. In a previous study, PC expression was markedly decreased in unambiguous lesions of melanoma compared to benign nevi, independent of other cell cycle variables. More recently it was demonstrated that the degree of graded cytologic severity in dysplastic nevi correlates with primary cilia loss. Building on these studies, the current study aimed to determine whether primary cilia expression in biopsy specimens could predict whether melanocytic lesions were benign nevi or melanoma based on their H&E diagnosis upon excision. Primary cilia were quantified in a biopsy and excision of the same lesion from 24 patients, and a model was built to analyze the sensitivity and specificity of the method. These results were compared to the Ki67 values for the same cohort of patients to assess the strengths of either method. Using the primary cilium as a biomarker, malignancy was able to be predicted from the biopsies with a sensitivity of 81.8% and a specificity of 80.0%.

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Acknowledgments

I would firstly like to acknowledge my mentor, Jiang Chen, for his support, guidance, and determination. Also Daniel Lozeau for his dermatopathologist's perspective and Elizabeth Venner for her help with statistics. Alejandra Moncayo helped immensely with the training cohort. Ning Yang and Yeun Ja Choi provided wonderful discussion. And of course my parents and fiancé for their endless support and love.

INTRODUCTION

Melanoma accounts for an overwhelming majority of mortality and morbidity from skin neoplasms, with over 65,000 diagnoses and 9,000 deaths in the US alone in 2012 (2015). The aggressiveness of melanoma necessitates accurate and efficient diagnosis, which currently relies on analysis of histological samples by dermatopathologists as the gold standard. However, this gold standard allows for many uncertain diagnoses along with increased morbidity and even mortality (Rigel *et al*., 2010).

Despite many recent advances in the molecular diagnosis of melanoma, the categorization of thin melanocytic lesions that share features of both nevi and melanoma remains a diagnostic challenge for dermatopathologists. Biomarkers for melanocytic differentiation (such as Melan-A/MART-1, MITF, and HMB45) have proven useful in delineating cell lineage as melanocytic, but often fail to differentiate benign from malignant neoplasms (Skelton *et al*., 1991). Markers such as Ki-67 and PHH3, which have been used for gauging the proliferative index of a tumor, have proven less useful in the context of thin atypical lesions (although may show some promise in lesions exhibiting subrabasalar spread of melanocytes) (Hall and LeBoit, 2014). Finally, given the frequency of thin atypical melanocytic lesions in clinical practice, molecular studies such as FISH and CGH are often costly and unwieldy for the low yield they provide in this context. Accordingly, H&E morphology (with or without a melanocytic stain) remain the gold standard in the diagnosis of dysplastic or Clark nevi and thin melanomas. Many histologically atypical nevi that are nevertheless benign are likely miscategorized as melanoma, and some thin melanomas may be missed.

Recently, studies of primary cilia (PC) have shown their expression to be reduced in cutaneous malignancies, including melanoma (Egeberg *et al*., 2012; Hassounah *et al*., 2013; Kim *et al*., 2011; Yuan *et al*., 2010). In a previous study, PC expression was markedly decreased in unambiguous lesions of melanoma compared to benign nevi, independent of other cell cycle variables (Snedecor *et al*., 2015). More recently it was demonstrated that the degree of graded cytologic severity in dysplastic nevi correlates with primary cilia loss (Lang *et al*., 2015). Building on these studies, the current study aimed to determine whether primary cilia expression in biopsy specimens could predict whether melanocytic lesions were benign nevi or melanoma based on their H&E diagnosis upon excision. Primary cilia were quantified in a biopsy and excision of the same lesion from 24 patients, and a model was built to analyze the sensitivity and specificity of the method. These results were compared to the Ki67 values for the same cohort of patients to assess the strengths of either method.

RESULTS

Biopsy and excision samples

A biopsy and excision of the same lesion were obtained from 24 patients (Table 1). A dermatopathologist [insert name] diagnosed the biopsies as "uncertain" and then diagnosed the excision as either "benign" or "malignant." The dermatopathologists diagnosed 11 excisions as benign and 13 excisions as malignant. These diagnoses were then applied back to the biopsies and compared to the primary cilia counts of the biopsies.

Primary cilia percentages stratify biopsies

We evaluated primary cilia in the biopsies of our 24 patient samples via immunofluorescence and microscopy. For the 11 biopsies whose resections were diagnosed as benign, the average number of melanocytes with a primary cilium was 26.73 ± 13.63%, whereas the average number of melanocytes and melanoma cells in biopsies whose resections were diagnosed as benign was 8.08 ± 3.67% (Figure 1 and Supp. Table 1). These groups were significantly different with a *P*-value of 0.0009.

Model uses primary cilia percentages to predict diagnoses of biopsies

Using a previously published set of data with 32 benign and 55 malignant samples as our training set, a cut-off value for primary cilia percentage was determined to be 9.5-10.5%. Using this cut-off, diagnoses for the biopsies (our testing set) were predicted: a biopsy with more than 10.5% ciliated cells would be predicted to be benign while a biopsy with less than 9.5% of its cells

ciliated would be predicted to be malignant (Figure 2). This model had an area of 97.7% under the receiving operating characteristic (ROC) curve and an odds ratio of 0.149 for every 5% decrease in the cilia percentage. According to this model, 9 biopsies were predicted to be benign, while 11 were diagnosed to be benign by their resection, and 8 biopsies were predicted to be malignant, while 10 were diagnosed to be malignant by their resection. This gave the model a sensitivity of 81.8% and a specificity of 80.0%.

Ki67 percentages stratify biopsies less significantly

To determine whether primary cilia would be a more effective diagnostic tool then pre-existing methods, we evaluated Ki67, a proliferation marker commonly used to assist diagnoses of melanoma, in the biopsies of our 24 patient samples via immunofluorescence and microscopy. For the 11 biopsies whose resections were diagnosed as benign, the average number of melanocytes expressing Ki67 was 0.49 ± 0.65%, whereas the average number of melanocytes and melanoma cells in biopsies whose resections were diagnosed as benign was 3.19 ± 2.50% (Figure 3). These groups were significantly different with a *P*-value of 0.01 – much less significant than the stratification via cilia counts (*P*=0.01).

Model using Ki67 percentages to predict diagnoses of biopsies is less effective

Using a previously published set of data with 10 benign and 25 malignant samples as our training set, a cut-off value for primary cilia percentage was determined to be 1.9-2.8% (Figure 4). This model had an area of 88.8% under the ROC curve and an odds ratio of 36.054 for every 5%

decrease in the cilia percentage. According to this model, 10 biopsies were predicted to be benign, all of which were diagnosed to be benign by their resection, and 4 biopsies were predicted to be malignant, while 7 were diagnosed to be malignant by their resection. This gave the model a sensitivity of 100.0% and a specificity of 57.1%. Overall, this model is less effective than the model using cilia percentage because of the large number of false negatives, which greatly increases the risk of mortality.

DISCUSSION

In this study we evaluated primary cilia in a cohort of 24 biopsies and their excisions and found that the rate of ciliation in the biopsies correlated with the diagnoses of their excisions: high ciliation in biopsies aligned with benign excisions and low ciliation in biopsies correlated with malignant excisions. This confirmed previous findings in which primary cilia loss correlated with malignancy in easily diagnosable cases (Snedecor 2015).

This finding gives promise for the use of primary cilia loss as a diagnostic marker for melanoma. Currently, the gold standard for melanoma diagnosis is histology and dermatopathologists (Rigel *et al*., 2010). However, even this standard is not as gold as it could be: a previous study had multiple dermatopathologists diagnose the same lesions and they had often called vastly different diagnoses (Braun *et al*., 2012). Since there is no quantitative biomarker available for diagnosing melanoma, this level of disagreement is not surprising, and using primary cilia has strong potential for aiding in accurate and efficient diagnoses. Furthermore, many lesions are difficult to diagnose from only a biopsy and require excision for diagnosis. With primary cilia labeling, lesions that would normally require excision for diagnosis can be diagnosed directly from their biopsy, thus eliminating the cost, stress, and scarring of excisions.

The specimens evaluated in the study were drawn from community private dermatology practices as well as an academic dermatology faculty practice, thus representing a cross-section of the type of atypical melanocytic lesions that are commonly biopsied. The results demonstrate that primary cilia expression discriminates benign nevi from thin melanomas with a high degree

of sensitivity and specificity. These findings support the recent observations of Lang et al, as well as the hypothesis that primary cilia expression may be a useful adjunct for dermatopathologists in the diagnosis of thin melanomas (Lang *et al*., 2015).

There are limitations to this study. Clinical follow-up data for the patients are not available, and the effect of patient age or other clinical factors is not known at this time. The mechanism of primary cilia loss in cutaneous malignancies is likewise not known, and there may be confounding factors relating to this knowledge gap. However, the findings are robust and warrant further study into the phenomenon of primary cilia loss in melanoma. As evaluation of primary cilia becomes technically feasible, reproducible, and cost-effective compared to other diagnostic modalities, it may become an important resource for dermatopathologists in an area where one has been sorely lacking.

MATERIALS AND METHODS

Training and testing cohorts

The training cohort contained 87 cases of melanocytic nevi and melanomas obtained from the archival collections of the Department of Pathology of Stony Brook Medicine between 2003 and 2011. These cases were previously analyzed for primary cilia and Ki67 percentages (Snedecor *et al*., 2015). Their use is approved by the IRB of Stony Brook University.

In the test cohort, paired samples, each consisting of a biopsy and its corresponding excision, were selected from the Thomas Jefferson University dermatopathology practice archives. Each of the biopsy specimens was diagnosed by a board-certified dermatopathologist as a "Clark nevus with atypical features" and was recommended for excision. Each excision specimen contained a residual melanocytic neoplasm that was subsequently diagnosed as either residual benign nevus or melanoma.

The mutational status is not known for either cohort.

Immunofluorescence and microscopy

Formalin-fixed paraffin-embedded sections were labeled for cilia, melanocytes/melanoma cells, and Ki67 via immunofluorescence, with EDTA antigen retrieval. Cilia were labeled with ARL13B (rabbit, 1:200, Proteintech, Chicago, IL), melanocytes and melanoma cells were

labeled with S100 (rabbit, 1:100, BioCare Medical, Concord, CA) or SOX10 (mouse, 1:200, Santa Cruz Biotechnology, Dallas, TX), and Ki67 was labeled with a mouse Ki67 antibody (BDPharmigen, Franklin Lakes, NJ). Secondary antibodies Alexa Fluor 488 and 594 from Thermo Fischer were used at a 1:250 dilution (Grand Island, NY). Coverslips were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and imaged on a Nikon Eclipse 80i microscope.

Cilia and Ki67 quantification

Cilia and Ki67 were counted using the ImageJ plugin, Cell Counter. For cilia, the number of SOX10 positive cells along with number of SOX10-positive cells containing a primary cilium (as shown by ARL13B staining) were counted and shown as a percentage of SOX10-positive cells containing a primary cilium. For Ki67, the number of S100-positive cells as well as the number of S100-positive cells also expressing Ki67 were counted and shown as a percentage of S100-positive cells also expressing Ki67. All data are displayed as percent (%) ± standard deviation. A students' T-test was used to determine significance, with $P < 0.05$ considered significant. Investigators were blinded to diagnosis until after counting was completed.

Predictive model building and testing

To compare how well cilia percentage and Ki67 percentage could predict the diagnosis of melanoma, two models were built and tested, one for each predictor. Previously published data were used to develop the models (Snedecor *et al*., 2015). The biopsy data for these predictors

were used as the test sets. All data analysis was done using SAS 9.3 (SAS Institute Inc., Cary, NC). SAS Proc Logistic was used to build each model, and each model's output was used to assess its ability to predict diagnosis, first with the training data (used to build the model) and then with the test data.

FIGURE LEGENDS

Table 1. Cases. Formalin-fixed paraffin-embedded sections of each case were obtained from the Thomas Jefferson University dermatopathology practice archives. Age, sex, and location of each case are listed. All biopsies were diagnosed as uncertain and then excised and diagnosed as benign or malignant. [*]atypical compound melanocytic nevus; [^]Clark type; [~]atypical junctional nevus; [+]residual; [$]melanoma *in situ* in association with a melanocytic nevus; [#]melanoma *in situ*.

Figure 1. Primary cilia are frequently lost in biopsies of malignant lesions. (**a–d**) Primary cilia and melanocytes/melanoma cells were labeled with ARL13B (green) and SOX10 (red), respectively, in a biopsy (left) and excision (right) of the same benign lesion (**a–b**) and in a biopsy (left) and excision (right) of the same malignant lesion (**c–d**). (**e**) Ciliated SOX10-positive cells shown as percentages. Samples were independently evaluated by three investigators with comparable results. Results obtained by one investigator are shown. Scale bar = 25 μm.

Figure 2. Predicted diagnoses of biopsies via primary cilia correlate with diagnoses of excisions. (**a**) The receiving operating characteristic (ROC) curve with an area of 97.7% under the curve. (**b**) Predicted probabilities curve. (**c**) Cilia percentages of biopsies. Biopsies predicted to be benign are shown in blue while those predicted to be malignant are shown in red. Diagnoses are based on excisions of the same lesions. Closed circles are matching predictions and diagnoses. Red hollow circles are false negatives and blue hollow circles are false positives. There is statistical significance when comparing the predicted benign group with the predicted malignant group (Student's T-test, $P = 0.0009$). (**d**) Predictive capabilities of the training cohort

and test (biopsy) cohort. The training cohort had a sensitivity of 92.7% and 84.4% and the test cohort had a sensitivity of 81.8% and a specificity of 80.0%.

Figure 3. Ki67 expression is frequently increased in biopsies of malignant lesions. (**a–d**) Ki67 and melanocytes/melanoma cells were labeled with Ki67 (red) and S100 (green), respectively, in a biopsy (left) and excision (right) of the same benign lesion (**a–b**) and in a biopsy (left) and excision (right) of the same malignant lesion (**c–d**). (**e**) S100-positive cells expressing Ki67 in biopsies shown as percentages. Samples were independently evaluated by three investigators with comparable results. Results obtained by one investigator are shown. Scale bar = 25 μm.

Figure 4. Predicted diagnoses of biopsies via Ki67 correlate with diagnoses of excisions. (**a**) The receiving operating characteristic (ROC) curve with an area of 88.8% under the curve. (**b**) Predicted probabilities curve. (**c**) Ki67 percentages of biopsies. Biopsies predicted to be benign are shown in blue while those predicted to be malignant are shown in red. Diagnoses are based on excisions of the same lesions. Closed circles are matching predictions and diagnoses. Blue hollow circles are false positives. There is statistical significance when comparing the predicted benign group with the predicted malignant group (Student's T-test, $P = 0.01$). (**d**) Predictive capabilities of the training cohort and test (biopsy) cohort. The training cohort had a sensitivity of 88.8% and 70.0% and the test cohort had a sensitivity of 100.0% and a specificity of 57.1%.

Supplemental Table 1. Cilia and Ki67 percentages in biopsies and matching excisions.

Values are shown as percentage ± standard deviation. "*" represents false positives and "**" represent false negatives.

Table 1. Cases

Case	Age	Sex	Location	Biopsy	Excision
1	47	F	thigh, right	Uncertain*	Benign+*
2	42	F	arm, right	Uncertain*	Benign+*
3	54	F	ear, right	Uncertain*	Benign+*
4	61	F	back, mid	Uncertain*	Benign+*
5	25	F	cheek, left	Uncertain*^	Benign+*
6	66	M	arm, left	Uncertain*^	Benign+*
7	39	F	back, right lower	Uncertain*^	Benign+*
8	55	M	back, upper	Uncertain*	Benign+*: superficial congenital pattern
9	35	F	forearm, right	Uncertain*	Benign: Residual intradermal melanocytic nevus
10	79	M	chest	Uncertain*	Benign: Residual intraepidermal melanocytic nevus
11	35	F	shoulder, left	Uncertain*	Benign+~
12	65	M	back	Uncertain~	Malignant: Favor melanoma in situ (residual intraepidermal proliferation)
13	46	F	back, upper central	Uncertain~	Malignant$
14	61	F	back	Uncertain~	Malignant$
15	73	M	back, right upper	Uncertain~^	Malignant$
16	65	M	back	Uncertain~^	Malignant$
17	50	M	arm, right	Uncertain~^	Malignant$
18	45	F	back, left	Uncertain*^	Malignant$
19	43	M	breast, right	Uncertain~	Malignant#
20	77	M	shoulder, right	Uncertain~	Malignant#
21	79	M	back, left upper	Uncertain~	Malignant#
22	71	M	back, right lower	Uncertain~^	Malignant#
23	60	M	clavicle, right	Uncertain~^	Malignant#
24	66	F	foot, right dorsal	Uncertain~^	Malignant: Melanoma 0.45mm

*atypical compound melanocytic nevus; ^Clark type; ~atypical junctional nevus; +residual; $melanoma *in situ* in association with a melanocytic nevus; #melanoma *in situ*.

Figure 1. Primary cilia are frequently lost in biopsies of malignant lesions.

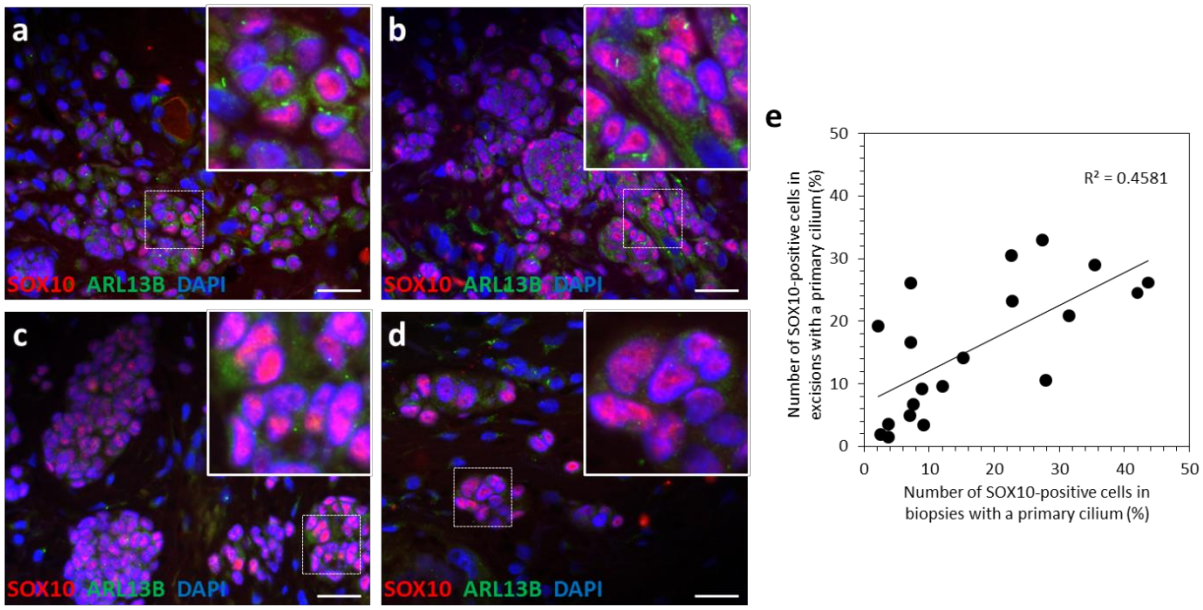


Figure 2. Predicted diagnoses of biopsies via primary cilia correlate with diagnoses of excisions.

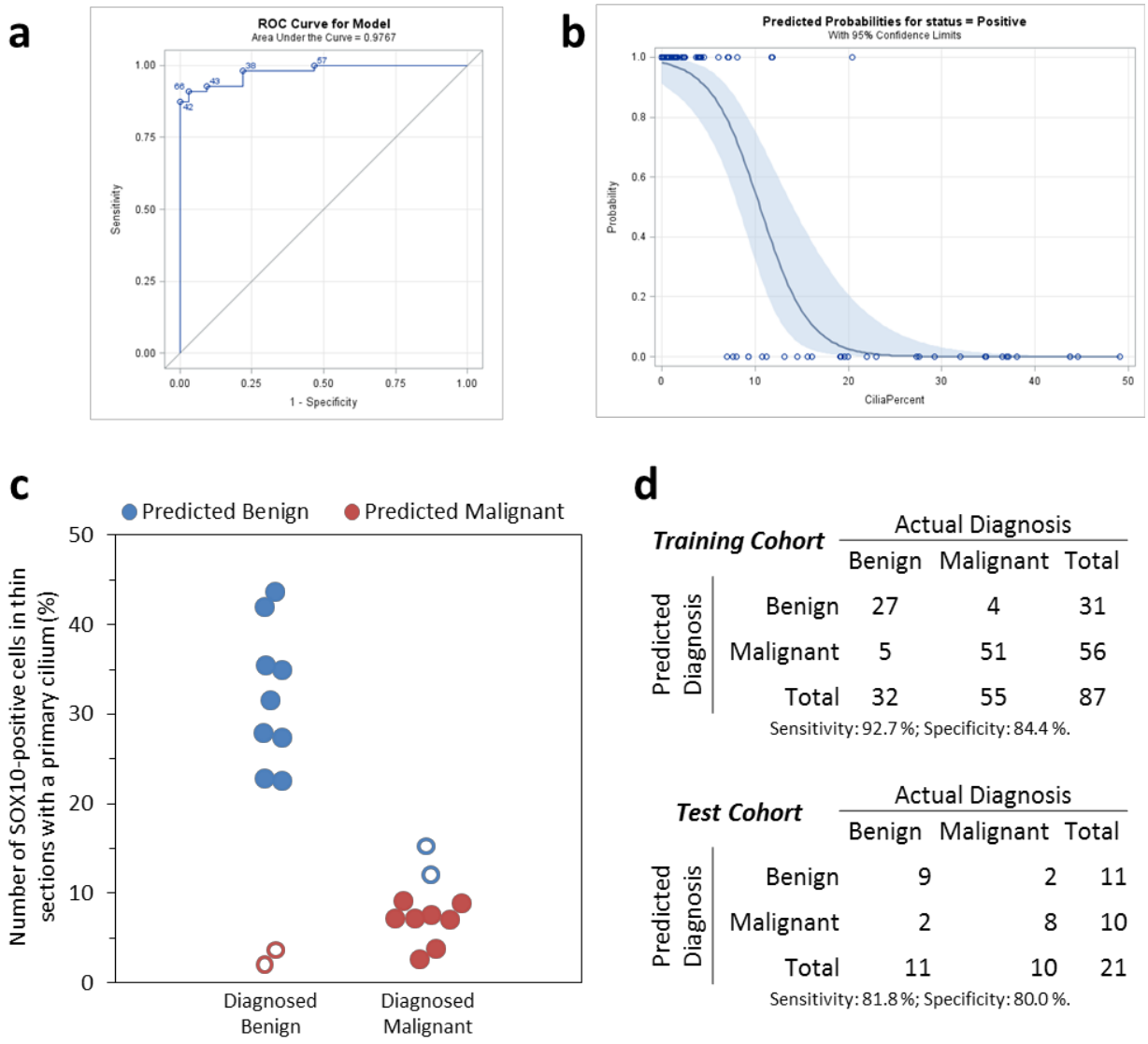
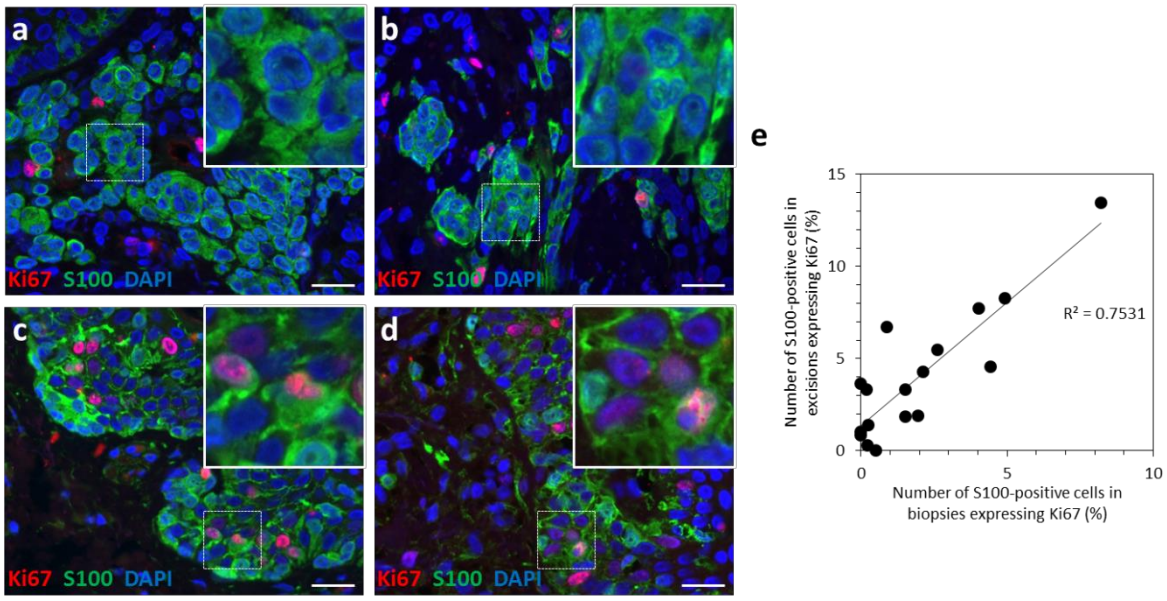


Figure 3. Ki67 expression is frequently increased in biopsies of malignant lesions.



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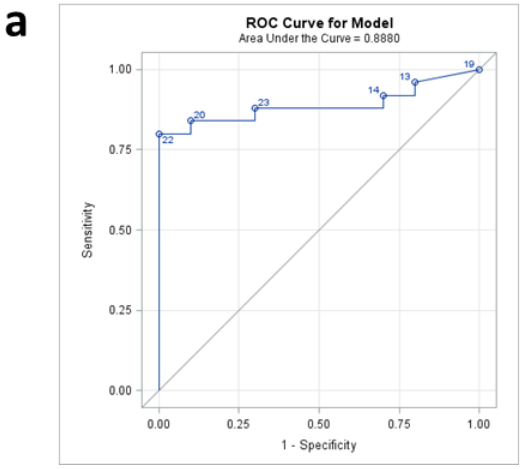

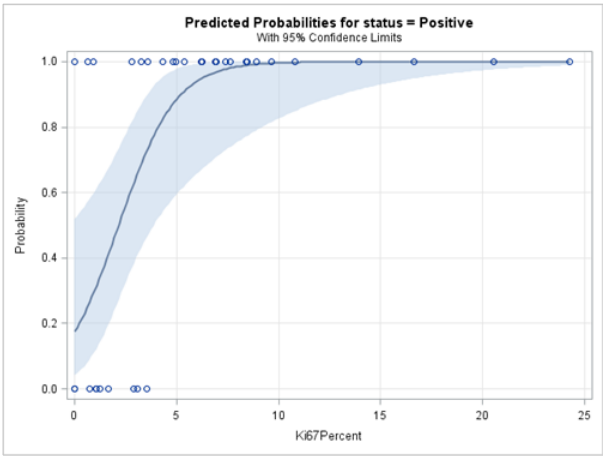

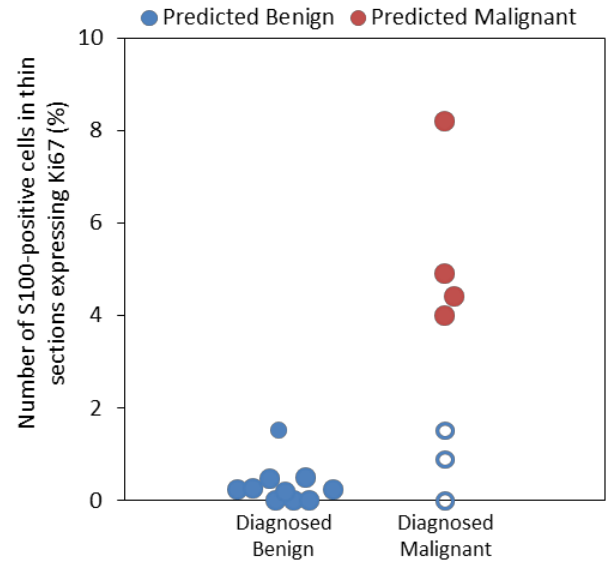

d

Training Cohort

Predicted Diagnosis	Actual Diagnosis: Benign	Malignant	Total
Benign	7	3	10
Malignant	3	22	25
Total	10	25	35

Sensitivity: 88.0 %; Specificity: 70.0 %.

Test Cohort

Predicted Diagnosis	Actual Diagnosis: Benign	Malignant	Total
Benign	10	3	13
Malignant	0	4	4
Total	10	7	17

Sensitivity: 100.0 %; Specificity: 57.1 %.

Supplemental Table 1. Cilia percentages in biopsies and matching excisions.

	Number of SOX10-positive Cells With a Primary Cilium (% ± Standard Deviation)		Number of S100-positive Cells Expressing Ki67 (% ± Standard Deviation)	
Case	Thin Section	Resection	Thin Section	Resection
1	2.08 ± 2.95*	19.29 ± 4.86	0.00 ± 0.00	0.85 ± 0.78
2	31.53 ± 22.71	20.96 ± 11.96	0.24 ± 0.42	n/a
3	3.7 ± 1.03*	3.57 ± 0.00	0.26 ± 0.45	1.39 ± 2.41
4	43.68 ± 6.59	26.17 ± 8.25	0.20 ± 0.35	3.31 ± 2.10
5	34.92 ± 3.76	n/a	0.00 ± 0.00	n/a
6	35.43 ± 2.25	28.95 ± 7.83	0.51 ± 0.87	0.00 ± 0.00
7	22.8 ± 3.71	23.26 ± 4.33	1.53 ± 0.35	3.31 ± 1.56
8	27.97 ± 4.57	10.53 ± 0.00	0.47 ± 0.81	n/a
9	22.58 ± 0.66	30.53 ± 10.09	1.94*	1.89 ± 1.12
10	27.4 ± 2.92	32.99 ± 13.22	0.23 ± 0.40	0.29 ± 0.50
11	41.97 ± 1.46	24.51 ± 12.74	0.00 ± 0.00	1.01 ± 1.75
12	7.09 ± 2.24	4.9 ± 5.30	4.92 ± 4.59	8.28 ± 9.20
13	8.94 ± 5.87	9.2 ± 8.07	2.61 ± 1.03	5.47 ± 1.55
14	n/a	n/a	n/a	3.53 ± 0.66
15	2.58 ± 3.13	1.91 ± 0.57	1.52 ± 0.43**	1.84 ± 0.82
16	n/a	7.79 ± 3.63	4.42 ± 2.97	4.55
17	7.14 ± 0.00	16.59 ± 5.79	8.20 ± 6.77	13.45 ± 3.31
18	7.59 ± 2.74	6.78 ± 0.75	0.90 ± 0.78**	6.70 ± 2.66
19	9.17 ± 8.11	3.49 ± 0.51	0.00 ± 0.00**	3.64 ± 3.11
20	3.76 ± 3.37	1.45 ± 1.34	n/a	2.94 ± 3.14
21	7.19 ± 2.93	26.06 ± 3.14	4.02 ± 3.43	7.73 ± 1.70
22	n/a	5.96 ± 3.04	n/a	n/a
23	15.26 ± 1.56**	14.15 ± 6.91	2.12 ± 0.79	4.31 ± 1.53
24	12.08 ± 1.37**	9.55 ± 4.38	n/a	n/a

*False positive; **False negative.

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