ACCSCIENCE PUBLISHING

Journal of Clinical and Translational Research

ORIGINAL ARTICLE

Flow cytometry in oral cytology: Improved brush biopsy-based delineation of oral malignant and potentially malignant lesions

Supplementary Files

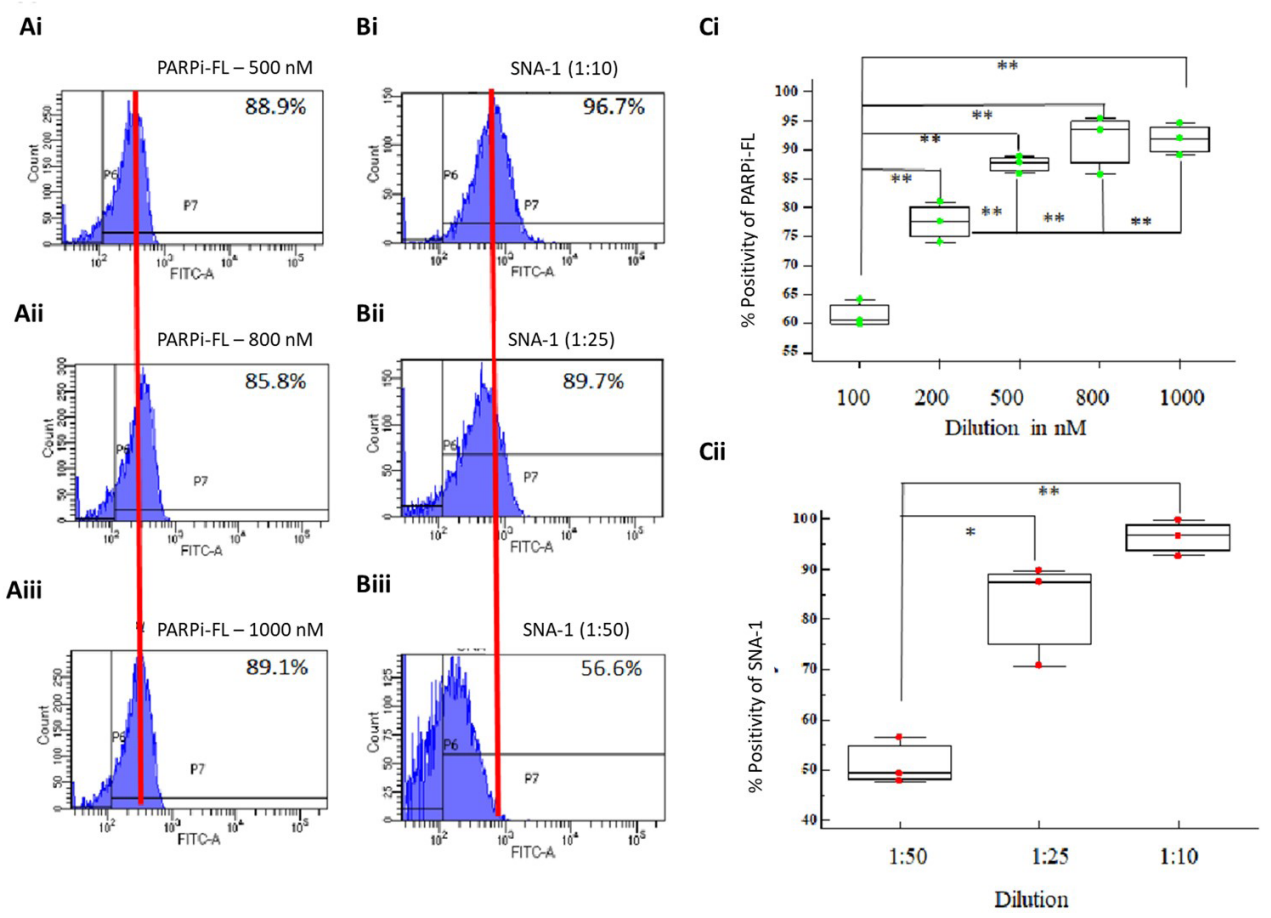


Figure S1. Optimization of the concentration of PARPi-FL and SNA-1 marker in the CAL-27 cell line. The graph depicts the percentage positivity of PARPi-FL (Ai–Aiii, Ci) staining in various dilutions in triplicate experiments. The histogram (Ai-Aiii) showed no significant increase in percentage positivity across the concentrations (500 nM, 800 nM, and 1,000 nM). Similarly, the percentage positivity of SNA-1 (Bi–Biii, Cii) marker dilution in 1:10, 1:25, and 1:50 was also depicted.

Notes: *$p < 0.05$; **$p < 0.005$.

Abbreviations: FITC A: Fluorescein isothiocyanate area; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; SNA-1: Sambucus–Nigra–Agglutinin-1.

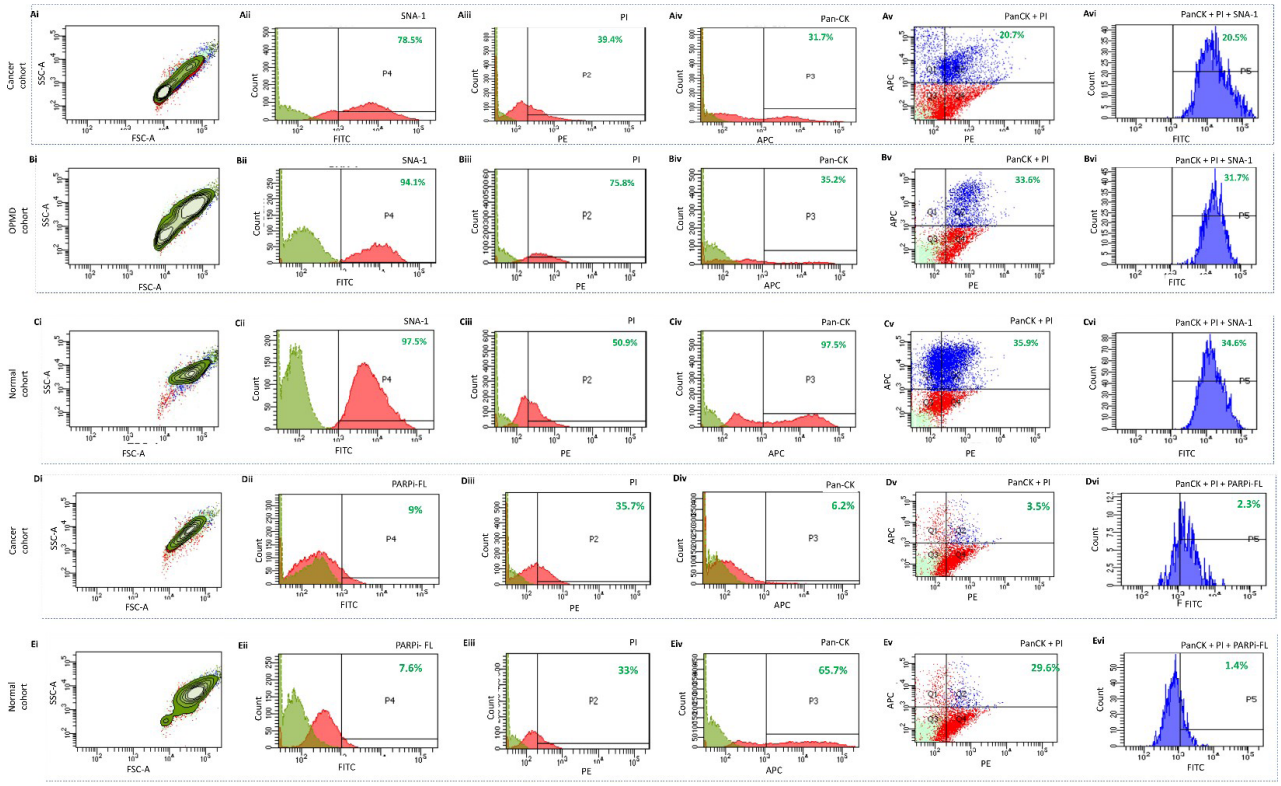


Figure S2. Representative flow cytometry images of marker profiling in pooled samples. Cohort-wise (A–E) distribution of SNA-1/Pan-CK/PI and PARPi-FL/Pan-CK/PI of Scatter plot, percentage positivity of single marker and multiplexing (i–vi).

Abbreviations: APC: Allophycocyanin; FITC: Fluorescein isothiocyanate; FSC-A: Forward scatter area; OPMD: Oral potentially malignant disorder; Pan-CK: Pan-Cytokeratin; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; PE: Phycoerythrin; PI: Propidium iodide; SNA-1: Sambucus–Nigra–Agglutinin-1; SSC-A: Side scatter area.

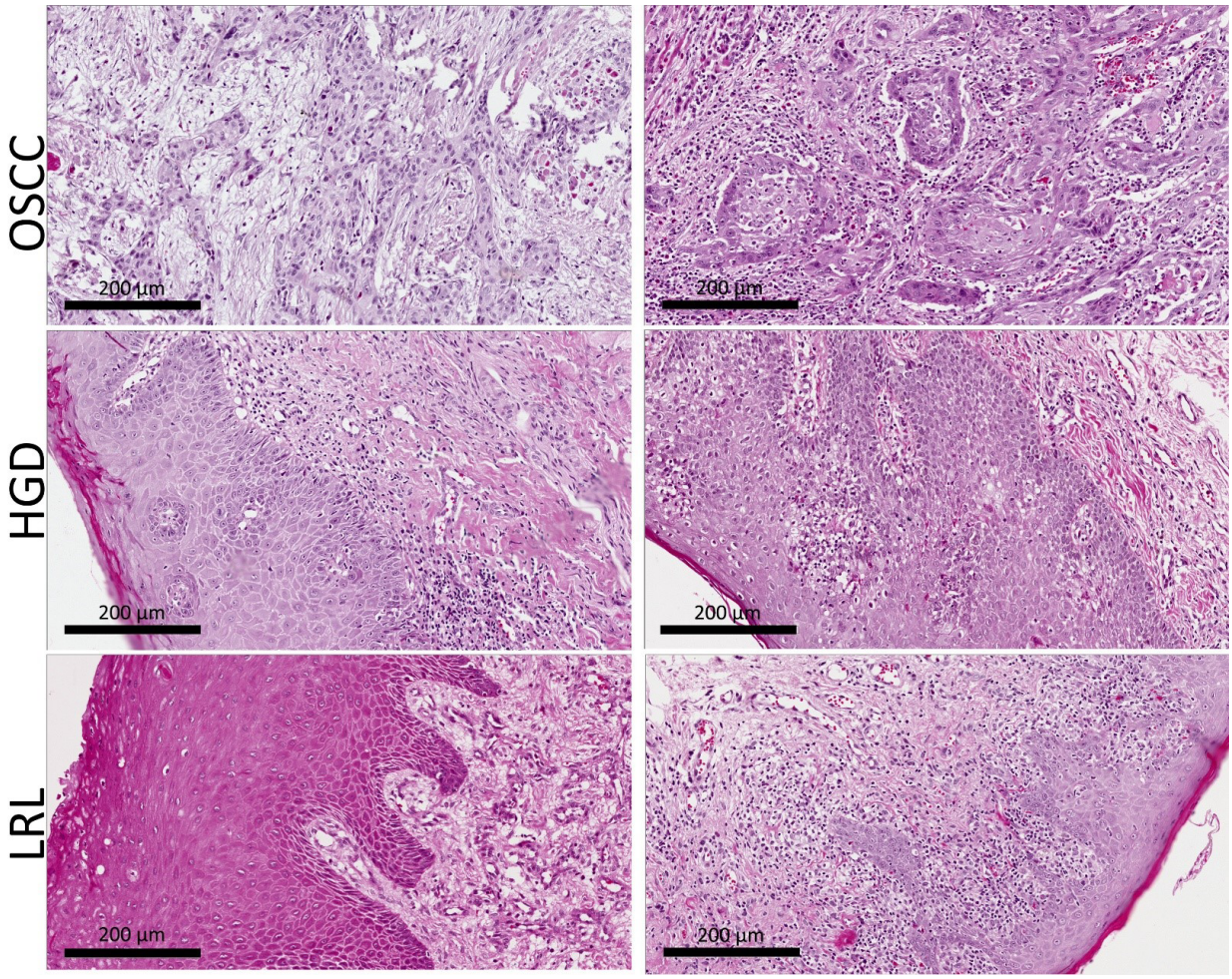


Figure S3. Representative histologic images, cohort-wise: Histologic images representing OSCC, HGD, and LRL, indicating the architectural changes. Scale bar: 200 µm; magnification: 10×.
Abbreviations: HGD: High-grade dysplasia; LRL: Low-risk lesion; OSCC: Oral squamous cell carcinoma.

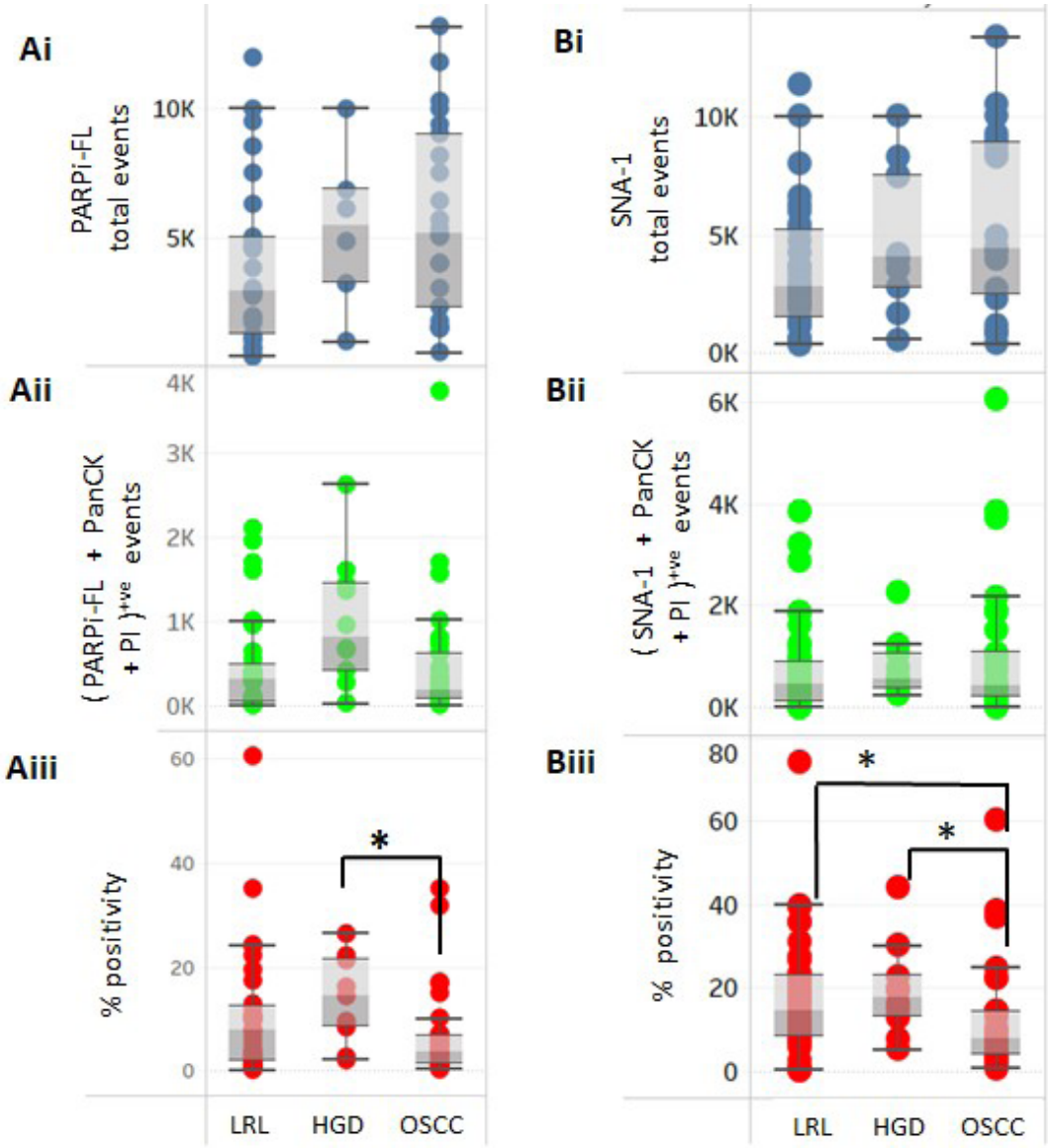


Figure S4. Distribution of total events, triple marker positive events, and percentage positivity of SNA-1 and PARPi-FL dataset. The box-and-whisker plot showed variation in total events across and between the cohorts (Ai, Bi). OSCC showed significantly ($p < 0.05$) less triple-marker positivity (SNA-1/PARPi-FL with Pan-CK and PI) in both datasets (Aii–iii, Bii–iii).

Abbreviations: HGD: High-grade dysplasia; LRL: Low-risk lesion; OSCC: Oral squamous cell carcinoma; Pan-CK: Pan-Cytokeratin; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; PI: Propidium iodide; SNA-1: Sambucus–Nigra–Agglutinin-1.

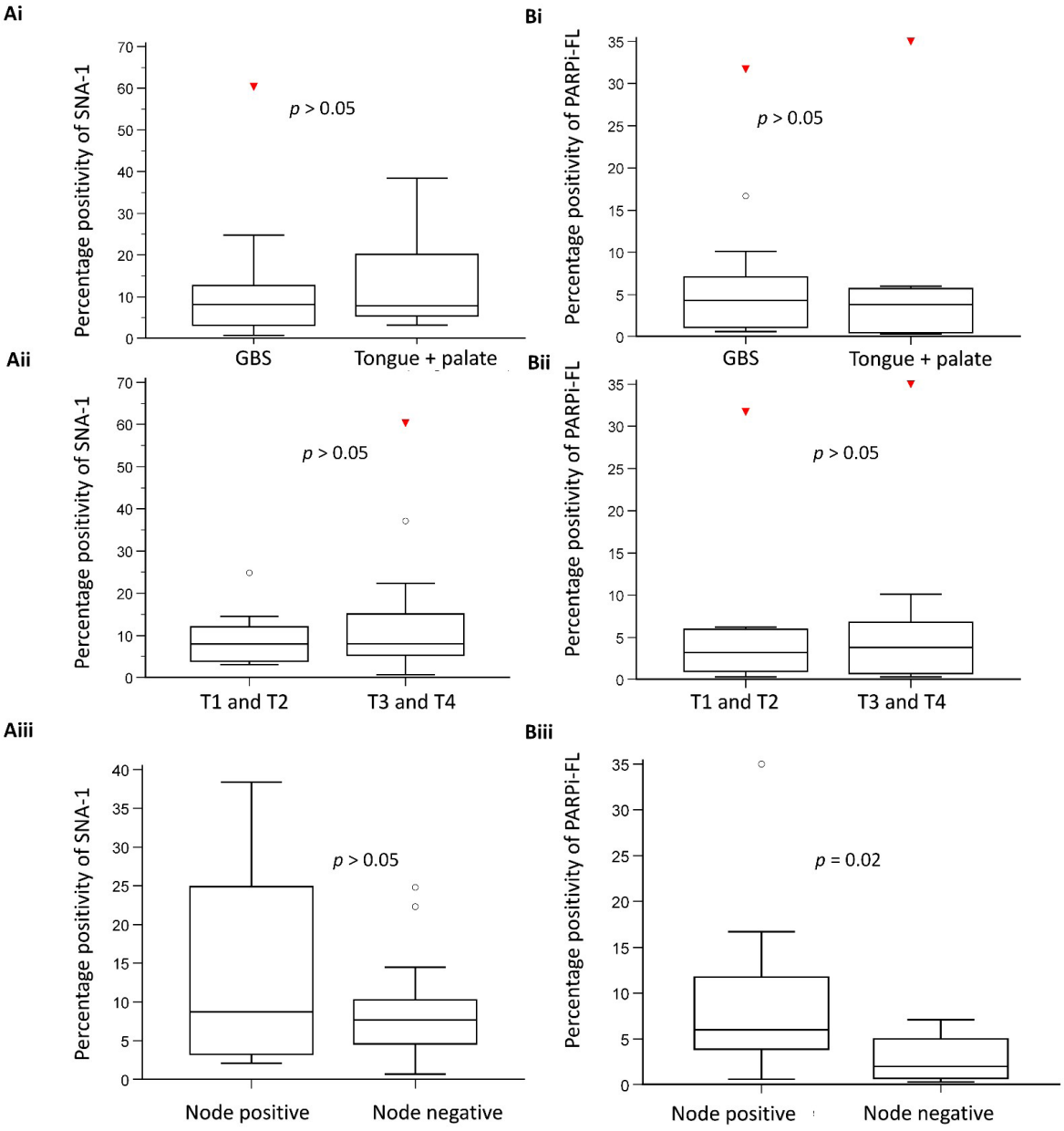


Figure S5. Clinicopathological correlation of OSCC cohorts with SNA-1 and PARPi-FL positivity. SNA-1 positivity in OSCC cohorts did not vary significantly with tumor site (Ai), T stage (Aii), and nodal status (Aiii). SNA-1 positivity shows an increasing trend in the node-positive group, although it is not statistically significant ($p > 0.05$). PARPi-FL positivity also did not differ by tumor site (Bi) or stage (Bii), whereas a significantly higher percentage of positivity was observed in node-positive patients (Biii).

Abbreviations: GBS: Gingivo-buccal-sulcus; OSCC: Oral squamous cell carcinoma; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; SNA-1: Sambucus–Nigra–Agglutinin-1.

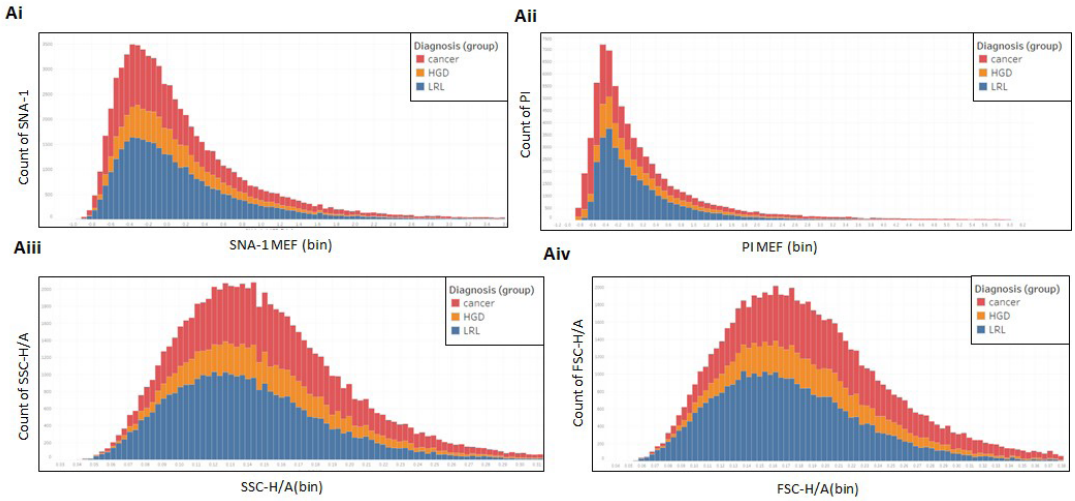


Figure S6. Distribution of SNA-1 dataset features. Histogram of MEF values of SNA-1 (Ai) and PI (Aii) shows a gradation in counts from cancer to HGD to LRL. Similar gradation is seen for ratio of scatter properties for SSC-H/A (Aiii) and FSC-H/A (Aiv) of the SNA-1 dataset.
Abbreviations: FSC: Forward scatter; H/A: Height/area ratio; HGD: High-grade dysplasia; LRL: Low-risk lesion; MEF: Molecular-equivalence fluorescence; SNA-1: Sambucus–Nigra–Agglutinin-1; SSC: Side scatter; FSC: Forward scatter.

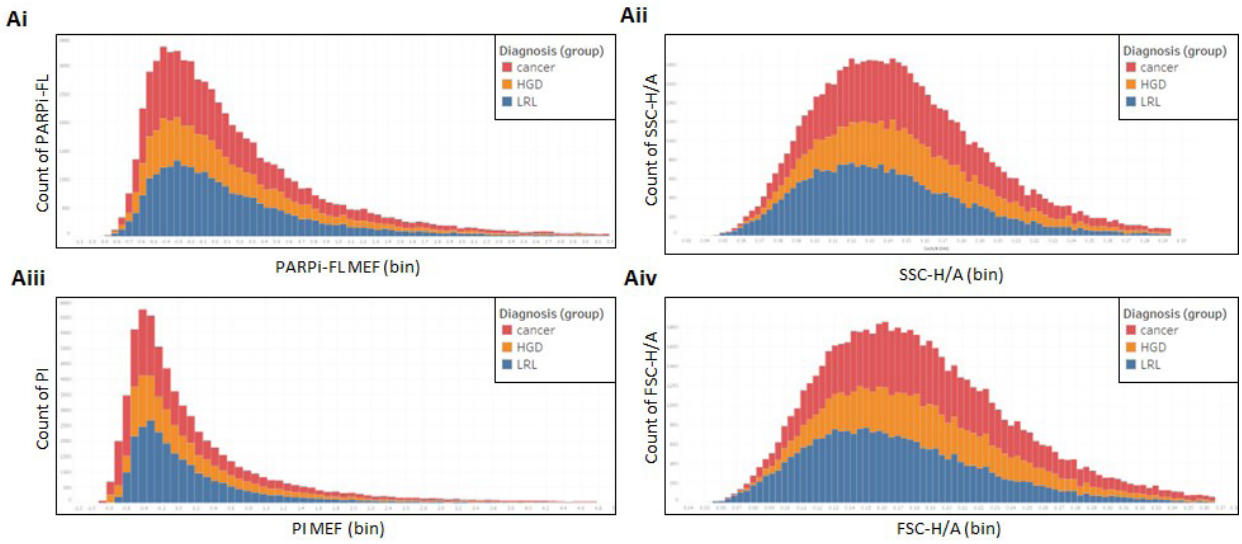


Figure S7. Distribution of PARPi-FL dataset. Histogram of MEF values of PARPi-FL (Ai) and PI (Aiii) shows gradation from cancer to HGD to LRL. Histogram of H/A ratio of scatter properties for SSC-H/A (Aii) and FSC-H/A (Aiii) of PARPi-FL dataset also shows similar pattern.
Abbreviations: FSC: Forward scatter; H/A: Height/area ratio; HGD: High-grade dysplasia; LRL: Low-risk lesion; MEF: Molecular-equivalence fluorescence; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; PI: Propidium iodide; SSC: Side scatter; FSC: Forward scatter.

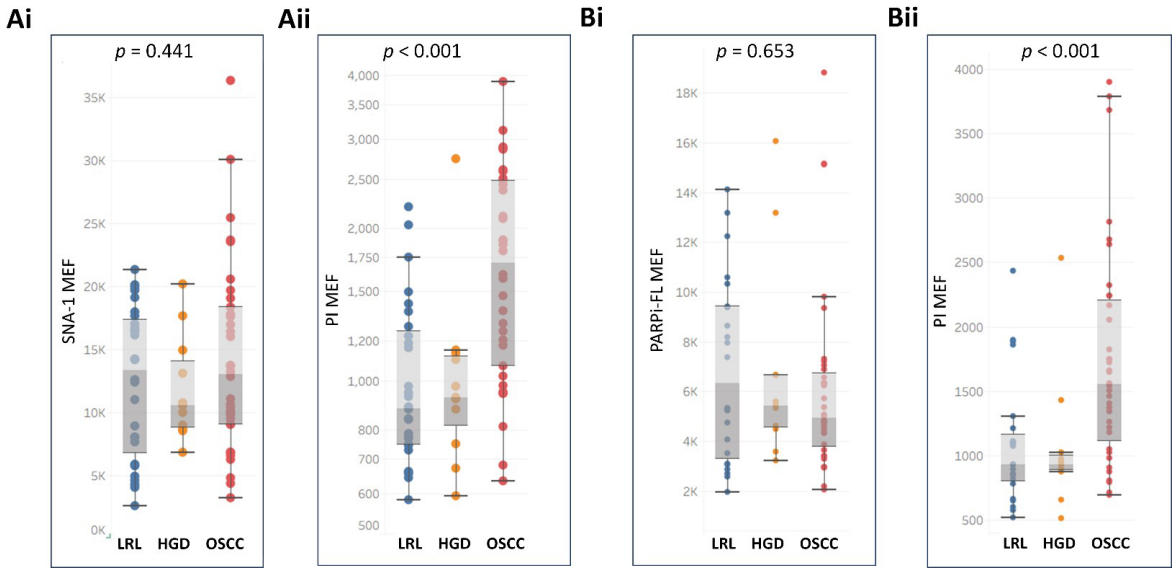
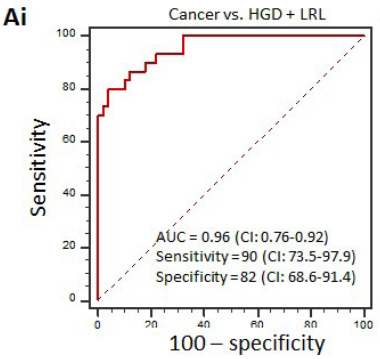


Figure S8. Mean fluorescence Intensity of SNA-1 and PARPi-FL dataset. (Ai–ii) The MEF values of mean fluorescent intensity showed no difference in SNA-1 expression, whereas the PI showed a significant difference across cohorts. (Bi–ii) No difference in MEF mean fluorescent intensities for PARPi-FL was observed, but significant differences were detected for PI.

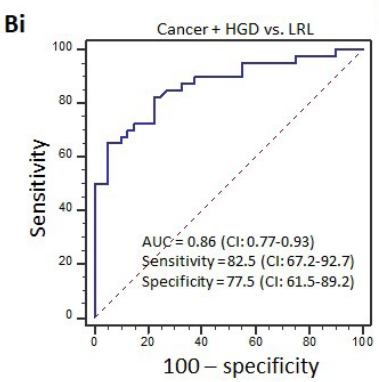
Abbreviations: HGD: High-grade dysplasia; LRL: Low-risk lesion; MEF: Molecular-equivalence fluorescence; OSCC: Oral squamous cell carcinoma; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; PI: Propidium iodide; SNA-1: Sambucus–Nigra–Agglutinin-1.



Aii

Cancer vs. HGD + LRL

Variable	Coefficient	Std. Error	*p*
SNA-1	6.5279	3.2602	0.0452
FSC-H/A	1,115.9551	538.3040	0.0382
PI	1.7773	0.6306	0.0048



Bii

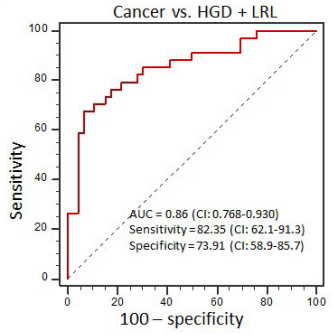
Cancer + HGD vs. LRL

Variable	Coefficient	Std. Error	*p*
SNA-1	0.1977	1.9290	0.9184
FSC-H/A	1,155.9944	435.6599	0.0080
PI	0.9837	0.4316	0.0227
Constant	-4.9709		

Figure S9. Multivariate analysis of the SNA-1 dataset. The combination of features achieved the highest AUC (0.96) for distinguishing oral cancer from HGD and LRL (Ai, ii). The AUC (0.86) decreased with less specificity in delineating cancer and HGD from LRL (Bi, ii). Three features (SNA-1, PI, and FSC-H/A) are significant in delineating cancer (Aii).

Abbreviations: AUC: Area under the curve; CI: Confidence interval; FSC: Forward scatter; H/A: Height/area ratio; HGD: High-grade dysplasia; LRL: Low-risk lesion; MEF: Molecular-equivalence fluorescence; PI: Propidium iodide; SNA-1: Sambucus–Nigra–Agglutinin-1.

Ai

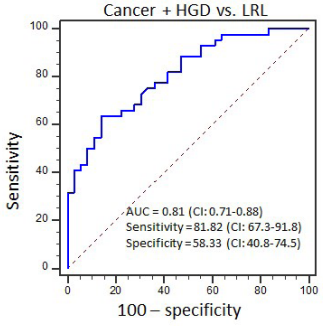


Aii

Cancer vs. HGD + LRL

Variable	Coefficient	Std. Error	P
PARPi-FL	1.3496	1.6108	0.4021
FSC-H/A	814.6370	626.7147	0.1937
PI	1.4384	0.4414	0.0011
SSC-H/A	-1,023.2394	1,073.4926	0.3405

Bi



Bii

Cancer + HGD Vs LRL

Variable	Coefficient	Std. Error	P
PARPi-FI	1.5459	1.5718	0.3253
FSC H/A	1,262.1399	653.3939	0.0534
PI	0.7910	0.3873	0.0411
SSC H/A	-1,343.3857	1,068.6789	0.2087

Figure S10. Multivariate analysis of PARPi-FL dataset. A combination of features achieved the highest AUC (0.86) for distinguishing oral cancer from HGD and LRL (Ai, ii). The AUC (0.81) decreased with less specificity in delineating cancer and HGD from LRL (Bi, ii). PI was significant in delineating cancer (Aii).

Abbreviations: AUC: Area under the curve; CI: Confidence interval; FSC: Forward scatter; H/A: Height/area ratio; HGD: High-grade dysplasia; LRL: Low-risk lesion; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; PI: Propidium iodide; SSC: Side scatter.

Table S1. Demographics and clinical parameters of subjects recruited according to selection criteria

Demographic and clinical parameters	*n*	%
Gender		
Male	86	63
Female	49	37
Age (21–77 years)		
≥40	88	65
<40	47	35
Habit history		
None	52	38
Tobacco	77	57
Alcohol	6	5
Histopathology/clinical diagnosis		
OSCC	55	34
OPMD	59	37
Normal/benign	46	29

Abbreviations: OPMD: Oral potentially malignant disorder; OSCC: Oral squamous cell carcinoma.

Table S2. Pan-CK and PI combination delineated the epithelial cells

S. No	Sample collected (oral subsites) and pooled (N_S)	Pan-CK%	PI%	% Pan-CK^{+ve} and PI^{+ve}	False positivity(Pan-CK^{+ve}/ PI^{-ve})
1	Normal mucosa of healthy participants (buccal mucosa: N_S = 2)	74.1	78.8	69.2	4.9
2	Normal mucosa of healthy participants (buccal mucosa: N_S = 1), normal mucosa of the contralateral site of oral cancer patients (floor of mouth: N_S =1, lateral border of tongue: N_S = 1)	53.9	45.2	30.0	23.9
3	Normal mucosa of the contralateral site of oral cancer patients (tongue: N_S = 1), cancer-buccal mucosa: N_S = 1, floor of mouth: N_S = 1	28.3	45.9	24.0	4.3
4	Cancer (buccal mucosa: N_S = 2), OPMD (buccal mucosa: N_S = 1), and normal (buccal mucosa: N_S = 2)	32.0	81.2	23.0	9.0

Abbreviations: NS: Number of samples; OPMD: Oral potentially malignant disorder; Pan-CK: Pan-Cytokeratin; PI: Propidium iodide.

Table S3. Percentage positivity of SNA-1, Pan-CK, and PI in pooled samples (cohort-wise)

S. No (Exp)	Cohort	Pan-CK %		PI %		SNA-1 %		SNA-1^{+ve}, Pan-CK^{+ve}, PI^{+ve}		Difference between I and II	SNA-1^{+ve}, Pan-CK^{+ve}	Difference between II and III (PI^{-ve})
		BC	AC	BC	AC	BC	AC	BC (I)	AC (II)		AC (III)	
1	Cancer (N_S = 3)	8.2	7.6	38.2	33.7	34.6	33.8	7.6	7.3	0.3	6.8	0.5
2	Normal (N_S = 4)	79.8	79.8	33.4	23.5	73.9	73.9	14.0	8.9	5.1	70.0	61.1
3	Cancer (N_S = 2)	32.9	32.9	85.3	85.3	93.1	93.1	31.7	31.7	0.0	34.2	2.5
4	OPMD (N_S = 3)	34.4	33.4	74.1	37.4	91.9	91.9	32.9	37.1	4.2	35.3	1.8
5	Normal (N_S = 4)	58.1	57.5	69.2	58.7	79.5	79.4	54.7	47.5	7.2	58.4	10.9
6	Cancer (N_S = 3)	31.4	31.4	60.9	39	31.4	77.8	27.7	20.5	7.2	32.7	12.2
7	Normal (N_S = 3)	60.3	60.3	77.6	49.1	94.1	94.1	53.8	34.6	19.2	61.7	27.1
8	OPMD (N_S = 3)	35.5	35.2	51.6	29.5	58.8	58.8	32.9	31.7	1.1	37.5	5.8

Abbreviations: AC: After compensation; BC: Before compensation; NS: Number of samples; OPMD: Oral Potentially Malignant Disorder; PI: Propidium iodide; Pan-CK: Pan-Cytokeratin; SNA-1: Sambucus–Nigra–Agglutinin-1.

Table S4. Percentage positivity of PARPi-FL, Pan-CK, and PI in pooled samples (cohort-wise)

S. No	Cohorts	Pan-CK %	PI %	PARPi-FL %	PARPi-FL^{+ve}, Pan-CK^{+ve} (I)	PARPi-FL $^{+ve}$, Pan-CK^{+ve}, PI^{+ve}(II)	PanCK^{+ve}, PI^{-ve} (false positive, difference between I and II)
1	Cancer (N_S = 2)	6.2	35.7	3.5	2.9	2.3	0.6
2	OPMD (N_S = 3)	22.1	44.8	14.4	11.5	8.9	2.6
3	Normal (N_S = 3)	38.6	65.2	35.7	22.5	20.1	2.4
4	Normal (N_S = 3)	65.7	33.0	7.6	7.1	5.7	1.4
5	Normal (N_S = 2)	86.0	37.2	8.8	8.3	6.9	1.4

Abbreviations: NS: Number of samples; OPMD: Oral potentially malignant disorder; Pan-CK: Pan-Cytokeratin; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; PI: Propidium iodide.

Table S5. Demographic and clinical characteristics of subjects recruited in the validation phase

Demographic and clinical parameters		*n*	%
Gender			
	Male	62	63.9
	Female	35	36.1
Age			
	>40	67	69.1
	<40	30	30.9
Risk factors			
	None	34	35.0
	Tobacco	60	61.9
	Alcohol	3	3.1
Histopathology/clinical diagnosis (sample-wise)			
	OSCC	41	37.6
	Severe/moderate dysplasia	12	11.0
	Mild dysplasia	8	7.4
	Normal/non-dysplasia	48	44.0

Abbreviation: OSCC: Oral squamous cell carcinoma.

Table S6. Median event counts and percentage of marker positivity for individual cohorts in the validation phase (NS = 80)

SNA-1	Median event counts (IQR)	Median Pan-CK^{+ve}PI^{+ve} events % (IQR)	Median Pan-CK^{+ve}PI^{+ve} SNA-1^{+ve} events % (IQR)
OSCC (N_S = 30)	10,000 (4,800)	11.5 (8.3)	6.8 (11.8)
HGD (N_S =10)	4,080 (4,600)	19.0 (3.7)	14.7 (10.9)
LRL (N_S = 40)	3,660 (4,170)	18.0 (12.5)	15.9 (15.3)
PARPi-FL	Median event counts (IQR)	Median Pan-CK^{+ve}PI^{+ve} % (IQR)	Median Pan-CK^{+ve}PI^{+ve}PARPi-FL^{+ve} % (IQR)
OSCC (N_S = 34)	6,960 (6,280)	11.0 (4.0)	3.5 (4.6)
HGD (N_S = 10)	8,420 (5,000)	22.3 (5.5)	7.1 (12.1)
LRL (N_S = 36)	4,600 (5,990)	13.0 (9.0)	11.9 (17.8)

Abbreviations: HGD: High-grade dysplasia; IQR: Interquartile range; LRL: Low-risk lesion; OSCC: Oral squamous cell carcinoma; NS: Number of samples; Pan-CK: Pan-Cytokeratin; PARPi-FL: Polyadenosine diphosphate-ribose polymerase inhibitor; PI: Propidium iodide; SNA-1: Sambucus–Nigra–Agglutinin-1.