Poster 2114

HPV-Driven Cancers Show Distinct Methylation Signatures in Cell-Free DNA (cfDNA)

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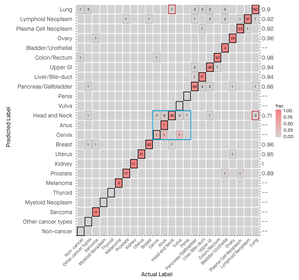
INTRODUCTION

- A noninvasive cell-free DNA (cfDNA)-based blood test designed to detect multiple cancers at pre-metastatic stages (stages I–III) could decrease cancer mortality.[1]
- For such a multi-cancer test to be effective at population scale, it should:
  - Detect clinically significant cancers in an elevated-risk population (eg, older than 50 years) with a fixed and low false positive rate (ie, very high specificity [>99%]) to limit overdiagnosis and unnecessary diagnostic workups;
  - Predict a specific tissue of origin (TOO) to direct appropriate diagnostic work-up for detected cancers[2,3];
  - Be validated by prospective, multi-center, longitudinal, population-scale studies, with a large number of control individuals.
- The Circulating Cell-free Genome Atlas study (CCGA; NCT02889978) is a prospective, multi-center, case-control, observational study with longitudinal follow-up to support development of a plasma cfDNA-based multi-cancer early detection test.
  - In the second CCGA substudy, classifiers trained on methylation states in targeted genomic regions were used to detect cancer and predict TOO using cfDNA, achieving 99.3% specificity and 55% sensitivity. TOO was predicted in 96% of cases with a cancer-like signal; of these, the prediction was accurate in 93% of cases.[4]
- Some systematic misclassifications of head and neck (H&N) cancer with other cancers suggest biological complexity.
  - High-risk human papillomavirus (HPV) infections have been implicated in the etiology of cervical cancer and other anogenital cancers, as well as cancers of the upper aerodigestive tract.[5,6]
  - Similarly, TOO misclassifications in the second CCGA substudy occurred between tissues commonly affected by HPV-associated cancers – anus, cervix, and clinically confirmed HPV-positive H&N. Additionally, the TOO for cancers of the vulva and penis was predicted as H&N. (Figure 1)
  - TOO misclassification was also observed between H&N and lung cancers; this could be driven by commonalities in cancer type and site (squamous cell carcinomas of the upper airways and larynx) and risk factor (exposure to carcinogens from smoking).

Figure 1. TOO Classification in the Second CCGA Substudy



Cells highlighted in blue show TOO misclassification between HPV-associated cancer types.
Cells highlighted in red show TOO misclassification between H&N and lung cancer.
CCGA, Circulating Cell-free Genome Atlas; TOO, tissue of origin

- In this post-hoc analysis of a subgroup of participants from CCGA, we aimed to (a) explore the hypothesis that TOO misclassifications among HPV-associated cancers are driven by epigenetic similarity due to underlying HPV infections, and (b) improve the accuracy of TOO predictions for HPV-associated cancer types.

METHODS

Detection of HPV DNA Fragments in Plasma cfDNA Samples

- Sample collection, accessioning, storage, and processing were conducted as previously described.[4] Additionally, the hybridization capture panel contained probes targeting the HPV16 and HPV18 genomes. Probes were designed to tile the entire genomes and target both methylated and unmethylated copies of each sequence (assuming uniform methylation status). (Note: For purposes of this investigation, we have only considered HPV16 and HPV18, the high-risk HPV types most commonly associated with cancer types such as cervical, anogenital, and H&N cancers.)
- Plasma cfDNA samples of all participants were assessed for presence of HPV DNA fragments by counting the number of unique fragments mapping to the HPV16 and 18 genomes.
- For a subset of participants (n=57), HPV status was established based on pathology reports.

Cancer Status Classification Using HPV DNA Fragments in Plasma cfDNA Samples Versus Methylation Features

- Classification of cancer status using HPV DNA fragments in plasma cfDNA samples was performed using a cross-validated cutoff on the number of unique cfDNA fragments aligned to HPV16 and HPV18 targets in a sample.
- Classification of cancer status and TOO was conducted as previously described using a methylation-based classifier.[4]

Visualization of Methylation Features Among Misclassified Tissues

- To create an informative embedding, we first subset methylation features that were selected by the classifier as discriminatory in pairwise comparisons among HPV-associated cancer types and pairwise comparisons to lung cancers.
- Selected features were used to create a UMAP embedding of participants with the cancer types of interest, using the subset of cancers used to train the TOO classifier.

Development of a Specialist Classifier for TOO Prediction of HPV-Associated Cancers

- As an addition to the original methylation-based TOO classifier, a 3-class logistic regression classifier was trained using the same methylation features but restricted to cervical, anal, and H&N cancers. This specialized classifier was applied to produce new predictions for samples predicted as any of the 3 cancers by the methylation-based TOO classifier.

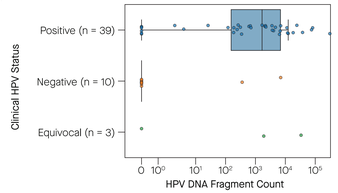
RESULTS

Detection of HPV DNA Fragments in Plasma cfDNA Samples

- In the overall population (N=3553; cancer, n=1530; non-cancer, n=2023), 72 had an HPV-associated cancer and 3481 did not have an HPV-associated cancer.
- HPV DNA fragment counts (HPV16 + HPV18 DNA fragment counts) in plasma cfDNA samples were mostly concordant with clinical diagnosis of HPV status, when available (Figure 2A).
- Of the tumor biopsies with HPV DNA fragments, HPV18 DNA fragments were most frequently observed in tumor biopsies of cervical cancer (84%); this aligned with reports of higher rates of HPV18 infection in cervical cancer versus anal and H&N cancer in literature (Figure 2B).[6,7]
- Among participants with H&N cancer, HPV DNA fragments were mainly detected in participants with tumors in the oropharyngeal region as opposed to tumors in the larynx and oral cavity; this aligned with reports of HPV-associated H&N cancers being more frequently observed in the oropharynx (Figure 2C).[6,7]

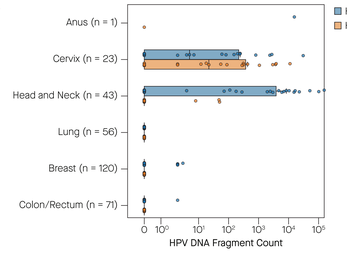
Figure 2. Clinical Observations From HPV DNA Fragment Counts. (A) HPV DNA Fragment Counts by Clinically Diagnosed HPV Status, (B) HPV16 Versus HPV18 DNA Fragment Counts in Tumor Biopsies by Tissue Type, (C) HPV DNA Fragment Counts in H&N Cancer Participants by Tumor Location

2A.



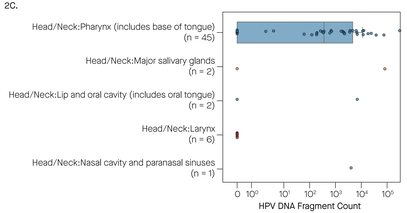
Presence of HPV DNA fragments in plasma cfDNA samples was more likely in participants with clinically confirmed HPV-positive status versus those with HPV-negative status.
Subset to high-signal plasma cfDNA samples and detected as having cancer.

2B.



HPV18 DNA fragments were most frequently observed in participants with cervical cancer. 84% (16/19) of tumor biopsies with non-zero HPV18 DNA fragment counts are cervical cancer.
Subset to tumor biopsy samples due to low number of plasma cfDNA samples from participants with cervical cancer.

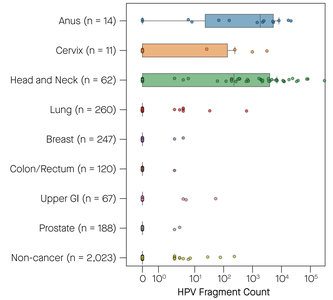
2C.



HPV DNA fragment counts were higher in participants with tumors in the oropharyngeal region versus those with tumors in the larynx and oral cavity.
Subset to high-signal plasma cfDNA samples and detected as having cancer.
HPV, human papillomavirus.

- Presence of HPV DNA fragments in plasma cfDNA samples was observed to be a highly specific indicator of HPV-associated cancer (Figure 3). In particular, HPV DNA fragments were detected in the plasma cfDNA samples of only 1.1% (40/3481) of participants with no reported HPV-associated cancer.

Figure 3. HPV DNA Fragment Counts in Plasma cfDNA Samples by Cancer Type



HPV DNA fragment counts in cfDNA samples were highest in participants with HPV-associated cancers such as H&N, cervical, and anorectal cancer.
Showing all cfDNA samples.
cfDNA, cell-free DNA; GI, gastrointestinal; HPV, human papillomavirus; H&N, head and neck.

Cancer Status Classification Using HPV DNA Fragments in Plasma cfDNA Samples Versus Methylation Features

- A cross-validated cutoff on the number of HPV DNA fragments in a plasma cfDNA sample (5.4 ± 1.2, across 6 folds) demonstrated high sensitivity for HPV-associated cancers at 99.8% specificity, achieving performance similar to the original methylation-based classifier for those cancer types (Table 1).
- The high specificity of HPV DNA fragments in plasma cfDNA samples for HPV-associated cancers, despite the prevalence of transient HPV infections in the US, was consistent with the lack of HPV viremia reported in the literature.[6,8-10]

Table 1. Comparison of Specificity and Sensitivity for Cross-Validated HPV DNA Fragment Cutoff and the Methylation-Based Classifier

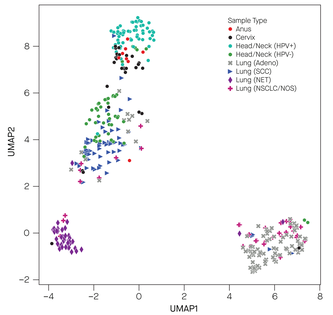
	HPV DNA Fragment Cutoff	Methylation-Based Classifier*
Specificity	99.8% (2018/2023)	99.6% (2015/2023)
Sensitivity		
Non-HPV-associated cancers	0.8% (11/1458)	53.8% (785/1458)
HPV-associated cancers	72.2% (52/72)	79.1% (57/72)
Anus	78.6% (11/14)	71.4% (10/14)
Cervix	36.4% (4/11)	45.4% (5/11)
HPV-positive H&N	81.1% (30/37)	97.3% (36/37)
Vulva	66.7% (6/9)	55.6% (5/9)
Penis	100% (1/1)	100% (1/1)

**Classifier same as that reported in Liu et al. (2020). Performance reported for the subgroup used for this post-hoc analysis.*
HPV, human papillomavirus; H&N, head and neck.

Visualization of Methylation Features Among Misclassified Tissues

- In the UMAP embedding (Figure 4), 4 distinct groups of participants were observed generally separated by lung cancer subtype and HPV signal (defined as presence or absence of HPV DNA fragments in plasma).
- Some notable exceptions to the participant clustering included
  - HPV signal-negative neuroendocrine cervical cancer (n=1) clustered with lung neuroendocrine tumor (NET; n=39) (cluster C)
  - HPV signal-negative cervical adenocarcinoma (n=1) and HPV signal-negative salivary gland cancers of the H&N (n=2) clustered with lung adenocarcinoma (n=79) and non-small cell lung cancer (NSCLC; n=26) (cluster D)
- There were 6 HPV signal-negative H&N cancer participants clustered with the HPV-associated cancers group (cluster A). Of these, 3 participants had sequenced tumor biopsies, all of which had non-zero HPV DNA fragments, indicating that the selected methylation features are informative for HPV signal in absence of observed HPV DNA fragments in plasma cfDNA samples.

Figure 4. UMAP Embedding of Detectable Cancers of the Anus, Cervix, Lung, and Head & Neck



	Cluster				
Cancer Type	A: HPV-Associated Cancers	B: Predominantly Non-HPV-Associated Squamous Cell Carcinomas	C: Predominantly Neuroendocrine Tumors	D: Predominantly Non-HPV-Associated Adenocarcinomas	TOTAL
Anus	7	1	0	0	8
Cervix	20	6	1	1	28
H&N (HPV signal +)	57	1	0	0	58
H&N (HPV signal –)	6	39	0	2	47
Lung (Adenocarcinoma)	0	12	0	79	91
Lung (NET)	0	1	39	2	42
Lung (NSCLC/NOS)	0	7	3	26	36
Lung (SCC)	2	57	0	5	64

Visualization of methylation features among misclassified tissues showed 4 distinct groups of participants generally separated by lung cancer subtype and HPV signal.
All data presented as n.
Subset to cancers used to train the TOO classifier.
H&N, head and neck; HPV, human papillomavirus; NET, neuroendocrine tumor; NOS, not otherwise specified; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

Development of a Specialist Classifier for TOO Prediction of HPV-Associated Cancers

- The development of a specialist classifier was motivated by the observation that despite the HPV-associated cancers forming a single cluster separate from HPV signal-negative samples, the HPV-associated cluster appeared to show some substructure and separation of H&N cancers from anal and cervical cancers.
- Applying the specialist classifier resulted in an increase in TOO prediction accuracy of anal cancers (Table 2).

Table 2. Comparison of TOO Prediction Accuracy for the HPV Specialist Classifier and the Methylation-Based Classifier

	Prediction Accuracy	
Cancer Type	Methylation-Based Classifier*	Methylation-Based Classifier + Methylation-Based HPV Specialist Classifier
Non-HPV associated cancers	89.8% (705/785)	89.8% (705/785)
Anus	10% (1/10)	100% (10/10)
Cervix	20% (1/5)	0% (0/5)
HPV+ H&N	97.2% (35/36)	94.4% (34/36)

**Classifier same as that reported in Liu et al. (2020). Performance reported for the subgroup used for this post-hoc analysis.*
H&N, head and neck; HPV, human papillomavirus; TOO, tissue of origin.

CONCLUSIONS

- HPV infection can induce similar epigenetic changes across multiple tissue types; although this could cause TOO misclassification, it indicates that the methylation-based classifier has learned to classify plasma cfDNA samples using epigenetic markers that reflect underlying biological signals and pathological processes.
- The presence of HPV DNA fragments in plasma cfDNA samples is a highly specific indicator of HPV-associated cancer; however, targeted sequencing of such DNA fragments does not improve cancer detection beyond that using a methylation-based assay able to detect multiple cancers independent of HPV status.
- Understanding the underlying cause of TOO misclassification can inform changes to classification architecture that could improve overall TOO prediction accuracy, furthering the goal of guiding effective clinical follow-up after signal detection from a multi-cancer early detection test.

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Disclosures

All authors are employees of GRAIL, Inc. with equity in the company. AJ holds equity in Illumina. JB holds equity in Roche. AMA is an advisor to and holds equity in Foresite Labs.

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