Next-generation sequencing analysis for detecting human papillomavirus in oral verrucous carcinoma.

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Manar Samman [1],*, Henry Wood[1,*], Caroline Conway[1], Stefano Berri[1], Monica Pentenero[2], Sergio Gandolfo[2], Adele Cassenti[3], Paola Cassoni[3], Abdul-Aziz Al Ajlan[4], A. William Barrett[5], Preetha Chengot[6], Kenneth MacLennan[1], Alec S. High[6], Pamela Rabbitts[1]

[1] Leeds Institute of Cancer and Pathology, University of Leeds, Leeds, United Kingdom
[2] Department of Oncology, Oral Medicine and Oral Oncology Unit, University of Turin, Turin, Italy
[3] Department of Medical Sciences, Pathology Unit, University of Turin, Turin, Italy
[4] Department of Pathology and Laboratory Medicine, National Guard Hospitals, Riyadh, Saudi Arabia
[5] Department of Histopathology, Queen Victoria Hospital, East Grinstead, United Kingdom
[6] Department of Histopathology, St James's University Hospital and Leeds Dental Institute, University of Leeds, Leeds, United Kingdom
*Joint first authors

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Objective

The etiology of oral verrucous carcinoma is unknown, and human papillomavirus 'involvement' remains contentious. The uncertainty can be attributed to varied detection procedures and difficulties in defining 'gold-standard' histologic criteria for diagnosing 'verrucous' lesions. Their paucity also hampers investigation. We aimed to analyze oral verrucous lesions for human papillomavirus (HPV) subtype genomes.

Study Design

We used next-generation sequencing for the detection of papillomavirus sequences, identifying subtypes and computing viral loads. We identified a total of 78 oral verrucous cases (62 carcinomas and 16 hyperplasias). DNA was extracted from all and sequenced at a coverage between 2.5% and 13%.

Results

An HPV-16 sequence was detected in 1 carcinoma and 1 hyperplasia, and an HPV-2 sequence was detected in 1 carcinoma out of the 78 cases, with viral loads of 2.24, 8.16, and 0.33 viral genomes per cell, respectively.

Conclusions

Our results indicate no conclusive human papillomavirus involvement in oral verrucous carcinoma or hyperplasia.

Statement of Clinical Relevance

We found that oral verrucous carcinomas and hyperplasias are rarely associated with human papillomavirus infection. Our next-generation sequencing methodology allowed us to test for all human viruses. We conclude that papillomavirus infections are not involved in the development of these tumors.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world, with an incidence of about 600 000 cases each year and a 5-year survival rate of only about 50%.[1] Around half of these cases occur in the oral cavity.[2] Most cases represent oral squamous cell carcinoma (OSCC), which is found mainly in older men exposed to tobacco and alcohol.[3] Verrucous carcinoma (VC) is a low-grade variant of squamous cell carcinoma (SCC) that rarely presents with distant metastasis.[4]

The association between human papillomavirus (HPV) and HNSCC is strongest among oropharyngeal squamous cell carcinomas (OPSCC), especially for cancers of the lingual and palatine tonsils.[5, 6, 7, 8]and [9] Furthermore, the risk of developing oropharyngeal cancer when adjusted for tobacco and alcohol use is substantially increased with high-risk HPV oral infections.[9] HPV has been identified in 45% to 95% of OPSCC[10, 11] and [12] and is believed to be an etiologic agent.[13] In 2012, Kansy et al.[14] conducted a systematic review encompassing 5338 patients to assess the role of high-risk HPV types in OSCC, and 30.1% revealed HPV-positive OSCC samples. Within this review, HPV type 16 was identified in 25.4% and type 18 in 18.1%. However, the role of HPV as a causative factor was not well defined.

In general, HPV-positive carcinomas have better prognosis and treatment response than HPV-negatives.[13, 15] and [16] Studies find a 2- to 3-year overall survival rate of about 60% for HPV-negative HNSCC, compared with about 85% for HPV-positives,[15] and [16] indicating the importance of accurate HPV detection and subtyping.

Oral verrucous carcinoma (OVC) accounts for 2% to 10% of all OSCC cases.[17] 'Verrucous' terminology is applied to lesions that show exophytic, keratotic surfaces made of blunt or sharp epithelial projections, filled with keratin invaginations, but without clear fibrovascular cores.[18] Histologically, OVC consists of thickened, club-shaped papillae and blunt stromal invaginations of well-differentiated squamous epithelium with marked keratinization (Figure 1, A, B), with the squamous epithelium lacking cytologic criteria of malignancy. OVC invades underlying stroma with a pushing, rather than infiltrating, front.[19] Oral verrucous hyperplasia (OVH) (Figure 2) and proliferative verrucous leukoplakia are both poorly defined but are believed to be histologic precursors of OVC and possibly OSCC.[20 and 21] Because a verrucous appearance suggests a viral etiology, this has prompted a number of investigations for HPV.[22] and [23] Patel et al.[24] recently concluded that verrucous carcinomas of the head and neck lacked transcriptionally active high-risk human papillomavirus, but they concluded with "our study numbers, although relatively generous for a tumor as rare as verrucous carcinoma, are still relatively small and may not reflect results on larger cohorts of these tumors."

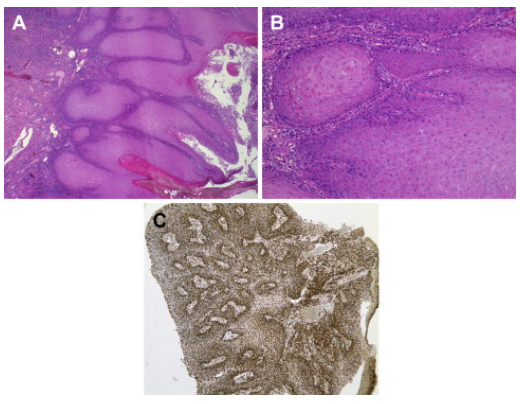

Fig. 1

A, Photomicrograph of oral verrucous carcinoma showing 'extension' into underlying mucous salivary glands. It 'retains' the bulbous rete seen in verrucous hyperplasia, but clearly has a 'pushing' advancing front that now extends considerably deeper than adjacent normal epithelium (hematoxylin-eosin, original magnification approximately × 100). **B**, A higher-power view of the deep margin from panel A. Note the lack of nuclear pleomorphism (hematoxylin-eosin, original magnification approximately × 400). **C**, Photomicrograph of $p16^{INK4a}$ immunohistochemical staining of the only HPV16-positive, tongue-based OVC identified by NGS. A majority of the nuclei are showing strong positive staining (original magnification approximately × 250) (antibody F-12: sc-1661; Santa Cruz Biotechnology Inc; using high-pH range settings on Dako Autostainer Link 48; Ely, United Kingdom). (*HPV*, human papillomavirus; *OVC*, oral verrucous carcinoma; *NGS*, next-generation sequencing.)

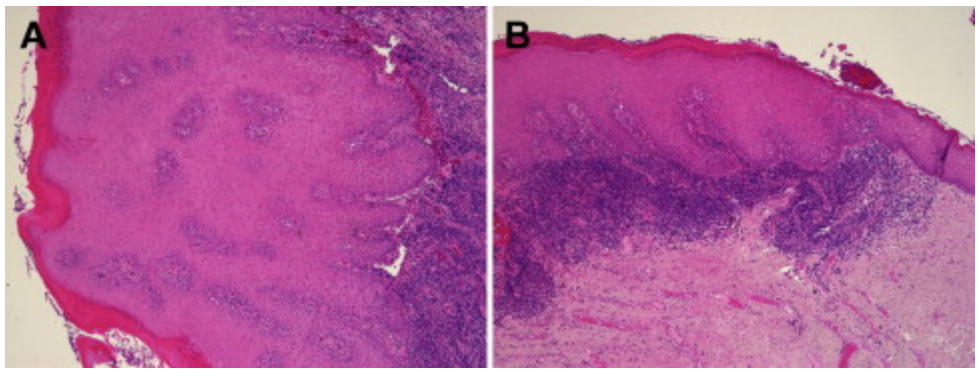

Fig. 2.

A, B, Photomicrograph of oral verrucous hyperplasia showing markedly acanthotic epithelium, with bulbous, club-shaped papillae lacking significant cellular atypia (A), adjacent to more normal epithelium (B) (A, B, hematoxylin-eosin, original magnification approximately ×250). In comparison with Figure 1, the affected epithelium does not show any significant 'deep extension.'

Most studies (TableI)[23, 25, 26, 27, 28, 29, 30 and 31] relied on polymerase chain reaction (PCR) or in situ hybridization for detection and did not identify HPV transcriptional activity markers, did not quantitate HPV viral load, or relied on a low sample number. Koskinen et al.[32] reported that 37 of 61 (61%) head and neck cancer samples were positive for HPV and that HPV-16 was the most frequently detected type (31 of 37, 84%).

Table I

Author	Year	Lesion location	Diagnosis	No. of cases	No. of HPV-positive lesions	HPV detection method	Identified HPV genotypes
Stokes et al.[25]	2012	Oral	VC	7	1/7	ISH, PCR	HPV-16
			Dysplastic verrucous lesions	13	5/13		
Adler-Storthz et al. [26]	1986	Oral	VC	9	3/9	ISH	HPV-2
Noble-Topham et al.[27]	1993	Oral	VC	25	12/25	PCR	HPV-6, 11, 16, 18
Shroyer et al.[28]	1993	Oral	VC	17	7/17	PCR, ISH	HPV-6, 11, 16, 18, 31, 33, 35
Balaram et al.[29]	1995	Oral	VC	15	10/15	PCR	HPV-6, 11, 16, 18
Mitsuishi et al.[30]	2005	Oral	VC	4	4/4	PCR	HPV-2, 18, 20, 27, 57, 62, and partial sequences of an unknown

							HPV type
Fujita et al.[31]	2008	Oral	VC	23	11/23	PCR, ISH	HPV-6, 11, 18, 33, 74
Del Pino et al.[23]	2012	Head and neck	VC	5	1/5	PCR	HPV-35, 45
		Head and neck	VH	1	0/1		

The aim of this study was to analyze a large cohort of oral verrucous lesions for the presence of HPV subtypes, as well as any other viral genomes, using next-generation sequencing (NGS). The cohort included VC and VH but excluded so-called hybrid lesions where VC and infiltrative SCC exist,[19] as well as lesions with distant metastases, as these lesions are not representative of 'classical' VC.

Materials and Methods

Sample selection

Of the samples included, [28] were collected from the Pathology Department, St James's University Hospital, Leeds, United Kingdom; [16] from the Pathology Division, Queen Victoria Hospital, East Grinstead, United Kingdom; [42] from the Pathology Division, University of Turin, Italy; and [8] from the Department of Pathology, National Guard Hospital, Saudi Arabia. Written informed consent and approval was obtained for all patients for the use of their tissue in this research (Leeds Research Ethics Committee reference 07/Q1206/30). Verrucous-appearing, but clearly 'invasive,' squamous lesions were classified as verrucous SCC and excluded.[19]

DNA extraction

Seven 10-µm sections were cut from each block onto plain glass slides. Premalignant or malignant areas were microscopically identified and marked on a corresponding hematoxylin-eosin-stained slide. Sections were dewaxed and macrodissected with a sterile scalpel blade to obtain >70% tumor cell content for DNA isolation, performed using Qiagen DNA extraction kits (Qiagen, Sussex, United Kingdom), according to the manufacturer's instructions. DNA extraction was successful in 78 cases.

Determining DNA concentration and purity

Nucleic acid concentration and purity were measured using the NanoDrop UV spectrophotometer NanoDrop-8000 (Thermo Scientific, Wilmington, DE, USA). Besides

the concentration, A260:230 and A260:280 ratios were obtained, which are used as indicators of the purity of the samples. Double-stranded DNA concentration was specifically quantified using the Quant-iT PicoGreen dsDNA BR assay (Invitrogen, Paisley, United Kingdom). Of 94 cases, 78 yielded sufficient DNA for library preparation and NGS analysis.

Library preparation and sequencing

Library preparation for Illumina Genome analyser GAIIx sequencing

Briefly, between 5 ng and 1 µg of DNA was sheared on a Covaris S2 Sample Preparation System (Covaris Inc, Woburn, MA, USA). Sheared DNA samples were checked for appropriate size distribution according to the manufacturer's instructions on an Agilent Bioanalyser DNA 1000 LabChip (Agilent Technologies Inc, Santa Clara, CA, USA). The End-It DNA End-Repair Kit (Epicentre Biotechnologies, Madison, WI, USA) was used to convert DNA with damaged ends to blunt-ended, 5′-phosphorylated DNA. Klenow DNA polymerase was used to add an A base to each blunt-ended DNA fragment so that adapters could be ligated. Six base pairs (bp) of unique oligonucleotide tag sequence (adapter) were ligated to the ends of the DNA fragments using methods previously published.[33] DNA fragments were size selected to 200 bp using magnetic beads. DNA was recovered using QiaQuick gel extraction kit (Qiagen, Venlo, Netherlands) standard protocols, after which a step comprising 12 enrichment PCR cycles was performed. DNA libraries were examined using Invitrogen's Quant-iT Picogreen dsDNA BR assay kit and Agilent Bioanalyser DNA 1000 LabChip to assess DNA concentration and quality, respectively (Agilent Technologies Inc). Equimolar amounts of each DNA library were pooled for cluster amplification and multiplexed up to 20 samples per lane for 76-bp Illumina single-end sequencing, where each read includes 6 bp of tagged adapter and 70 bp of genomic DNA sequence.

Library preparation for Illumina HiSeq 2000 sequencing

For sequencing using HiSeq 2000, DNA libraries were prepared using NEBNext DNA Library Prep Master mix as described for Illumina (New England BioLabs Inc, Hitchin, United Kingdom). Between 200 ng and 300 ng of DNA was sheared on a Covaris S2 Sample Preparation System (Covaris Inc) and checked for appropriate size distribution on an Agilent TapeStation D1K High Sensitivity ScreenTape (Agilent Technologies Inc). After purification, DNA fragments were end repaired to convert DNA containing damaged ends to blunt-ended, 5′-phosphorylated DNA according to the manufacturer's instructions. End-repaired DNA fragments were dA-tailed and ligated with the NEBNext adaptor. Adaptor-ligated DNA fragments were size selected to 200 bp using magnetic beads (Agencourt AMPure; Beckman Coulter Inc, Danvers, MA, USA). Adaptor-ligated DNA fragments were tagged to 96 different indexed primers designed by our group (Integrated DNA Technologies Inc, Glasgow, United Kingdom) and PCR-enriched using

15 enrichment PCR cycles. All reagents used, with the exception of the indexed primers, were part of the NEBNext DNA Library Prep Master mix with a NEB adaptor as described for Illumina. Libraries were then examined using Invitrogen's Quant-iT Picogreen dsDNA BR assay kit and Agilent TapeStation D1K High Sensitivity ScreenTape to assess DNA concentration and quality, respectively (Agilent Technologies Inc). Samples were pooled, up to 50 samples per lane, and paired-end sequenced (2 × 100 bp) on an Illumina HiSeq 2000.

Data analysis

Alignment and sequencing

Reads were split into separate files according to tag and aligned to the human reference genome (University of California Santa Cruz version GRCh37/hg[19], http://genome.ucsc.edu) and to all known viral genomes, including HPV subtypes, downloaded from the National Center for Biotechnology Information (http://www-ncbi-nlm-nih-ov.offcampus.dam.unito.it/genomes/GenomesHome.cgi?taxid%20=%2010239).

Human genomic copy number analysis

Human DNA copy number analysis was calculated and analyzed as recently described.[34] Sample reads were arranged and organized by chromosome and position. The ratio of test to control reads was calculated across the genome in equally sized windows, averaging 200 tumor reads. A control sample was pooled from a group of 20 data sets for normal individuals downloaded from the 1000 Genomes Project[35] (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp).

Viral genomes, HPV subtype detection, and load measurements by sequencing

Viral load was measured as previously described by Conway et al.[36] This technique has been found to have good sensitivity and specificity, with the additional benefits of providing information on viral subtype and load but also informing genomic copy number. The number of reads aligning to the human genome was used to calculate read depth, in terms of reads per Kb. (For example, 1 million human reads in a 6-Gb diploid human genome equates to 1 read for every 6 Kb of human DNA.) Next, the number of reads uniquely aligning to viral genomes was counted. This was equated to a certain number of Kb viral sequence per human genome and hence the number of viral genomes per human genome. (For example, if 5 HPV reads are detected, and we have previously calculated that there is 1 read every 6 Kb, then there is likely to be around 30 Kb of viral DNA per cell. This is equivalent to 30/7.9 = 3.8 viral genomes per cell.) We can also calculate the probability of detecting 1 copy of the HPV genome per cell, as follows:

p=1-e (0-(7900-bp viral genomex number of human reads)/6 billion-bp diploid human genome)

So a sample with 1 million human reads has a 73% chance of detecting a single copy of the HPV genome per cell, whereas a sample with 3 million reads has a 98% chance. Higher viral loads will be easier to detect. Given a certain number of human reads, the possible viral load that could be detected with 95% confidence is as follows:

(0-log(1-0,95)x6-Mb diploid human genome)/(7.9-Kb viral genomexnumber of human reads)

So, for example, only 456 000 reads are needed to detect a load of 5 viral copies per cell with 95% confidence.

We used HPV sequencing data from a previous study (from our group) that included positive and negative controls.36 NGS in HPV-positive cases was compared with the gold standard of p16 immunohistochemistry (antibody F-12: Sc-1661; Santa Cruz Biotechnology Inc) using high-pH range settings on Dako Autostainer Link 48 (Ely, United Kingdom) and found to be comparable in sensitivity and specificity.

Results

A total of 78 patients, including 41 women and 37 men (mean age, 67 years; range, 38-96) were included (Table II). Full data are given in Supplemental Table S1, available at www.oooojournal.net. Tumor DNA was extracted from a total of 94 samples, and 78 (83%) of these yielded sufficient DNA for library preparations and NGS. Neither the age of the formalin-fixed, paraffin-embedded block nor the amount of tissue sampled correlated with low yields.

Table II.

Summary of lesion types and patients

Type of lesion	n	Age	Male	Female
OVC	62	38-96 (mean = 67.2)	33	32
OVH	16	52-80 (mean = 67)	5	8

OVH, oral verrucous hyperplasia; OVC, oral verrucous carcinoma. HPV

Sequencing libraries were prepared from 78 samples. Table III lists the range of human and viral reads. HPV-16 sequence was identified in 1 OVH and 1 OVC, and HPV-2 sequence was detected in 1 OVC out of 78 samples at 95% confidence level with 2.24, 8.16, and 0.33 viral genomes per cell, respectively. The standard deviation of reads taken from all samples was 4 257 556.901 [ranging from 296 655 to 115 682 098].

Table III.

Viral load determined by next generation sequencing in human oral verrucous carcinomas (n = 62) and hyperplasias (n = 16)

Patient ID	Species	Virus length	Human reads	Virus reads	Read density Kb per read	Kb virus sequence	Viral load	Detectable HPV load at 95% confidence	Probability of detecting 1 copy of HPV per cell
V-2-2-C1	Herpesvirus 5	235646	296655	1	20.23	20.23	0.09	7.6696	0.3233
V-2-2-C1	Herpesvirus 1	152261	296655	2	20.23	40.45	0.27	7.6696	0.3233
V-3-1-A1	-	0	431502	0	13.9	0	0	5.2728	0.4334
V-4-1-E9	-	0	589117	0	10.18	0	0	3.8621	0.5396
V-7-1-A	Herpesvirus 6A	159322	671946	8	8.93	71.43	0.45	3.3860	0.5872
V-7-1-A	Herpesvirus 1	152261	671946	1	8.93	8.93	0.06	3.3860	0.5872
V-8-1-C	-	0	479578	0	12.51	0	0	4.7443	0.4682
V-10-1-1	-	0	1556862	0	3.85	0	0	1.4614	0.8712
V-14-1-6	-	0	1288201	0	4.66	0	0	1.7662	0.8166
V-15-A3	-	0	1554350	0	3.86	0	0	1.4638	0.8708
V-19-I4	-	0	1182923	0	5.07	0	0	1.9234	0.7893
V-20-3	-	0	4253014	0	1.41	0	0	0.5350	0.9963
V-22-1-4	HPV-16	7905	2368058	7	2.53	17.74	2.24	0.9608	0.9558
V-23-1-5	-	0	378361	0	15.86	0	0	6.0134	0.3924
V-25-1-	-	0	668260	0	8.98	0	0	3.4047	0.5852

Patient ID	Species	Virus length	Human reads	Virus reads	Read density Kb per read	Kb virus sequence	Viral load	Detectable HPV load at 95% confidence	Probability of detecting 1 copy of HPV per cell
D2									
V-26-1-A4	-	0	666770	0	8.99	0	0	3.4123	0.5844
V-29-2-A3	-	0	840256	0	7.14	0	0	2.7078	0.6692
V-30-1	Herpesvirus 1	152261	2943604	4	2.04	8.15	0.05	0.7729	0.9793
V-31-1-B1	-	0	583436	0	10.28	0	0	3.8997	0.5361
V-33-1-4	Herpesvirus 6A	159322	1182195	18	5.08	91.36	0.57	1.9246	0.7891
V-33-1-4	Herpesvirus 6B	162114	1182195	1	5.08	5.08	0.03	1.9246	0.7891
V-38-1-B	-	0	9163412	0	0.66	0	0	0.2483	1.0000
V-40-1	-	0	7611248	0	0.79	0	0	0.2989	1.0000
V-41-1-G	-	0	2324407	0	2.58	0	0	0.9788	0.9531
V-42-1-G	Herpesvirus 1	152261	14884005	6	0.4	2.42	0.02	0.1529	1.0000
V-44-1-3J	Herpesvirus 1	152261	4793286	2	1.25	2.5	0.016	0.4747	0.9982
V-46-1-2	Herpesvirus 6B	162114	12047262	150	0.5	74.71	0.46	0.1889	1.0000
V-60-1	-	0	289026	0	20.76	0	0	7.8721	0.3165
V-61-1-4	-	0	4901408	0	1.22	0	0	0.4642	0.9984

Patient ID	Species	Virus length	Human reads	Virus reads	Read density Kb per read	Kb virus sequence	Viral load	Detectable HPV load at 95% confidence	Probability of detecting 1 copy of HPV per cell
V-62-1-B	Herpesvirus 6A	159322	5978774	2	1	2.01	0.13	0.3806	0.9996
V-63-1-2	-	0	6794116	0	0.88	0	0	0.3349	0.9999
V-65-1-D1	-	0	7769042	0	0.77	0	0	0.2929	1.0000
V-66-1-B	-	0	4446770	0	1.35	0	0	0.5117	0.9971
V-67-1-B1	-	0	6100026	0	0.98	0	0	0.3730	0.9997
V-68-1	Herpesvirus 5	235646	2984540	4	2.01	8.04	0.03	0.7623	0.9803
V-69-1-D	Herpesvirus 5	235646	8960354	2	0.67	1.34	0.01	0.2539	1.0000
V-70-1-B	Herpesvirus 7	153080	6371664	2	0.94	1.88	0.01	0.3571	0.9998
V-71-1-5	-	0	7896410	0	0.76	0	0	0.2881	1.0000
V-72-1-4	-	0	6091470	0	0.99	0	0	0.3735	0.9997
V-73-1	-	0	7774162	0	0.77	0	0	0.2927	1.0000
V-74-1-A	-	0	8256130	0	0.73	0	0	0.2756	1.0000
V-75-1	-	0	1527565	0	3.93	0	0	1.4895	0.8662
V-77-1	-	0	7883782	0	0.76	0	0	0.2886	1.0000
V-78-1-A	Herpesvirus 5	235646	6029256	4	0.99	3.98	0.017	0.3774	0.9996
V-79-1-B	Herpesvirus 6B	162114	6483082	2	0.93	1.85	0.01	0.3510	0.9998

Patient ID	Species	Virus length	Human reads	Virus reads	Read density Kb per read	Kb virus sequence	Viral load	Detectable HPV load at 95% confidence	Probability of detecting 1 copy of HPV per cell
V-80-1	-	0	3288128	0	1.83	0	0	0.6920	0.9868
V-83-1-C	-	0	5666808	0	1.06	0	0	0.4015	0.9994
V-84-1	-	0	4969566	0	1.21	0	0	0.4578	0.9986
V-85-1	-	0	5348678	0	1.12	0	0	0.4254	0.9991
V-86-1	-	0	15107456	0	0.4	0	0	0.1506	1.0000
V-87-1	-	0	5178734	0	1.16	0	0	0.4393	0.9989
V-88-1	Herpesvirus 1	152261	6584298	2	0.91	1.82	0.01	0.3456	0.9998
V-89-1-A	-	0	6898174	0	0.87	0	0	0.3298	0.9999
V-90-1	-	0	4493076	0	1.34	0	0	0.5064	0.9973
V-91-1	Herpesvirus 1	152261	8733136	2	0.69	1.37	0.01	0.2605	1.0000
V-92-1	Herpesvirus 1	152261	10556564	4	0.57	2.27	0.02	0.2155	1.0000
V-94-1	HPV-2	7860	4614998	2	1.3	2.6	0.33	0.4930	0.9977
V-94-1	Herpesvirus 1	152261	4614998	4	1.3	5.2	0.03	0.4930	0.9977
V-95-1-B	Herpesvirus 1	152261	11163878	4	0.54	2.15	0.01	0.2038	1.0000
V-95-1-B	Herpesvirus 6B	162114	11163878	4	0.54	2.15	0.01	0.2038	1.0000
V-97-1	-	0	9483722	0	0.63	0	0	0.2399	1.0000
V-98-1-	-	0	7454606	0	0.81	0	0	0.3052	0.9999

Patient ID	Species	Virus length	Human reads	Virus reads	Read density Kb per read	Kb virus sequence	Viral load	Detectable HPV load at 95% confidence	Probability of detecting 1 copy of HPV per cell
D									
V-99-1-4	-	0	6585616	0	0.91	0	0	0.3455	0.9998
V-100-1-B	-	0	4716241	0	1.27	0	0	0.4824	0.9980
V-101-1-F	-	0	4412444	0	1.36	0	0	0.5156	0.9970
V-102-1	-	0	6610388	0	0.91	0	0	0.3442	0.9998
V-104-01	Herpesvirus 1	152261	10385806	8	0.58	4.62	0.03	0.0114	1.0000
V-105-01	Herpesvirus 1	152261	10847800	2	0.55	1.10	0.01	0.0109	1.0000
V-106-01	-	0	9112970	0	0.66	0	0	0.2497	1.0000
V-108-01	-	0	13893548	0	0.43	0	0	0.1638	1.0000
V-109-T	Herpesvirus 4	171823	14969258	6	0.40	2.41	0.01	0.006988318	1.0000
V-110-01	-	0	12720402	0	0.47	0	0	0.1789	1.0000
V-111-01	-	0	9047886	0	0.66	0	0	0.2515	1.0000
V-113-T	-	0	4086390	0	1.47	0	0	0.5568	0.9954
V-114-T	-	0	7797994	0	0.77	0	0	0.2918	1.0000
V-115-01	-	0	15682098	0	0.38	0	0	0.1451	1.0000

Patient ID	Species	Virus length	Human reads	Virus reads	Read density Kb per read	Kb virus sequence	Viral load	Detectable HPV load at 95% confidence	Probability of detecting 1 copy of HPV per cell
V-116-T	-	0	10659704	0	0.56	0	0	0.2134	1.0000
V-118-01	-	0	15473750	0	0.39	0	0	0.1471	1.0000
V-120-T	-	0	8762276	0	0.68	0	0	0.2597	1.0000
V-122-01	-	0	12558750	0	0.48	0	0	0.1812	1.0000
V-123-T	-	0	11502764	0	0.52	0	0	0.1978	1.0000
V-124-T	Herpesvirus 6B	162114	9115790	142	0.66	93.46	0.58	0.0122	1.0000
V-125-T	HPV-16	7905	10041734	108	0.60	64.53	8.16	0.2264	1.0000

HPV, human papillomavirus.

Human herpesvirus

Human herpesvirus (HHV) sequences were detected in 22 of 78 samples (6 OVHs and 16 OVCs) with viral loads ranging from 0.01 to 0.58 viral genomes per cell (see Table III) and with 0.19 viral loads SD. There were 12 samples positive for HHV-1, 1 for HHV-4, 4 for HHV-5, 5 for HHV-6B, 3 for HHV-6A, and 1 for HHV-7. Four cases had double HHV infections (HHV-1 and -5, HHV-1 and -6A, HHV-1 and -6B, and HHV-6A and -6B).

Discussion

We have described the use of NGS as a validated, powerful, high-throughput method to investigate the presence of all characterized human viral genome loads and subtypes, in the largest, specifically oral, verrucous sample cohort described to date. Using NGS, testing for all HPV subtypes found HPV-16 positivity in only 1 OVH and 1 OVC sample and HPV-2 positivity in 1 OVC sample out of 78 oral verrucous lesions with 2.2, 8.1, and 0.3 viral genomes per cell, respectively. Although it is difficult to accurately predict the exact viral load with only a very small number of aligning viral reads, loads in this study were much lower than loads in our previous study of HNSCC.[36] There the standard

deviation of the viral loads was 37.7, suggesting that the virus was not contributing to disease etiology. We have calculated the probability of detecting a viral load of 1 HPV genome per cell, given our read numbers. Of our 78 samples, 60 have sufficient depth to detect 1 HPV genome per cell. All but 4 samples have sufficient depth to be able to detect 4 copies per cell with 95% confidence.

HPV DNA may degrade in paraffin-embedded tissues. Sequencing may be less affected by this than PCR. The standard PCR test for HPV requires a 120-bp fragment to be amplified. We size select our libraries to be around 200 bp to ensure that enough fragments of <100 bp are sequenced. If an HPV sequence is in one of these, it would be picked up by sequencing but not by PCR.

The recent article by Patel et al.24 used HPV reverse transcription PCR (RT-PCR) testing, but they had a significant subset of cases in which insufficient RNA from the formalin-fixed, paraffin-embedded tumor sections precluded HPV RT-PCR testing. Nevertheless, they concluded that “active HPV in (head and neck) verrucous carcinomas is rare enough to likely be clinically inconsequential.”

In 2012, del Pino et al.[23] investigated the prevalence of HPV in 18 verrucous lesions. Using PCR, and finding that only 1 head and neck VC was positive for HPV infection, the authors concluded that VC is “unlikely to be related to HPV infection.” In addition, Stokes et al.[25] studied the role of HPV in malignant and dysplastic oral verrucous lesions and suggested that although high-risk HPV DNA was identified in 1 of 7 carcinomas and 5 of 13 dysplasias, the oncogenic process is not enhanced by HPV oncoproteins, as p16 overexpression was lacking; they concluded that further work is needed on a larger cohort to determine HPV's biologic significance in VC development.

We have used NGS for the detection of HPV in verrucous samples on the largest cohort of oral verrucous carcinomas to date. In addition, we used histologic diagnostic criteria defined by the World Health Organization,[19] suggesting (unlike Patel et al.[24]) that oral verrucous lesions with frank stromal invasion in the bulk of the tumor should be classified as SCCs with verrucous architecture and therefore should be excluded from study.

Our method using NGS was validated previously (on our control sample set) by detecting HPV sequences using PCR and by evaluating p16 expression as a marker for HPV infection.[36] We found in our previous study that NGS has a high specificity and sensitivity for HPV detection when compared with PCR and p16. It has been previously suggested that PCR methods can be oversensitive,[37] whereas our method can provide better specificity, as demonstrated by our observation that all p16-positive samples were also positive for HPV-16 by sequencing.[36] NGS has also been validated for detecting papilloma subtypes and loads not identified by other means in cervical samples.[38]

In this study, we searched for all human viruses with sequenced genomes. HHV types were detected in one-third of lesions, although they were unconfirmed using any other

diagnostic test. NGS tells us nothing about transcriptional activity, so it is not possible to speculate further on the clinical significance of this finding. In addition, we scanned the control samples used in our previous study[36] for all human virus sequences and identified 8 positive cases (34.78%). In general, herpes simplex virus–related infections are common, affecting approximately two-thirds of adults. Two HHV types known to be associated with cancer, Kaposi sarcoma–associated herpesvirus and Epstein-Barr virus,[39] were not detected. Herpes infection may not be the cause of the disease in our patients, but future studies of a similar nature may reveal previously unsuspected oncoviruses to be common in a different tumor type. The fact that our read depth is enough to detect 1 HPV copy per cell with 95% confidence in most of our samples and a modest infection of 5 copies per cell in all but 5, combined with our previous ease of detecting HPV in oropharyngeal cancer, confirms that the absence of detection of HPV in this large sample is not a technical error but a real biologic finding.

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Appendix

Supplemental Table S1.

Next-generation sequencing analysis for detecting human papillomavirus in oral verrucous carcinoma

Patient ID	Sex	Age (y)	Lesion location□	Diagnosis
V-2-2-C1	F	60	Mandible and BM	OVH
V-3-1-A1	F	62	Mandible and BM	OVH
V-4-1-E9	M	77	Mandible and BM	OVC
V-7-1-A	F	86	Lower lip	OVC
V-8-1-C	F	38	FOM	OVC
V-10-1-1 e1	M	62	BM	OVC
V-14-1-6	M	62	BM	OVC
V-15-1-A3	F	75	HP and BM	OVH
V-19-1-I4	F	63	BM	OVC

Patient ID	Sex	Age (y)	Lesion location	Diagnosis
V-20-3	M	54	SP	OVC
V-22-1-4	F	67	BM	OVH
V-23-1-5	F	78	BM	OVC
V-25-1-D2	F	80	Tongue	OVH
V-026-01-A4	M	72	BM	OVC
V-29-2-A3	F	52	HP	OVH
V-30-1	F	80	BM	OVH
V-31-1-B1	M	72	BM	OVH
V-33-01-4	M	72	BM	OVH
V-38-1-B	M	63	BM	OVC
V-40-1	F	63	Maxilla	OVH
V-41-1-G	M	67	Maxilla	OVH
V-42-1-G	F	70	Tongue	OVH
V-44-1-3J	M	54	Tongue	OVH
V-46-1-2	M	60	HP	OVH
V-60-1	M	65	Tongue	OVC
V-61-1-4	F	96	Tongue	OVC
V-62-1-B	F	73	Tongue	OVC
V-63-1-2	F	66	BM	OVC
V-65-1-D1	M	78	Upper lip	OVC
V-66-1-B	M	73	BM	OVC
V-67-1-B1	F	61	FOM	OVC
V-68-1	M	61	BM	OVC

Patient ID	Sex	Age (y)	Lesion location	Diagnosis
V-69-1-D	M	66	Lower lip and BM	OVC
V-70-1-B	M	49	BM	OVC
V-71-1-5	F	82	Mandible	OVC
V-72-1-4	F	82	BM	OVC
V-73-1	F	88	Mandible	OVC
V-74-1-A	F	88	HP	OVC
V-75-1	F	49	BM	OVC
V-77-1	M	81	BM	OVC
V-78-1-A	M	74	BM	OVC
V-79-1-B	F	77	HP/SP	OVC
V-80-1	F	63	Mandible	OVC
V-83-1-C	F	72	Mandible and BM	OVC
V-84-1	F	61	BM	OVC
V-85-1	M	73	BM	OVC
V-86-1	M	70	Tongue	OVC
V-87-1	F	53	BM	OVC
V-88-1	M	74	BM	OVC
V-89-1-A	M	53	BM	OVC
V-90-1	M	61	BM	OVC
V-91-1	F	60	HP	OVC
V-92-1	M	57	BM	OVC
V-94-1	M	46	BM	OVC
V-95-1-B	M	53	BM	OVH

Patient ID	Sex	Age (y)	Lesion location	Diagnosis
V-98-1-D	F	85	Tongue	OVC
V-99-1-4	F	81	Tongue	OVC
V-100-1-B	M	69	BM	OVC
V-101-1-F	F	80	SP	OVC
V-102-1	F	55	BM	OVC
V-104-01	F	78	Tongue	OVC
V-105-01	M	47	Tongue	OVC
V-106-01	F	76	Tongue	OVH
V-108-01	F	78	Tongue	OVC
V-109-T	F	82	HP	OVC
V-110-01	F	82	BM	OVC
V-111-01	F	58	Lower lip	OVC
V-112-T	F	79	HP	OVC
V-113-T	M	64	Maxilla	OVC
V-114-T	M	53	Tongue	OVC
V-115-01	M	43	Tongue	OVC
V-116-T	M	60	Hard palate/maxilla	OVC
V-118-01	M	58	BM	OVC
V-120-T	F	45	BM	OVC
V-122-01	M	76	FOM	OVC
V-123-T	M	60	Hard palate/maxilla	OVC
V-124-T	M	66	SP	OVC
V-125-T	M	64	Tongue	OVC

BM, buccal mucosa; FOM, floor of mouth; HP, hard palate; SP, soft palate; OVH, oral verrucous hyperplasia; OVC, oral verrucous carcinoma.

Mandible and maxilla refer to alveolus.

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