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HPV73 a nonvaccine type causes cervical cancer

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HPV73 is classified as possibly oncogenic. It is neither routinely evaluated in HPV screening, nor covered by any of the prophylactic vaccines. We sought to investigate the carcinogenic characteristics of HPV73. Molecular studies were performed on eight cervix cancer biopsy specimens containing HPV73 from a cross-sectional cancer cohort of 590 women referred to the National Cancer Institute in Rio de Janeiro, Brazil. Transcriptional activity of HPV73 was evaluated by detection of spliced transcripts of E6/E6* and E1^E4 in cDNA created from RNA isolated from fresh tissue. Disruption of viral E1 and E2 genes in the tumor DNA was assessed by overlapping PCR amplification. Evaluation of viral integration was performed using a customized capture panel and next-generation sequencing, and an in-house bioinformatic pipeline. HPV73 E6/E6* transcripts were found in 7/7 specimens with available RNA, and three also had HPV73 E1^E4 transcripts. Disruption of E1 and E2 genes was observed in 4/8 specimens. Integration of HPV73 sequences into the cancer cell genomes was identified in all cervix cancer tissues. These results provide evidence that HPV73 is an oncogenic virus that can cause invasive cervix cancer. With current molecular screening and HPV vaccination, not all cervix cancers will be prevented.

Introduction

Human papillomavirus (HPV) type-specific oncogenicity is based primarily on the prevalence of HPV DNA types identified in cervix cancer tissues[1] with supporting molecular evidence that includes the presence of specific HPV alternate spliced transcripts, integration of HPV DNA into the cancer cell genome, and expression of E6 and E7 oncoproteins.[2,3]

HPV73 is currently classified as possibly carcinogenic based on its global prevalence of 0.5% of invasive cervix cancers (CxCa).[1] However, HPV73 prevalence differs considerably across geographic regions[4]; its prevalence may also vary depending upon detection and typing methods.[5,6] Given that HPV73 is neither generally screened for in cervix cancer prevention programs nor is it covered by any of the existing HPV vaccines,

Key words: HPV, cervical cancer, oncogenic type, transcriptionally active, integration

Abbreviations: ATCC: American Type Culture Collection; BWA: Burrows–Wheeler Aligner; cDNA: complementary DNA; CxCa: invasive cervical cancer; EINSTEIN: Albert Einstein College of Medicine; HPV: human papillomavirus; IHC: immunohistochemistry; INCA: Instituto Nacional de Cancer; NGS: next-generation sequencing; SCC: cervical squamous cell carcinoma

Additional Supporting Information may be found in the online version of this article.

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What's new?

Several HPV viruses classified as "possibly" oncogenic are not routinely evaluated in HPV screening; nor are they covered by current prophylactic vaccines. In this study, the authors examined one such virus, HPV73, which occurs with a serologic prevalence as high as 14% in some regions. They found that HPV73 can, indeed, cause cervical cancer. It is likely that, due to these low-prevalence HPVs, current screening and vaccines will fail to eliminate a proportion of cervical cancers. Population specific prevention efforts may thus need to be reevaluated.

we sought to evaluate the molecular evidence of HPV73's oncogenicity in CxCa tissues.

Materials and Methods

Case specimens

Between June 2011 and March 2014, 590 patients were referred to the National Cancer Institute (INCA, *Instituto Nacional de Cancer*), Rio de Janeiro, Brazil, for treatment of CxCa. A total of eight (1.4%) women had HPV73-positive CxCa specimens detected by PCR and Sanger sequencing as previously reported and were designated SA162–SA169.[7] All subjects gave informed consent and completed an epidemiological questionnaire. Our study was approved by the institutional ethics committee (CAAE: 53398416.0.0000.5274).

HPV 73 confirmation and identification of transcriptional activity

DNA and RNA were isolated from CxCa biopsy specimens using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA). HPV73-positive cervix specimens were retested at the Albert Einstein College of Medicine (EINSTEIN, Bronx, NY) using a validated MY09/11 primer PCR protocol and type-specific oligonucleotide hybridization technique, able to detect the presence of more than 40 HPV types, as described previously.[8]

Complementary DNA (cDNA) was synthesized using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA) from RNA isolated from CxCa specimens as described above. Type-specific PCR assays were developed to detect the spliced forms of HPV73 (E6* and E1^E4) and HPV16 (E6*), followed by Sanger sequencing on an ABI Prism® 3730 platform (Applied Biosystems, Foster City, CA) for confirmation of the transcript sequences.

Immunohistochemistry for $p16^{ink4a}$ expression

Five of the eight HPV73-positive tumors (62.5%) had formalin-fixed paraffin-embedded cervical tissue available for immunohistochemistry (IHC) assay. All tissue sections were hematoxylin and eosin-stained and reviewed by an expert pathologist (FCBM). Three micrometers sections of each paraffin-embedded biopsy were stained for the qualitative detection of $p16^{ink4a}$ protein with CINtec p16 Histology (Ventana Medical System, Oro Valley, AZ) according to standard protocols and were visualized using 3,3′-diaminobenzidine (DAKO, Glostrup, Denmark) with an exposure time of 3 min. Positive controls included five HPV16-positive CxCa specimens that were matched by Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) staging to the HPV73-positive CxCa cases to compare the expression of the $p16^{ink4a}$ marker.

HPV integration analysis

One hundred nanograms of genomic DNA from the eight cervix biopsy specimens were mechanically fragmented to 250 base pair (bp) using the Covaris S2 Focused-ultrasonicator (Covaris, Woburn, MA), followed by preparation of individual libraries using the KAPA HyperPlus Kit (KAPA Biosystems, Boston, MA) according to the manufacturer's instructions. Four HPV73-positive exfoliated cell samples from women without detectable cervical precancer or cancer were included and similarly processed as controls.

Positive and negative controls included SiHa HPV16-positive (American Type Culture Collection [ATCC®] HTB-35™), HeLa HPV18-positive (ATCC® CCL-2™) and HEK-293 HPV-negative (ATCC® CRL-1573™) cells maintained for 20 passages in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). The cell lines tested negative by e-Myco™ Mycoplasma PCR Detection Kit 2.0 (iNtRON Biotechnology, Kyungki-Do, North Korea) March 2017. DNA extraction was performed using QIAamp DNA Blood Mini Kit (Qiagen).

A customized DNA capture probe panel was designed using the reference and variant genome sequences of HPV16, 18, 45 and 73[9] (SeqCap EZ Developer, Roche Diagnostics, Indianapolis, IN), and contained a total of 1,209 RNA probes approximately 100 bp in size designed to tile across each of the included viral genomes. The capture reactions were performed using SeqCap EZ HyperCap (Roche Diagnostics) following the manufacturer's instructions. Briefly, the libraries from the eight HPV73-positive CxCa specimens, cell line controls and HPV73 noncancer samples were pooled in two batches (i.e., two precapture library mixes) followed by hybridization with the SeqCap EZ Probe pool of biotinylated oligonucleotides (Roche Diagnostics) for 20 hr at 47°C. The captured molecules were then recovered with Streptavidin beads (Invitrogen), washed, purified and subjected to 15 cycles of enrichment PCR. Captured libraries were sequenced on an Illumina MiSeq (250 bp paired-end protocol) at the EINSTEIN Epigenomics Core Facility (Bronx, NY).

Bioinformatics analysis

Illumina sequencing data files were initially processed to remove small sequences (<50 bp), trim low quality reads and merge paired-end reads. The HPV DNA reference sequence

panel was combined with the human genome assembly GRCh38/hg38 (from the University of California, Santa Cruz [UCSC] genome browser,[10] Dec 2013) to which the paired-end reads were aligned using the Burrows–Wheeler Aligner algorithm (BWA-MEM). The viral–human chimeras were extracted by identifying reads aligned to both HPV genomes included in the HPV capture panel and human sequences within GRCh38/hg38. The viral integration sites were assigned using a minimum of 10 chimeric reads per junction and plotted using R v3.3.1 with the gglot2 v2.2.1 package. HPV73 variant classification and phylogenetic analyses were performed using the consensus viral sequence from captured reads after alignment and a total of 10,000 bootstrap iterations, in which HPV34 rooted the maximum likelihood tree using RAxML.

Results

HPV73-positive cervix cancer cases

Table 1 shows the age at diagnosis, histologic type, grade and stage of cervix cancer upon presentation for treatment. HPV genotyping was repeated on the eight CxCa specimens identified as single type HPV73-positive in Brazil. All specimens were confirmed to contain HPV73 and two specimens were also found to contain HPV58 and HPV16 upon retesting.

To determine if HPV73 was transcriptionally active in the tumor tissues, we converted RNA transcripts into cDNA and amplified specific viral fragments that were confirmed by Sanger sequencing (Fig. 1). The presence of HPV73 E1^E4 transcripts (306 bp) was observed in four of the CxCa cases (Fig. 1, Panel *a*), while HPV73 E6* (139 bp) transcripts were detected in all seven CxCa specimens with available cDNA (Fig. 1, Panel *b*). The nonspliced form of HPV73 E6 (321 bp) was also detected in four specimens (Fig. 1, Panel *b*). Since HPV16 is the predominant type found in CxCa, we evaluated the specimens for HPV16 activity but were unable to find evidence of HPV16 E6/E6* transcripts even in the one sample (SA164) containing HPV16 DNA (Table 1; Fig. 1, Panel *c*).

A PCR assay was initially used to evaluate viral genome interruption of HPV73 E1 and E2 genes; four specimens (SA162, SA164, SA167 and SA169) had disruption of these regions as evidenced by lack of at least one of the PCR fragments (Supporting Information Fig. S1 and Table S1). All specimens with HPV73 E1 and/or E2 genome disruption (4/4) did not have detectable HPV73 E1^E4 transcripts. Capture and sequencing of the HPV73 genomes provided definitive evidence of viral genome disruption as described below.

Immunohistochemistry

Five formalin-fixed paraffin-embedded cervix cancer tissues were available for analysis of $p16^{ink4a}$ expression (SA162, SA164, SA166, SA168 and SA169). The $p16^{ink4a}$ staining demonstrated intense diffuse dispersion with nuclear and cytoplasmic localization (Fig. 2). These cases were compared to five CxCa tissue specimens that were HPV16-positive and no difference was observed in $p16^{ink4a}$ expression and pattern between HPV73-positive and HPV16-positive cancers (data not shown).

Analysis of HPV73 integration into the host genome

DNA from tumor tissue was fragmented, captured and sequenced by next-generation sequencing (NGS) as described in the Materials and Methods. A total of 40 chimeric junctions containing HPV73 and human chromosomal DNA in single molecules (see Fig. 3 for an example) were identified in the eight CxCa specimens (Table 2; Supporting Information Fig. S2). The primary integration sites described in Table 2 were detected and identified with a range of 17–2,224 reads per chimeric junction, after removal of duplicated reads. Among the eight cancers, 20 primary integration sites (1–5 per sample) were localized to 12 human chromosomes including chromosome 2 (2q22.3 and 2q36.3), chromosome 4 (4p11, 4q13.3, 4q31.21 and 4q31.3) and chromosome 13 (13p11.1 and 13q21.31; Table 2; Supporting Information Fig. S2). To validate the viral integration sites, a PCR assay was performed

Table 1. Clinical and molecular characteristics of HPV73-positive cancers

							cDNA transcript analysis			
Sample	**Age**	**Histology**	**Grade**	**Staging**	**Specimen**	**HPV types**	**HPV73 E1^E4**	**HPV73 E6***	**HPV16 E6***	**$p16^{ink4a}$ IHC**
SA162	58	SCC	G2	IB1	DNA/cDNA	73, 58[1]	neg	**pos**	neg	**pos**
SA163	40	SCC	G2	IIB	DNA/cDNA	73	**pos**	**pos**	neg	NA
SA164	39	SCC	G3	IIIB	DNA/cDNA	73, 16[1]	neg	**pos**	neg	**pos**
SA165	49	SCC	G2	IIIB	DNA/cDNA	73	**pos**	**pos**	neg	NA
SA166	38	SCC	G2	IIA	DNA/cDNA	73	**pos**	**pos**	neg	**pos**
SA167	32	SCC	G2	IIB	DNA[2]	73	NA	NA	NA	NA
SA168	69	SCC	NA	IIA	DNA/cDNA	73	**pos**	**pos**	neg	**pos**
SA169	45	SCC	G2	NA	DNA/cDNA	73	neg	**pos**	neg	**pos**

Pos represents the presence of transcript indicated at the top of the column, Neg represents absence of the transcript at the top of the table.
[1]Detected only upon retesting with MY09/11.
[2]cDNA not available.
Abbreviation: SCC, squamous cell carcinoma; NA, information/specimen not available.

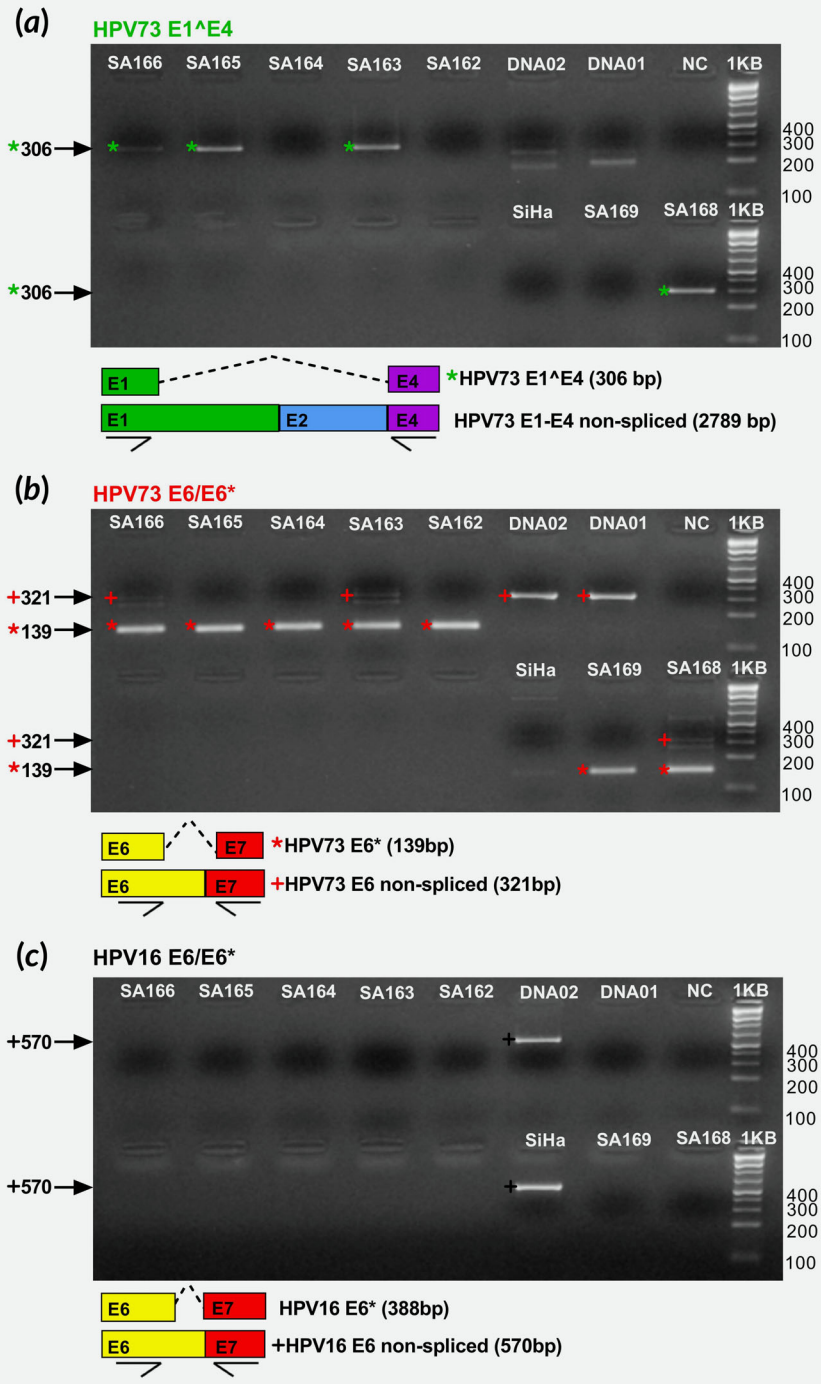

Figure 1. Identification of HPV73 transcriptional activity in cervix cancer tissues. (Panels *a*–*c*) show ethidium bromide stained gels demonstrating the presence of HPV73 E1^E4, HPV73 E6/E6* and HPV16 E6/E6* amplicons, respectively. (Panel *a*) HPV73 E1^E4 (306 bp, green asterisks) was identified in four (SA163, SA165, SA166 and SA168) of seven cDNA specimens. (Panel *b*) HPV73 E6/E6* transcripts (139 bp, red asterisk) were present in all cDNA specimens, and three also contained the amplified product of the nonspliced form of E6 (321 bp, red cross). (Panel *c*) HPV16 E6/E6* PCR products were absent in all cDNA specimens from HPV73-positive tumors whereas the nonspliced form of E6 was present in the HPV16-positive controls (DNA02 and SiHa DNA, black cross). A schematic map showing primer positions (black arrows) for detection of spliced and unspliced forms (dash lines) of the viral region are indicated under each panel. DNA from exfoliated cells with HPV73 (DNA01), HPV16 (SiHa DNA) and HPV73 coinfected with HPV16 (DNA02) were used as positive controls; DNase-free water was used as a negative control (NC).

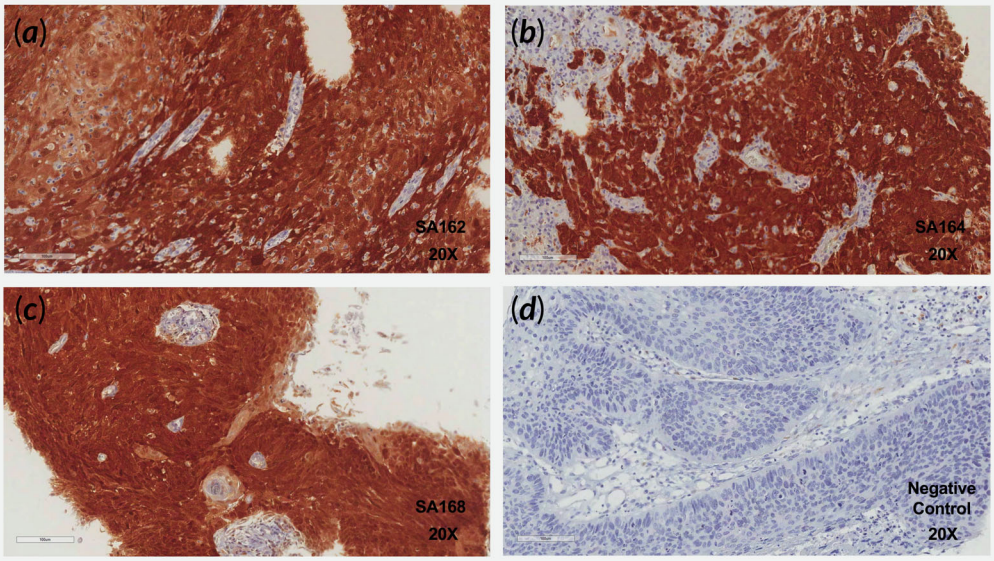

Figure 2. Expression of $p16^{ink4a}$ in HPV73-positive cervix cancer specimens. (Panels *a*–*d*): invasive cervix cancer epithelium, showing strong and diffuse expression of $p16^{ink4a}$ (brown stain).

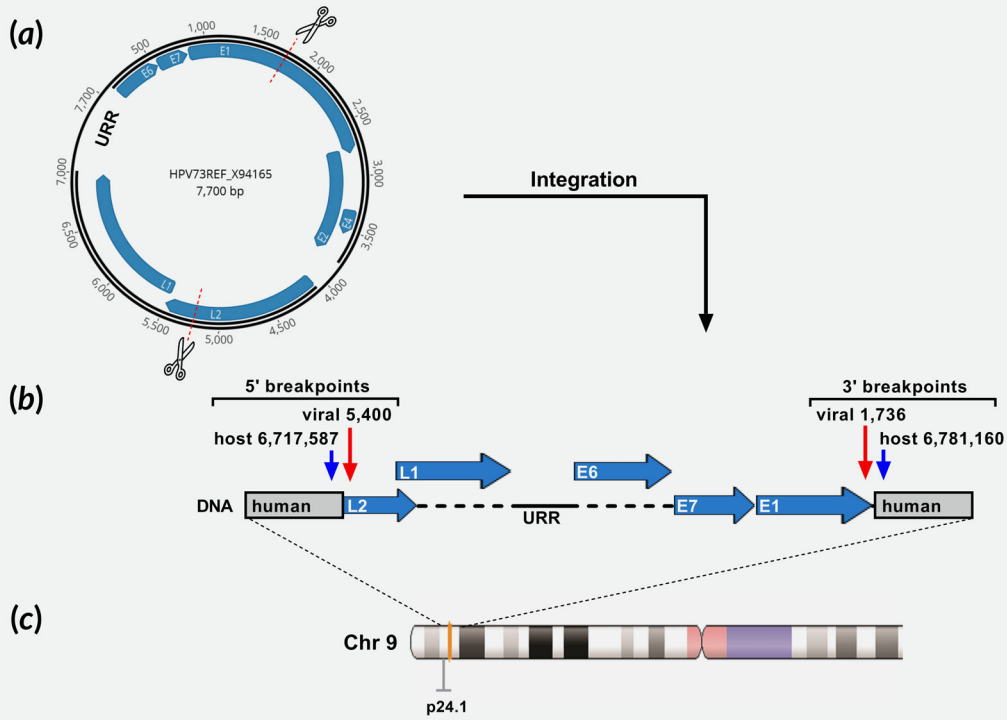

Figure 3. Pictorial representation of HPV73 integration in cervix cancer tissue (SA162). (Panel *a*) HPV73 reference circular genome (X94165) illustrating the deleted segment (red dash lines and scissors); (Panel *b*) HPV73 integration site, the viral-human junctions display the 5′ and 3′ regions of human host and viral joining (blue and red vertical arrows, respectively); (Panel *c*) chromosomal region of HPV73 integration at 9p24.1 (orange line represents deleted segment of human genome where HPV73 has inserted). The blue arrows in (panels *a* and *b*) show the organization of the HPV73 genome open reading frames (ORFs).

Table 2. Characterization of HPV73 integration sites identified by DNA capture NGS

Specimen	Total HPV reads	HPV-human reads	Sanger confirmation	Viral genome breakpoints[1]	Viral genes disrupted[2]	Human genome breakpoints (start–end)	Human genome location[3]	Candidate human genes affected
HEK-293	0	0	–	–	–	–	–	–
SiHa (HPV16)	803,129	224	–	3,132–3,384	E2/E4	73,214,729–73,513,425	13q22.1	LINC00393
HeLa (HPV18)	365,073	349	–	2,497–5,736	E1–L1	127,218,387–127,229,187	8q24.21	MYC
				3,100–7,857	E2/E4–URR	127,221,122–127,222,010	8q24.21	MYC
SA162	85,308	2,224	Yes	1,736–5,400	E1–L2	6,717,587–6,781,160	9p24.1	KDM4C
SA163	2,254,869	870	Yes	3,944–4,035	E5	226,422,324–226,522,308	2q36.3	–
		55	Yes	664–6,407	E7–L1	60,408,391–60,532,189	3p14.2	FHIT
		91		3,672–7,556	E2–URR	60,546,679–60,546,796	3p14.2	FHIT
		73	Yes	976–1,739	E1	64,606,478–64,622,870	13q21.31	–
		254		1,739–5,600	E1–L1	64,749,515–64,798,406	13q21.31	–
		261	Yes	1,586–5,785	E1–L1	88,781,555–88,821,135	15q26.1	ACAN
		82		3,204–3,949	E2/E4–E5	88,765,412–88,766,187	15q26.1	–
SA164	116,417	1,760	Yes	2,525–4,804	E1–L2	145,675,812–145,705,980	2q22.3	–
SA165	58,888	623	Yes	2,002–2,255	E1	245,483,898–245,491,529	1q44	KIF26B
SA166	209,361	444	Yes	1,659–4,883	E1–L2	184,829,647–184,829,746	1q25.3	FAM129A
		17	Yes	1,140–1,509	E1	16,658,594–16,690,000	13q11.1	–
SA167	25,308	675	Yes	2,282–3,228	E1–L2	73,664,158–73,728,281	4q13.3	–
SA168	6,903,435	70	Yes	3,376–4,804	E2/E4–L2	49,098,639–49,099,775	4p11	–
		706	Yes	3,762–5,554	E2/E4–L2	145,884,433–145,885,178	4q31.21	ZNF827
		868	Yes	3,469–4,346	E2/E4–L2	152,428,380–152,428,931	4q31.3	FBXW7
		28	No	4,804–5,249	L2	49,658,356–49,782,022	5q11.1	–
		20	No	4,804–5,207	L2	41,859,979–41,906,549	10q11.21	–
SA169	7,641	175	Yes	2,558–3,085	E1–E2	73,593,191–73,593,193	4q13.3	RASSF6
CIN2	192,402	0		Episomal	NA	Undetectable	–	–
CIN1	13,290	0		Episomal	NA	Undetectable	–	–
WNL_1	22,375	0		Episomal	NA	Undetectable	–	–
WNL_2	29,163	0		Episomal	NA	Undetectable	–	–

[1]The region between the viral genome breakpoints represents deletion of viral sequences.
[2]HPV73 Reference genome X94165 used to landmark nucleotide positions.
[3]UCSC genome browser GRCh38/hg38.
Abbreviations: CIN, cervical intraepithelial neoplasia; NA, not applicable; WNL, within normal limits.

with tumor DNA using primers designed from each NGS-identified viral–human chimeric junction and 90.0% (18/20) of the integrations were confirmed by Sanger sequencing. In addition to viral integration, the capture also provided viral genome sequence content, which revealed that all eight specimens contained HPV73B variant isolates (Supporting Information Fig. S3).

SiHa and HeLa cells were used as positive controls for methodological validation of the HPV capture-NGS-bioinformatic assay.[11,12] Consistent with previous studies, two chimeric junctions were observed in SiHa cells constituting the described integration site at loci 13q22.1[13–15]; and five chimeric concatenated junctions were observed for HeLa cells at loci 8q24.21 representing the four known concatenated viral copies (Table 2; Fig. 3).[12,13,15]

Discussion

The current study documents the detection of HPV73 in eight CxCa specimens and provides molecular evidence that HPV73 was associated with development of cervix cancer in these women. One of the key requirements to assign causality to a specific oncogenic HPV type is the presence of viral DNA and transcriptional activity in cancer tissues. In our study, we detected E6* spliced transcripts from HPV73 that could encode the E7 protein which results in activation of $p16^{ink4a}$ through retinoblastoma protein silencing. Consistent with this observation, we show that $p16^{ink4a}$ protein was overexpressed in the HPV73-positive cancers. It has also been previously shown that HPV73 E6 could degrade p53 in a biochemical assay.[16]

The detection of viral–human chimeric molecules in all eight CxCa cases indicated HPV73 was integrated into the cancer cell genome. Moreover, HPV73 integrations were found in genomic regions previously shown to contain HPV integrations: 2q22.3 in cervical squamous cell carcinoma (SCC), and 1q44, 3p14.2 and 9p24.1 in head and neck squamous cell carcinoma.[17] Taken together, these data suggest HPV73 employs similar molecular mechanisms of cervical epithelial cell transformation observed in HPV16-associated cervix cancers.[3]

HPV73 is classified as a member of the species group *Alphapapillomavirus11* and forms a clade with the highly oncogenic HPV16-containing species group *Alphapapillomavirus9*.[18] Isolates of HPV73 form two main lineage groups[19] and surprisingly, all viral isolate genomes identified in our study were of the HPV73B lineage (Supporting Information Fig. S3). Another study from Rio de Janeiro identified four HPV73 isolates that were classified as lineage A2.[20] Thus, multiple lineages of HPV73 circulate in Rio de Janeiro and it is possible that the B lineage has an increased oncogenicity, but more data will be required to establish this point.

The prevalence of HPV73 in cervix cancer varies by DNA testing method and geographic region, for example, 0.5% worldwide[1] to 1.4% in Rio de Janeiro, Brazil.[7] HPV73 has also been detected in 11% of adenosquamous cancers,[21] and a recent report found HPV73 DNA in three out of 10 cervical SCCs that were negative by Hybrid Capture 2 testing.[6] The prevalence of HPV73 in high-grade disease has been reported in multiple locations with substantial levels in Iran (25%, 4/16),[4] Northern Germany (4.3%, 13/301)[22] and Edinburgh (10.6%, 10/94).[23] HPV73 was reported as the fourth most common HPV type in cervix cancer in Oceania.[1] The overall prevalence of HPV73 by a specific serologic assay in Swedish women was 14%.[24] The carcinogenic potential for HPV73 is also suggested by its detection in penile cancer[25] and in multiple independent reports of periungual and digital squamous cell cancers.[26,27] These later reports indicate oncogenicity of HPV73 beyond cervix cancer. Based on the data in this report and others, HPV73 should be upgraded from being possibly carcinogenic (Group 2B) to being a carcinogen (Group 1) by International Agency for Research on Cancer (IARC).[28]

Currently, HPV73 is neither in HPV screens for cervix cancer prevention nor in the current HPV vaccines. HPV73 is also not distinguished in many common HPV tests worldwide, including SPF10/LiPA25 and Cobas®.[5,29] Thus, HPV73 and other nonvaccine covered oncogenic HPV types will continue to be a clinical concern and will not be recognized using current US Food and Drug Administration (FDA)-approved HPV tests. In addition, the possibility of changes in HPV type distribution in vaccinated women requires continued monitoring of cervical HPV prevalence and distribution. For example, regional differences in HPV prevalence of oncogenic types that are neither in HPV tests nor in vaccines should alert physicians to the possibility of cervix precancer and cancer in individuals. It will be up to public health workers to evaluate the cervix cancer screening strategies at the population level based on cotest performance and costs. Low prevalence HPVs associated with cancer can be addressed by the development of new diagnostics and/or broader coverage vaccines. Nevertheless, individual women could be at substantial risk outside the screening strategy recommended for the public and how to deal with such personalized risk needs to be considered in the future.

In summary, HPV73 prevalence varies by geographic location worldwide. The cohort from which these cases emerged had a higher or equivalent prevalence of HPV73 compared to HPV31 and HPV33, two *Alphapapillomavirus9* types included in both the Gardasil-9 HPV vaccine and detected in most HPV DNA screening assays.[30] This report is thus an example of an oncogenic HPV type from a population with a substantial prevalence of HPV73 in cervix cancer. There are many regions and specific populations that have "personalized" risks for a particular HPV type that will require rethinking screening and vaccination as we expand beyond HPV16 and HPV18. The data presented here should alert physicians and public health decision makers that HPV73 should be considered oncogenic and a continued risk for women that need monitoring, particularly in populations with elevated HPV73 prevalence in cervix cancer.

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Author Contributions

Study concept and design: Drs. Amaro-Filho SM, Gradissimo A and Burk RD had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Experiment development: Amaro-Filho SM, Gradissimo A. Bioinformatic pipeline design: Usyk M. Immunohistochemistry experiments: Moreira FCB. Acquisition, analysis, or interpretation of data: Amaro-Filho SM, Gradissimo A, Usyk M, Moreira MAM, Burk RD. Epidemiological data collection: de Almeida LM. Figure design: Amaro-Filho SM, Usyk M. Critical revision and proofreading of the manuscript: Amaro-Filho SM, Gradissimo A, Usyk M, Moreira FCB, de Almeida LM, Moreira MAM, Burk RD. Drafting of the manuscript: Amaro-Filho SM, Gradissimo A, Burk RD. Obtained funding: Moreira MAM, Burk RD.

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