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Keywords: Cervical cancer, Human papillomavirus, HPV 16, Variants

Posted Date: January 10th, 2020

DOI: <https://doi.org/10.21203/rs.2.20515/v1>

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Role of HPV 16 variants among cervical samples from Northeastern Brazil

Molecular variants of HPV 16 in Northeastern Brazil

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Abstract

Background: Cervical cancer is the fourth most common type of cancer affecting women globally. In Brazil, it is the third most frequent type of cancer in women and HPV is present in approximately 90% of cases. Evidence suggests that variants of HPV 16 can interfere biologically and etiologically during the development of cervical cancer. **Methods:** Cervix tumor fragments were collected, their DNA was extracted, and nested PCR was used to detect HPV. Positive samples were sequenced to determine the viral genotype. To characterize the HPV 16 strains, positive samples PCR was used to amplify the LCR and E6 regions of the HPV 16 virus. **Results:** Data from 120 patients with cervical cancer were analyzed. Most women were between 41 and 54 years of age, had schooling until primary school, a family income between 1 and 2 times the minimum wage and were married/in a consensual union. There was no statistically significant association between HPV or socio-demographic variables and risk factors for cervical cancer ($P < 0.05$). HPV was present in 88 women (73%). The most prevalent types were HPV 16 (47/54%), HPV 18 (12/13.8%), HPV 35 (6/6.9%) and HPV 45 (5/5.7%). Of the 47 HPV 16 positive cases, variant A (49%) was present in 23 samples, followed by variant D in 20 cases (43%), and variants B and C in 2 cases each (4%). The most prevalent

histological type of HPV 16 tumors was epidermoid carcinoma, followed by adenocarcinoma. There was a statistically significant association between HPV 16 variants and the tumors' histological types ($P < 0.001$). **Conclusions:** Knowledge of HPV 16 variants will provide data on their influence on the pathological and oncogenic aspects of cervical lesions.

Keywords: Cervical cancer; Human papillomavirus; HPV 16; Variants.

Background

Cervical cancer is the fourth most common cancer type affecting women worldwide, with more than 265,000 estimated deaths annually and more than 80% of cases occurring in developing countries [1,2]. According to INCA, cervical cancer is the third most common cancer type in the Brazilian female population. They estimate that there will be 16,370 new cervical cancer cases for the biennium from 2018-2019 [3].

It is estimated that in the state of Maranhão there will be around 1,090 new cervical cancer cases for 2018, with 240 of them in the capital, São Luís, with a gross rate of incidence of 30.55 cases per 100,000 inhabitants [3].

Human Papillomavirus (HPV) is the main etiological factor for cervical cancer and is associated with the development of diseases ranging from benign warts to invasive cancer [4]. Although approximately 80% of women acquire HPV infection by the age of 50, less than 1% of persistent infections progress to invasive cervical cancer [5].

1 The reason why only some uterine cervical lesions associated with high-
2 risk genotypes progress to invasive cancer remains unknown. Evidence
3 suggests that variants of the same HPV type can interact biologically and
4 etiologically during cancer development [6,7].

5 The HPV16 is the most carcinogenic HPV type followed by HPV18 and
6 most infections are asymptomatic [8]. A few studies have been conducted in
7 Brazil on HPV 16 variants. This type of study has never been conducted in
8 Maranhão. Interest in this topic has been growing in recent years, with variation
9 in carcinoma prognosis at different stages of the disease depending on the viral
10 variant found attracting special interest. Even in research carried out other
11 places in the world, many of these questions remain unclear and many of the
12 findings are contradictory, making it necessary to develop new and continuous
13 approaches to this topic.

14 Despite of the relationship between different HPV type and the
15 development of cancer being well established, evidence suggests that genetic
16 variations between the same viral type may influence infection potential, viral
17 persistence, the development of precursor lesions and the progression to
18 invasive cancer [9-12]. Due to the high prevalence of HPV 16 in cases of
19 cervical cancer, the association between HPV 16 and cancer has been studied
20 at the level of intratype variants, and several lines of study have attributed a
21 higher risk of invasive cervical cancer to some HPV 16 strains.

22 Studies on HPV variants have been developed with the objective of
23 understanding their association with pathological and oncogenic aspects of
24 cervical lesions. Factors influencing HPV infection in cervical cancer are not
25 completely clear, but it is believed that HPV 16 variants play a fundamental role

in cervical carcinogenesis and are currently recognized as an important marker for research on viral transmission, persistence and carcinogenicity [5]. Variation in these aspects may contribute to disparities in cervical cancer incidence.

Methods

Type of Study

This is descriptive, prospective, and transversal study.

Period and Location of study

The study was conducted from January 2016 to December 2017, at the High Complexity Care Unit (UNACON) of the Cancer State Hospital of Maranhão and in the High Complexity Care Center in Oncology (CACON) of the Aldenora Bello Cancer Hospital.

Population and Sample

The study population was composed of 120 women with a diagnosis of cervical cancer, who were treated at the previously mentioned hospitals.

Inclusion criteria

Women older than 18 years diagnosed with cervical cancer who agreed to participate in the study by signing an Informed Consent Form (ICF).

Exclusion criteria

Women who presented with surgical indications as an initial treatment or who presented with small lesions, in which biopsy could interfere with staging. Women in psychiatric treatment were also excluded.

Sample Calculation

According to estimates by the National Cancer Institute (INCA), the number of cases of cervical cancer malignant neoplasms in São Luis is 230 new cases for 2016. Since is a finite sample of less than 100,000 individuals we used the following formula:

$$n = \frac{n_0}{1 + \frac{n_0 - 1}{N}}$$

Legend:

n_0 = sampling intensity;

N = Population Sample Size.

with n_0 equal to:

$$n_0 = \frac{z(k)^2}{4d^2}$$

Legend:

$z(k)^2$ = value in the Student's t-test table with (n-1) degrees of freedom for the confidence interval;

d= error

If we adopt as a population, the incidence estimates of new cases for São Luís population by INCA, with a confidence interval of 95% and sample error of 5% we have the following values:

$$n_0 = \frac{1.96^2}{4(0.05)^2} = 384.16$$

Substituting n_0 into the equation, we have:

$$n = \frac{384.16}{1 + \frac{(384.16-1)}{230}} = 144.15$$

Thus, based on our population, 144 samples are indicated.

Collection instrument and data evaluation

Initially, the patients were referred for outpatient care at the Oncology Gynecology Service of the Cancer State Hospital of Maranhão and the Aldenora Bello Cancer Hospital and were invited to participate in the study by signing an Informed Consent Form (ICF). A questionnaire was supplied to collect socio-demographic, reproduction, and smoking data.

Cervical tumor fragments identified by gynecological examination were then placed in microtubes containing 1 mL of RNA Later solution (Life Technologies) at 4°C and were transported in thermal boxes to the Multiuser Laboratory in the Biobank of Tumors and DNA of Maranhão of the Federal University of Maranhão, University Hospital of the Federal University of

1 Maranhão-HUUFMA. After 24 hours, the samples were removed from the RNA
2 Later solution and stored in a freezer at -80°C until use.

3 **Experimental Procedures**

4 **DNA Extraction**

5 DNA was extract with a Dneasy Blood &Tissue Kit (QIAGEN Ltd., UK)
6 according to the manufacturer's directions. Briefly, up to25mg of tumor tissue
7 was sectioned with a sterile razor and deposited in 2 mL tubes, where they
8 were resuspended in 180 µL of lysis buffer (ATL) and 20 µL of Proteinase K.
9 The tubes were incubated at 56 °C in a thermomixer for 24h for tissue lysis.

10 200 µL of AL Buffer was then added to the mixture which was
11 homogenized in a vortex for 15s. The tubes were then incubated at 90 °C for 1
12 hour in a thermomixer, 200 µL of ethanol P.A. (Merck, BR) was added to the
13 sample and it was again homogenized in the vortex.

14 The mixture was transferred to a tube containing a silica column
15 (QIAamp MinElute) and centrifuged at 8,000 rpm for 2 minutes. After
16 centrifugation, the column was repositioned in a new collector tube, and the
17 filtrate was discarded.

18 The column was then washed successively with 500 µL of wash buffer 1
19 and wash buffer 2 centrifuging at 8,000 rpm for 2 minutes between washes. The
20 column was then incubated with 100 µL of AE Buffer at room temperature for 5
21 minutes before being centrifuged at 14,000 rpm for 4 minutes to obtain the
22 DNA. The samples were identified and stored at -20°C for later use in PCR
23 reactions.

DNA Quantification

The extracted DNA was quantified by reading absorbance in a Nanovue spectrophotometer (GE) at a wave length of 260nm. The purity of the DNA was verified by reading at 280nm to detect possible protein contamination. The samples were considered pure when the A260/A280 ratio was between 1.6 and 1.9.

Detection of HPV DNA by Nested PCR

HPV DNA in the cervical tumor samples was identified by Nested PCR using a Veriti 96 Well Thermal Cycler (Applied Biosystems, Thermo Scientific, California, USA). This technique consists of amplifying the DNA of interest in two rounds and using specific primers for round. With two rounds of PCR, the first amplification is more general and the second reaction, which uses the Amplicon produced in the first round as a template, leads to amplification of smaller and more specific sequences of viral DNA.

In the first-round, the primers PGMY 09 and 11 (Table 1) were used to amplify 450 bp sequences of the L1 region of viral DNA; in the second round, the primers GP +5 and GP+6 were used to amplify 190bp sequences of the L1 region of the viral DNA [12]. We used samples known to be positive as a positive control and water as a negative control.

To minimize the possibility of external contamination, the reactions were prepared in a laminar flow chapel exposed to ultraviolet light for approximately 15 minutes, along with all plastic material used.

- 1 **Table 1.** Primer sequences used for the Nested PCR reaction to identify HPV
 2 DNA.

Primer	Sequence 5' - 3'	
PGMY11	PGMY11-A	GCA CAG GGA CAT AAC AAT GG
	PGMY11-B	GCG CAG GGC CAT AAT AAT GG
	PGMY11-C	GCA CAG GGA CAT AAT AAT GG
	PGMY11-D	GCC CAG GGC CAC AAC AAT GG
	PGMY11-E	GCT CAG GGT TTA AAC AAT GG
PGMY09	PGMY09-F	CGT CCC AAA GGA AAC TGA TC
	PGMY09-G	CGA CCT AAA GGA AAC TGA TC
	PGMY09-H	CGT CCA AAA GGA AAC TGA TC
	PGMY09-Ia	G CCA AGG GGA AAC TGA TC
	PGMY09-J	CGT CCC AAA GGA TAC TGA TC
	PGMY09-K	CGT CCA AGG GGA TAC TGA TC
	PGMY09-L	CGA CCT AAA GGG AAT TGA TC
	PGMY09-M	CGA CCT AGT GGA AAT TGA TC
	PGMY09-N	CGA CCA AGG GGA TAT TGA TC
	PGMY09-Pa	G CCC AAC GGA AAC TGA TC
	PGMY09-Q	CGA CCC AAG GGA AAC TGG TC
	PGMY09-R	CGT CCT AAA GGA AAC TGG TC
	HMB01b	GCG ACC CAA TGC AAA TTG GT
GP+5/6	GP+5	TTT GTT ACT GTG GTA GAT ACT AC
	GP+6	GAA AAA TAA ACT GTA AAT CAT ATT C

The reaction conditions using primers PGMY09/11 were initial denaturation for 2 minutes at 95°C followed by 40 cycles of denaturation for 40 seconds at 95°C, annealing for 40 seconds at 55°C, and extension for 40 seconds at 72°C. The reaction mixture consisted of a final volume of 25 µL containing 10 pmol of each *primer*, 2.5 µL of 10x reaction buffer, 0.5 µL of magnesium chloride at 50 Mm, 10 Mm of dNTP and 0.2 µL of Taq polymerase Platinum (Invitrogen) [15].

The reaction conditions for the second round of the Nested PCR with primers GP5 +/-GP6+ was an initial denaturation of 4 minutes at 95°C followed by 45 cycles of denaturation for 45 seconds at 95°C for 1 min, annealing for 1 minute at 40 °C and extension for 1 minute at 72°C. The reaction mixture consisted of a final volume of 25 µL, containing 10 pmol of each primer, 2.5 µL of 10x buffer, 1.5 µL of magnesium chloride at 50 Mm, 10 Mm of dNTP and 0.3 µL of Taq polymerase Platinum (Invitrogen) [15].

Visualization of amplified products

5 µL aliquots of each reaction with loading buffer (Sigma-Aldrich, USA) and 0.1% Gel Red dye were visualized by electrophoresis in 1.5% agarose gel in TBE 1X buffer.

Purification of PCR products

The PCR products were purified with a Genelute PCR Clean up Kit according to the manufacturer's protocol (Sigma-Aldrich, USA). Briefly, 500 µL of capture buffer was added to 100 µL of PCR product in each microtube. The material was centrifuged and 2 µL of purified DNA was run in a 2% agarose gel to evaluate the purified sample.

Automatic sequencing

HPV genotypes were determined by automated sequencing of the PCR products using a1000 MegaBACE sequencer (GE Healthcare, UK) at the Molecular Biology Laboratory of the State University of Maranhão located in the Center of Higher Studies of Caxias (CESC-UEMA).

Sequencing was performed with an ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, UK), according to the manufacturer's protocol. Each reaction contained 2 µL of the purified PCR product, 40 ng of specific oligonucleotides for the specific exon (sense or antisense) and 2 µL of the Big Dye kit.

The Chromas program was used to obtain electropherograms of the HPV DNA sequences present in the samples. To identify the HPV type the nucleotide sequences were compared to the Genbank Nucleotide Sequence Database using the BLAST program (NCBI).

Identification of HPV 16 variants

After determination of the HPV 16 strains, the LCR regions and E6 virus gene of the positive samples were amplified with specific primers. The reaction mixture consisted of a final volume of 25 µL, with 1X PCR Buffer, 2.5mM of MgCl₂, 0.25 µM of each dNTP, 100 pmol/L of each primer, 50 – 100 ng of DNA, and 2.5 U of Platinum Taq Polymerase. The PCR reaction consisted of denaturation at 95°C for 10 min, followed by 40 cycles of 95 °C for 1 min; annealing temperature for 1 min and 72 °C for 1 min, followed by a final extension step for 15 min.

The PCR product was subsequently purified and sequenced according to the above protocol. The consensus sequences were merged using the Geneious software (Biomatters Ltd.) and all the sequences generated were aligned with HPV 16 specific strains, using the reference sequences proposed by Burk *et al* (2013), using the MEGA Software (version 6.0, www.megasoftware.net). BLAST online (Available at: <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) was then used to identify the HPV types.

Statistical analyses

Descriptive statistical analysis was performed using the Stata program (version 14.0). The χ^2 (Chi-square) test was used to verify the association between HPV and sociodemographic and clinical variables, and P values ≤ 0.05 were considered statistically significant. The values corresponding to don't know/did not respond were excluded from the association analysis.

Phylogenic Analysis

The phylogenetic tree of the HPV 16 strains was constructed from the 1,300bp sequences from the E6 and LCR regions using the "neighbor joining" method with p-distance (obtained with pairwise deletion) using the program Mega 4.1. The references proposed by Burk *et al*. (2013) [20] were included. The analysis of the HPV 16 variants was performed at the National Cancer Institute (INCA) under the supervision of Dr. Miguel Ângelo Martins Moreira.

RESULTS

Sociodemographic and clinical data

Among the women diagnosed with cervical cancer, HPV was present in 88 (73.3%). The majority of these women were in the age group 40 to 49 years of age (34/28.33%), and self-declared with mixed-race (84/70%), had schooling up to elementary school (51/42.50%), had a family income between 1 and 2 times the minimum wage (66/55%) and were married or in a consensual union (62/51.67%). There was no statistically significant association between the sociodemographic variables and the presence of HPV ($p < 0.05$) (Table 2).

Table 2. Association between sociodemographic factors and the presence of Human Papillomavirus.

Sociodemographic Variables	HPV						p-value
	Total		Negative		Positive		
	N	(%)	n	%	N	%	
Age (Years)							
≤ 29	8	(6.67)	4	50.00	4	50.00	0.421
30 to 39	20	(16.67)	5	25.00	15	75.00	
40 to 49	34	(28.33)	9	26.47	25	73.53	
50 to 59	19	(15.83)	7	36.84	12	63.16	
60 to 69	19	(15.83)	4	21.05	15	78.95	
≥ 70	20	(16.67)	3	15.00	17	85.00	

Ethnicity

European-descent	20	(16.67)	3	15.00	17	85.00	0.377
African-descent	13	(10.83)	2	15.38	11	84.62	
East Asian- descent	3	(2.5)	1	33.33	2	66.67	
Mixed-race	84	(70)	26	30.95	58	69.05	

Marital Status

Single	39	(32.5)	12	30.77	27	69.23	0.774
Married/Consensual Union	62	(51.67)	14	22.58	48	77.42	
Divorced/Separated	6	(5)	2	33.33	4	66.67	
Widow	13	(10.83)	4	30.77	9	69.23	

Family Income

Less than the minimum wage	29	(24.17)	7	24.14	22	75.86	0.797
1 to 2 times the minimum wage	66	(55)	18	27.27	48	72.73	
Above 2 times the minimum wage	20	(16.67)	4	20.00	16	80.00	
Don't Know/Didn't Answer	5	(4.16)	3	60.00	2	40.00	

Education

None	41	(34.17)	13	31.74	28	68.29	0.399
Adult Education	4	(3.33)	0	0.00	4	100.0	
Primary Education/Primary Level	51	(42.50)	11	21.57	40	78.43	
High School/Secondary Level	20	(16.67)	8	40.00	12	60.00	
Higher Incomplete	1	(0.83)	0	0.00	1	100.0	
Higher Complete	1	(0.83)	0	0.00	1	100.0	

Don't Know/Didn't Answer 2 (1.67) 0 0.00 2 100.0

Of the risk factors associated with cervical cancer, for most women first sexual intercourse occurred between 10 and 19 years of age (87/72.50%), a first pregnancy between 16 and 21 (41/34.17%) and 1 to 3 children (37/30.83%) were most common. Having only one sexual partner during their lifetime (38/31.67%) was most common followed by more than 3 partners (36/30%). Most women reported never having used contraceptive methods (70/58.33%) (Table 3).

Table 3. Association between cervical cancer risk factors, reproductive history and presence of HPV

Risk Factors	HPV						p-value
	Total		Negative		Positive		
	n	%	N	%	N	%	
Onset of sexual activity (years)							
10 to 19	87	(72.50)	25	28.74	62	71.26	0.738
20 to 29	9	(7.50)	3	33.33	6	66.67	
Above 30	2	(1.67)	0	0.00	2	100.0	
Don't Know/ Did not answer	22	(18.33)	4	18.18	18	81.82	
Age at 1 st pregnancy							
11 to 15	11	(9.17)	1	9.09	10	90.01	0.268
16 to 21	41	(34.17)	14	34.15	27	65.85	

22 to 27	13	(10.83)	5	38.46	8	61.54	
Above 27	2	(1.67)	0	0.00	2	100.0	
Don't Know/ Did not answer	53	(44.17)	12	22.41	41	77.36	
Number of pregnancies							
1 to 3	37	(30.83)	11	29.73	26	70.27	
4 to 6	26	(21.67)	6	23.08	20	76.92	
7 to 9	32	(26.67)	8	25.00	24	75.00	0.859
10 to12	13	(10.83)	4	30.77	9	69.23	
Above 12	8	(6.67)	1	12.50	7	87.50	
Don't Know / Did not answer	4	(3.33)	2	50.0	2	50	
Use of contraceptive							
Yes. Actually Use	7	(5.83)	2	28.57	5	71.43	
Yes. Already Used	40	(33.33)	12	30.00	28	70.00	
No	70	(58.33)	18	25.71	52	74.29	0.887
Don't Know /Did not answer	3	(2.50)	0	0.00	3	100	
Number of sexual partners							
1	38	(31.67)	11	28.95	27	71.05	
2	27	(22.50)	4	14.81	23	85.19	0.406
≥ 3	36	(30.00)	8	22.22	28	77.78	
Don't know/Did not answer	19	(15.83)	9	47.37	10	52.63	

1

2 Most women (84/70%) reported having undergone examination before
3 their diagnosis with cervical cancer and most (44/36.67%) said they received

1 preventive exam annually. Most women reported no tobacco use (69
2 /57.50%) (Table 4).

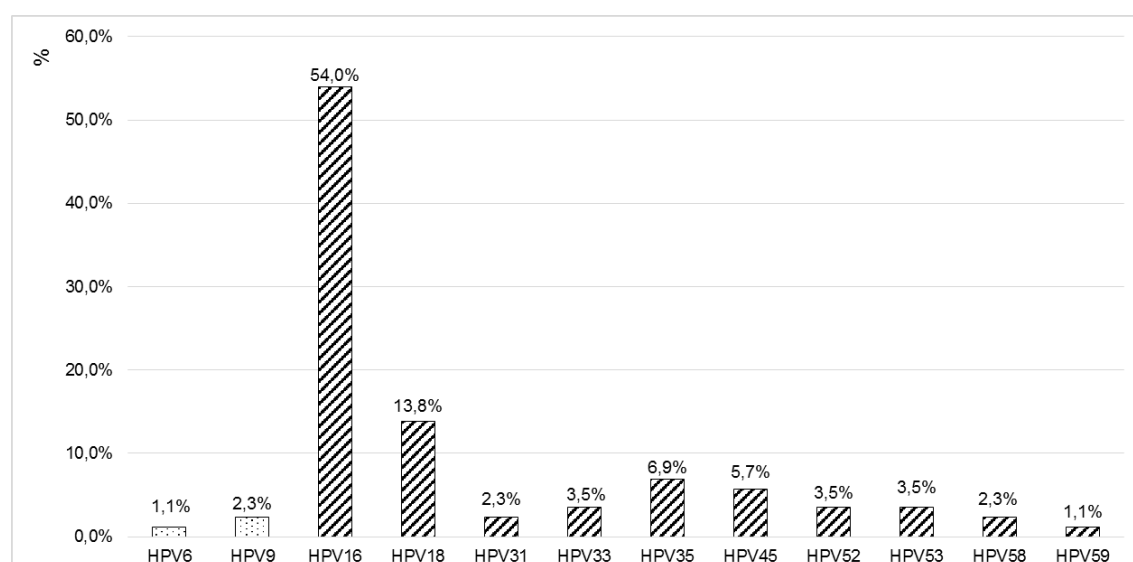
3 **Table 4. Association between preventive examination, smoking and the**
4 **presence of Human Papillomavirus**

Risk Factors	HPV						p-value
	Total		Negative		Positive		
	n	%	n	%	n	%	
Preventive examination before diagnosis							
No	29	(24.17)	12	41.38	17	58.62	0.070
Yes	84	(70.00)	20	23.81	64	76.19	
Don't Know/ Did not Answer	7	(5.83)	0	0.00	7	100.00	
Frequency of Pap test							
Every Year	44	(36.67)	12	27.27	32	72.73	0.153
Every 2 Years	9	(7.50)	1	11.11	8	88.89	
Every 3 Years	1	(0.83)	1	100.0	0	0.00	
4 years or more	1	(0.83)	1	100.0	0	0.00	
Without Regularity	32	(26.67)	8	25.00	24	75.00	
Don't Know/ Did not answer	33	(27.50)	9	27.27	24	72.73	
Smoking							
No	69	(57.50)	22	31.88	47	68.12	0.208
Yes	43	(35.83)	9	20.93	34	79.07	

Don'tKnow/ Did not answer 8 (6.67) 1 12.50 7 87.50

Identification of HPV type

HPV was present in 88 women (73.33%). HPV 16 was the most prevalent type among the analyzed cases and combined with HPV 18 accounted for 68.17% of cases. Most of the samples had HPV types considered high risk, while less than 3.4% had HPV types considered to have low oncogenic risk (Fig 1).



Histological types of analyzed tumors

The most prevalent types of tumor histological types were epidermoid carcinoma (EC) with a total of 95 cases (79.1%) and 11 (9.1%) cases of adenocarcinomas. Of the 120 women in this study, 45 (37.5%) had stage IIIB tumors, in cases where HPV 16 was present, stage III was predominant with 23 samples.

HPV 16 Variants

Of the 47 samples identified as HPV 16, 23 samples were variant A (49%), 20 (43%) were variant D, while variants B and C were present in two samples each (4%).

The mean age for women with variant A of HPV 16 was 50.6 years, 58.5 years for variant B, 58.5 years for variant C and 50 years for those with variant D. The most prevalent histological type in tumors with HPV 16 was squamous cell carcinoma (SCC) in 38 samples, followed by adenocarcinoma in 4 samples (11%).

There was a statistically significant association between histological type and HPV variant ($p < 0.001$), regarding squamous cell carcinoma and HPV 16 variant “A” and “D”. Staging also changed depending on the HPV 16 variant. Among patients with variant A, stage III (9 patients) was predominant followed by stage II (8 cases). For those with variant D, clinical stage III (10 cases) was predominant, followed by stage II (4 cases).

Treatment response also depended on the HPV 16 variant. For example, 12 cases with variant A showed complete remission, compared to only 7 cases with variant D. Likewise, there were 2 deaths with variant A, and 3 who had variant D. In 12 cases it was not possible to evaluate disease status at the end of the first treatment.

A reference sequence described by Burk *et al.* (2013) was used to construct the phylogenetic tree. Among HPV 16 cervical samples classified as belonging to variant A, 21 belonged to sublineage A1, 1 belonged to sublineage

1 A2 and 1 belonged to sublineage A4. Of the samples belonging to variant B, 1
2 belonged to sublineage B1 and 1 belonged to sublineage B2.

3 Of the HPV 16 cervical samples belonging to variant D, 1 belonged to
4 sublineage D1, 1 belonged to sublineage D2 and 18 belonged to sublineage
5 D3. Variant C, with 2 cases, does not have any sublineages (Fig 2).

Discussion

Cervical cancer is associated with low socioeconomic indices, presenting a higher prevalence in regions with high poverty, high illiteracy rates and precarious hygiene habits [13,14].

The virus was highly prevalent (88/120 – 73%) in the tumor samples examined. The prevalence of HPV in invasive cervical cancer samples can range from 70% to 100%, which may be associated with different techniques used to detect the virus [15-17].

HPV 16 is the most prevalent in cervical cancer worldwide, followed by HPV 18. However, the frequency of HPV types may vary according to the geographic region of the population under analysis. Other studies point out that types 16, 18, 31, and 45 as the four most prevalent HPV types in South and Central America [18,19].

Studies have sought to evaluate the role of intratype variants of HPV16 and HPV18 in the persistence of viral infection, the risk of cervical intraepithelial neoplasia development, and the development of invasive cervical cancer [11,20].

For both HPV 16 and HPV 18, the distribution of variants worldwide is influenced by geographic and ethnic factors.

In a case-control study conducted by Hang *et al.* (2016) [5], we attempted to evaluate the association between HPV 16 variants and the risk of cervical cancer in 298 women with HPV 16 in China and found that variant A was predominant.

1 In the study conducted by Volpini *et al.*, (2017) [21] in Brazil on 24
2 women positive for HPV 16, with and HIV diagnosis, but anormal Pap smear,
3 variant A accounted for 70.8% of cases (17/24), followed by the C and D
4 variants, which together totaled 29.2% of the samples (7/24). Variant B was not
5 found in that study.

6 In a study conducted in Brazil by Vidal *et al.* (2016) [12] on a cohort of
7 594 women with invasive cervical cancer, 334 women had HPV 16. Of these,
8 217 (65%) belonged to variant A, 97 (29%) belonged to variant D. Variants B
9 and C had 10 cases (3%) each.

10 A study by Villa *et al.* (2000) [22], also in Brazil, examined the geographic
11 differences in intratype variations of HPV 16 and their associations with the
12 development of cervical cancer precursor lesions. Variant A (54%) was the
13 most frequent, followed by variant D in 22% of the cases. It also emphasizes
14 the strong association between persistence and the presence of the non-
15 European variants B, C and D when compared to the European prototype A.

16 In the present study in São Luís do Maranhão, the most prevalent strain
17 in the population studied was variant A for HPV 16, followed by variant D.

18 Studies also indicate a variation in the distribution of HPV types among
19 histological types. HPV 16 has been associated with squamous cell carcinoma
20 and HPV 18 has a higher prevalence in adenocarcinoma than HPV 16.
21 However, few studies have attempted to identify associations between HPV 16
22 variants and the histologic type of cervical tumors [23,24]

23 In the study by Hang *et al.* (2016) [5], it was observed among the cases
24 of women with cervical cancer infected with HPV 16, 289 (97%) had a pattern of

squamous cell carcinoma, followed by adenocarcinoma (2.3%) and adenosquamous carcinoma (0.7%).

Few studies have been conducted to evaluate the effect of HPV 16 variants on the response to oncologic treatment. After analyzing 155 cases of HPV16 positive cervical cancer (132 cases of variants E x 23 cases of NE variants) Zuna *et al* (2011) [25] came to the conclusion that the non-European variants showed less aggressive behavior in relation to mortality than the European variants. It should be pointed out that the data should be evaluated with caution due to the small number of cases of NE variants in the study.

In our study we found a greater number of NE variants (B, C, and D). Of the 47 cases we found 23 with variant A (E) and 24 with variants B, C, and D. The mortality of patients with the NE variants was 13% (3/24), while it was 9% (2/23) in those with variant A (E). The number of cases of complete remission was also lower with the NE variants (33% x 52%). These findings can be explained by the large number of cases of variant D in our study (20 cases). A study by Burk *et al* (2013) [20], showed the specific aggressiveness of this variant.

We observed the predominance of sublineage A1 (21 cases), followed by sublineage D3 (18 cases). In the study by Hang *et al* (2016) [5], the A4 sublineage was associated with a significantly higher risk of cervical cancer than the A1-A3 sublineages (OR = 1.72, 95% CI).

In a study by Alfaro *et al.*, (2016) [26] of 462 women with cervical cancer, HPV 16 was identified in 50.9% of the cases. Of these, the A1/2 (31.4%) sublineage was predominant, followed by the D2 (10.4%) and D3 (9.1%)

sublineages. However, AA variants accounted for 38.7% of the HPV 16 positive cases.

A study by Nicolas-Parraga *et al.* (2017) [27], sought to explore the prevalence of HPV 16 variants in patients with cervical cancer from Europe, South-Central America, Asia and Africa. 118 cases of squamous cell carcinoma, 120 of adenocarcinoma, and 53 of adenosquamous carcinoma were observed. Also, the highest prevalence of HPV 16 was observed in patients with squamous cell carcinoma. When examining HPV 16 variants, the A1 – 3 sublineages were predominant in squamous cell carcinomas (76.9 to 97% for different geographic regions) and there was a large variation for variant D depending on the geographic region in adenocarcinoma (28.6 to 63.3% for different geographic regions), and adenosquamous carcinoma (12.5 to 61.5% for different geographic regions).

The data also showed an increase in the prevalence of A1 – 3 in Europe (67.9 to 97% for all histological types), variant D in South-Central America (61.5 to 63%, for adenocarcinoma and adenosquamous carcinoma), A4 in Asia (from 11.5 to 27.6% for all types) and variants B and C for Africa (from 28 to 66.7% and from 12.3 to 37.5% for ECC and ADC) [27]

These results show a differential association between HPV 16 lineages and the histological features of cervical tumors in different geographic regions. Molecular differences among viral lineages and interactions with tumors may be one of the factors associated with the observed variation in prevalence.

There have also been studies on socioeconomic aspects. A study by Wang *et al.* (2015) [28] reported that women who received Pap smear test

tended to have higher education levels, corroborating the results of other studies, and that this may be associated with a lack of information and delays in searching for treatment. In a study conducted by Manga *et al.* (2015) [29] on 209 women who sought care for cervical cancer screening, the mean age was 39.6 years old, and only 15% of the women were not literate, 88% were married and 48% had a paid activity.

Conclusions

In this study conducted in São Luís-MA, it was shown that variant A was the most prevalent variant in HPV 16 patients, corroborating data from the literature that shows a higher prevalence of variant A in mixed populations, as is the case of the Brazilian population.

Further studies are needed to understand the origin and progression of cancer, as well as the relationship between HPV 16 and its variants with the development of lesion, a cervical cancer precursor. The data presented here may help the development of future epidemiological studies on HPV 16 variants, as well as in the creation of strategies to combat the types that remain circulating, and which were not included in the currently available vaccines against HPV.

Abbreviations

DNA: deoxyribonucleic acid; HPV: Human Papillomavirus; ICF: Informed Consent Form; INCA: National Institute of Cancer; RNA: ribonucleic acid

Declarations section

Ethics approvals and consent to participate

This project was approved by the Research Ethics Committee of the Federal University of Maranhão (CEP-UFMA), under Consolidated Opinion N° 1.289.419/2015. All women have signed an Informed Consent Term to participate of this research.

Consent to publish

All women have signed an Informed Consent Term for data publication.

Availability of data and materials

All data is included in the manuscript file. The data is available with the researches in their own data bank.

Competing interests

The authors declare that there are any conflicts of interest in this work

Funding

Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Estado do Maranhão (FAPEMA). EDITAL UNIVERSAL-00668/15.

FAPEMA has supported the material and equipments acquisition for this research.

Author's contributions

RLS, ZSB, FCBV and MDSBN participated in interpretation of data, drafted and critically revised the manuscript. RLS, MAMM, FVF, ZSB, LMOB, MCLB and MDSBN contributed to study design, interpretation of data, and critically revised the manuscript. MACNS, GRB, APAC, LOC and LAP analyzed and assisted in interpretation of the data and assisted in drafting the manuscript. WEMF, DFS, MCB and ECF contributed to interpretation of data and critically revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank to National Institute of Cancer, Genetic Division to HPV variants analyses.

References

1. Bahls L, Yamakawa R, Zanão K, Alfieri D, Flauzino T, Delongui F, et al. Human Leukocyte Antigen Class I and Class II Polymorphisms and Serum Cytokine Profiles in Cervical Cancer. *Int J Mol Sci*. 2017 Aug 31;18(9).
2. Barroeta JE, Adhikari-Guragain D, Grotkowski CE. Cervical cancer screening in the era of HPV vaccination: A review of shifting paradigms in cytopathology. *Diagn Cytopathol*. 2017 Oct;45(10):903-914.

3. Instituto Nacional de Câncer. Estimativa 2018: incidência de câncer no Brasil / Instituto Nacional de Câncer José Alencar Gomes da Silva. Coordenação de Prevenção e Vigilância. – Rio de Janeiro: INCA, 128p. 2017.
4. Doorbar J, Egawa N, Griffin H, Kranjec C, Murakami I. Human papillomavirus molecular biology and disease association. *Rev Med Virol.* 2015 Mar;25 Suppl 1:2-23
5. Hang D, Yin Y, Han J, Jiang J, Ma H, Xie S, et al. Analysis of human papillomavirus 16 variants and risk for cervical cancer in Chinese population. *Virology.* 2016 Jan 15;488:156-61
6. Bernard HU, Calleja-Macias IE, Dunn ST. Genome variation of human papillomavirus types: phylogenetic and medical implications. *Int J Cancer.* 2006 Mar 1;118(5):1071-6.
7. Xi LF, Koutsky LA, Hildesheim A, Galloway DA, Wheeler CM, Winer RL, et al. Risk for high-grade cervical intraepithelial neoplasia associated with variants of human papillomavirus types 16 and 18. *Cancer Epidemiol Biomarkers Prev.* 2007 Jan;16(1):4-10.
8. Chen W, Molijn A, Enqi W, Zhang X, Jenkins D, Yu X, et al. The variable clinicopathological categories and role of human papillomavirus in cervical adenocarcinoma: A hospital based nation-wide multi-center retrospective study across China. *Int J Cancer.* 2016 Dec 15;139(12):2687-2697
9. Bernard HU. The clinical importance of the nomenclature, evolution and taxonomy of human papillomaviruses. *J Clin Virol.* 2005 Mar;32 Suppl 1:S1-6.

- 1 10. Tamegão-Lopes BP, Sousa-Júnior EC, Passetti F, Ferreira CG, de Mello
2 WA, Duarte Silvestre RV. Prevalence of human papillomavirus infection and
3 phylogenetic analysis of HPV-16 E6 variants among infected women from
4 Northern Brazil. *Infect Agent Cancer*. 2014 Aug 5;9:25.
- 5 11. Chen AA, Gheit T, Franceschi S, Tommasino M, Clifford GM; IARC HPV
6 Variant Study Group. Human Papillomavirus 18 Genetic Variation and Cervical
7 Cancer Risk Worldwide. *J Virol*. 2015 Oct;89(20):10680-7.
- 8 12. Vidal JP, Felix SP, Chaves CB, Patury P, Franco VF, de Moraes EA. Genetic
9 diversity of HPV16 and HPV18 in Brazilian patients with invasive cervical
10 cancer. *J Med Virol*. 2016 Jul;88(7):1279-87
- 11 13. Castellsagué X, Schneider A, Kaufmann AM, Bosch FX. HPV vaccination
12 against cervical cancer in women above 25 years of age: key considerations
13 and current perspectives. *Gynecol Oncol*. 2009 Dec;115(3 Suppl):S15-23
- 14 14. Pinho-França JR, Chein MBDC, Thuler LCS. Patterns of cervical cytological
15 abnormalities according to the Human Development Index in the northeast
16 region of Brazil. *BMC Womens Health*. 2016 Aug 12;16:54.
- 17 15. Coutlée F, Gravitt P, Kornegay J, Hankins C, Richardson H, Lapointe N, et
18 al. Use of PGMY primers in L1 consensus PCR improves detection of human
19 papillomavirus DNA in genital samples. *J Clin Microbiol*. 2002 Mar;40(3):902-7.
- 20 16. Vidal FCB, Nascimento MDSB, Ferraro CTL, Brito, LMO. Análise crítica dos
21 métodos moleculares para detecção do papilomavírus humano: revisão da
22 literatura. *Femina*. 2012. 40(5):264–7.

- 1 17. Schiffman M, Doorbar J, Wentzensen N, de Sanjosé S, Fakhry C, Monk BJ.
2 Carcinogenic human papillomavirus infection. *Nat Rev Dis Primers*. 2016 Dec
3 1;2:16086.
- 4 18. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive
5 women in cervical cancer screening. *J Clin Virol*. 2016 Mar;76 Suppl 1:S49-
6 S55.
- 7 19. Serrano B, Brotons M, Bosch FX, Bruni L. Epidemiology and burden of
8 HPV-related disease. *Best Pract Res Clin Obstet Gynaecol*. 2018 Feb;47:14-26
- 9 20. Burk RD, Harari A, Chen Z. Human papillomavirus genome variants.
10 *Virology*. 2013 Oct;445(1-2):232-43.
- 11 21. Volpini LPB, Boldrini NAT, de Freitas LB, Miranda AE, Spano LC. The high
12 prevalence of HPV and HPV16 European variants in cervical and anal samples
13 of HIV-seropositive women with normal Pap test results. *PLoS One*. 2017 Apr
14 20;12(4):e0176422.
- 15 22. Villa LL, Sichero L, Rahal P, Caballero O, Ferenczy A, Rohan T, Franco EL.
16 Molecular variants of human papillomavirus types 16 and 18 preferentially
17 associated with cervical neoplasia. *J Gen Virol*. 2000 Dec;81(Pt 12):2959-68.
- 18 23. Altekruze SF, Lacey JV Jr, Brinton LA, Gravitt PE, Silverberg SG, Barnes
19 WA Jr, et al. Comparison of human papillomavirus genotypes, sexual, and
20 reproductive risk factors of cervical adenocarcinoma and squamous cell

- 1 carcinoma: Northeastern United States. *Am J Obstet Gynecol.* 2003
- 2 Mar;188(3):657-63.
- 3 24. Burk RD, Chen Z, Van Doorslaer K. Human papillomaviruses: Genetic basis
- 4 of carcinogenicity. *Public Health Genomics.* 2009;12(5-6):281-90.
- 5 25. Zuna RE, Tuller E, Wentzensen N, Mathews C, Allen RA, Shanesmith R, et
- 6 al. HPV16 variant lineage, clinical stage, and survival in women with invasive
- 7 cervical cancer. *Infect Agent Cancer.* 2011 Oct 28;6:19.
- 8 26. Alfaro A, Juárez-Torres E, Medina-Martínez I, Mateos-Guerrero N, Bautista-
- 9 Huerta M, Román-Bassaure E, et al. Different Association of Human
- 10 Papillomavirus 16 Variants with Early and Late Presentation of Cervical Cancer.
- 11 *PLoS One.* 2016 Dec 30;11(12):e0169315.
- 12 27. Nicolás-Párraga S, Alemany L, de Sanjosé S, Bosch FX, Bravo IG; RIS
- 13 HPV TT and HPV VVAP study groups Differential HPV16 variant distribution in
- 14 squamous cell carcinoma, adenocarcinoma and adenosquamous cell
- 15 carcinoma. *Int J Cancer.* 2017 May 1;140(9):2092-2100
- 16 28. Wang B, He M, Chao A, Engelgau MM, Saraiya M, Wang L, Wang L.
- 17 Cervical Cancer Screening Among Adult Women in China, 2010. *Oncologist.*
- 18 2015 Jun;20(6):627-34.
- 19 29. Manga MM, Fowotade A, Abdullahi YM, El-Nafaty AU, Adamu DB, Pindiga
- 20 HU, et al. Epidemiological patterns of cervical human papillomavirus infection

1 among women presenting for cervical cancer screening in North-Eastern
2 Nigeria. Infect Agent Cancer. 2015 Oct 2;10:39.

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6 **Figure Legends**

7 **Figure 1:** Prevalence of HPV types in cervical cancer tissues

8 **Figure 2:** Phylogenetic tree of HPV 16 with lineages. The tree was constructed
9 using Neighbor-Joining with *pairwise deletion*. The numbers at each node are
10 bootstrap values (with 1000 replicates).

11