IJMS
Vol 46, No 4, July 2021

Original Article

Detection of Human Papillomavirus in Papillary Thyroid Carcinoma and its Association with Tumor Staging and Pathologic Features

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Received: 07 September 2019
Revised: 18 April 2020
Accepted: 29 April 2020

Abstract

Background: The role of human papillomavirus (HPV), as a common infection, has been evaluated in many cancers such as the cervix and squamous cell carcinoma of the head and neck. To the best of our knowledge, for the first time, the association of HPV with papillary thyroid carcinoma (PTC) and its pathologic features are investigated.

Methods: A retrospective cross-sectional study was conducted from May 2014 to January 2018 in several hospitals affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. Thyroid tissue specimens of patients diagnosed with PTC (n=82) and benign thyroid nodules (n=77) were collected using the consecutive sampling method. The presence of HPV in PTC, adjacent normal tissue, and benign thyroid nodules was evaluated using the polymerase chain reaction (PCR) method. The frequency of HPV positivity in PTC tissues was compared with benign thyroid nodules and adjacent normal tissue. Association of pathologic features of PTC with HPV positivity was also investigated. Data were analyzed using SPSS version 21.0, and P values less than 0.05 were considered statistically significant.

Results: HPV PCR positivity was observed in 3.8% of benign thyroid nodules and 13.4% of PTC samples but in none of the adjacent normal tissues. After adjustment for age and sex, the prevalence of HPV PCR positivity in the PTC tissues was significantly more than the benign thyroid nodules (P=0.015). The prevalence was also significantly higher than the adjacent normal tissues (P<0.001).

Conclusion: There was a significant association between PTC and HPV positivity. Further studies are required to determine the cause and effect of the association between these two conditions.

Please cite this article as: Archin Dialameh P, Saki F, Monabbati A, Dehghanian AR, Valibeigi B, Soveid M. Detection of Human Papillomavirus in Papillary Thyroid Carcinoma and its Association with Tumor Staging and Pathologic Features. Iran J Med Sci. 2021;46(4):256-262. doi: 10.30476/ijms.2020.83135.1191.

Keywords ● Papillomaviridae ● Thyroid cancer ● Papillary ● Thyroid neoplasms

What's Known

- Known risk factors for the development of papillary thyroid carcinoma are exposure to ionizing radiation, high iodine intake, autoimmune thyroid diseases, and genetic predisposition.
- The role of parvovirus B19, herpesvirus, and polyomavirus in the pathogenesis of papillary thyroid carcinoma has been suggested. Human papillomavirus causes various cancer types.

What's New

- For the first time, we demonstrated the presence of human papillomavirus DNA in thyroid tissue and its association with papillary thyroid carcinoma.

Introduction

Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy comprising 50-90% of the differentiated (follicular cell) thyroid carcinomas.[1] Despite many research studies, the etiology of this common thyroid malignancy remains unclear.[2] Possible risk factors for PTC are exposure to ionizing radiation, high

iodine intake, autoimmune thyroid disease, and genetic predisposition.[2] The role of parvovirus B19 in the pathogenesis of PTC has also been suggested.[3, 4] A study reported the presence of a high proportion of parvovirus B19 nucleic acids and proteins in PTC tissues.[3] In addition, a correlation between parvovirus B19 and all types of thyroid cancer has been reported.[4] A probable effect of parvovirus B19 on the activation of nuclear factor-kappa light chain enhancer of activated B cells (NF-kB) has been indicated as a factor in the pathogenesis of PTC.[4] In contrast to previous studies, Adamson and others reported no association between parvovirus B19 and PTC.[5] A recent study, however, showed that thyroid cancer tissues harbored viral DNA or viral gene products of both the polyoma and herpes family viruses.[6]

Human papillomavirus (HPV) is a non-enveloped, double-stranded DNA virus from the papillomavirus family, which has more than 170 HPV genotypes.[7, 8] HPV is divided into two groups, based on the ability to cause neoplastic transformation, first is the benign type and the second is high-risk HPV types (HPV 16, 18, 31, 33, 35, 45, 51, 52, and 56).[9] They play an important role in cancers of the anus and genital organs.[9] A previous study has shown that persistent HPV infection can lead to precancerous lesions.[10] Recently, HPV has been implicated in head and neck carcinomas, especially oropharyngeal cancer.[7] HPV was detected in 25% of the head and neck squamous cell carcinoma cases.[11] In addition, it is suggested that HPV vaccines can have a significant health benefit in preventing oropharyngeal cancers.[11] However, it is unclear whether this virus can also play a role in other cancers of the head and neck, such as thyroid cancer.[10, 11]

Despite a relatively high prevalence of thyroid cancer and the availability of HPV vaccines for cancer prevention, studies on the role of HPV infection in thyroid cancers are scarce. The present study aimed to evaluate the association between HPV infection and PTC in comparison with benign thyroid nodules and normal thyroid tissues. In addition, the association between HPV infection and PTC severity was evaluated using tumor staging criteria and pathologic features. To the best of our knowledge, this is the first time such a study has been conducted.

Materials and Methods

A retrospective cross-sectional study was conducted from May 2014 to January 2018 in hospitals affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (code: IR.SUMS.REC.1396 S90).

Sample Size

In the absence of any previous studies on the association between PTC and HPV, data from studies on the association between PCT and other viruses were used. The sample size was calculated (95% confidence interval, 5% significance level) using the MedCalc statistical software (Ver. 19.1.7; Med Calc software Ltd., Ostend, Belgium).

The calculated sample size was 44 for each group, but it was increased to 82 PTC samples and 77 benign thyroid nodules.

Tissue Samples

Thyroid tissue specimens from patients diagnosed with PTC and benign thyroid nodules were collected using the consecutive sampling method. A total of 175 paraffin-embedded thyroid tissues with the diagnosis of PTC and benign thyroid nodules were collected. All thyroid tissues were re-examined by two expert thyroid pathologists to confirm the diagnosis. Medical records of the patients were evaluated to exclude samples from patients, who were immunocompromised, had a history of head and neck radiation or underwent chemotherapy. Accordingly, 16 samples from patients with Hashimoto thyroiditis or Graves' disease were excluded from the study. Eventually, 82 PTC and 77 benign thyroid nodules (adenomatous and colloid nodules) specimens were included. In PTC specimens, samples from adjacent normal non-tumoral thyroid tissue were also taken. The data was anonymized throughout the study using irreversible encryption.

Tumor Staging and Pathologic Criteria

In accordance with the classification by the World Health Organization (WHO),[12] PTC histological types were classified into six groups: classic, encapsulated follicular, infiltrative follicular, tall cell, cribriform, and diffuse sclerosing variants. Tumor staging was determined according to the American Joint Committee on Cancer and tumor-node-metastasis staging system.[13] Pathologic features were evaluated according to the criteria specified by the College of American pathologists.[14]

Sample Preparation and Analysis

Tissue specimens were cut in 7 µm thickness, deparaffinized in xylene (GeNet Bio, South Korea), and then washed in ethanol (GeNet Bio, South Korea). DNA extraction was performed

using the PrimePrep Genomic DNA isolation kit (GeNet Bio, South Korea).

All DNA samples were amplified with both primer pairs as described in table 1. The first-round polymerase chain reaction (PCR) amplification was as follows: 12.5 µL Taq 2X Master Mix (BioFact, South Korea), 0.4 µm of each MY09 and MY11 primers (Metabion, Germany), 20 ng of DNA, and up to 25 µL PCR-grade water. The second-round PCR (nPCR) was carried out using $GP5^{+}/6^{+}$ primers and a housekeeping gene (β-actin) to assess DNA integrity with forward primer 5`-ATCATGTTGAGACCTCCAA-3`and reverse primer 5`-CATCTCTTGCTCGAAGTCCA-3`. The nPCR reaction contained 12.5 µL Taq 2X Master Mix, 0.3 µm of each $Gp5^{+}/6^{+}$ primers, 0.2 µm of each of the forward and reverse β-actin primers, 2 µL of PCR product of the first-round PCR, and 8 µL of the PCR-grade water. All PCR amplifications were performed on a 96-well thermal cycler (Applied Biosystems, Foster, CA, USA) using a PCR.

PCR products were electrophoresed on a 2.5% agarose gel (BioFact, South Korea)in 1X TAE buffer (BioFact, South Korea), stained with gel Red EcoDye™ Nucleic Acid Staining Solution (BioFact, South Korea), and visualized with a UV trans-illumination (UVItec Ltd, UK). The DNA amplification was 450 bp for the first-round PCR, 150 dp for the nPCR, and 317 dp for the β-actin gene. Uterine cervix tissue of a known HPV positive specimen, earlier analyzed during nPCR, was used as a positive tissue control to evaluate the success of the amplification. Blood samples of babies suspicious of kala-azar were used as negative controls (figure 1).

Statistical Analysis

Data were analyzed using SPSS software (version 21.0, Chicago, IL, USA). HPV positive tests were compared in two groups of PTC and

Table 1: Human papillomavirus nested polymerase chain reaction primer sequences

Name	Sequence (5'-3')	Size (base pair)
MY9	GTCCMARRGGAWACTGATC	450
MY11	GCMCAGGGWCTATAAYAATGG	
GP5+	TTTGTTACTGTGGTAGATACYAC	140
GP6+	GAAAAATAAACTGTAAATCATATTC	

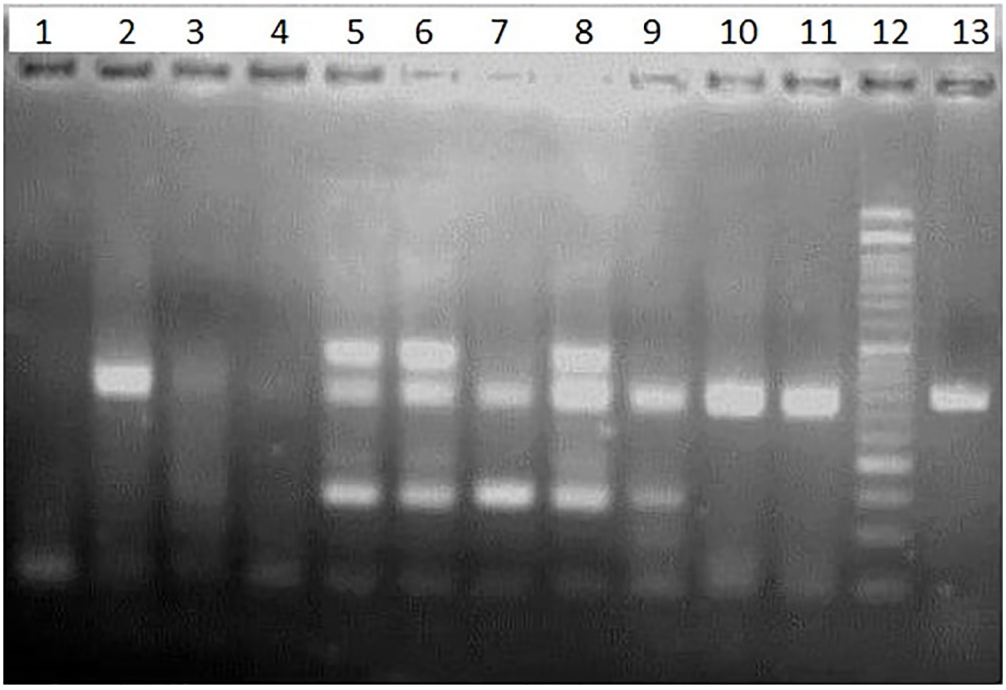

Figure 1: Agarose gel electrophoresis image of common human papillomavirus DNA in a nested multiplex polymerase chain reaction in a papillary thyroid cancer specimen. The length of amplicons generated with MY09/011* primers is 450 base pairs, GP05/06* is 150 base pairs, and β-actin primers 317 base pairs in size. Lane 1 is distilled water control. Distilled water in place of the DNA template was used as a negative control. Lane 2 is a negative control sample (317 base pairs β-actin) for the true negative result of common human papillomavirus detection. Lanes 5-6 are positive samples for first and nested human papillomavirus polymerase chain reaction and β-actin. Lanes 7 and 9 are positive samples for the nested and β-actin polymerase chain reaction. Lane 8 is positive control consisted of DNA from vaginal condyloma acuminatum. Lanes 10, 11, 13 are negative samples for the first and nested human papillomavirus polymerase chain reaction. Lane 12 is DNA Ladder: 50 base pairs. *MY09/011 and GP05/06 are primers for the detection of human papillomavirus in the polymerase chain reaction.

thyroid nodule, and PTC and adjacent normal tissue using Chi-squared and Fisher's exact tests. These tests were also used to evaluate the association between the qualitative pathologic features of PTC and the presence of HPV. The independent sample *t* test was used to compare quantitative variables such as age and tumor diameter. P values less than 0.05 were considered statistically significant.

Results

Of the 159 samples, 77 tissue specimens were benign thyroid nodules and 82 were PTC. A total of 30 samples were from male patients and 129 from female patients. The benign thyroid nodules are composed of 32 colloids (42%) and 45 (58%) adenomatous nodules (table 2). The prevalence of HPV PCR positivity in the PTC tissues was significantly higher than the benign thyroid nodules. Logistic regression analysis ("enter" method) was used to eliminate age and sex as the two probable confounding factors (table 3). The results showed that the prevalence of HPV positive PCR in the PTC group remained significantly higher than the benign thyroid nodules group (P=0.015). HPV was not detected in any adjacent normal non-tumoral thyroid tissue samples and the difference with the PTC group was significant (P<0.001).

The association between the pathologic characteristics of patients with PTC and HPV positivity was evaluated (table 4). Although the positive rate (%) of mitosis, lymph node involvement, capsular invasion, and tumor diameter in HPV positive PTC was higher than

Table 2: Comparison of sex, age, and human papillomavirus positivity in specimens with papillary thyroid carcinoma and benign thyroid nodule

Variable		PTC N (%)	Thyroid nodule N (%)	Total	P value
		82 (51.5)	77 (48.5)	159 (100)	-
Sex	Male	21 (25.6)	9 (11.6)	30 (18.8)	0.025*
	Female	61 (74.4)	68 (88.3)	129 (81.2)	
HPV+		11 (13.4)	3 (3.8)	14 (8)	0.034*
Age (year)		40.7±14.9§	46.9±13.3§	-	0.006**

*Chi-squared test, **Independent sample *t* test, §Mean±standard deviation; PTC: Papillary thyroid carcinoma, HPV: Human papillomavirus

Table 3: The results of logistic regression analysis

Variables	Regression coefficient	Wald test result	P value	Odds ratio	95% confidence interval	
					Lower	Upper
Age	-0.34	6.97	0.008	0.96	0.94	0.99
Sex	-1.10	5.66	0.017	0.33	0.13	0.82
HPV	1.56	4.76	0.029	4.77	1.17	19.40
Constant	2.26	9.54	0.002	9.63		

HPV: Human papillomavirus

Table 4: Association between human papillomavirus polymerase chain reaction positive results and pathologic characteristics of patients with papillary thyroid carcinoma

Pathologic characteristics	HPV-positive papillary cancer N (%)	HPV-negative papillary cancer N (%)	Total	P value
	11 (13)	71 (87)	82 (100)	-
Mitosis	2 (18.2)	6 (8.5)	8 (10)	0.291*
Atypia	0 (0)	6 (8.5)	6 (7)	0.409*
Encapsulation	8 (72.7)	41 (57.7)	49 (60)	0.346*
Capsular invasion	7 (87.5)	22 (53.6)	29 (35)	0.07*
Lymph vessel invasion	3 (27.3)	19 (26.8)	22 (27)	0.613*
Blood vessel invasion	3 (27.3)	16 (22.5)	19 (23)	0.495*
Extra thyroid extension	1 (9.1)	11 (15.5)	12 (15)	0.495*
Surgical margin involvement	0 (0)	7 (9.9)	7 (8.5)	0.35*
Multi-center tumor	3 (27.3)	16 (22.5)	19 (23)	0.496*
Lymph node involvement	2 (18.2)	8 (11.3)	10 (12)	0.401*
Diameter of tumor (mm)	32.3±11.4§	29.5±18.5§	-	0.295**

*Chi-squared and Fisher test, **Independent sample *t* test, §Mean±SD; N (%): Number (percentage), HPV: Human papillomavirus

HPV negative PTC, it was not significant. By comparing the prevalence of HPV positivity in benign thyroid nodules and normal thyroid tissue, no significant association between HPV presence and benign thyroid nodules was observed (P=0.11).

Fifty-six percent of PTC were classic type, 23.2% were follicular variant, and 14.6% were micropapillary carcinoma. The prevalence of oncocytic, sclerosing, Warthin tumor, and tall cell types was 2.4%, 1.2%, 1.2%, and 1.2%, respectively. There was no significant association between the PTC types and HPV positivity (P=0.384). Of all PTC tissue samples, 72% were in stage I, 13.4% in stage II, 13.4% in stage III, and 1.2% in stage IV. Of the 71 HPV negative samples, 51 (71.8%) were in stage I, 9 (12.6%) in stage II, 10 (14%) in stage III, and 1 (1.4%) in stage IV. In 11 HPV positive samples, the numbers and frequencies in stages I, II, III, and IV were 8 (72.7%), 2 (18.1%), 1 (9%), and 0 (0%), respectively. There was no significant association between HPV positivity and tumor staging (P=0.728).

Discussion

To the best of our knowledge, this is the first study that evaluated the association between HPV infection and PTC, as well as PTC severity, based on tumor staging and pathologic features. An association between HPV and PTC was found, and HPV was more significantly present in tissues involved with PTC than the benign thyroid nodules and normal thyroid tissues. This association was independent of age and sex. In addition, there was a trend in favor of capsular invasion in HPV positive PTC, but this association was not statistically significant.

Several factors, such as viral infections, might be involved in the development of PTC.[2] Previous studies have suggested a possible role of parvovirus B19, herpesvirus, and polyomavirus.[3-5] In the present study, we demonstrated a probable role of HPV as a newly identified risk factor for the development of some PTC cases. Some studies have shown the role of HPV in the pathogenesis of cancer, especially oropharyngeal, anogenital, and head and neck cancers.[7, 10] The reported prevalence of oral HPV infection in healthy adults is 4.5%. However, this rate is higher in human immunodeficiency virus-infected patients.[10] It has been shown that the prevalence is higher in men, particularly in older adults.[10] Two studies have shown that primary tumors in HPV-positive head and neck squamous cell carcinoma (HNSCC) were loco-regionally more advanced and had extended lymph node involvement.[15, 16] Although these tumors were more aggressive than the HPV-negative HNSCC, patients had better survival and better response to treatment.[15, 17-19] Keller and others showed the possible role of HPV in HNSCC of unknown origin.[20] They also showed an association between HPV infection and tumor staging and extra-capsular extension. Masand and others reported the role of HPV in some cases of adenosquamous carcinoma of the head and neck and concluded that this subset of patients had better survival.[21] Hartley and others did not find any association between small cell neuroendocrine carcinoma of the lung and HPV infection in high-risk HPV population.[22]

Another group of studies evaluated the effect of HPV in genital carcinomas.[23-25] Based on epidemiologic and molecular studies, persistent infection with high-risk HPV variants is the leading cause of uterine cervical cancer.[23] Ghedira and others showed an association between infection with high-risk variants of HPV and squamous intraepithelial lesions of the cervix in Tunisian women.[24] Another study in the United States showed the association between HPV infection and penile carcinogenesis.[25] However, Fonseca and others found no correlation between HPV infection and histological findings suggestive of poor prognosis or lymph metastasis in penile carcinoma.[26] Frega and others revealed that HPV-mRNA, as a marker of persistent HPV infection, is a risk factor for the onset of metachronous lesions in intraepithelial neoplasm of the lower female genital tract.[27]

HPVs are epithelial viruses, which spread through respiratory secretion by close mucosal or skin contact.[9, 10] They can also be transferred by peripheral blood mononuclear cells,[28] and as in our study, may reach the thyroid through the blood. Multiple mechanisms such as gene mutation and gene structural alteration are suggested for HPV tumorigenesis.[10, 15] For instance, some HPV-associated tumors reveal an E2F1 amplification (20q1), which is necessary for cell cycle proliferation. Another important structural change in HPV infection is the deletion of TRAF3, that occurs in about 20% of HPV-associated tumors. This gene plays an important role in antiviral immunity and is a regulator for NF-kB. Therefore, HPV may play a role in the activation of NF-kB in PTC.[15] NF-kB regulates genes involved in cell growth and proliferation.[29] In thyroid cancer cells, the activity of NF-kB is increased and may be involved in thyroid carcinogenesis.[29]

In the present study, for the first time, we showed the presence of HPV DNA in thyroid tissue, and an association between PTC and

HPV. The main limitations of our study were the lack of resources and laboratory equipment, and the fact that we did not use other methods for HPV detection (e.g., immunohistochemistry, in situ hybridization). These may have resulted in an underestimation of HPV positivity rates in our samples. Considering that PTC is a common thyroid carcinoma, and HPV vaccines are currently available and have shown to be effective in preventing some HPV-associated cancers, further studies in this field are strongly recommended.

Conclusion

For the first time, a link between HPV and some cases of PTC was established. However, the role of infection in cancer initiation could not be proven. Further studies with large sample size and among different populations are required to determine the exact role of HPV in the pathogenesis of PTC.

Acknowledgment

The present manuscript was derived from the subspecialty thesis of Pardisa Archin Dialameh. The study was financially supported by the Vice-Chancellor of Research of Shiraz University of Medical Sciences (grant number: 95-01-01-12927). We would like to thank the staff in the Pathology Department of Shiraz University of Medical Sciences for their cooperation in collecting the samples.

Conflicts of Interest: None declared.

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