Molecular Detection of HPVgenotype-16 in a Group of Patients with Grade 1 Invasive Papillary Thyroid Carcinoma

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ABSTRACT:

BACKGROUND:

Thyroid cancers are neoplasms originating from follicular thyroid cells thought to be related to a number of environmental and genetic predisposing factors. Human Papilloma Virus (HPV) is one among their associated infective agents.

OBJECTIVE:

To determine the frequency of human papilloma virus genotype-16 in infection in a group of iraqi patients with papillary thyroidcell carcinoma

MATERIALS AND METHODS:

A total number of 64 patients with different thyroid lesions were collected during 2008-2013; 30 have thyroid cancers, 22 benign thyroid tumors while12 healthy thyroid tissues were used as control group. In situ hybridization(ISH) was used to localize high risk HPV-genotype 16 in these tissues.

RESULTS:

Among malignant thyroid tumors 53.3% patients had HPV-16 while 27.3% HPV-16 positivity was detected in benign thyroid tumor group. None of healthy thyroid tissues revealed ISH reactions. There was 38.5% female had HPV-16, while there was 28% male had HPV-16. No significant statistical associations were noticed between the presence of HPV-16 and the age of those patients.

CONCLUSION:

Human papilloma virus genotype-16 could share a role in pathogenesis of this group of patients with thyroid cancers.

KEY WORDS: HPVgenotype-16; thyroid carcinoma; in situ hybridization.

INTRODUCTION:

Head and neck cancers arise in the nasal cavity, sinuses, mouth, lips, salivary glands, throat, larynx and thyroid gland, are predominately squamous cell carcinomas. Globally, head and neck cancers are the sixth most common type of cancer, where more than 70% of cases occurring in developing countries[1]. They are rare in the United States, accounting for 5% of all cancer cases [2].Thyroid cancer is the most common cancer of the endocrine organs accounting for up to 2.5% of all malignancies [2,3] .

Incidence is, in fact, rising faster than all cancers, where females are more frequently affected than males. It is thought that this is in part the result of earlier detection; however, the increase in mortality as well as the increase in incidence of larger thyroidtumors suggests that there may be additional factors behind this increase [1] .The majority of thyroid cancers develop from follicular epithelial cells and according to their morphology and biology are divided grossly into two groups; the first group, well differentiated thyroid cancers, which include the usually slowly growing papillary thyroid carcinoma (PTC) and the follicular thyroid carcinoma (FTC). The PTC accounts for approximately up to 80% of thyroid tumours, whereas the FTC accounts for 5-15% of diagnosed thyroid malignancies [4]. These cancers are usually curable and in contrast to the second group which compromised the anaplastic thyroid cancers (ATC), which represent the poorly differentiated thyroid cancers and comprises only 1-2% of all diagnosed thyroid malignancies [5]. ATC is characterized by rapid and invasive growth resulting in fulminant disease course and poor outcome .In thyroid, approximately 5% of thyroid malignancies are diagnosed as medullary carcinoma, which in turn originates from the para follicular C cells[6].

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From 2000 to 2009, incidence rates among men have decreased for five of the 17 most common cancers: prostate, lung-bronchus, colorectal, stomach, and larynx. In contrast, rates among men during the same time interval increased for six cancers: kidney, pancreas, liver, thyroid, melanoma of the skin, and myeloma. Among women, incidence rates have decreased from 2000 to 2009 for seven of the 18 most common cancers: lung, colorectal, urinary bladder, cervix uteri, oral cavity and pharynx (all tumors regardless of their potential association with HPV infection), ovary, and stomach. Incidence rates among women have increased from 2000 to 2009 for seven cancers: thyroid, melanoma, kidney, pancreas, leukemia, liver, and uterus [6] .

Although there are approximately a dozen oncogenic HPV types, HPV 16 and 18 are the most common HPV types , are found in approximately 70% of cervical cancers. Human papilloma virus 16 is found in approximately 90% of the non-cervical cancers like oropharyngeal cancer (head and neck cancers) that are often associated with HPV infection [7].

Experts have long suspected a link between HPV and oro-pharyngeal cancer (OPC) with an increasing body of evidence supporting this association. A case-control study of 100 patients with newly diagnosed oro-pharyngeal cancers (as compared to 200 matched controls) has found HPV-16 strain in 72% of these cancerous tumors [8]. Recent trends continue speculation that by 2020 year HPV will cause more cases of oro-pharyngeal cancers each year than cervical cancers [9].

Exposure to HPV is common through sexual contact, and most infections resolve over time. However, persistent infection with oncogenic HPV types is etiologically linked to cervical cancer [10], as well as cancers of the oropharynx [11], anus [12], vagina and vulva [13], and penis [14]. Virtually, all cervical cancers are due to HPV infection, along with 90% of anal cancers, more than 60% of certain subsets of oro-pharyngeal cancers, and 40% of vagina, vulva, and penile cancers [7] .

Oral HPV16 DNA is common among female patients with HPV-OPC, but not among their spouses. Spouses of HPV-OPC female patients may have an elevated risk or history of cervical cancer [15] .

This research work, and up to our best knowledge, is the first in Iraq, that study the percentage of HPV16 genotype in a group of Iraqi patients with thyroid lesions.

MATERIALS AND METHODS:

The study was designed as a retrospective one at the virology and molecular Laboratories of Communicable Diseases Research Unit /College of Medicine/University of Baghdad, as well as Pathology and Virology Laboratories of College of Science /University of Babylon . It has recruited 64 selected formalin fixed, paraffin embedded thyroid tissue blocks were obtained ,among them (30) tissue biopsies with grade I invasive papillary thyroid carcinoma and (22) benign thyroid tissue blocks as well as (12) apparently normal thyroid tissue autopsies which were collected from the archives of Forensic Medicine Institute / Baghdad and used as thyroid healthy control groups. The diagnosis of these tissue blocks were based on their accompanied records.

In one hand, the detection of HPV16 by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4µm paraffin embedded tissue sections using digoxigenin-labeled oligo-nucleotides probe which targets HPV16 DNA. One section was mounted on ordinary glass slide and stained with hematoxyline and eosin, while another slide was mounted on charged slide to be used for ISH for detection of HPV16.

For the in situ hybridization procedure, the slides were placed in 60c hot-air oven over night then the tissue sections were de-paraffinized and then treated by graded alcohols according to the standard methods and the details of processes for performing ISH reaction with this probe were applied according the instructions of the manufacturing company (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany). The main steps for ISH procedure are:

Incubation of slides for min at 70°C(e.g. on hot plate), then Incubation of slides for 5 min in xylene. After that incubation for 5 min in 100% Ethanol (alternatively, dewaxing protocols routinely used in immunohistochemistry procedures, *e.g.* 2-5 min xylene, 2-5 min 100% Ethanol, 2-5 min 96% Ethanol, 1-5 min 70% Ethanol, can be used. Air drying of sections. Then application (dropwise) Pepsin Solution(ES1) to the tissue/cell section and incubate for 20-30 min at 37°C in a humidity chamber. After that we immersed slides in distilled water and drain off the water, air dried sections, then add the probe to the center of a cover slip and place cover slip upside down on target area. Denaturation of the slides at 75°C for 5 min. on hot plate, then transferred the slides to

a humidity chamber and hybridize for 60 min at 37°C for DNA-targeting probes. The post-hybridization and detection process included removing the cover slip by submerging in 1x wash buffer TBS, then washed for 5 min in 1x wash B\buffer TBS (prepared by using WB5) at 55°C. Then application of AP-Streptovidin (AB9) drop wise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber. Then washed in wash buffer TBS (prepared by usingWB5) and then twice times for 1 min in distilled water and application of NBT/BCIP(SB4) drop wise (4 drops per slide) to the slides and incubated for 40 min at 37°C in humidity chamber .Then checking the color development in intervals of approx, 5-10 min using microscope. Lastly, washing three times for min. in distilled water. After that covering the sections and were embedded in an aqueous embedded medium, then final evaluation by light microscope.

Technical Analysis:

According to the specification of the kit, proper use of this ISH detection system gives an intense blue signal at specific sites of the hybridization probe in positive test tissues (by using light microscope).

The signal was evaluated under light microscopy using × 100 lens for counting the positive cells. The ISH results were given intensity and percentage scores based on intensity of positive signals and number of cells that gave these signals, respectively.

Positive cells were counted in 10 different fields of 100 cells for each sample and the average percentage of positive cells within the 10 fields was determined. A scale of 0-3 was used for relative intensity with 0 corresponding to no detectable ISH reaction, and 1, 2, 3 equivalents to low, moderate, and high intensity of reaction respectively. Cases were assigned to one of the following percentage score categories: 1%–25% (score 1), 26%–50% (score 2) or > 50% (score 3) (16). The chi-squared test was used to detect the significance between groups. The statistical analysis was done using SPSS program, version 17, and differences were considered significant when $P < 0.05$.

Statistical Analysis:

Chi –square test was used to detect the significance between variables of this study. All the statistical analysis was done by SPSS program(Version– 17)&P-value was considered significant when $p < 0.05$.

RESULTS:

Table-1- shows the consistency between the tumor types of thyroid cancer and control group with gender. There was 66.7% female and 33.3% male had malignant thyroid lesions, also there was 59.1% female and 40.9% male had benign thyroid lesions while both females and males in the control group did not have any type of thyroid lesions. By applying Chi-square test , there were no significant differences between the presence of tumor and the gender.

Table 1: Distribution of Patients with Thyroid Lesions According To Their Gender.

*P-value >0.05.

Gender		Malignant thyroid tumors	Benign thyroid tumors	Control thyroid tissues	Total*
Female	Count	20	13	6	39
	%within Gender	51.3%	33.3%	14.6%	100%
	%within Group	66.7%	59.1%	50.0%	60%
Male	Count	10	9	6	25
	%within Gender	40%	36%	24%	100%
	%within Group	33.3%	40.9%	50%	39.1%
Total	Count	30	22	12	64
	%within Gender	46%	34.4%	18.8%	100%
	%within Group	100%	100%	100.0%	100%

P<0.01 when chi-square was applied to test the correlation between the presence or absence of HPV-16 and type of thyroid lesions, it was found that there was significant correlation between the type of thyroid lesion and control group and the presence or absence of HPV-16, since there was 53.3% had malignant thyroid lesions and had HPV-16 and 27.3% had benign thyroid tumors, while there was 46.7% had malignant thyroid lesions and did not have HPV-16 while 72.7% had benign thyroid lesions and did not had HPV-16. Control group does not had HPV-16 .

Table 2: Percentage of HPV 16-ISH Results According to the Type of Thyroid Lesions.

		The Studied Groups*			
Negative		Malignant	Benign	Control	Total
	Count	14	16	12	42
	% within HPV16 + or -	33.3%	38.1%	28.6%	100%
	% within group	46.7%	72.7%	100%	65.6%
Positive	Count	16	6	0	22
	% within HPV + or -	72.7%	27.3%	0%	100%
	% within group	53.3%	27.3%	0%	34.4%
Total	Count	30	22	12	64
	% within HPV16 + or -	46.9%	34.4%	18.8%	100%
	% within group	100%	100%	100%	100%

*(P<0.01).

When one way analysis of variance is applied to compare the age of the patients in the three groups (malignant tumors, benign tumors, and control), it was found that the mean age of patients with malignant thyroid lesions was 40.9 years, the mean age of patients with benign thyroid lesion was 40.6 years, while the mean age of control group was high 54.7 years. There was statistically significant differences between the mean of age of the patients in the three groups (P<0.01).

The results of the present study (Table-3) show that statistically there were no significant differences between female and male about the presence or absence of HPV16. There were 38.5% females had HPV-16 and 28% males had HPV-16.

Table 3: Distribution of HPV-16 -ISH Results According To Gender of Patients.

HPV-16 ISH results		Gender*		
		Female	Male	Total
Negative	Count	24	18	42
	%within HPV-16 + or –	57.1%	42.9%	100.0%
	%within Gender	61.5%	72.0%	65.6%
Positive	Count	15	7	22
	%within HPV-16 + or –	68.2%	31.8%	100.0%
	%within Gender	38.5%	28.0%	34.4%
Total	Count	39	25	64
	%within HPV-16 + or –	60.9%	39.1%	100.0%
	%within Gender	100.0%	100.0%	100.0%

*(P>0.05).

Chi-square was done to test the scoring of HPV16-ISH signals of thyroid lesion (Table-4& Figure 1), it was found that there was 37.5% thyroid lesions had low scoring in malignant thyroid lesions group and 50% thyroid lesions had low scoring in benign thyroid lesions group. While there was 31.3% thyroid lesions have moderate scoring in malignant thyroid lesions group and 33.3% thyroid lesions have moderate scoring in benign thyroid lesions group. Finally, there were 31.3% thyroid lesions have strong scoring in malignant thyroid lesions group and 16.7% thyroid lesions have strong scoring in benign thyroid lesions group.

Table 4: Scoring of HPV16 Results According to Tumor type of Thyroid Lesions.

Scoring of HPV16-ISH Signals		Group Malignant	Benign	Total
Low	Count	6	3	9
	%within Scoring	66.7%	33.3%	100.0%
	%within Group	37.5%	50.0%	40.9%
Moderate	Count	5	2	7
	%within Scoring	71.4%	28.6%	100.0%
	%within Group	31.3%	33.3%	31.8%
Strong	Count	5	1	6
	%within Scoring	83.3%	16.7%	100.0%
	% within Group	31.3%	16.7%	27.3%
Total	Count	16	6	22
	%within Scoring	72.7%	27.3%	100.0%
	%within Group	100.0%	100.0%	100.0%

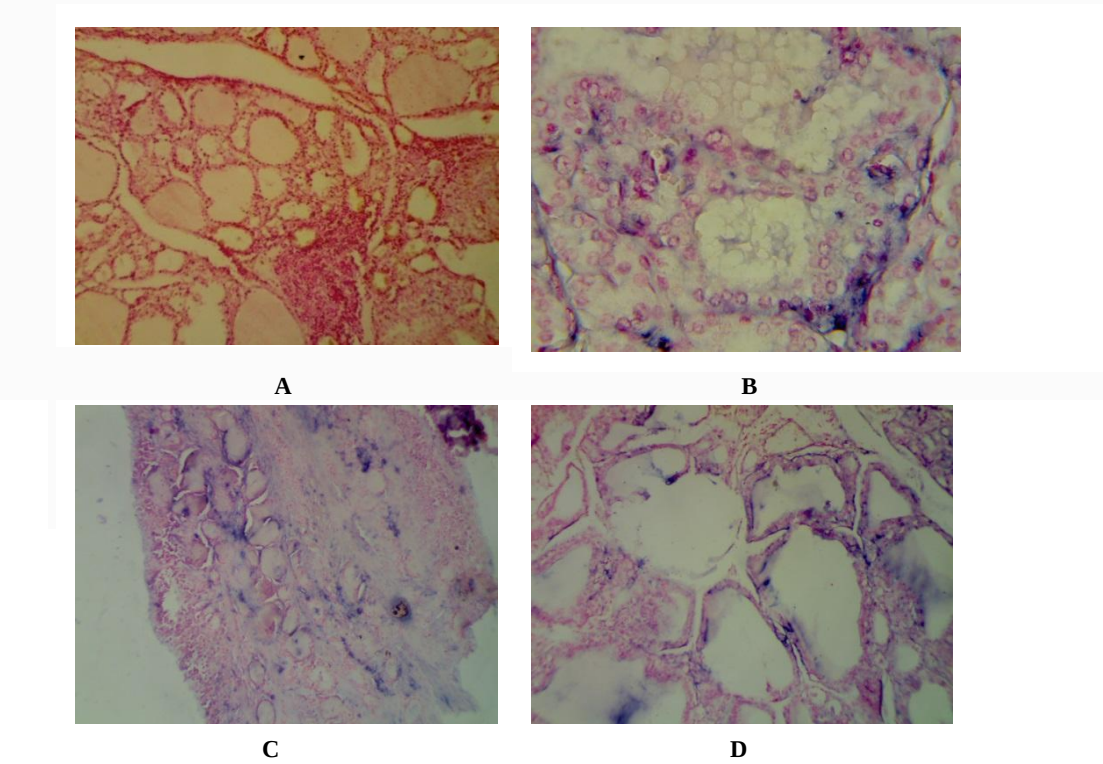

Figure 1:In Situ Hybridization(ISH) for HPV-16 Detection in Infiltrative Thyroid Cancers Using Biotinylated -Labeled HPV-16Probe;Stained with NBT/ BCIP (Blue signals)and Counter Stained by Nuclear Fast Red (Red signals). A-Thyroid Cancer with negative HPV16 –ISH reaction (20X) B-Positive HPV16-ISH reaction with moderate score and high signal intensity (20X).C-Positive HPV16-ISH reaction with high score and low signal intensity (240X). D-Positive HPV16 -ISH reaction with moderate score and moderate signal intensity (20X).

Chi-square was done for evaluation of signals intensities of HPV16-ISH results according to type of thyroid lesions (malignant thyroid lesions, benign thyroid lesions groups) (Table-5 & Figure -1),there was 12.5% thyroid lesions had low intensity in malignant thyroid lesions group and 33.3% thyroid lesions had low intensity in benign thyroid lesion group. While there was 37.5% thyroid lesions have moderate intensity in malignant thyroid lesion group and 50% thyroid lesions have moderate intensity in benign thyroid lesions group. The percentage of patients that had high intensity signals of HPV16-ISH in malignant thyroid lesions group was 50% whereas there was 16.7% patients had high intensity signals of HPV16-ISH in benign thyroid lesions group.

Table 5: Evaluation of Signals Intensities of HPV16-ISH Results According to Type of Thyroid Lesions.

Signals intensity of HPV16-ISH reaction	Group Malignant	Benign			Total
Intensity	Low	Count	2	2	4
		%within intensity	50.0%	100.0%	100.0%
		%within Group	33.3%	18.2%	18.2%
	Moderate	Count	6	3	9
		%within intensity	66.7%	33.3%	100.0%
		%within Group	37.5%	50.0%	40.9%
	High	Count	8	1	9
		%within intensity	88.9%	11.1%	100.0%
		%within Group	50.0%	16.7%	40.9%
Total		Count	16	6	22
		%within intensity	72.7%	27.3%	100.0%
		%within Group	100.0%	100.0%	100.0%

DISCUSSION:

The majority of molecular events in the genesis of oral squamous cell carcinoma are unknown [17].

Although recent evidence has implicated viruses in the regulation of epithelial –to- mesenchymal transition and tumor progression, little is known regarding viral infections in thyroid malignancies [16].

In the current research, the rate of detection of positive results of HPV 16 DNA-ISH reactions in the group of malignant thyroid tumors was 53.3 % (16 of total 30) while in the benign thyroid tumors was 27.3%(6 of total 22).

Up to our best knowledge, this study is the first in Iraq that investigate the HPV16 genotype in a group of Iraq patients with malignant & benign thyroid lesions. In addition, and on reviewing the available scientific research works in this entity, we found an extreme shortage of the articles in this respect. However, the present results are in disagreement with a work done by Stamatiou*et al*. [17],who have examined and who did not able to find HPV 16 on examination of their but were not able to find HPV16 in the examined thyroid lesions.

Regarding the copy number of viral infection in the examined thyroid lesions of this study, the present found signal scoring is referring to the wide spread of the viral invasion in relation to the surface area to examined tissues. However, the present study has found an in equal percentage of distribution of the 3 scores of HPV16-ISH in the examined thyroid tissues.

Uterine cancer ranked fourth among women of each racial and ethnic group except API women, in whom thyroid cancer was the fourth most common cancer. Beyond the three commonly diagnosed cancers for men and four most commonly diagnosed cancers for women, cancers ranking have varied by race and ethnicity [9].

Despite great variability in the HPV detection rates worldwide, the majority of HPV types that have been detected were the high oncogenic risk group (HPV -16 & HPV-18) [18].

By analogy, it was found that the present findings of HPV16 in Iraqi patients with thyroid cancers have an equal proportions of consistency to those studies done in Iraq [19], [20] , [21] and [22] who found that the HPV 16 as the most prevalent type in their studied group of patients with esophageal, oral, prostate and breast cancers, respectively. However, other types of HPV, such as HPV18, 31 and 33 were found to have probable roles in such cancers. High-risk HPV encodes a series of proteins, some of which have oncogenic potential [23].

Although it is known and widely accepted for many years that integration of DNA of high-risk HPV types into cellular genome as the major contributing factor in cervical carcinogenesis [24], recently is also linked to a common head & neck cancers [25].

The transmission routes of HPV detected in thyroid cancers are still unclear.

However, the researchers have indicated that infection with HPV is a risk factor for oro-pharyngeal carcinoma (OPC). HPV is most commonly passed from person to person during sexual activity, including oral intercourse. However, the risk of developing HPV-positive OPC is low for spouses [1].

HPV is most commonly passed from person to person during sexual activity, including oral intercourse. Michael Douglas [26] recently related HPV16-associated throat cancer to oral sex as a possible cause of their studied throat cancers.

High viral load of HPV has been be associated with the microscopical diagnosis of a concurrent lesions as well as an indication to progress to precancer and cancer [27]. Studies concerning HPV association with cancer, especially cervical cancers, have revealed that an increase in HPV load is a significantly associated with cervical carcinoma [28].). However, it has been well documented that the episomal viral DNA frequently integrates into the host genome as HPV-infected lesions progress to cervical cancer [29].

High proliferation of the infected host tissues has been considered to be crucial for the persistence of HPV that infects a basal layer cell of the cervical stratified squamous epithelium, the HPV DNA only replicated during cell divisions of the host before the differentiation of the infected cells. This allow the copies of viral DNA preserves in a large number in premature cells [30 &31]. In a similar way, infection of mammary epithelial cells ,that partly loses control in proliferation the HPV DNA copies may be preserved in nearly every clone of its first host that may further interact with other carcinogenic factors and involve in the later carcinogenic steps of breast cancer [31].

In an infected cell, vegetative viral replication ending with release of the virus from cell, where this is definitely against its transformation into a malignant one [32]. Only E6 and E7 genes remain in the host genome during HPV DNA integration

and therefore, their presence in tumor tissues may better represent the real HPV participation in carcinogenesis [29].

Although in the present study, a respective percentage of HPV-16 infection was found in tissues obtained from thyroid cancers as well as a highest percentage of HPV-16 ISH – reactions with low signal scoring, for this reason, ISH signals of HPV DNA ought to be of low intensity while the high intensity could reflect and indicate for viral replication inside the cells and is as such have rush an additional conflict for HPV participation in thyroid cancer and more researches could be forwarded to explore their actual role in that carcinogenesis .

The fact that we have not found the Human papilloma viral DNA in healthy thyroid specimens could support, to certain extent, the hypothesis that the virus might play a role in the etiology of thyroid cancer in only a sub set of population of patients. On the other hand, it is logical to believe that the presence of high-risk types of HPV alone is not sufficient to implement full process of tumorigenesis and that further changes should be accumulated over time in a step-wise manner to cause the disease.

CONCLUSION:

The present results that Human papilloma virus genotype-16 could share a role in pathogenesis of this group of patients with thyroid cancers.

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