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Original Article

Chromogenic in Situ-Hybridization of HPV16/18 DNA in Relation to the Over-Expressed Protein of P73-Gene in Tissues from a Group of Thyroid Carcinoma

Kadhim Salim, N[1] *, Hasan Mohammed Ali, S[2], Mahdi Al-Sudani, I[3]

1. Ministry of Health, Baghdad, Iraq
2. Clinical Communicable Diseases Research, College of Medicine, University of Baghdad, Baghdad, Iraq
3. Ibn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq

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Corresponding Author: munaf.2t@yahoo.com

Abstract

Thyroid cancer has been related to many environmental, genetic, and viral factors. Human Papilloma Viruses (HPV) are epitheliotropic viruses infecting cutaneous and mucosal tissues, leading to a variety of benign and malignant tumors. The p73-gene expresses two important isoforms from the N-terminal end with two opposite activities in the regulation of cell fate. The present study aimed to assess the histopathological expression of tissues from thyroid cancers in relation to the over-expression of the p73 gene with HPV 16/18 infection. A total of 116 thyroid tissues were examined for HPV 16/18-DNA and P73-gene protein expression. The samples belonged to 36 patients diagnosed with thyroid carcinoma, 40 thyroid adenoma tissues blocks, and 40 apparently normal thyroid tissues. The detection of HPV 16/18-DNA was performed by in situ hybridization (ISH), whereas P73 gene expression was carried out by immunohistochemistry (IHC). The HPV16/18 DNA-ISH reactions in thyroid cancers were found in 72.2% tissues, 35% HPV16/18- positivity was detected in the thyroid adenoma tissues group, and 27.5% of healthy thyroid tissues revealed ISH reactions. Statistically, the difference of the HPV16/18 in thyroid cancers and control was highly significant. The p73 was detected in 66.7% and 57.5% of thyroid cancer and adenoma thyroid tissues, respectively, while 45% of the examined healthy thyroid tissues revealed IHC-reactions. The difference between the p73-protein expression percentages detected in tissues of thyroid tumors and the control group was non statistically significant. The presence of HPV16/18, as well as an over-expressed p73-gene, in thyroid cancer patients, suggests that the virus, as well as this protein, may play an etiologic role in thyroid carcinogenesis.

Keywords: Benign thyroid adenoma, HPV genotype 16/18, IHC, ISH, P73, Thyroid Cancers

1. Introduction

Cancers as non-communicable diseases are presently ranked as the leading cause of global deaths and expected to be the single most important barrier to the enhancement of life expectancy in the 21st century across the globe (1, 2). Regarding the Eastern Mediterranean Region, the predicted burden of cancers could be doubled by 2030 (3). Thyroid cancer has been more common over the world during the last three decades. High-income countries, on the other hand, may account for more than 60% of thyroid cancer diagnoses due to enhanced access to healthcare, improved diagnostic technologies, and increased monitoring (4, 5).

Thyroid cancers are grossly assigned to two groups based on their biology and morphology:1) well-differentiated thyroid cancers [slowly growing papillary thyroid carcinoma (PTC) accounts for approximately

85% of thyroid tumors) and the follicular thyroid carcinoma (FTC) (accounts for 5-15% of thyroid malignancies)] (6-8), 2) compromised the rapid and invasive anaplastic thyroid cancers (ATC), (poorly differentiated thyroid cancers comprising 1%-2% of all diagnosed thyroid malignancies). Approximately 5% of thyroid malignancies originating from the Parafollicular C cells are diagnosed as medullary carcinoma (7).

Worldwide, thyroid cancers ranked the 9th in incidence, representing 5% of cancer diagnoses in 2018, and the incidence was much higher among females than males, especially in high-incidence regions (1). In females, these cancers are estimated to account for 90% of newly diagnosed cases in South Korea; 70%-80% in the United States, Italy, France, and Australia; and 50% in Japan, the Nordic countries, England, and Scotland (9). The exact cause of thyroid cancer is still unknown. In Iraq, according to the ministry of health in 2016, the incidence of this cancer was 4.39% in both genders (2).

Papillomaviridae family members are human papillomaviruses (HPV), which include both tissue- and species-specific (10). These epitheliotropic viruses infect cutaneous and mucosal tissues, causing infections ranging from asymptomatic, benign warts, and cancers (11). Infection with HPV affects a range of different tissues, such as the respiratory tract, oral cavity, anogenital tract, and urethral mucosal tissues (10). To date, the *Papillomaviridae* family comprises more than 450 different types of HPV and over 200 animal papillomaviruses (12-14). These viruses are categorized into high-risk and low-risk varieties based on their oncogenic potential, with 25 HPV types linked to various benign and malignant tumors (15). Globally, HPV type16 and 18 were identified as a prevalent source of sexually transmitted viral infections that were associated with >95% of cervical malignancies etiologically (16, 17).

The thyroid gland as a key part of the endocrine system consists of two lobes connected by the isthmus and a third lobe (called a pyramidal lobe) which is found in 28%-55 % of the population (18-21). The p73 which is a transcription factor belonging to the p53 family has two isoforms with opposite activities in terms of their participation in carcinogenesis (22). The p73 is substantially weaker than p53. On the other hand, unlike p53, TAp73 rarely mutated and is overexpressed in a variety of malignancies, suggesting that it may have an anti-tumor effect (17). While p53 mutations are found in more than 50% of all malignancies, p73 mutations are observed in less than 0.5 % of all tumors, making p73 a promising therapeutic target. Other actions of p73 include its association with chronic inflammation and its requirement for embryonic development (23).

In light of the aforementioned issues, the present study aimed to determine the percentage of common and important high-oncogenic risk types of human papillomavirus (genotypes 16/18), and assess the effects of the expression of certain tumor suppressor genes (p73) in archival tissues specimens from thyroid cancers and benign tumors.

2. Materials and Methods

A total of 116 formalin-fixed paraffin-embedded thyroid tissues blocks were used in this study, including 36 malignant thyroid tumors divided into three stages (I, II & III), 40 thyroid adenoma tissues, and 40 healthy thyroid tissues (used as the control group). The choice of cases for testing depended on two features to find the sufficient component for testing, and re-diagnosis for confirmation of cases by the advice of an expert histopathologist.

One section was mounted on an ordinary glass slide and stained with hematoxylin and eosin, while another slide was mounted on charged slide to be used in in situ hybridization (ISH) for the detection of HPV and immunohistochemistry (IHC) to detect p73 protein. The detection of HPV16/18 by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4µm paraffin-embedded tissue sections using a digoxigenin-labeled oligo-nucleotides probe which targets HPV 16/18-DNA. An immunohistochemistry detection system (Abcam,

England) was used to demonstrate the p73 protein of tumor suppressor genes.

2.1. Statistical Analysis

Pearson Chi-square test was used to detect the significant relationship among the variables of the current study. All the statistical analyses were performed in SPSS software (Version 27), and a *P*-value less than 0.05 was considered statistically significant.

3. Results

The mean age scores of patients in thyroid carcinoma, thyroid adenoma, and control groups were reported as 37.9±14.5, 40.3±13.4, and 39.4±12.9 years, respectively. No statistically significant differences were noticed among the research groups (*P=0.840*). The most affected age group among all the examined groups of patients was 40-49 years (Table 1).

Table 1 displays that 77.8%, 80%, and 77.5% of patients in thyroid carcinoma, thyroid adenoma, and the control groups were female, respectively. The differences across study groups were not statistically significant(*P=0.840*); moreover, there were non-significant differences in the gender distribution of each of the analyzed groups.

Based on table 2 which presents the tumor staging of thyroid carcinoma tissues, 80.6%, 11.1%, and 8.3% of patients had stages I, II, and III, respectively.

The HR-HPV16/18 DNA was detected in tissue blocks from 72.2%, 35%, and 27.5% of the thyroid carcinomatous, thyroid adenomatous, and thyroid normal (control)tissues, respectively. The statistical differences in percentages are highly significant (*P=0.0001*) (Table 3).

Table 3 displays the score and signal intensities of positive HPV 16/18-CISH where 52.8% have high intensities and 58.3% with score 3 in thyroid carcinoma tissues, 7.5% with high intensities and 17.5% have score 3 in thyroid adenoma tissues, while 12.5% have high intensities and score 3 in apparently healthy control tissues. Statistically significant differences were observed among the studied groups.

Table 1. Studied groups according to the mean age and gender

		Thyroid Carcinoma		Thyroid Adenoma		Apparently Healthy Control		*P*-value
		No	%	No	%	No	%	
Age (years)	10-19	2	5.6	2	5.0	1	2.5	0.840
	20-29	10	27.8	6	15.0	9	22.5	
	30-39	7	19.4	9	22.5	8	20.0	
	40-49	11	30.6	13	32.5	15	37.5	
	50-59	2	5.6	7	17.5	3	7.5	
	=>60years	4	11.1	3	7.5	4	10.0	
	Mean±SD	37.9±14.5		40.3±13.4		39.4±12.9		
Gender	Male	8	22.2	8	20.0	9	22.5	0.957
	Female	28	77.8	32	80.0	31	77.5	

Table 2. Tumor staging of thyroid carcinoma group

		Thyroid Carcinoma	
		No	%
Stage	[I]	29	80.6
	[II]	4	11.1
	[III]	3	8.3

Table 3. Colorimetric in situ hybridization detection of DNA HR-HPV (16 /18) in the studied groups

Colorimetric in situ hybridization		Thyroid Carcinoma		Thyroid Adenoma		Apparently Healthy Control		*P*-value
		No	%	No	%	No	%	
HPV16/18	Positive	26	72.2	14	35.0	11	27.5	0.0001*
	Negative	10	27.8	26	65.0	29	72.5	
HPV16/18 Intensity	low	3	8.3	9	22.5	1	2.5	0.0001*
	moderate	4	11.1	2	5.0	5	12.5	
	high	19	52.8	3	7.5	5	12.5	
HPV16/18 Score	1	2	5.6	-	-	-	-	0.0001*
	2	3	8.3	7	17.5	6	15.0	
	3	21	58.3	7	17.5	5	12.5	

Table 4 shows the physical status of the DNA-expressed patterns where the means of HPV 16/18 DNA expression as an integrated form in thyroid carcinoma tissues was 71.9, in thyroid adenoma tissues, the mean of HPV 16/18 DNA expression as an integrated form was 67.1, and the mean of HPV 16/18 DNA expression as an integrated form of apparently healthy control tissues was 32.8. Statistically significant differences were detected among the studied groups.

The IHC technique was used in the current study to detect changes in p73 expression in the cytoplasm of the analyzed thyroid tissues in the study groups. Table 5 illustrates that positive p73-IHC results were detected in 66.7% and 57.5% of thyroid carcinoma, and thyroid adenoma groups, respectively. Finally, in the healthy control tissues group, positive p73-IHC results were observed in 45% of cases. Non-significant differences (*P=0.160*) were observed among the studied groups.

Immunostaining for all the proteins was classified on the basis of the percentage of stained cells as follows: 0, negative; +1, <10 percent, +2, 10 to 49 percent, +3, ≥50 percent (Figure 1).

Table 4. Colorimetric in situ hybridization detection of DNA HR-HPV (16 /18) in the studied groups

Colorimetric in situ hybridization		Thyroid Carcinoma		Thyroid Adenoma		Apparently Healthy Control		*P*-value
		No	%	No	%	No	%	
HPV16,18 Integrated pattern	<10	-	-	-	-	3	27.3	0.001*
	10-19	-	-	1	7.1	3	27.3	
	20-29	1	3.8	2	14.3	1	9.1	
	30-39	3	11.5	-	-	-	-	
	40-49	5	19.2	1	7.1	1	9.1	
	50-59	1	3.8	-	-	-	-	
	60-69	-	-	-	-	1	9.1	
	70-79	-	-	2	14.3	-	-	
	80-89	2	7.7	4	28.6	-	-	
	=>90	14	53.8	4	28.6	2	18.2	
	Mean±SD (Range)	71.9±29.4 (20-100)		67.1±29.0 (15-95)		32.8±36.0 (3-98)		
HPV16,18 Diffused pattern	<10	12	46.2	1	7.1	2	18.2	0.0001*
	10-19	2	7.7	6	42.9	-	-	
	20-29	2	7.7	1	7.1	-	-	
	30-39	-	-	2	14.3	-	-	
	40-49	-	-	-	-	1	9.1	
	50-59	1	3.8	-	-	-	-	
	60-69	6	23.1	1	7.1	1	9.1	
	70-79	2	7.7	1	7.1	1	9.1	
	80-89	1	3.8	2	14.3	-	-	
	=>90	-	-	-	-	6	54.5	
	Mean±SD (Range)	28.2±29.2 (2-80)		32.9±29.0 (5-85)		67.3±35.8 (3-97)		

*Significant difference between percentages using Pearson Chi-square test (χ2-test) at 0.05 level.

Table 5. Immunohistochemistry-expression of p73 protein among studied groups

Immunohistochemistry		Thyroid Carcinoma		Thyroid Adenoma		Apparently Healthy Control		*P*-value
		No	%	No	%	No	%	
P73	Negative	12	33.3	17	42.5	22	55.0	0.160
	Positive	24	66.7	23	57.5	18	45.0	
P73 Score	1	2	5.6	-	-	-	-	0.047*
	2	4	11.1	1	2.5	-	-	
	3	18	50.0	22	55.0	18	45.0	
P73 Intensity	Low	6	16.7	5	12.5	1	2.5	0.225
	Moderate	5	13.9	8	20.0	4	10.0	
	High	13	36.1	10	25.0	13	32.5	

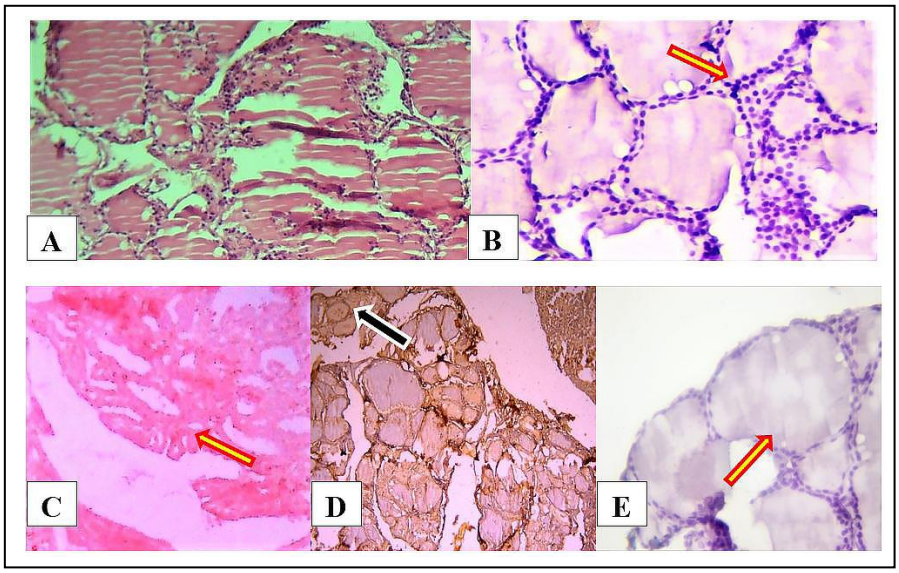

Figure 1. Representative histologic, In Situ Hybridization and immunohistochemistry results. **(A)** thyroid tissue (hematoxylin & eosin stain); **(B)** HPV16/18-ISH shows predominantly blue staining Positive result; **(C)** HPV16/18-ISH shows predominantly Red staining negative result; **(D)** P73 immunostaining positive result (dark brawn); and (E) P73 immunostaining negative result (purple hematoxylin stain)

4. Discussion

The malignant head and neck tumors ranked the 6th most common type of malignant tumors worldwide, and more than 70% of them have been reported in third world countries (24). In Iraq, thyroid cancer ranked the 8th among the 10 most common cancers in females. Among several kinds of human cancers, the incidence rate of thyroid tumors has increased during the last three decades (25). All ages can be affected by thyroid tumors, and annually, more than 560,000 new cases of thyroid cancer are diagnosed across the globe. Relatively, thyroid nodules are widespread in the population and 90%-95% of them are benign (26).

The increased thyroid cancer rates around the world emphasize the need for finding novel prognostic biomarkers so as to follow up the rationale of treatment. Scientists strive to identify any possible link between viral infection and human diseases, particularly cancer virus infection is one of the most critical factors in tumor progression among several infectious agents (27). Regarding thyroid carcinogenesis, it was recently discovered that biological carcinogens, such as viral infections, are among the most important causes (1, 8). Only a few studies have looked into the link between viral infections and thyroid cancer risk (8).

The results of this study revealed a statistically significant difference in the detection rates of HPV 16/18 between the analyzed thyroid tumors and apparently healthy thyroid tissues using the CISH technique, in conjunction with the histopathological analysis of thyroid tumors (thyroid carcinoma and thyroid adenoma). Mostafaei, Keshavarz (8) disclosed that papillary carcinoma was the most commonly related cancer to viral infections among thyroid tumor types, which is likely due to the fact that papillary cancer is the most frequent kind of thyroid carcinoma, accounting for nearly 85% of cases. As illustrated in table 3, HR-HPV 16/18 DNA positive results were found in 72.2%, 35%, and 27.5% of thyroid carcinomatous, thyroid adenomatous, and control thyroid tissues, respectively. Highly significant differences were detected among the studied groups (*P=0.0001*).

In Iraq, Mohammed, Alajeely (28) conducted a study in 2018 to identify the expressed HPV-18 antigen in thyroid tissues by using immunohistochemistry technique and found that 56.7% of patients had HPV-18 antigen, and while 35% of benign thyroid tumors have HPV-18 positive-IHC tests, none of the healthy tissues in the control tissues had HPV-18 positive- IHC reactions. These findings are in agreement with the results of the present study; nonetheless, those researchers studied the HPV 18- expressed antigens in these tissues, while the present study searched for HPV 16/18 DNA localization where the differences in rates are referred for the difference in technique and the physical states of HPV 18 in both studies.

In 2021, Dialameh, Saki (29), (from Shiraz, Iran) considered their study to be the first in Iran that link HPV to thyroid cancers (particularly papillary thyroid carcinoma). They enrolled 82 papillary thyroid carcinoma (PTC) and 77 benign thyroid nodules and used DNA amplifying nested polymerase chain reaction technique (two rounds) to determine the presence of a broad spectrum of HPV genotypes. They found HPV PCR positivity in 13.4% of PTC samples and 3.8% of benign thyroid nodules, while none of the neighboring normal tissues showed PCR positive results. The use of different methods, such as chromogenic ISH and IHC, was strongly suggested for the detection of HPV in the stated study.

In both studies, it could be suggested that the infecting particles of HPV can possibly reach the thyroid gland via the bloodstream, where HPVs are epitheliotropic viruses that spread by mucosal or skin contact. Nonetheless, non-sexual HPV transmission was described to be transmitted by mononuclear cells in the peripheral circulation (29-31). The integration of HPV into the chromosomes of the host cell is thought to be a significant step in the progression of intraepithelial lesions to an invasive cancer (32, 33). The diffuse pattern was typically observed in low-grade intraepithelial lesions, whereas the punctate pattern was detected in invasive malignancy. The integration of the viral genome into the host cell chromosome in the well-consented process of transformation and cancer formation is the most crucial phase for transformation and cancer development (multi-step carcinogenesis) (32).

The p73 protein belongs to the p53 family of transcription factors. These proteins act as molecular foci of an extensive and strong signaling network in stress response or carcinogenic cellular stimulations; moreover, they organize cellular proliferation, apoptosis, and specialization among many other crucial cellular functions (34, 35). The p73 has two isoforms: one with transactivation domain (TP73) that works as a pro-apoptotic, while those isoforms without that domain (ΔNP73) work as an anti-apoptotic, inhibiting p53 and transactivating p73 proteins from responding. Tumor protein p73 is possibly involved in p53-like tumor suppressor activities (22, 36).

The findings of the present study are in accordance with those obtained by Rufini, Agostini (37) who reported that both TAp73 and ΔNp73 isoforms were overexpressed in the majority of thyroid carcinomas, and comparable findings have been found in head and neck squamous cell carcinomas, with a substantial association between ΔNp73 levels and poor prognosis.

Manzella, Stella (38), revealed that the p73 expression has been found in human thyroid cancers in several investigations. The TAp73 and ΔNp73 transcripts were also detected in a substantial amount of human papillary, follicular, and undifferentiated thyroid carcinoma. Increasing evidence suggests that members of the p53 family are involved in the initiation of a spectrum of thyroid malignancies, and a significant number of therapeutic compounds are developed to target these proteins. Cancer cells have manipulated many ways to decline the function and/or expression of p73, from the promotion of hypermethylation to ratio modulation between its isoforms (anti- and pro-apoptotic); therefore, the balance between the two isoforms could be cell-fate determined (22).

Recent studies have demonstrated that HPV early protein2 (E2) inhibited the transcription of oncogenes E6 and E7 which participate in the tumorigenesis process in the first steps of HPV infection. These two proteins (E6 and E7) promote tumor growth by inactivating two types of tumor suppressor proteins, retinoblastoma (pRb), and tumor protein 53 (gene TP53), which manage the cell cycle switch from G1 to S phase. Non-phosphorylated pRb forms a complex with E2F regulatory proteins in the absence of cell growth, blocking the cell cycle in the G1 phase. Finally, it is believed that the retinoblastoma protein cascade is related to carcinogenesis by inactivating cell cycle regulators, including cyclin D1, CDK6, p21WAF-1, and p16 INK4A. Through a 3D complex composed of E6, E6AP (E6 associated protein), and TP53, the E6 contributes to the cleavage of the "wild" type of p53. The E6 also blocks the action of the tumor protein (p73), which is a homologous protein to p53 (14, 39).

The purchased kit of IHC primary antibody of p73 (Abcam, England), and as many other previous researchers concluded, the total p73 cannot differentiate the 2 isoforms (TAp73 and ΔNp73); therefore, it is difficult to clearly conclude from present results in the three studied groups about the actual role of the p73 protein overexpression. Nevertheless, since it belongs to the p53 family, researchers discovered that they could not investigate the wild type of p53 in normal cells since it appeared for a few minutes and then faded away. Therefore, it is suggested that p73 protein overexpression could be the result of a mutation in the p73-gene, leading to the defection of proteins with no anti-cancer functions.

In conclusion, the considerable identification of HPV16/18, along with the protein over-expressed from the P73-gene in patients with thyroid cancer in the present study, could suggest an etiologic involvement for that virus and this protein in thyroid carcinogenesis. As evidenced by the results of this study, it is recommended that future studies be conducted on large sample size and more prognostic clinicopathological features (type, grade, and stage) in various thyroid tumors to evaluate positive HPV16/18 infection needed to determine the role of these viral genotypes in thyroid tumor carcinogenesis (initiation and progression). Furthermore, it is suggested to use the most up-to-date laboratory molecular techniques and materials to distinguish between the various isoforms of p73 and investigate the roles of these isoforms and their importance in the survival of thyroid gland cells in benign and malignant tumor tissues in order to accurately predict diseases and select effective therapeutic patterns.

Authors' Contribution

Study concept and design: N. K. S.
Acquisition of data: N. K. S.
Analysis and interpretation of data: S. H. M. A.
Drafting of the manuscript: S. H. M. A.
Critical revision of the manuscript for important intellectual content: I. M. A.
Statistical analysis: I. M. A.
Administrative, technical, and material support: S. H. M. A.

Ethics

All The procedures were approved by the human ethics committee of the Ministry of Health, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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