Molecular Localization of Human Papilloma Viral 16/18 DNA in Adenoctomized Tissues from a group of Iraqi Patients

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Abstract

Background: Low- and high- oncogenic risk human papilloma viral infections have been related to the genesis of a variety of human benign and malignant tumors in the oral cavity and nasopharyngeal tissues. High- risk types of human papilloma virus are now well established as major etiologic factors of head and neck cancers, including tonsillar carcinomas. **Objective:** The current prospective case- control study aimed to unravel the frequency of HPV genotype 16/18 DNA detection rates in the adenoctomized tissues from patients with nasopharyngeal tonsillar adenoid hypertrophy. **Materials and Method:** A sixty (60) nasal as well as nasopharyngeal adenotonsillar tissues obtained from patients with adenoid hypertrophy via adenoctomies were enrolled. Forty (40) adenotonsillar tissues were from patients with adenoid hypertrophy, and (20) normal nasal tissue specimens were obtained from pediatric patients following trimming operations of inferior nasal turbinates' with unremarkable pathological changes (as an apparently healthy control group). A recent version of chromogenic in situ hybridization (CISH) method for HPV detection were performed by using specified DNA probes for DNA of high- risk HPV 16/18 genotypes. **Results:** Among nasopharyngeal adenotonsillar tissues group, 15 out of 40 have revealed positive CISH signals for DNA of HPV 16 / 18 genotype, constituting 35% of the total screened adenoid hypertrophied tissues. No positive–CISH reactions were detected in the control nasal tissues. Statistically, the results obtained in this study showed significant difference when compared to the control tissues group. **Conclusions:** The significant rate of such high- oncogenic HPV genotypes detection in those nasopharyngeal adenotonsillar tissues criticizes searching its further importance, as HPV 16/18 genotypes are critically correlated to many pre- neoplastic as well as malignant lesions.

Key Words: *Adenoid hypertrophy; Nasopharyngeal adenotonsillar tissues; HPV 16/18; CISH.*

Introduction

Waldeyer's ring consists of submucosal and subepithelial lymphatic tissues localized in the pharynx and comprises the tubal, pharyngeal, palatine, and lingual tonsils. The pharyngeal tonsils, also known as adenoids, located at the posterior superior nasopharynx [(1)].

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Adenoidal hypertrophy is an increase in adenoids size that might be associated with or without an acute or chronic infection[(2)]. An array of viral agent were recognized in association with the etiology of adenoid hypertrophy, such as herpes simplex virus, Epstein-Barr virus, , cytomegalovirus, adenovirus, coronavirus, coxsackie virus,,human boca virus, para influenza virus, rhinovirus, Parvovirus B19, and novel KI and KU polyomaviruses [(3-6).]

Among over 100 different types of Human papillomavirus (HPV) that are capable of infecting skin or stratified epithelial cells, at least 13 are cancer-causing types deemed 'high-risk'; the most common of

these being HPV 16 and 18 The remaining 'low-risk' types, causing non-cancerous lesions, of which HPV 6 and 11 are most common [7]. During the last 2 decades, an increasing evidence has suggested that human papillomaviruses (HPVs) can also infect the tonsillar epithelium,[3,4] and similar to other mucosal sites, have been associated with malignant transformation of tonsillar epithelia[8-10].

Since the incidence of tonsillar SCC has increased, whereas simultaneously the frequency of smokers has declined, additional risk factors probably exist for this malignancy [11,12]. Previous studies have suggested a substantial proportion of tonsillar SCCs to be associated with oncogenic HPV infections(13). The prevalence of HPV-positive related oropharyngeal SCC has been progressively increased and the tonsils were the most commonly affected sites [14] . Of note, >90% revealed HPV 16 [15] . However, to delineate when tonsillar HPV infection is first acquired as well as how long it remains latent, this goal could be approached by evaluating its prevalence in the pediatric population [7].

To our knowledge, this is the first molecular analysis that shade light on the association rate of HPV 16/18 infection in pediatric patients with tonsillar adenoid hypertrophy in Iraq.

Material and Method

In this prospective case- control study, sixty nasopharyngeal adeno-tonsillar tissue specimens from patients with adenoid hypertrophy have enrolled that including 40 nasopharyngeal adeno-tonsillar tissues from patients with adenoid hypertrophy and other 20 normal nasal tissues (as an apparently healthy control group since have unremarkable pathological changes). CISH detection of HPV 16\18 DNA, a specified four- (4) µm tissue section was tested by a digoxigenin-labeled, oligonucleotide probe to target this HPV 16\18-DNA, via using specific CISH kit (purchased from Zyto Vision GmbH. Fischkai, Bremerhaven. Germany).

For the statistical analysis, Chi–square test has used to calculate the statistical significance of the studied parameters, and the SPSS-23 package has been performed to analyze the relationship between the variables, where the p value of less than 0.05 points for a significant relationship between the studied parameters.

Results

Distribution of patients with nasopharyngeal adenoid hypertrophy according to their Age:

In this study, the specimens were collected from patients with nasopharyngeal adenoid hypertrophy whom ages were ranging from 4 to 9 years with a mean of 5.77 ± 3.73 years. The mean age of those apparently healthy individuals (A.H. control) was 6.35 ± 5.66 years and their age ranged from 5- 12 years (Table 1) .

Table (1): Distribution of studied patients with nasopharyngeal adenoid hypertrophy according to the mean and range of their age

Studied groups (Age / Year)	N	Mean	Std. Deviation	Std. Error	Range		ANOVA test (P-value)
					Minimum	Maximum	
Nasopharyngeal Adenoid Hypertrophy	40	5.77	3.73	1.11	4	9	P=0.4
A.H. Control	20	6.35	5.66	2.14	5	12	
Total	60						

Gender distribution of the patients with nasopharyngeal adenoid hypertrophy:

Males **with nasopharyngeal adenoid hypertrophy** was higher (60%: 24) than their female counter parts (40%: 16) .Also, in the control group , the males were higher (60%: 12) than females (40%: 8). The statistical analysis showed significant difference (P<0.01) among the studied groups (Table 2).

Table (2): Distribution of the studied patients with nasopharyngeal adenoid hypertrophy according to their gender.

Gender			Studied Groups		Pearson Chi-Square (P-value)
			Apparently Healthy Control	Nasopharyngeal Adenoid Hypertrophy	
	Male	N	12	24	
		%	60%	60%	P=0.007 Sign. (P<0.01)
	Female	N	8	16	
		%	40%	40%	
Total		N	20	40	

CISH expression of Human Papilloma Viral types 16\18 DNA **in nasopharyngeal adenoid hypertrophied tissues**

I-Positive HPV 16\18 DNA- CISH signal scoring

The nasopharyngeal adenoid hypertrophied tissues have revealed 35% positive signals which represented 14 out of 40 tissues in this group. None of control tissues group presented positive signals for HPV 16\18-CISH test. The low signal scoring was noticed in (15%) whereas (12.5%) and (7.5%) have moderate and high signals scoring, respectively (detailed in Table 3). However, in comparison to the percentage of HPV 16\18-DNA in healthy control group, the differences are statistically highly significant (P value = < 0.0001).

Table (3): Distribution of signal scores of HPV 16\18-DNA-CISH reactions.

HPV 16\18 scores		Studied groups		
		A.H. Control	Nasopharyngeal Adenoid Hypertrophy	P-Value
Negative	N	20	26	
	%	100%	65%	
Positive	N	0	14	
	%	0.00%	35%	
Low	N	0	6	
	%	0.00%	15%	
Moderate	N	0	5	P=0.000 Highly Sign. (P<0.01)
	%	0.00%	12.5%	
High	N	0	3	
	%	0.00%	7.5%	
Total	N	20	40	
	%	100%	100%	

Signal intensity of HPV 16\18- CISH testing:

Regarding signal intensities of HPV 16\18-CISH signal detection in nasopharyngeal adenoid hypertrophied tissues, the weak signal intensity was noticed in (20 %) whereas (10%) and (5%) have in both moderate and strong intensity, respectively. Statistically, significant differences were recorded between studied groups at (P<0.01) (detailed in Table 4).

Table (4): Distribution of signal intensities of HPV 16\18-DNA-CISH reactions.

HPV 6\11 intensity		Studied groups		
		A.H. Control	Nasopharyngeal Adenoid Hypertrophy	P-Value
Negative	N	20	26	P=0.004 Highly Sign. (P<0.01)
	%	100%	65%	
Positive	N	0	14	
	%	0.00%	35%	
Weak	N	0	8	
	%	0.00%	20%	
Moderate	N	0	4	
	%	0.00%	10%	
Strong	N	0	2	
	%	0.00%	5%	
Total	N	20	40	
	%	100%	100%	

Physical state assessment of HPV-16/18 DNA as an integrated and episomal forms

Regarding episomal and integrated forms of HPV 16\18-CISH signal detection in adenoid hypertrophy tissues group, the episomal was noticed in (28.6 %) whereas (71.4%) have an integrated phase (Table 5).

Table 5: Integrated and episomal forms of HPV-16/18

HPV 16\18	Positive (No.)	%
Episomal	4	28.6
Integrated	10	71.4

V. Spearman's Rho statistical testing of age, gender, and HPV 16\18-CISH to evaluate the studied markers in nasopharyngeal adenotonsillar hypertrophied tissues.

A strong positive relationship (with highly significant correlation) was found between HPV 16\18 and age of patients with nasopharyngeal adenotonsillar hypertrophy (r = 0.248, P = 0.003). However, there are no significant correlation between adenoid hypertrophy and gender (and as illustrated in table 6).

Table 6 .Spearman's Rho statistical testing of age, gender, and HPV 16\18-CISH to evaluate the studied markers in nasopharyngeal adenotonsillar tissues.

Spearman's rho		Age groups (years)	Gender	HPV 16\18
Age groups (years)	r		0.050	
	P		0.898	
Gender	r			0.186
	P			0.342
HPV16\18	r	0.248		
	P	0.003*		

***Correlation is highly significant (P<0.01).**

Discussion

While tonsillar and/or adenoidal hypertrophy are prevalent otolaryngology disorders in children, their pathogenesis are largely unknown [(16).] Normal tonsillar tissues have been assessed in only a few studies, where by the end of year 2002, 8.5% (17 of 200) of the tonsillitis samples contained HPV DNA, and out of them 12 samples of HPV 16[(1)] . However, the current rate of HPV infection in the tonsillar and adenoid hypertrophied tissues of the pediatric population remains poorly defined [(7).]

In the current study, 14out of 40 nasopharyngeal adenotonsillar tissues have revealed positive CISH signals for DNA of HPV 16 / 18 genotype, constituting 35% of the total screened group of tissues. The detecting of such important HPV 16/18 genotypes in these adenotonsillar tissues criticizes searching their further importance.

Based on a systematic review of the literature by Wojtera et al, [(7)], the range of prevalence of HPV in pediatric tonsillar tissues was 0 - 21%. However, an interesting parallel in the pathogenesis of HPV infection of the cervix, is that persistent and often asymptomatic HPV infection can lead to intraepithelial neoplasia and the eventual accumulation of mutations, resulting in cancerous invasion and metastasis. A similar cycle may occur with tonsillar HPV infection, emphasizing the importance of early detection of HPV[(17).]

Geographical distribution of HPV infection (in general) as well as HPV-related cervical lesions are alternate possibilities for supporting the notion of such variation in the prevalence of pediatric tonsillar- HPV infection [(18,19).]

In the present study, the CISH results of HPV DNA detection showed 15% low scores and 20% weak signal intensity.

In the present, and as all previous studies, the obtained tissues from tonsillar hypertrophy and chronic tonsillitis cases were the indications for tonsillectomies. The current findings support these authors that such subclinical HPV infections detected in childhood may represent a pre-malignant lesion with a long-term course, and as well as a risk factor for the development of tonsillar cancer in adulthood.

Of value in this respect to note that, up to 2003, a total world literature has reported presence of HPV DNA in 51% of the analyzed tonsillar SCCs via a variety of detection techniques .In addition, the most prevalent HPV type was HPV-16 (84%). Moreover, HPV-33 has been found in only 4.6% whereas HPV types 5, 12, 31, 35, and 59 have been detected in occasional tonsillar carcinomas. However, four studies have enrolled 1941 patients have failed to detect HPV[1].

These contradictable results were suggested to be related to a potential sampling or selection bias since were done on a smaller scale [20,21].

For being RT-qPCR as a gold standard [22], and since the unreliability of conventional PCR (that use broad-spectrum primers for HPV) as well as lack of controls (in some studies), some of the observed high prevalences in majority of the previous studies may be explained [23].

In the present study, the CISH results to analyze the physical state of HPV DNA detection in the tonsillar tissues have showed that 71.4% have an integrated forms while episomal forms constituting 28.6% of the tissues.

Only few studies have systematically analyzed the physical state of HPV in the tonsillar carcinoma(1,24). Interestingly, integration of HPV-18 into the chromosome at 10q24 in tonsillar SCC cases has also been reported [25].

It was also described an episomal forms of HPV-16 as well as integrated or both (the episomal and integrated) forms of HPV 33- positive tonsillar carcinomas[8]. One possible explanation, for being HPV is mostly in the episomal form in tonsillar carcinomas, could be a genetic alteration in the long control region of extra chromosomal HPV, that leads to dysregulation of the viral oncogenes[1].

It was also reported an episomal (but deleted) HPV-16 in tonsillar carcinomas, where the biological implications of such deletions remained obscure [8]. The pathogenesis of HPV- induced tonsillar carcinoma is different from cervical carcinoma, where HPV is mostly in the cervical carcinoma of an integrated form [1]. Whether these pediatric HPV 16 /18 infections are representing a transient and eventually eliminated by the host's immune system, or remains as a latently viral status, or eventually progresses to symptomatic disease, pre-malignancy states and ultimately lead to a malignant lesions is currently unknown and to be determined, too.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

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