P53-tumor suppressor gene overexpression in human papilloma virus-infected patients with oral squamous cell carcinoma

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ABSTRACT

Background: Via molecular DNA hybridization, more than 120 different genotypes of human papilloma virus have been confirmed. Many studies have described an association of high risk-HPV genotypes and overexpression of mutated P53 gene with a variety of oral benign tumors as well as malignant squamous cell carcinomas. This study aimed to: 1) Determine the prevalence of HPV DNA in archival tissue specimens with a range from apparently healthy tissue to invasive oral S.C.C by using one of the recent versions of insitu hybridaization. 2) Define the genotypes of the obtained HPV and to find out rational significance and relation of such genotypes to the severity of underlying lesions. 3) Study the correlation of over expressed products of mutant p53 genes with HPV-negative and HPV-related oral cancers.

Materials and methods: A total number of 72 tissue specimens were collected from 41 patients with oral squamous cell (OSCC) and 31 individuals with apparently-healthy oral tissues (AHOT). The molecular detection methods for HPV detection and genotyping were performed by in situ hybridization using cocktailed- and specified high- risk HPV DNA probes, respectively. Immunohistochemical method was used to demonstrate the prevalence of P53 overexpression in those oral cancers.

Results: Among oral OSCC group, 16 archived tissue blocks were found to contain HPV DNA related to the cocktailed HPV genotypes. This result constituted 39% of the total oral SCC screened for HPV DNA. HPV-18 positive oral SCC tissue blocks constituted (68.75%) whereas HPV genotypes 16& 31/33 constituted (43.75%) & (12.5%), respectively. Mixed infection of HPV genotypes was found in 31.3%. Interestingly, HPV DNA detection was documented in 3.2% of those appeared as healthy tissues on histopathological examinations. Among oral SCC group, 22(53.7%) showed over expression of P53 tumor suppression gene. Interestingly, the co-occurrence of mutated P53 overexpression and high oncogenic risk HPV genotypes was documented in 75% of Iraqi patients with OSCC.

Conclusions: The significant prevalence of high oncogenic HPV genotypes detection in those patients with OSCC indicates a herald marks for the spread of such important sexually transmitted infection among Iraqi general population. Both of mutated p53 genes as well as high-oncogenic risk HPV genotypes could play an important role in oral carcinogenesis.

Key Words: Oral Squamous Cell Carcinoma; Human Papilloma Virus; P53; In Situ Hybridization. (J Bagh Coll Dentistry 2011; 23(sp. issue):70-76).

INTRODUCTION

Although many attempts have been made, yet successful tissue culture system for propagation of papillomaviruses has not been developed. Therefore, HPVs had been characterized by molecular hybridization and recently phylogenetic relationships, based on nucleotide and amino acid sequence alignment, had gained wider acceptance and replaced the classic phenotypic classification [(10)].

It was not until mid 1970s that HPV has been singled out to be the most likely causative candidate for cervical cancer, whereas during 1983 - 1986, HPV-16, - 18, -31, and -33 were isolated from the precursors cervical cancer [(3)].

The link between oral squamous cell cancer and HPV seems logical, given the viral propensity for epithelial cell involvement. This connection was first proposed by Syrjanen et al., (1983) [(21)] when cytopathic effects of HPV (koilocytosis) were noted on light microscopy in oral lesions.

Oral Scc is the commonest malignant tumor of the oral cavity, accounting for more than 90% of these malignancies. In situ hybridization later confirmed the presence of HPV DNA in oral pre-malignancies and oral carcinomas, thereby suggesting a causal association of HPV and carcinogenesis in oral lesions as well [(16)]

It is clear that such high-risk HPV types, most notably HPV16 and HPV18, are predominantly found in both cervical and oral cancers and are able to transform both cervical and upper aerodigestive tract epithelia via similar mechanisms, notably E6 (early protein 6)-induced inactivation of p53, the protein encoded by the tumor suppressor gene that serves as a gatekeeper against carcinogenesis [(6)].

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The present study,up to our knowledge, represents the first in Iraq that highlighting for a possible etiological role of HPV infections in Iraqi patients with oral cancers and to elucidate the human papillomaviral interaction with P53 tumor suppressor gene in oral carcinogenesis.

MATERIALS AND METHODS

A total number of 72 oral tissues was enrolled in this study. From the archives of histopathology laboratories of college of Dentistry/Baghdad University as well as many private laboratories at Baghdad, forty one (41) formalin-fixed, paraffin embedded oral tissues blocks were obtained from patients who had undergone surgical operation or biopsies from different oral malignant lesions during the period from December 2006 till June 2007 .Thirty one (31) Oral biopsies were collected as apparently normal control group for this study.

At the histopathological department of Teaching laboratories / Medical City, each oral formalin-fixed paraffin-embedded blocks from was subjected to cut as serial thin sections of (4μm) thickness and were sticked on charge slides. In order to prevent carry-over DNA contaminations from one tissue sample to another, only one disposable cutting knife, which was specified for each tissue block, was used and then each section was sticked on a single charged slide. The 1st and 2nd tissue slides were specified for hematoxyline and eosin staining whereas many subsequent 4μm thickness-paraffinized tissue sections were specified for the following procedures of in situ hybridization and immunohistochemical staining.

It was feasible to include tissue-containing charged slides in each experiment as positive and negative HPV controls by using cervical tissue blocks, proved by PCR to have both cocktailed and high risk-oncogenic HPV genotypes, as a positive controls, as well as negative control from those apparently healthy cervical tissues, that were also proved by PCR technique to be negative for HPV.

Molecular detection and genotyping of HPV DNA in those tissue blocks was performed by a recent generation of in situ hybridization (ISH), using a specific biotinylated DNA probes for high oncogenic-risk HPV genotypes including 16, 18, 31/33. The detailed instructions of the processes for performing in situ hybridization method for detection and genotyping HPV in this study were done according to the manufacturing company (Dako cytomation Code No. K0601).

Immunohistochemical method that was used to demonstrate the prevalence P53 overexpression in those oral cancers was don according to the manufactoring company (Dako, Denmark Code K0673).

T, ANOVA, and Chi square tests were applied for statistical analysis of all results obtained in this research.

RESULTS

The Results of In situ hybridization for detecting HPV DNA in oral squamous cell carcinoma showed highly significant difference when the total percentage (39%) ; (16 out of 41) of HPV DNA detection in the total group of OSCC compared with its percentage in healthy-control group(3.2% ; 1 out of 31) (Table 1 & Figure 1).

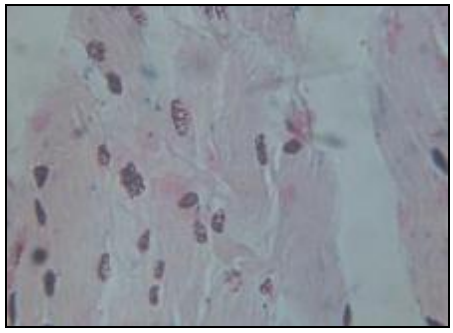

Figure 1: Positive in situ hybridization reaction showing HPV-DNA (using cocktaile of probes) within the cells of tissue block from a patient with OSCC; BCIP/NBT-chromogen stained & counter stained by nuclear fast red (X40)

Sex distribution of HPV-associated OSCC revealed higher percentage of HPV DNA detection in males than females, yet statistical analysis of difference was non-significant neither regarding HPV detection rate in males versus females nor its rate of detection among each group (male or female) (table 2)

Following the use of specific HPV DNA probes for three different HPV genotypes; HPV16, HPV18 and HPV31/33, the genotyping results of HPV-positive OSCC tissue blocks revealed that HPV DNA of genotype 16 was detected in 43.75% (8 out of 16), HPV18 in 68.75% (11 out of 16 and HPV31/33 in 12.5% (2 out of 16) of these HPV-positive oral cancers (Table 3)

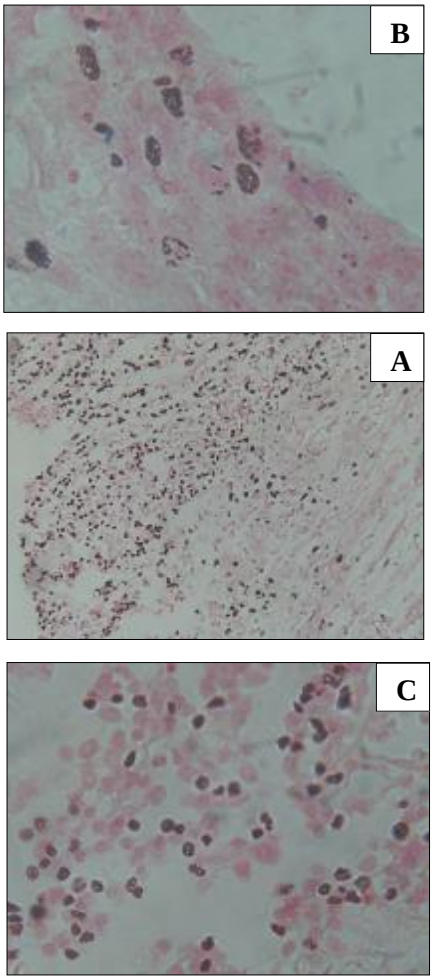

Figure 2: Formalin-fixed, paraffin embedded oral squamous cell carcinoma tissue blocks shows: A) HPV genotype 16-positive ISH reaction, BCIP/NBT-Chromogen stained and counter stained with Nuclear Fast Red (X 10) .B) HPV genotype 18-positive ISH reaction, BCIP/NBT-Chromogen stained and counter stained with Nuclear Fast Red (X 40). C) HPV genotype 31/33-positive ISH reaction, BCIP/NBT-Chromogen stained and counter stained with Nuclear Fast Red (X 20).

The percentage of mutated P53 overexpression has decreased with the proceeding of grade of differentiations of those infected with oncogenic high risk- HPV types. A similar trend of decreased grading of OSCC was found in their counterparts OSCC tissues that neither shared mutated P53 overexpression nor high-risk HPV genotypes infection. Also, similarly, such situation was faced with the rest two groups of OSCC cases that have either mutated P53 overexpression but were negative for any high-risk HPV infection or having infection with high oncogenic–risk HPV genotypes but negative for P53 mutation (table 4 & figure 3).

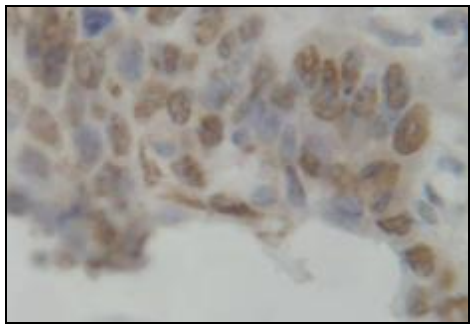

Figure 3: Immunohistochemical reaction of p53.OSCC tissue stained by DAB chromogen (brown), counterstained with Mayer's heamatoxylin (X40).

DISCUSSION

The screening of wide spectrum-HPVs in oral SCC:

Human papillomavirus has become well known to be the causative agent of both cervical adenocarcinoma and squamous cell carcinoma [23].

It was furtherly revealed that high risk HPVs have the ability to immortalize oral keratinocytes in vitro .In this respect, a theory has been raised on the importance of HPV among many viral infections in the development of oral SCC. [22].

In view of theses facts and speculations, this study subjected forty-one (41) tissue blocks from patients with oral squamous cell carcinoma to be examined by a recent generation of ISH technique to detect the DNA of the following wide spectrum HPV genotypes HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-45, HPV- HPV-51, and HPV-52 as well as specific IHC tests for detection of HPV16,HPV18 and HPV13/33 genotypes.

In Iraq, and up to our knowledge, this is the 00first study that performed ISH technique for detection HPV infection in archived histopathological specimens (FFPE) with oral SCC that were obtained from different anatomical sites in the oral cavity.

The results of the present study appear to be relatively similar to the results of Correnti et al. [5] who revealed that the HPV-DNA in 50% (eight of 16) of the SCC cases in a study for detection human papillomaviruses (HPV) in oral squamous cell carcinoma (OSCC) in a Venezuelan population. The small size of the sample included as well as the quality & sensitivity of the used technique (PCR) in this study are clearly responsible for this relative difference in the percentage of detection HPV DNA in OSCC.

The genotyping results of HPV DNA-positive oral squamous cell carcinoma lesions revealed that the most frequent HPV genotypes, predominantly found alsin oral cancers, are HPV16 and HPV18.The present results are in agreement with the results of Zhang et al.[28]. From the these results we found also that the HPV18 is more predominant than HPV16, and the present results are in agreement with Giovannelli et al. [9] who found that HPV-18 was the most frequent genotype, followed by HPV-6, -16, -33, and -53 in a study for HPV detection in potentially malignant (oral leukoplakia [OL], oral lichen planus [OLP]) and malignant oral squamous cell carcinoma [OSCC] lesions. These results may be due to the fact that HPV 18 has the ability to induce a more rapid transition to malignancy than what HPV 16 does [4]. However, This result is in disagreement with results of Furrer et al. [8]. On the other hand, Xiaofei et al. [27] found equal prevalences of both HPV 16 and 18 through their study on HPV prevalence in Japanese and North-east Chinese with oral sequamous cell carcinoma.

Regarding the present results of HPV31/33, the other studies also found that the HPV31/33 is not frequent in oral SCC. The present result agrees with [24].It is clear that the same high-risk HPV types, most notably HPV16 and HPV18, are predominantly found in both cervical cancer and oral cancers ,and that these HPV types are able to transform both cervical and upper aerodigestive tract epithelia via similar mechanisms [13] .

Detection of high percentage (31.3%) of mixed HPV genotypes in oral squamous cell carcinoma is in agreement with the finding that over 20% of genital HPV lesions are infected by more than one HPV type [21]. In Iraq, when we comparatively viewed the results of the present study regarding HPV genotypes 31/33 and mixed HPV genotypes, they were well supported by the results of another Iraqi researchers [15,2] who had found multiple/mixed HPV infections in Iraqi patients with preinvasive and invasive cervical neoplasia by using PCR and ISH, respectively .In addition,Al-Suraihi,2006 (using ISH) had also found multiple/mixed HPV infections in Iraqi patients with laryngeal carcinoma [1] .

Immunohistochemical evaluation of mutated-P53 overexpression in oral squamous cell carcinoma:

The p53 protein detected in this study could represents mutated or wild-type p53 protein since the monoclonal antibody used in the present study for the detection of p53 (DO7) can have the ability to react with both the mutant and the wild types of p53. However, the current study as other studies [11,25] was undertaken with assumption that the immunohistochemical detection of p53 with the DO7 monoclonal antibody (Dako Co., Denmark) is almost associated with the presence of mutated forms of p53 alleles. Based on the knowledge that wild-type p53 protein possessing a short half-life, ranging up to 30 minutes, hence not accumulating to an immunohistochemically-detectable levels, and on the idea that mutant forms having longer half-lives that providing a good chance for IHC detection in many instances [7].

The changes in the half-life of P53 proteins can either follow exposure of p53 gene to agents which damage or delete its DNA or could be due to functional inactivation by the binding P53 protein to other cellular proteins (mdm-2) or to a viral products (such as E6 and E7 antigens of HPV) [19].

As such, it could be proposed that the P53-negative OSCC cases in the present study which constituted (47.6%) of the total OSCC cases might resulted from biallelic deletion of TP53 gene, very low levels of mutant or wild-type p53, a nonsense mutation or a truncated p53 protein in its N-terminal portion, which would not be recognized by DO7 antibody [17]. Another possible explanation would be an accumulation of Mdm2 protein that caused by gene amplification, promoting p53 degradation and non-detection by immunohistochemistry [14].

On the other hand, it is now known that the wild-type p53 protein level can be increased to IHC-detectable levels during cellular damage, non-mutational p53 stabilization by cellular protein mdm2 or viral proteins such as large T antigen of SV40 and / or the lack of functional E6 expression of HPV [12].

The association of HPV genotypes with the mutated- p53 overexpression in cases with OSCC

The researchers had noticed that mutated p53 appeared to occur preferentially in those HPV-negative carcinomas but hardly, if at all, in HPV-containing carcinomas. This has strengthened the idea that inactivation of wild-type p53 function is essential for carcinogenesis and that the presence of high risk HPV E6 proteins obviates the requirement for mutational p53 inactivation and vice versa [20].

In agreement with this study, a report by Wiest et al [26] who showed that among HPV-positive tumors of the head and neck regions, most tumors of the oropharynx had expressed the viral oncogenes E6 and E7 and presented the wild type of p53, whereas most tumors arising from other oral regions presented a mutated p53 and did not

express the viral oncogenes E6& E7. Pintos et al. [18] on the other hand, supported those findings and concluded that p53 mutations were very common among both HPV- negative tumors and HPV - positive tumors that did not express the E6 gene.

CONCLUSIONS

1) The obvious high percentage of mutated P53 overexpression, as reflected by abnormal P53 gene product, among patients with OSCC indicates the presence of an important role of such genetic mutations in the carcinogenesis of that case of OSCC.
2) Despite mutated P53 appeared to occur preferentially in HPV-negative carcinomas& hardly in HPV-containing carcinomas, the high percentage of combined presence of inactivation of wild type P53 function and/or mutated P53 genes as well as the high percentage of detection of high oncogenic risk-HPV genotypes 16&18 in our Iraqi patients with OSCC strengthened a theory that both these factors could played an important role in their carcinogenesis.

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Table1: Percentage of (cocktailed) HPV DNA in OSCC

HPV –DNA ISH tests		Healthy control	squamous cell carcinoma patients	Comparison of Significance	
				P-value	Sig.
Positive	N	1#	16	0.000	Highly Sig. (P<0.01)
	%	3.2	39		
Negative	N	30	25		
	%	96.8	61		
Total	N	31	41		
	%	100	100		

Table 2: The prevalence of generic HPV DNA in OSCC according to sex

HPV –DNA ISH Tests		Gender		Comparison of Significance	
		Male	Female	P-value	Sig.
Positive	N	10	6	0.467	Non Sig. (P>0.05)
	%	41.7	35.3		
Negative	N	14	11		
	%	58.3	64.7		
Total	N	24	17		
	%	100°	100*		

Table 3: The genotyping pf HPV DNA in the studied groups

HPV –DNA Tests			Healthy control	squamous cell carcinoma patients	Comparison of Significance	
					P-value	Sig.
Type 16 HPV	Positive	N	0	8	0.031	Sig. (P<0.05)
		%	0	19.5		
	Negative	N	31	33		
		%	100	80.5		
	Total	N	31	41		
		%	100	100*		
Type 18 HPV	Positive	N	0	11	0.001	Highly Sig. (P<0.01)
		%	0	26.83		
	Negative	N	31	30		
		%	100	73.17		
	Total	N	31	41		
		%	100	100*		
Type 31/33 HPV	Positive	N	0	2	0.634	Non Sig. (P>0.05)
		%	0	4.88		
	Negative	N	31	39		
		%	100	95.12		
	Total	N	31	41		
		%	100	100**		

Table 4: The co-existence of wide spectrum HPV-ISH reaction with P53-IHC reaction in patients with OSCC

Results of P53-IHC Reaction				
Wide spectrum-HPV ISH reaction		+	-	Total
	+	12 (29.3%)	4 (9.7%)	16 (39%)
	-	10 (24.4%)	15 (36.6%)	25 (61%)
	Total	22 (53.7%)	19 (46.3%)	41 (100%)