Original Article

Molecular Detection and Genotyping of Human Papilloma Virus Infections in Iraqi Patients with Esophageal Carcinoma.

Intisar M. Jumaa * **PhD**
Saad H. Mohammed Ali ** **PhD**
Tural Y. Bakir * **MSc**
Delaram M. M. Ardalan *** **MSc**

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Summary:

Background: Molecular DNA hybridization has confirmed more than 120 different human papilloma virus (HPV) genotypes. A small group of them have high- risk oncogenic potential. Many studies have described an association of such high risk-HPV genotypes with a variety of esophageal benign tumors as well as malignant squamous cell carcinomas.

Patients and Methods: A total number of 90 tissue specimens were collected from 50 patients with esophageal squamous cell (SCC), adenocarcinoma (AC) and carcinoma in situ (CIS); 20 patients with squamous acanthosis (SA); and 20 individuals with apparently-healthy esophageal tissues (AHET). The molecular detection methods for HPV detection and genotyping were performed by in situ hybridization using cocktailed- and specific high- risk HPV DNA probes, respectively.

Results: The overall percentage of HPV in the total group of esophageal carcinoma was 20%.The percentage of HPV DNA in the subgroup of SCC and AC was 26.7% and 13.3%, respectively,. However, neither HPV DNA was detected in CIS subgroup nor in both control groups (SA and AHET).The overall genotyping results showed that HPV 18 constituted the majority of the detected high-risk oncogenic HPV genotypes, followed by HPV 16 then HPV 31/33.

Conclusions: Despite the low prevalence of HPV infection and rarity of invasive esophageal carcinoma in the general Iraqi population, the detection of high percentage of such high oncogenic risk- HPV genotypes in these carcinomas indicating for a relevant importance in esophageal carcinogenesis.

Key Words: Esophageal Carcinoma; Human Papilloma Virus; In Situ Hybridization.

Introduction:

Esophageal cancer ranks the ninth among the most common cancers world wide and represents approximately 4% of the total cancers. It remains one of the leading causes of cancer mortality. It ranks the sixth most common of cancer-related deaths (1-3). An association of some viruses with the occurrence of esophageal cancer has been described, like: Herpes Simplex Virus (HSV), Cytomegalovirus, Epstein Barr virus (EBV) and Human Papilloma Virus (HPV) (4, 5). The papilloma viruses have a specific tropism for squamous epithelial cells and their full productive cycle is only supported in these cells (6). Update more Than 100 different HPV types have been described; of these, nearly 30 can infect the anogenital tract some of These HPVs are frequently associated with cancer and are considered high risk genotypes (for e.g. HPV type 16 and 18), whereas others give rise to warts and benign lesions and are considered as low and intermediate risk genotypes (for e.g. HPV type 6, 11, 31, 33 and 35) (4, 5, 7).

** College of Medical and Health Technologies*
*** College of Medicine / University of Baghdad*
**** Teaching laboratories / Medical City*

The association of HPV infections in squamous cell precancerous lesions of the uterine cervix has been well established since the late 1970 (8) Oncogenic HPV types are regarded as the most important etiological factor of cervical SCC.(9, 10, 11). The early 1980s have witnessed the rapid expansion of HPV research from genital tract to cover the other squamous cell epithelia, thus widening the scope of HPV-associated human tumors to involve the oral cavity, pharynx, larynx, upper airway, and esophagus (8, 10, and 12). The squamous cell lining of the oral mucosa is in direct continuity with the esophagus and the first descriptions on HPV lesions in the oral mucosa were slightly preceded by reports suggested by Syrjanen in 1982 that there is an association between HPV with both benign and malignant squamous cell lesions of the esophagus (13). Hille and co-workers were the first who used IHC in 1986 for the demonstration of HPV antigens in esophageal SCC (14). Currently, PCR and DNA hybridization are used for HPV genotyping (15). Following these pioneering observations, HPV research has resulted in rapidly expanding literature on both benign and malignant esophageal lesions in different geographical regions (16, 17, and 18). In addition, the evidence accumulated during the past 20 years is strongly suggestive of a

causal role for HPV in esophageal carcinogenesis (7, 19, and 20). However, data concerning the involvement of HPV in esophageal cancers are still controversial and the exact mechanism by which HPV contributes to carcinogenesis has yet to be elucidated in the context of the esophagus (21, 22, and 23). This type of cancer is rare in Iraq, where is represent 0.7% among other cancers (24). The present study represents the first report in Iraq that designed to study the prevalence as well as the genotypes of HPV infection in Iraqi patients with esophageal cancers to elucidate a possible etiological role for HPV in esophageal carcinogenesis.

Materials and Methods:

Subjects: This study was designed as a retrospective research. A total number of (90) formalin-fixed, paraffin embedded esophageal tissue blocks were collected during the period from August 2004 to March 2005.These patients had undergone esophageal biopsy. They were collected from the archives of histopathology laboratories of different general hospitals, including Specialized Surgeries Hospital laboratories in the Medical City, AL-Yarmook Teaching Hospital Laboratories, Al-Kadhemiya Teaching Hospital Laboratories, Gastroenterology and Liver Diseases Teaching Hospital Laboratories, Al-Kendi Teaching Hospital Laboratories, Baquba Public Hospital Laboratories, Azadi (Kirkuk)Public Hospital, as well as from many private laboratories, including Dr.Raji Al-Hadethi private laboratory, Dr.Loui AL-Khuri private laboratory, Dr.Nawal Alash private laboratory and Dr.Faris Khairo private laboratory. Fifty esophageal malignant samples has been collected from 30 patients with squamous cell carcinoma (SCC) and 15 specimens from adenocarcinoma (AC) and 5 from those with carcinoma in situ .In addition, 20 tissue samples from squamous acanthosis cases were included, as a related esophageal disease control group, as well as 20 esophageal tissue samples with normal histological appearance (i .e without any significant pathological changes) were also included, as healthy control group for this study. The diagnosis of these tissue blocks were based on the obtained histopathological records attached to each esophageal biopsy in the relevant hospital files that accompanied these specimens. But following trimming process of these tissue blocks, a confirmatory histopathological re-examination of each obtained tissue blocks was done by further consultant histopathologist.

Methodology: 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.

Tissue Sectioning and Slide Preparation: At the histopathological department of Teaching laboratories / Medical City, formalin-fixed paraffin-embedded blocks from each esophageal biopsy was subjected to cut as serial thin sections of (4-5µm) 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 4-5µm thickness-paraffinized tissue sections were specified for the following procedures of in situ hybridization. As positive and negative HPV controls, it was feasible to include such tissue-containing charged slides in each experiment 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.

In Situ Hibridization for Detection Cocktailed (Generic) – HPV Genotypes: 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 corporation Co./ Denmark) (25).

In Situ Hybridization of HPV Genotype 16, 18, 31/33 Biotinylated and Probes: The same procedures for in situ hybridization for detection of cocktailed probes of HPV (Dako cytomation Code No. K0601) were followed, except for the step of the temperature for stringent washing of these steps was changed to 58^{o}C for 20-40 minutes. Assessment of the Result: Within 2 hours, the obtained result was assessed by examining the processed slides under light microscope; a deposition of a soluble blue purple product at the sites of hybridization of the probes to their targets is a positive indicator for the presence of the questioned group of HPV. Statistical Analysis: T test, and ANOVA test, and Qui square were applied for statistical examination of all results obtained in our research.

Results:

1. Detection in the total group of esophageal carcinoma was 20%, while its percentage in the SCC group was 26.7% and in the AC group was 13.3%. No HPV DNA was detected in both squamous acanthosis and healthy control group (Table 1 & Figure 1).

Table1: Percentage of cocktailed HPV DNA Detection in esophageal carcinoma

Study Groups	No. of Tested Cases	Cocktailed HPV DNA Detection Via In Situ Hybridization			
		Positive		Negative	
		No.	%	No.	%
Carcinoma In Situ	5	0	0.0	5	100.0
Squamous Cell Carcinoma	30	8	26.7	22	73.3
Adenocarcinoma	15	2	13.3	13	86.7
Squamous Acanthosis	20	0	0.0	20	100.0
Healthy Control	20	0	0.0	20	100.0

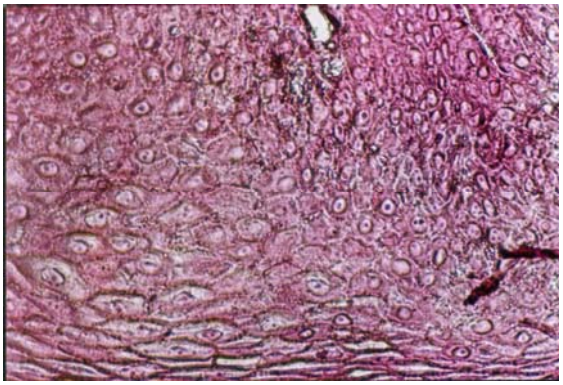

Figure1: Positive in situ hybridization reaction showing HPV-DNA (Generic) within the cells of tissue blocks from patients with esophageal cancer. BCIP/NBT-chromogen stained & counter stained by nuclear fast red (X100)

2. Sex Distribution in HPV-Associated Esophageal Carcinoma:

None of the cases with carcinoma in situ had showed cocktailed / generic HPV DNA-ISH reaction, whereas HPV DNA was detected in 6 males (20%) out of 30 SCC and in 2 females (6.6%) out of 30 SCC. In those cases with AC, HPV DNA was detected in only 2 males (13.3%) out of 15 AC specimens (table 2).

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

Study Groups	Total	Male				Female			
		HPV positive		HPV negative		HPV positive		HPV negative	
		No.	%	No.	%	No.	%	No.	%
Carcinoma In Situ	5	0	0.0	3	60.0	0	0.0	2	40.0
Squamous Cell Carcinoma	30	6	20.0	14	46.7	2	6.6	8	26.7
Adenocarcinoma	15	2	13.3	6	40.0	0	0.0	7	46.7
Squamous Acanthosis	20	0	0.0	12	60.0	0	0.0	8	40.0
Healthy Control	20	0	0.0	14	70.0	0	0.0	6	30.0

3. Genotyping Results of HPV- Positive Esophageal Carcinoma:

Three different HPV genotypes were found in cases with esophageal carcinoma of the present study. The genotype HPV16 was detected in 5 out of 8 (62.5%) of HPV-positive SCC tissue blocks. However, no HPV 16 was detected in any AC specimens (Table3 & Figure 2).

Table3: Frequency of HPV- genotype 16 detection in esophageal carcinoma

Study Groups	No. of HPV -Positive- tested cases	In Situ Hybridization for HPV genotype 16 Detection			
		Positive		Negative	
		No.	%	No.	%
Squamous Cell Carcinoma	8	5	62.5	3	37.5
Adenocarcinoma	2	0	0.0	2	100.0
Total Esophageal Carcinoma Group	10	5	50.0	5	50.0

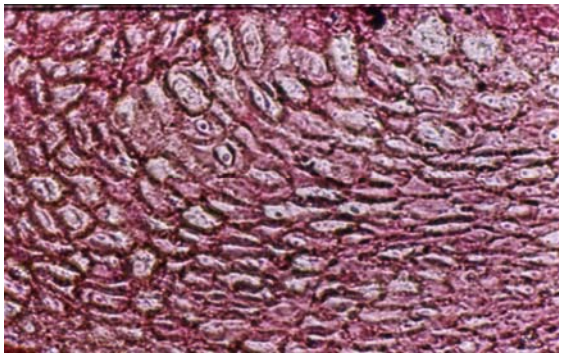

Figure2: Positive in situ hybridization reaction showing HPV genotype 16 DNA within the cells of tissue blocks from patients with esophageal cancer. BCIP/NBT-chromogen stained & counter stained by nuclear fast red (X200).

On the other hand, HPV18 was detected in half (50%) of the HPV-positive SCC specimens, whereas all adenocarcinoma specimens were positive for HPV18 (Table 4).

Table4: Frequency of HPV- genotype 18 in esophageal carcinoma.

Study Groups	No. of HPV-Positive Tested Cases	In Situ Hybridization for HPV genotype 18 Detection			
		Positive		Negative	
		No.	%	No.	%
Squamous Cell Carcinoma	8	4	50.0	4	50.0
Adenocarcinoma	2	2	100.0	0	0.0
Total Esophageal Carcinoma Group	10	6	60.0	4	40.0

The HPV31/33 in the current study was detected in 2 out of 8 (25%) of SCC cases and in half (50%) of adenocarcinoma cases (table 5).

Table 5: Frequency of HPV genotype 31/33 in esophageal carcinoma

Study Groups	No. of HPV-Positive Tested Cases	In Situ Hybridization for HPV genotype 31/33 Detection			
		Positive		Negative	
		No.	%	No.	%
Squamous Cell Carcinoma	8	2	25.0	6	75.0
Adenocarcinoma	2	1	50.0	1	50.0
Total Esophageal Carcinoma Group	10	3	30.0	7	70.0

Lastly, it was found in this study tissue blocks with mixed- HPV genotypes infections in 4 out of 8 (50%) of HPV-positive esophageal carcinoma cases (table 6).

Table 6: Frequency of mixed HPV genotypes Detection in esophageal carcinoma

Study Groups	No. of HPV-positive Tested blocks	Mixed HPV		HPV Geno-type 16+18	HPV Geno-type 16+ 31/33	HPV Gen-otype 18+ 31/33	HPV Gen-otype 16+18 +31/33
		No.	%	Freq-uency	Freq-uency	Freq-uency	Freq-uency
Squamous Cell Carcinoma	8	4	50.0	1	1	1	1
Adeno-carcinoma	2	1	50.0	0	0	1	0

Discussion:

Results of HPV Detection in Esophageal Carcinoma:

Data concerning the involvement of HPV in esophageal cancers are controversial (23). This type of cancer is rare in Iraq. It represents 0.7% among other cancers. The present study represents the first report regarding the prevalence of HPV infection in Iraqi patients with esophageal cancer to elucidate a possible etiological role for HPV in esophageal carcinogenesis.

In the current study, the esophageal tissue samples, which were collected from histopathology laboratories of different general hospitals as well as many private laboratories, are including the following specimens:-

A. Five carcinoma in situ samples; none of them were positive for HPV using recent generation of ISH techniques. This could be related to the small number of biopsies that were studied as well as to the very tiny size of these biopsies that were endoscopically biopsied (i.e. they are tiny pieces) and are not like those surgically resected specimens and thus could lead to a missing HPV-positive esophageal pre-cancerous cases. In consistence with the present results, only one study that examined both endoscopic biopsies and surgically-resected specimens and found a lower incidence of HPV DNA in endoscopic biopsies (26). However, other studies were able to demonstrate a high incidence of HPV DNA but they had relied exclusively on large numbers of biopsies (27).

B. The HPV DNA are detected in 8 out of 30 (26.7 %) of invasive SCC cases whereas only 2 out of 15 (13.3%) of invasive AC cases are HPV- positive. In SCC, the present HPV- positive results are as double higher as those positive results of HPV in AC cases. This could be related to the fact that HPV are epitheliotropic viruses which require squamous epithelial tissue as micro- environment for their replication and to continue their life cycle (28).On reviewing and discussing the relationship of HPV to esophageal AC, it was noticed that only very limited researches in this field were present and only few researchers had directed their efforts in this field; one of them, in Turkey, who scored HPV in 4 out of 10 (40%) of AC cases by using PCR technique (3). Another study in China, had also detected the HPV score but in 22 out of 62 (35.5%) of the AC of the gastric corpus by using ISH technique which is a solid tumor similar to AC of the esophagus (29). The frequency of HPV infection, reported in the present study, is placing our country among countries in the moderate risk region especially when we compared the detected prevalence with those in high and low risk regions of HPV infection. In high- risk regions (such as China and South Africa) it had reported HPV prevalence that ranged from 40 to 71%) (30). Chang and co-workers(16) reported the presence of HPV in 43% of Chinese esophageal carcinoma cases whereas HPV-DNA was detected in 52%, 65% and 71% of esophageal carcinoma cases from South Africa (30). Moreover, lower frequencies (2% and 6.7%) were reported in western countries and North America, respectively (26). In these low- risk regions, most studies have failed to find any association between HPV and esophageal cancer (31). The present results are ranked between the results of very high risk regions of HPV infection and the other extremity (zero% of HPV detection). The examples of studies from these very high risk regions are those of Chen *et al* (32) who had demonstrated HPV positivity in 60% of SCC cases from high risk areas of China and study of Williamson *et al* (33) who also reported HPV DNA positivity in 71% of esophageal cancer tissue. While reports from Japan, North Europe and United States show little or no evidence of HPV in esophageal SCC (34, 35). A potential shortcoming of this study, as well as other retrospective studies, are their limited ability to find out a clear-cut explanation for the association between HPV and those cases of esophageal cancer, whether HPV has a hit-and-run mechanism in this anatomic site or not. By analogy, bovine papilloma virus-4(BPV-4) causes diffuse alimentary tract papillomatosis in cattle. This infection is usually a self-limited, unless the cattle were

ingesting broken fern. It was observed that the bracken fern contained both immunosuppressant and a DNA damaging agent (called quercetin). Those immunosuppressed cattle are unable to clear the BPV-4-infected cell and thereby promote tumor progression (36). Despite its presence in benign papilloma, the BPV is rarely found in malignant tumors and this notice is suggesting an important role for the virus in tumor initiation and promotion but not maintenance of the malignant phenotype (12). However both the absence of HPV in benign esophageal lesions and the lack of an association between HPV L1 seroactivity and esophageal cancer risk argue against this possibility in western cultures, here, further case series and case-control studies, especially in high-incidence areas, such as Africa and China were warranted. Such studies should include detailed evaluations of human dietary or medical exposures that may be analogous to broken fern in cattle (12). No infection was revealed by ISH techniques in both squamous acanthosis group and healthy control group in this study. Moreover, marked variations in the prevalence of esophageal HPV infections have been reported by different authors. These differences might be, in part, due to the differences in the sensitivity and specificity of the methods used. Furthermore, most researchers who made relation between HPV infection and esophageal cancer had especially studied the relationship between HPV and esophageal SCC that are, in one hand, based on the observation of characteristic histopathological findings (koilocytic changes), suggesting the presence of HPV in benign esophageal epithelia and malignant esophageal tumors and, in the other hand, are based on the morphological similarities between esophageal SCC and cervical SCC (13). The consumption of tobacco and alcohol could explain more than 90% of cases of esophageal SCC (37). Also health registries had indicated that the consumption of tobacco by Iraqi people is very high which in turn may enhance the possibility of SCC of the esophagus (24).In addition, in some areas with a high risk of esophageal cancer, like China and Latin America, the consumption of very hot beverages (hot mate or boiling water) and the consumption of fermented fish have been identified as probably carcinogenic factors for esophagus (38).It is worthy to mention that Iraqi people have a similar disposition to those in southern east of Asia in respect to same habits of eating spicy foods as well as consumption hot beverages (like tea) which were well recognized as one of the risk factors.

Genotype Results of HPV-Positive Esophageal Carcinoma: The mechanisms through which HPV16 and HPV18 can induce epithelial neoplasia have been extensively studied (39, 40). Some of their viral proteins, notably E6 and E7 are oncoprotiens that immortalize various human cell types, inactivate host protein (such as p53 or Rbp) and induce mutations in the host cell DNA (41). Since the most prevalent HPV genotypes in different studies were related to the class of high oncogenic types (i.e. HPV16 and HPV18), most of these studies had suggested a possible etiological role for HPV in esophageal cancer .The other reported HPV genotypes were supposed to represent a lower oncogenic HPV risk types, these were HPV6, HPV11, HPV31 and HPV 33 (30). On comparing the results of the present study with 2 separate studies in Turkey and Iran, which had also reported the presence of HPV16 and HPV18 in both esophageal SCC and AC by PCR technique; in Turkey, the researchers had detected HPV16 and /or HPV18 in 10 out of 30 (33.3%) of SCC samples and 1 out of 10 (10%) of AC samples (3) .In the Iranian study, HPV16 and HPV18 have been found in 5 out of 38 (13.2%) and in 3 out of 38 (7.9%) of esophageal SCC cases, respectively (23). The differences noticed in these percentages of HPV16 and HPV18 between these studies when we compared them to our results they may be due to different techniques used in each study. Moreover, in those studies there may be other HPV genotypes in the examined materials but they were not detected because they could be out of the range of the ability of detection level of the used HPV probes. The results of this study are similar to the abroad one where it was observed that this disease is common in men more than women. SCC had noticed to occur most commonly in African American men compared with Caucasian men, African American women, and Caucasian women. Although SCC occurs more commonly in men than women in areas of low incidence, however, high incidence areas did not show such strong sex difference (42). Esophageal AC is the fastest rising malignancy among Caucasian men. Men were 6-8 fold more likely than women to suffer from esophageal AC and Caucasians were 3-4fold more likely than African Americans (43). Also the effect of such sex differences was noticed when HPV genotyping of HPV positive cases were done in our study especially HPV genotypes 16, 18 and 31/33 where in this respect, all of them were higher in males more than females. These results were consistent to the results of (42). In this study, positive HPV-results were detected in old aged patients. For SCC, the age range was between (40-80 years) for males and for females was (65-75 year). However, there were no HPV-positive results in females. So far, our results are in good agreement with the results of Yang and Davis who demonstrated an age-related increase risk for the development of SCC and AC (31). The incidence for each of these cancers is extremely low under the age of 40 years. After the age of 40, an increased incidence had been observed with each decade of life (31, 44).

Conclusion:

1. Despite a low prevalence of HPV infection and the rarity of invasive esophageal carcinoma in the general Iraqi population, the detection of differed oncogenic

HPV genotypes in that esophageal cancer could have a relevant importance, among other risk factors, in the process of its carcinogenesis.

2. The very lower percentage of HPV DNA in pre-invasive esophageal neoplastic lesions in the present study (than previous studies elsewhere) could be in part, due to other HPV genotypes (but not included with the range of cocktailed-genotypes probes used for in situ hybridization in this study) or due to different other agents that have oncogenic potential or have some role in the carcinogenesis of HPV-negative esophageal carcinoma. In addition, carcinoma in situ group that showed a negative result for generic HPV infection could also be a reflection that most of these HPV infections are transient and did not persist or might be obstacle by the very low number of specimens or are not present in these very tiny pieces (endoscopic biopsies) used in these experiments.

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