Human Papillomavirus and Esophageal Carcinoma: A Study in China

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***Abstract*—Eesophagealsquamous cell carcinoma (ESCC) is considered as the ninth most common malignancy in the world. There are a number of suspected casual reasons of this type of carcinoma. The association of viral infection with EC (esophageal carcinoma) has been reported in last 30 years. Human papillomavirus (HPV) is said to be a major aetiology in areas with high incidence of esophageal carcinoma, but the prevalence and the role of HPV virus in the aetiology of esophageal squamous cell carcinoma (ESCC) is still uncertain. In this study we designed to evaluate the prevalence of HPV in EC cases diagnosed in pathology department of Hebei, China. In this study 170 cases that were pathologically diagnosed as esophagealcarcinoma were obtained from department of pathology files at Hebei provincebetween2011-2013. DNA material was extracted from formalin-fixed paraffin-embedded tissues (FFPET) and PCR was performed to detect HPV genome. In this study negative and positive control were used for HPV 16/18 and beta-globin PCR as internal control. More than 95% of FFPETs had acceptable result in DNA qualification PCR test. Overall prevalence of HPV in tumour tissues was 81.17% in GP6+/GP5+ PCR, 40.58% by HPV16 and 49.41% for HPV18. The presence of HPV DNA in esophagus tumours (high risk HPV types 16 and 18) implicates HPV as one of the possible aetiology factors in esophageal carcinoma.**

***Index Terms*—ESCC (oesophagealsquamous cell carcinoma), FFPET (formalin fixed paraffin embedded tissue), PCR (Polymerase chain reaction), HPV (human papillomavirus), EC (oesophagealcarcinoma).**

I. Introduction

Oesophageal cancer is the 8th most common cancer and the 6th most common cause of deathin the world [1]. Human papillomavirus (HPV) is a non- enveloped, double-stranded DNA virus with more than 100genotype .To date, molecular and epidemiological studies have demonstrated that HPV infection, mainly high-risk HPV, play a main role in the development of uterine cervical cancer [2], [3]. In malignant transformation of cervical epithelia, integration of HPV DNA into the host cell genome is considered as an important step [4].

A number of studies reported HPV DNA detection in extra-genital cases as well, but the aetiological involvement of HPV in these cancers is still controversial [5], [6]. The association between HPV and ESCC (oesophageal squamous cell carcinoma) was firstly reported by Syrijnen in 1982. Since then, HPV infection has received attention as a possible risk factor for ESCC development (Syrjanen 1982) [7]. An extensive review by syrjanen published in 2002 showed that HPV was positive in 22.9% of 1485 ESCC cases analysed by in situ Hybridization (ISH) and in 15.2% of 2020 ESCC cases analysed by PCR (Syrjanen 2002) [8]. Malignant oesophageal tumours usually arise from epithelial layer of the oesophagus. Worldwide, squamous cell carcinomas (SCC) constitute 90% of oesophageal cancers. Although in some regions such as USA their incidence is comparable to that of adenocarcinomas [9].

With regard to incidence and prevalence, oesophageal cancer exhibits geographical variations due to unknown factors between countries, as well as between different regions of some countries. According to the world health organization (WHO), incidence–rate areas located between West Africa, at the low-risk end, and China at the high-risk end. [10] Among Latin America countries, Mexico and Peru have the lowest mortality rate for oesophageal carcinoma in both males and females, whereas Brazil, Argentina and Chile have the highest mortality rate [11]. A number of studies have done in some areas of China but the results are controversial. Some studies were conducted in Anyang, Shandong and Gansu area in China showed the detection of HPV, particularly high-risk genotypes. [12] However the finding wasn't confirmed by another study conducted among high-ESCC population in Linxian, China [13]. Studies on the presence of HPV in oesophageal cancer have generated conflicting results, with the prevalence rates ranging from 0% to 71% [14]. These differences probably because of variations in the specificity and sensitivity of the analytical techniques were used. In compare with other techniques, polymerase chain reaction (PCR) is simple, rapid and sensitive method for detection of HPV DNA in tissue samples. Furthermore, the use of suitable primers is an advantage in PCR based studies because the primers can detect a wide spectrum of HPV types [15]. Because collecting fresh particular cancerous tissues in large scale is too hard and in the other hand by examining of fresh tissues we just can evaluate samples of short period of time, so, in this study,

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formalin-fixedparaffin-embedded archived tissue were used. There are some methods to examining PETs (paraffin embedded tissues) such as IHC (immuno-histochemistry method or the other methods to examine DNA or RNA of PETs sections after extraction of DNA or RNA. Among different methods, polymerase chain reaction (PCR) has confirmed to be a rapid and particularly sensitive method for examining DNA from PETs. Adequate storage of samples and undertaking a procedure to produce high quality extracted DNA, are important factors for obtaining successful results from PCR[16].

II. Material and Methods

A. Tissue Collection

Cases with a pathological diagnosis of oesophageal carcinoma were obtained from files of department of pathology at Hebei province during the period between2011-2013. Samples weretotally170 formalin-fixed paraffin embedded tissue sections of histologically confirmed oesophageal carcinoma.

B. DNA Extraction

20μmsections of formalin-fixed paraffin embedded tumours were de-waxed by 2 hours incubation in pure xylene with every 45 minutes mixing tube continents. This step repeated for 7 times and then de-waxed samples were washed and dehydrated with 100% ethanol for 6 times and then were dried in room temperature. Deparaffinised samples were digested with 600μl of lysing buffer was made as follow: Tris-HCL 10 mmol/L PH8.0, EDTA 0.1 mol/L PH8.0, SDS 0.5% and RNA-aseA 20μg/ml. then added 20μl proteinase k 20μg/ml and after mixing, tubes were incubated in 55℃ for 12 hours, with remixing contents of tubes every 2 hours. After complete digestion, DNA was extracted with phenol/chloroform precipitation. This process was done 2times for every sample and then followed by 1 time phenol/chloroform precipitation. DNA was precipitated with 100% ethanol and incubated in -20℃ for one night. After precipitating of DNA, tubes centrifuged in high speed (13000 rpm for 15min) and then rewashed with 75% ethanol and dried in room temp. 80μl TE buffer added to every tube and tubes were incubated in 4℃ for a few hours to dissolving precipitated DNA.

C. Polymerase Chain Reaction

To test DNA extraction performance, the obtained DNA was amplified for beta-globin gene with usage of Takara PCR kit and primers of PC04/GH20 [Table I]. In this study GP5+/GP6+ set primer (Table I) used as a non-degenerated primer set that detects a wide range of HPV types using a lower annealing temperature during PCR and produces a PCR product of approximately 150bp [17], [18].For specialize detection of HPV 16 and 18, two set primers (Table I) were used that annealing temperature and PCR product of these two sets are mentioned in table1. Each batch of samples included negative control, containing water, and positive control, DNA from an HPV positive cervical carcinoma. PCR products were analysed on 1.0% Agar gel and visualised by staining [DMSO].

D. Sequencing of PCR Products

To identify the HPV types and any variation in sequence, all positive PCR products were subjected to direct DNA sequence analysing by the T7 sequence version 2.0 DNA sequencing kit. The nucleotide sequences were subsequently subjected to basic local alignment search (BLAST), which is a set of similarity search programmers designed to explore all of the available sequence databases (ncbi.nin.gov).

III. Results

First of all we should mention that to confirm the existence of PCR product of interest, all PCR processes were done twice. The first time PCR products were used as the template of the secondary PCR. In the second time PCR, amount of all PCR mixture materials except to template of 8μl were similar to the first time PCR process. (Table II) summarized the result of every PCR process of 170 ESCC cases examined in this study. All results are shown in this table are the second time PCR results.

A. Beta globinPCR

In this study DNA extracting method seems to be more effective than other methods previously reported. After DNA extraction, of all 170 cases of ESCC in more than 163 cases (more than 95%) DNA quality was adequate for PCR analysis to detect HPV DNA sequences, as demonstrated by beta-globin gene amplification. PCR using the PC04/GH20 primer pair resulted in very clear differentiate seized DNA fragment of 150bp (Fig. 1).

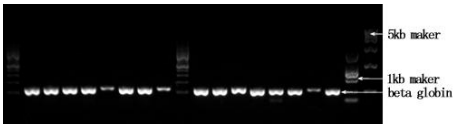

Figure 1. Beta globin PCR result and clear differentiated seized DNA fragment of 150bp

B. GP5+/GP6+ HPVPCR

After beta-globin PCR, all 170 samples were examined by using GP5+/GP6+ primer set to visualize HPV-positive cases in agar gel. PCR using the GP5+/GP6+ primer pair resulted in a very clear differentiate seized DNA fragment of 150bp (Fig. 2). Of all 170 specimens tested, 138 (81.17%) were positive for HPV, as seen by the presence of the 150bp PCR fragment.

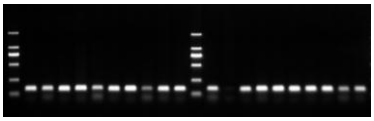

Figure 2. GP5+/GP6+ PCR and very clear differentiate seized DNA fragment of 150bp

C. Type specific PCR

All samples were amplified with the HPV16 and 18 specified primer sets of JHPV16E6F/JHPV16E6Rfor

HPV 16 and X18E6F/X18E6R for HPV 18 with the sequences mentioned in Table I. These two PCR processes yielded intense band of the correct size of 350bp for HPV 16 and 350bp for HPV 18 (Fig. 3-Fig. 4). Of all 170samples tested, 69 (40.58%) were positive for HPV16 and 84 (49.41%) for HPV 18, as seen by the presence of corresponding PCR fragment (Table II).

TABLE I. PRIMERS USED IN PCR AMPLIFICATION OF HUMAN PAPILLOMAVIRUS DNA SHOWING THE CORRESPONDING ANNEALING TEMPERATURE AND PCR PRODUCT SIZES

Primers	Sequences	Annealing temperature	PCR product size
PC04	5'-CAACTTCATCCACGTTCACC-3'	62 ℃	150bp
GH20	5'-GAAGAGCCAAGGACAGGTAC-3'		
GP5+	5'-TTTGTTACTGTGGTAGATACTAC-3	55 ℃	150bp
GP6+	3'CTTATACTAAATGTCAAATAAAAAG-5		
16E6 F	5'-CAACAAGACATACATCGACC-3'	60 ℃	350bp
16E6 R	5'-CAACAAGACATACATCGACC-3'		
18E6 F	5'-CACTTCACTGCAAGACATAGA-3'	55 ℃	350bp
18E6 R	5'-GTTGTGAAATCGTCGTTTTTCA-3'		

TABLE II. PCR HPV DETECTION FREQUENCY ACCORD-ING TO DIFFERENT PRIMERS

Set of primers	Total	Positive cases	% of cases
PC04/GH20	170	165	>95%
GP5+/GP6+	170	138	81.17%
HPV 16	170	69	40.58%
HPV 18	170	84	49.41%
GP+&HPV16/18-*	170	11	6.4%
HPV 16&18	170	45	26.47%
HPV16or18	170	105	61.76%
GP&HPV16&18	170	38	22.35%
GP+/HPV16+/ HPV18+**	170	157	92.3%

*GP5+/GP6+ positive but HPV16/18 negative

**GP5+/GP6+ positive or HPV16 positive or HPV18 positive

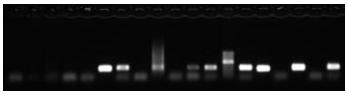

Figure 3. HPV16 PCR and 350bp DNA fragment in the result

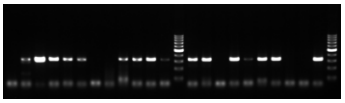

Figure 4. HPV18 PCR and 350bp DNA fragment in the result

D. Sequencing PCR Products

To identify the HPV types, all HPV-positive PCR products were subjected to DNA sequence analysis with DNA man and compared with the known HPV sequences in the DNA database using BLAST (basic local alignment search). The HPV18 was the most common subtype (49.41%) whereas the HPV16 occurred at lower frequency (40.58%) (Table II). DNA analysing software results confirmed the presence of HPV DNA and a few mutations in virus DNA (Fig. 5-Fig.6).

Figure 5. Sequencing result HPV16E6 in three samples with some mutation

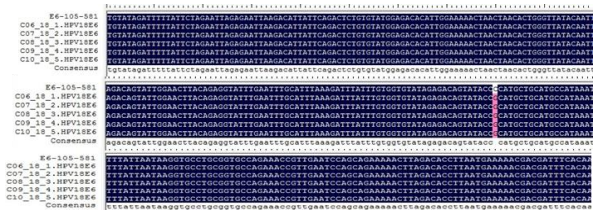

Figure 6. Sequencing results of HPN18E6 in three samples with one mutation

IV. DISCUSSION

Oesophageal squamous cell carcinoma is one of the most common causes of cancer death world- wide. In western countries, where the risk of ESCC is generally low, tobacco usage and consumption of alcohol could explain great majority of the causes of ESCC. However in ESCC high incidence regions like China and in some regions such as north of Iran, which have a high rate of ESCC, only a small proportion of ESCC cases could be attributed to smoking or alcohol consumption. So, other risk factors must be responsible for the high incidence of ESCC in such as these areas (for example Iran and China). The main suspected risk factors include low intake of fruits and vegetables, drinking hot tea, consumption of opium products and tobacco, Helicobacter pylori infection of the stomach, drinking contaminated water source and genetic susceptibility [19]. The main suspected mutagens are polycyclic aromatic hydrocarbons and N-nitroso compound [20]. SinceSyrjanen studies in the 1982, several studies have been conducted in different countries and in different geographical regions of the same country, to identify HPV DNA in ESCC samples. Several studies during the past two decades have shown the presence of HPV in DNA isolated from patients with oesophageal cancer [21]-[25]. Studies have generated conflicting and often contradictory data, which may be attributed to the geographical location, with respect to either low or high incidence areas [21]-[25]. In addition, variations in the prevalence rates of HPV from the same geographical areas have also been reported. For example in Iran, a high incidence rate in north of Iranaround 36.8% HPV infection in ESCC was reported by Farhadi and colleges

[26] but another study in that country in other city (Shiraz) on 92 cases of ESCC showed no HPV infection. For another example we can mention China. The presence of HPV DNA has been confirmed in patients with oesophageal cancer in Shan dong, China [27]. Whereas another study carried out in the same area [28], found no evidence of HPV DNA. Similarly, prevalence rates of 50% and 23% have been reported in patients with oesophageal cancer from Australia; in both instances the same technique was used [29], [30]. By using molecular methods, the majority of these studies have shown the presence of high- risk HPV in a variable Proportion of cases[31]-[34]; however, have failed to demonstrate HPV etiologic EC, even from highly prevalent regions [35], [36]. Differences in such studies could be attributed to the sensitivity and specificity of molecular methods employed to detect HPV DNA. It is widely accepted that PCR is the most sensitive method for detecting HPV DNA and can detect as few as 20 copies or less [37]. In this study we tested 198 cases with a pathological diagnosis of oesophageal squamous cell carcinoma. In this evaluation we found HPV DNA with high prevalence around totally 62% of total cases, include 40.58% and 49.41% for HPV16 and 18 respectively. This frequency in compare to some previous studies in other regions of this country (China) is clearly high. For example one study, was done in Shantou region of China showed a relatively high prevalence of HPV DNA in formalin fixed paraffin embedded tissues as 77.2%, 62.2% and 11.1% for the screening of HPV L1, HPV 16 and HPV18 respectively [38]. On the other hand another study in the other regions of China, Shandong and Gansu, indicated not so similar results. In that study, evaluation methods were almost the same, PCR, and samples were also the same type (paraffin embedded cancerous tissues) but the results weren't as high as the evaluation was done in the other region, Shantou. HPV prevalence in this study was 19% HPV positive samples in compare to prevalence of 77.2% detection of HPV DNA in previous evaluation in the other region (Shantou) [39], [40]. With regard to studies previously have done in high incidence areas all around the world such as in China or the other high incidence regions in other countries, HPVDNA was detected in almost samples and these results can implicate HPV virus as a suspected causative agent beside of the other etiologies, environmentalor genetic. About the controversial results in different countries or sometime in different regions of the same country, there are several suspected reasons that we can mention the sample preparation (includes all process from cutting suspected tissue by surgeon during surgery to DNA extraction process and DNA analysing for virus genome), evaluation methods sensitivity (any item related to analysing HPV genome, for example instruments sensitivity and using high quality materials during DNA analysing in PCR procedure) and regional diet habits or other special costumes. In conclusion, a potential role of HPV in the development of oesophageal squamous carcinoma has emerged as a result of the HPV-like histological changes in mucosa of patients with oesophageal cancer and the presence of HPV antigens and HPV DNA in cancerous tissues. Although various types of HPV were been detected by PCR amplification or other methods, clear evidence of etiologic significance of HPV in oesophageal squamous cell cancer is still lacking. With regard to this fact that in Tangshan region of Chinahasn't done any study about relation between HPV and oesophagus cancer, we hope this study leads to do other efforts to confirm etiologic significance of HPV in oesophagus squamous cell carcinoma.

V. Command

Since 1982, for first time HPV virus was introduced as one of the possible agents in esophageal carcinoma, until now many studies have done to prove this relation ; but results were controversial. In this study we focused on China as one of high incidence areas of esophagealcarcinoma. In China we can also find conflicting results from different regions in the range of 0% to 71%. One possible reason for these differences is using different evaluation methods. In this study the most sensitive and common method (PCR) was used to evaluate cancerous tissues. One problem in this evaluation was finding negative control, normal oesophagus tissue is too difficult to obtain. This study can give others new idea to use another methods or other studies in other regions to clarify etiologic significance of HPV in esophagus cancer.

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G. HRV Drowsiness Indicators

A linear regression model was applied using the normalized HRV data HF and LF/HF ratio, which reached a goodness of fit R^2=0.04 (see Table III). Both covariates for the linear regression model were significant. However, the number of observations dropped by a factor of 6 in comparison with the analysis of alpha bursts and eye parameter due to many invalid data. In addition, the linear mixed models for HF as well as LF/HF ratio, in relation to TOR, were not significant (see Table II). Based on these findings, HRV data were not included in the overall regression model, described in the next section.

TABLE III. COMBINATION OF PARAMETERS AND THEIR STRENGTH OF ASSOCIATION TO TOR AS WELL AS SIGNIFICANCE BASED ON LINEAR REGRESSION MODEL

Drowsiness Predictor	Reg. Coefficient	t	p	R^2
EEG Alpha Band Normalized				
MPSR C4	1.179 [0.928; 1.430]	9.21	<0.001	0.21
MPSR O2	2.034 [1.824; 2.244]	18.97	<0.001	
Alpha Bursts Normalized (minimum four waves)				
Duration C4	0.102 [0.052; 0.151]	3.09	<0.001	0.30
Duration O2	0.304 [0.229; 0.379]	7.70	<0.001	
Mean amplitude C4	-0.017 [-.022; -0.011]	-5.36	<0.001	
Mean amplitude O2	-0.016 [-0.022; -0.010]	-5.48	<0.001	
Relative amplitude C4	0.649 [0.521; 0.778]	9.77	<0.001	
Relative amplitude O2	0.587 [0.477; 0.696]	10.38	<0.001	
Spectral EEG Analysis and Alpha Bursts combined				
Parameters EEG alpha band and alpha bursts (as above)				0.30
Eye Parameter (algorithm based)				
Average duration blink<0.5s	4.398 [4.030; 4.766]	23.42	<0.001	0.48
Total duration blink/eye movement ≥0.5s	0.056 [0.047; 0.064]	13.17	<0.001	
ECD blink/eye movement	1.946 [1.572; 2.321]	10.19	<0.001	
AECS blink/eye movement	-73.20 [-95.82; -50.61]	-6.37	<0.001	
Amplitude blink/eye movement normalized	-3727 [-4103; -3351]	-19.34	<0.001	
HRV Data Normalized				
HF	-5e-5 [-7e-5; -3e-5]	-4.72	<0.001	0.04
LF/HF ratio	-0.054 [-0.079; -0.031]	-4.53	<0.001	
Eye Parameter (manual scoring)				
Average eye blink/movement duration	0.851 [0.809; 0.892]	40.55	<0.001	0.27
Log of average eye blink/movement duration	0.573 [0.555; 0.592	65.54	<0.001	0.46

H. Alpha Burst and Eye Parameter combined

A linear regression model based on alpha bursts and eye parameter characteristics was created using only significant covariates. Table IV illustrates the strength of associations between the drowsiness predictors and TOR as well as SDLP and provides an overview of the respective p-values from the linear regression model and the linear mixed model. The combination of eye and alpha burst parameters resulted in an increase of R^2 to 0.54. R^2 of eye and alpha burst parameter with SDLP (0.15) approached that of TOR (0.17).

TABLE IV. ALPHA BURSTS NORMALIZED (MINIMUM FOUR WAVES) & EYE PARAMETERS (ALGORITHM BASED)

Drowsiness Predictor	TOR				SDLP			
	LRM	LMM			LRM	LMM		
Alpha Burst and Eye Parameter combined	R^2= 0.54				R^2= 0.15			
	t	z	p>|t|	p>|z|	t	z	p>|t|	p>|z|
Duration C4	3.24	2.93	<0.001	<0.001	3.89	0.05	< 0.001	0.960
Duration O2	4.06	5.07	<0.001	<0.001	4.16	5.63	< 0.001	< 0.001
Relative amplitude C4	5.02	7.15	<0.001	<0.001	-0.43	1.37	0.668	0.170
Relative amplitude O2	7.55	7.05	<0.001	<0.001	-3.27	-2.13	< 0.001	0.033
Average duration blink<0.5s	19.9	11	<0.001	<0.001	6.30	0.59	< 0.001	0.553
Total duration blink/eye movement ≥0.5s	7.00	8.04	<0.001	<0.001	6.36	4.23	< 0.001	< 0.001
ECD blink/eye movement	11.24	6.73	<0.001	<0.001	3.03	3.46	< 0.001	0.001
AECS blink/eye movement	-6.9	-8.75	<0.001	<0.001	-5.14	-1.24	< 0.001	0.216
Amplitude blink/eye movement normalized	-14.43	-2.43	<0.001	<0.001	-2.71	-2.48	< 0.001	0.013
Error SD estimates		constant=0.265 residual=0.367				constant=0.098 residual=0.162		

All p-values in the linear regression model as well as the linear mixed model are < 0.05; hence there is a significant relationship between TOR and drowsiness predictors. However, a significant association can also be seen between the covariates and SDLP in the linear regression model but not in the linear mixed model. When LMM was used, the standard deviation (SD) of constant subject error was markedly smaller than the SD of the residual error, confirming appropriateness of using R^2 from LRM for comparing contributions of different covariates.