e-ISSN:2320-3528
p-ISSN:2347-2286

Research & Reviews: Journal of Microbiology and Biotechnology

Prevalence of HPV Proteins in Esophageal Squamous Cell Carcinoma from Tang Shan of China: An Immunohistochemistry Study

Mohammadreza Mohammadzad Mehryar[1,2], Fan Li[1], Jintao Li[1,2*], Shu Ying Li[4], Hongwei Liu[3], Fang Zhang[1], Yubai Zhou[1] and Yi Zeng[1,2*]

[1]Beijing Key Laboratory of Environmental and Viral Oncology, College of Life Science and Bio-Engineering, Beijing University of Technology, Beijing100124, China

[2]National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, and State Key Laboratory for Infectious Disease Prevention and Control, Beijing100052, China

[3]Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education) College of Life Sciences, China West Normal University, Nanchong, China

[4]Department of Pathogenic Biology, College of Basic Medicine, Hebei United University, Tangshan, Hebei province.

Research Article

Received date: 08/04/2015
Accepted date: 22/05/2015
Published date: 26/05/2015

***For Correspondence**

Jintao Li, Yi Zeng, Beijing Key Laboratory of Environmental and Viral Oncology, College of Life Science and Bio-Engineering, Beijing University of Technology, Beijing, China, Tel: 0086-13717736576

Email: ljt2000593@163.com; zengyicdc@sina.com; m.mehryar@hotmail.com

Keywords: Immunohistochemistry, Esophageal squamous-cell carcinoma, Esophageal carcinoma, Human papillomavirus, Formalin fixed paraffin-embedded tissue

ABSTRACT

The presence of HPV DNA in cases of esophageal squamous-cell carcinoma has been reported repeatedly from Tangshan area of China and other regions with a high incidence of esophageal carcinoma. In current study, to investigate the presence of human papillomavirus in esophageal squamous cell carcinoma, 112 formalin fixed paraffin-embedded tissue samples of ESCC from Tangshan were evaluated for HPV16/18E6, HPV16/18E7, P16 and P21 proteins by Immunohistochemistry method. 112 samples divided to two HPV PCR positive and negative groups of 83 and 29 cases respectively. From 83 slides of PCR positive group 73(87.95%), 48(57.83%), 75(90.36%) and 72(86.74%) of slides had positive reaction for HPV16/18 E6, HPV16/18E7, P21 and P16 respectively. From 29 PCR negative cases 14(48.27%) and 15(51.72%) showed positive staining reaction for P16 and P21 respectively. Results showed a good direct relation between expression of two common cancerous proteins, P16 and P21, and HPV specific proteins by higher positive slides of 72/83 (86.74%) for P16 and 75/83 (90.36%) for P21 in group of positive HPV PCR, in compare with the lower positive result in negative HPV PCR group of 15/29 (51.72%) and 14/29 (48.27%) for P16 and P21 respectively. In this study, P16 and P21 were implicated as two good markers with high expression in ESCC cancerous tissue with HPV infection probability. Findings in this study raise the possibility that HPV is involved in esophageal carcinogenesis. Further investigation with a large sample size over different areas may be warranted.

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) occurs worldwide and has a variable geographic distribution [1]. China considered as a high-risk area of esophageal carcinoma. Tangshan area of China like other areas of China (Linxian, Anyang, Shantou) has a high incidence of esophageal carcinoma (EC) [2]. Esophageal cancer is one of common types of cancer and 6th cause of death from cancer [3].

Human papillomavirus (HPV) is a non-enveloped, double-stranded DNA virus with more than 200 genotypes. Molecular and epidemiological studies confirmed the relation between cervical cancer and high risk HPV virus and it has recently also been considered as a risk factor for non-cervical carcinoma [4].

The relation between Human papillomavirus and EC has been reported in the recent decades, especially in the areas with a high prevalence of EC. An association between HPV and esophageal squamous cell carcinoma was initially suggested in 1982 when the esophageal squamous cell carcinoma in some cases showed the similar phenomenon of lesions caused by HPV in the genital tract [5]. However, because of different infection rates of HPV, varying from 0% to 68%, the role of HPV in non-cervical carcinoma is still controversial, compare with cervical carcinoma that its association with high risk HPV almost confirmed [6]. The usage of different methods to evaluate infection rates is one of possible reasons for this variation. Polymerase chain reaction (PCR), amplification targeting different fragments of the virus DNA, southern blot, *in situ* hybridization and immunohistochemistry are the common used methods to investigate presence of virus. Previous studies have shown large variations in the prevalence of HPV among patients with ESCC depending on geographic area, ethnic group and method used for viral detection [7]. Even, studies by the usage of PCR, same methodology, reported different HPV DNA prevalence in ESCC samples [7]. Also, there is a wide range of HPV prevalence reports in esophageal tumors in the same country [8]. A few probable reasons for different results between studies include small study sizes, different detection methods, inter-laboratory variability, incorrect sample collection that leads to trans-contamination [8-10]. Contamination of specimens during collection, processing and evaluation can cause false positive results [10,11]. Prevention of contamination is considered as the most important part of studies on HPV because this virus is a common human infection that can involve in a variety of epithelial tissues and DNA is resistant to detergent used for cleaning [12-14].

Among the HPV types have been reported in human ESCC samples, HPV-16 is the most common that followed by other high-risk HPV types such as HPV-18, HPV-31 and HPV-35 [15,16].

There are a few methods to identify HPV infection in suspicious tissue samples, detection of DNA or proteins expressed by cells infected by HPV virus. The most common method used for this purpose is PCR but in this method, as mentioned before, non-contaminated procedure is very important. Because of this fact, using just one method to confirm of HPV virus infection is not enough. Due to less false positive results, the second best method to detect HPV virus infection is immunohistochemistry method, detect HPV virus related proteins expressed by infected cells.

In this study we evaluated 112 paraffin embedded tissues from Tang Shan area of china as high incidence area of esophagus carcinoma. Samples divided in two groups: one group consist of 83 samples that had positive PCR result for HPV DNA and the other group, 29 samples, didn't have any result in PCR evaluation. Our purpose of this study are firstly investigating samples for proteins expressed by cells have shown the presence of HPV virus, already confirmed by PCR test, to confirm results of PCR, and secondly finding a relation between HPV infection and some famous cancerous proteins expressed by infected cells, in this study P16, P21.

MATERIALS AND METHODS

In this study 112 pathologically diagnosed ESCC paraffin embedded tissue samples were obtained from files of pathology department at Tang Shan 2008-2013. In another study, all samples had tested for HPV DNA and according to that evaluation, samples divided to two groups of HPV PCR positive of 83 samples and HPV PCR negative of 29 samples. In this study samples were analyzed for proteins expression, three HPV gene specialized proteins, HPV 16 and 18, for HPV PCR positive group and two proteins, P16 and P21, for HPV PCR negative group. Specimens were collected and tissue slides prepared by using a standard protocol to minimize the probable tissue trans-contamination by other tissue blocks. For this purpose, we used one microtome blade for each block and at the end of tissue slide preparing for each block microtome and water bath were cleaned carefully. Tissue sections (4 µm) were mounted onto slides and stained with specific monoclonal antibodies specified to desired proteins, HPV 16 and 18 E6, HPV 16 and 18 E7, P16 and P21, according to SABC method. In this study we used monoclonal antibodies for HPV 16 and 18 E6 and HPV 16 and 18 E7 from Santa Cruz. Biotechnology, lot.10606 and D2709 and antibodies pre diluted in 1:200. For P16 and P21 antibodies were from ZSGB-Bio, lot ZM-0205, with the dilution of 1:60.

In brief, slides were baked at 60º for 30min and deparaffinised in xylene for two 15 min periods of time, and endogenous peroxidase was inactivated by immersing the slides in a 0.3% H_2O_2 solution for 10 min at room temperature. Antigen retrieval was done in retrieving buffer (CB) at 90º for 15min. Serum blocking solution (BSA) was added to each tissue section (100µl) and then anti-HPV monoclonal antibody to protein of interest, HPV 16 and 18 E6, HPV 16 and 18 E7, P16 and P21, was added to each section and tissues incubated for one night. After rinsing slides, secondary antibody and SABC solution were added according to the protocol recommended by the supplier (Boster Biological Technology Ltd, lot. SA1021) and DAB solution was used to stain slides (ZSGB-BIO Technology Ltd, lot.ZLI-9017). Finally for visualization of nuclei, all slides were stained by haematoxylin stain and slides were mounted after final dehydration.

In this study monoclonal antibodies to HPV specific proteins include HPV-16 and 18 E6, HPV-16 and 18 E7 and common cancerous proteins, P16 and P21, were chosen to test cancerous tissues [17]. Antibodies used to detect HPV-18 and 16 were mouse monoclonal antibodies and P16 and P21 specific antibodies were rabbit monoclonal antibody.

To determine whether the expression of these proteins might have an influence on esophageal malignant progression and to find possible relationship between special clinical characteristic, all parameters (Table 1) and IHC results (Table 2) were analyzed

by sex, age, type of cancer and grade by simple correlation analyze method. The level of statistical significance was set at 0.05 (2-sides) and all analyses involved usage of SPSS v16.0 (SPSS Inc., Chicago, IL).

Table 1: Brief information of cases in different group.

Parameter	Group 1 HPV PCR positive	% of group 1	Group 2 HPV PCR negative	% of group 2
Male	75/83	90.36%	22/29	75.86%
Female	8/83	9.63%	7/29	24.13%
Age <60	45/83	54.21%	14/29	48.27%
Age >60	38/83	45.78%	15/29	51.72%
ESCC*	53/83	63.85%	10/29	34.48%
Adenocarcinoma	30/83	36.14%	19/29	65.51%
Grade 1	20/83	24.09%	8/29	27.58%
Grade 2	41/83	49.39%	17/29	58.62%
Grade 3	22/83	26.50%	4/29	13.79%

Table 2: IHC HPV detection frequency with different antibody in different groups.

Monoclonal antibody	Group 1 HPV PCR positive	% of group 1	Group 2 HPV PCR negative	% of group 2
HPV-18/16 E6	73/83	87.95%	-	-
HPV-18/16 E7	48/83	57.83%	-	-
P 21	75/83	90.36%	14/29	48.27%
P 16	72/83	86.74%	15/29	51.72%
HPV-18/16 E6 and E7	46/83	55.42%	-	-
HPV-18/16 E6 or E7	75/83	90.36%	-	-
P21 and P16	70/83	84.33%	8/29	27.58%
P21 or P16	78/83	93.97	20/29	68.96%
P 21+P16+HPV- 18/16 E6/E7*	42/83	50.60%	-	-
P 21+P16 or HPV-18/16 E6/E7**	78/83	93.97%	-	-
***HPV-18/16 E6/E7 positive and P21/16 positive **P 21/16 positive or HPV-18/16 E6/E7 positive**				

RESULTS

The current study includes 112 cases with esophagus carcinoma. Histologically the specimens were classified in two groups of esophageal Squamous cell carcinoma (ESCC), 56.25% (63/112), and adenocarcinoma, 43.75% (49/112). Brief information of cases includes age, sex, grade and type of cancer are shown in table 1 for two different groups separately. According to these data we have clear differences in sex and cancer type prevalence in two HPV PCR positive and negative groups. In HPV PCR positive and negative groups we can see the majority of male but in cancer type, situation is different in HPV PCR positive and negative group, with high majority of SCC, 63.85% (53/83), in PCR positive group in contrast to high majority of adenocarcinoma, 65.51% (19/29), in PCR negative group. Our data also showed the majority of cancer grade 2 in both groups.

After IHC evaluation, our data showed higher degree HPV-16/18 E6 gene expression of 87.95% (73/83) in HPV PCR positive samples but there were no big differences between P16 and P21 expression among groups. All positive samples for any kind of monoclonal antibody were shown two kinds of cytoplasmic and nuclear staining reaction. And with regard to microscopy analyze, different types of cell included basal, pre-basal and inner cells were shown to be involved in positive reactions. Results of each antibody are briefly shown in table 2 for different group of samples.

After analyzing of IHC results and clinical information of cases by SPSS statistic analyze software, simple correlation analyze method, we found no meaningful relation between prevalence of any kind of proteins and clinical characteristics include sex, age, cancer type and grade.

HPV-18/16 E6

From 83 slides of group one, 73 (87.95%) slides with positive staining reaction of two types of nuclear and cytoplasmic staining reaction, were found in different slides but in different frequency (Figure 1). This result in compare with HPV DNA evaluation of these genes fragment that has done in another study was almost the same [18].

HPV-18/16 E7

Among 83 slides of group one, the positive result of this antigen, HPV-18/16 E7, was 48(57.83%) that there was lower than HPV-16/18 E6 prevalence with positive results of 73/83(87.95%). Like HPV-16/18 E6 positive slides, two types of nuclear and cytoplasmic staining reaction were found in positive slides but not as strong as staining reaction in positive slides of HPV-16/18 E6 (Figure 2).

The positive slides for these antigens were 72/83(86.74%) for P16 and 75/83(90.36%) for P21 in group one, 15/29(51.7%) and 14/29(48.2%) for P16 and P21in group 2 respectively. Like other antigens, two types of staining reaction were observed in

e-ISSN:2320-3528
p-ISSN:2347-2286

positive samples (Figure 3).

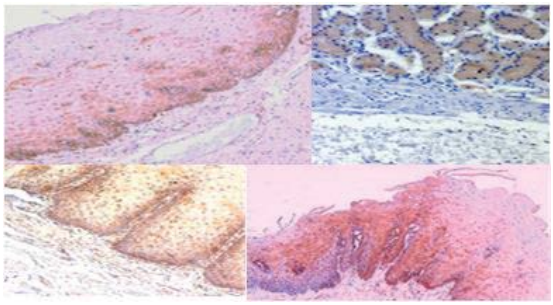

Figure 1: Immunohistochemistry results targeting HPV-16/18 E6 in tumor samples. The positive expression of HPV, indicated by brown immunohistochemistry signals, located mainly in the cytoplasm of cancer cells in different grades.

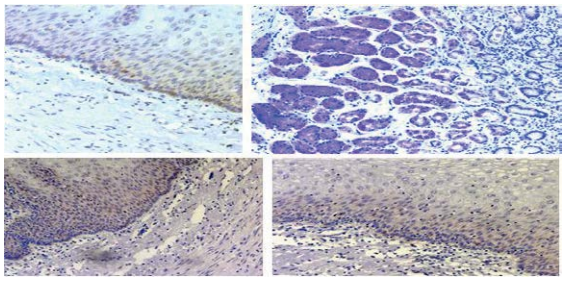

Figure 2: Immunohistochemistry results targeting HPV-16/18 E7 in tumor samples. The positive samples, indicated by immunohistochemistry signals,located mainly in different parts of the tissues.P16 and P21.

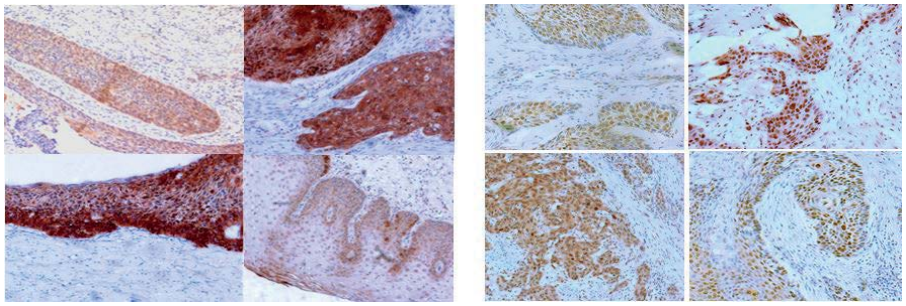

Figure 3: (left and right) Immunohistochemistry results targeting p16 and p21 antigens respectively in tumor samples. The positive expression of HPV, indicated by brown immunohistochemistry signals, located mainly in the cytoplasm of cancer cells in different anatomical parts of cancerous tissues.

DISSCUSION

Over the past twenty years many studies have done on the HPV involvement in esophageal cancer but the results have been shown the HPV infection rates from 0 to 67% [19-22]. Even there are different infection rates in studies on samples from different areas of the same country [23,24].

Sampling method, demographic and ethnic factors, disease status and sensitivity of different detection methods have been considered as main causes of this variety.

The association between HPV infection and age, gender, tumor TNM stage, tobacco smoking and alcohol consumption has been analyzed. Compared with HPV negative subjects, just alcohol consumption can be considered as a risk factor for esophageal cancer. However there are no statistical evidences of such relationship [25].

HPV has been detected infrequently in esophageal carcinomas collected from western countries, whereas there are more reports of HPV involvement in esophageal cancers from regions of high HPV incidence, particularly in China and South Africa and part of Middle East countries such as Iran. It therefore has been proposed that HPV infection may play a role in esophageal carcinogenesis only in high incidence regions [26,27].

As mentioned, in many studies there are variable results of HPV detection rate in different areas of the same high incidence region. For example in China as a high incidence region of interest in current study, many studies have done on HPV prevalence in esophagus cancer samples but reports have shown different results from different parts of this high incidence region as listed below: Gansu, Shandong, Anyang, Shantou, Xinjiang, Lin xian and Tangshan with prevalence of 65%, 16%, 51.8%, 60.2%, 53.8%, 1.1%, 50% respectively [28,29]. In another study in Anyang as a region with high prevalence of ESCC (esophageal squamous cell carcinoma), HPV-16 infection rate was much higher in the high incident village (72%) than that of the low incident village (37%)

[17]. However, the involvement of HPV remains controversial, since different studies have demonstrated wide range of infection rates. There are some reasons that maybe cause these differences that one of the most important factors is the use of different methods to measure infection rates. For evaluation of HPV in ESCC tissues polymerase chain reaction (PCR), amplification targeting different fragments of the virus DNA, Southern Blot, *in situ* Hybridization and Immunohistochemistry have all been used to develop the data (17).

PCR method is considered as the most popular and sensitive method to evaluation of HPV in tissues. But in paraffin embedded tissue, preparing tissue sections and extract DNA maybe be lead to contamination or missing part of DNA and sometime quality of DNA extraction maybe causes DNA destruction and consequently the result of PCR maybe will not represent the real situation of virus DNA in the tissue samples.

In current study to evaluate protein expression in cancerous tissues and using another method to investigate HPV virus infection, we used immunohistochemistry (IHC) method.

Tang Shan is considered as one of high incidence area of ESCC in china but there are no large scale studies about ESCC in this region. In current study we got 112 samples of this area that have been tested for HPV DNA by polymerase chain reaction. Samples were divided to two groups of DNA positive and DNA negative. In this study expression of HPV specialized proteins for DNA positive group and two other proteins, P16 and P21, for both groups were checked.

Our data confirmed the presence of HPV virus in the DNA positive group, by high positive result of 73/83 (87.95%) for HPV specific protein expression. High positive result of IHC evaluation also can be considered as good confirmation for the accuracy of HPV PCR procedure, in this case previous study [18]. In this study also we had high expression of HPV-16 and 18 E6 of 87.95% (73/83) in compare with HPV-16 and 18 E7, 57.83% (48/83). There are two probable reasons for this situation; the first is E7 gene late expression in compare with E6 gene and secondly, maybe the time wasn't enough for E7 gene to express, most of cases showed grade 2 of cancer in their clinical characteristics, so, in these cases HPV DNA will be positive in contrast to IHC. And on the other hand expression of E7 gene normally is lower than E6.

Analyzing of result showed a good direct relation between expression of two common cancerous protein, P16 and P21, and HPV specific proteins by higher positive slides of 72/83 (86.74%) for P16 and 75/83 (90.36%) for P21 in group one with positive HPV PCR, in compare with the lower positive result of the second group with negative HPV PCR, 15/29 (51.72%) and 14/29 (48.27%) for P16 and P21 respectively. After analyzing results to find any relationship between clinical characteristics of patients and protein expression, our data didn't show any special relation between clinical characteristics, table 1, and expression of any of four proteins, HPV16/18 E6, HPV16/18 E7, P16 and P21.

We recognized that there is a limitation of cancerous tissue quality in this study. Because these cancerous tissues recruited from different places of Tangshan area and some of them have been stored for many years and probably the quality of preparing FFPE were not same at different centers. This factor may limit the sample's representativeness for inference.

In conclusion, this study firstly was evaluation of samples with using another method to check result differences between two methods and secondary, compare the result of IHC method with that of PCR results to check and confirm the quality of results of the pervious study[18].On the other hand in this study, P16 and P21 were introduced as two good markers with high expression in ESCC cancerous tissue with probable HPV infection.

ACKNOWLEDGEMENT

Work partially supported by a grant from National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, and State Key Laboratory for Infectious Disease Prevention and Control.

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