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The relationship between HPV16 and expression of CD44v6, nm23H1 in esophageal squamous cell carcinoma

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Summary. The esophageal squamous cell carcinoma (ESCC) has high incidence in Shaanxi Province of China. More and more researches indicated that human papillomavirus type 16 (HPV16) might play an important role in carcinogenesis of ESCC but the relationship between HPV16 and CD44v6, nm23H1 has not been elucidated. HPV16 was detected by amplifying HPV16 E6 gene through polymerase chain reaction (PCR) method and the expression of CD44v6, nm23H1 in 40 ESCCs and fifteen normal esophageal mucosa (NEM) from Shaanxi Province was examined by Streptavidin-Peroxidase (SP) method using monoclonal antibody specific to CD44v6 and nm23H1. The positive rates of HPV16 E6 gene, CD44v6 and nm23H1 were 60% (24/40), 65% (26/40) and 45% (18/40) respectively in ESCCs and 26.67% (4/15), 33.33% (5/15) and 86.67% (13/15) respectively in NEMs. There exited statistical difference for HPV16, CD44v6 and nm23H1 between NEMs and ESCCs respectively ($p < 0.05$). The relationship between HPV16 and the expression of CD44v6 in ESCCs was statistical significance ($P = 0.021$), but no significant correlation was found between HPV16 and the expression of nm23H1 ($P = 0.436$) in ESCCs. The infection rate of HPV16 had no statistical difference in all pathological features we observed, but the expression rates of CD44v6 and nm23H1 had statistical correlation with invasion ($p = 0.001, 0.013$) and lymph nodes metastasis ($p = 0.014, 0.002$) respectively. In different histology grade of ESSCs, the relationship between HPV16 and CD44v6 was statistical significance in grade I ($p = 0.044$) but was not in grade II ($p = 0.165$) and grade III ($p = 0.658$), however as to the expression of nm23H1 there exited no statistical significance in all histology grades of

ESCC ($p > 0.05$). The expression rates of CD44v6 and nm23H1 were statistically different between grade I and II ($p = 0.004$, 0.016) respectively and between grade I and grade III ($p = 0.014$, 0.020), but not statistically different between grade II and III ($p = 0.792$, 0.943) respectively. Our data firstly suggested that there existed the statistical relationship between the infection of HPV16 and the expression of CD44v6 in ESCCs and that HPV16 may be involved in invasion and metastasis of ESCC.

Introduction

The esophageal squamous cell carcinoma (ESCC) is one of the upper-digestive tract neoplasms and has high incidence, high mortality and poor prognosis in China. There exited high incidence for ESCC mainly aggregated in North China such as Shaanxi Province, as well as Henan and Shanxi Provinces. The confirmed causes and the mechanism of ESCC have not been elucidated yet. The variation of geographical distribution suggests a dominant role of environmental factors in the etiology of ESCC such as nitrate salt, nitrite salt and second-degree amine in drinking water, which can cause transformation of epithelium cells. In addition, other risk factors such as nutrition imbalance (lack or absence of vitamins and minerals such as vitamin A, B2 and C) and improper life style (cigarette smoking and consumption of pickled food) give rise to the damage, proliferation and inflammation of esophageal mucosa cells. These may result in weakness of esophageal mucosa cells against microbiology. Moreover the factor of genetic susceptibility to microbiological infection may not be neglected [4, 33].

Human papillomavirus (HPV), especially high risk HPV16 is regarded as one kind of important tumor-related virus, which was firmly recognized in cervical cancer. HPV16 E6 and E7 can interfere normal cell cycles by degrading tumor suppressor protein P53 and Rb respectively in cervical carcinoma; moreover the virus causes instability of host genome through integration of viral DNA into host genome randomly and increases centrosome number in cervical carcinoma [32, 35]. The role of HPV16 in ESCC becomes a research hotspot on ESCC with more and more reports about the relationship between HPV16 and ESCC [8, 17, 19, 26, 37–39]. But in past two years most reports suggested that HPV16 was associated with carcinogenesis of ESCC especially in some areas of china although there were some reports that no relationship was found between HPV and ESCC in other countries and regions such as Germany and so on [1, 11, 14, 30, 31]. According to the precious study results through different methods, types of HPV in ESCC were mainly some high risk HPV such as HPV16, HPV18, but HPV16 was the most common HPV type which was implicated in ESCC [4, 33, 39]. The more important is that there were some reports that about HPV18 E6 and E7 gene can immortalize fetal esophageal epithelial cell in ESCC in terms of DNA level, mRNA level and cellular level, which supported that high risk HPV might have an important role in ESCC [25, 26, 39]. Considering the poor prognosis and high mortality of ESCC in China, it is very necessary to investigate the role of HPV16 in invasion

and metastasis of ESCC, which may help us further understand the function of HPV16 in ESCC.

CD44v6 and nm23H1 were involved in the invasion and metastasis of ESCC [6, 36], but the relationship between HPV16 and CD44v6, nm23H1 has not been elucidated. In this paper HPV16 E6 gene was detected from ESCC patients in Shaanxi Province by polymerase chain reaction (PCR) method and the expression of CD44V6 and nm23H1 was examined by Streptavidin-Peroxidase (SP) method. We reported the discovery that the relationship between HPV16 and the expression of CD44v6 was statistically significant in grade I of ESCCs but was not in both grade II and grade III. In addition the relationship between HPV16 and nm23H1 expression was not statistically significant. To investigate these may help medical virologist diagnosis ESCC earlier and provide them new data about the prevention from and treatment on ESCC.

Materials and methods

Tissue samples

Forty primary tumors from ESCCs patients and fifteen normal esophageal mucosa (NEM) were obtained in first and second Hospitals of Xi'an JiaoTong University and Renmin Hospital during 1997–2000. No patient received preoperative or adjuvant chemotherapy or radiotherapy. Pathological examination of ESCCs and NEMs was strictly performed at the Department of Pathology in corresponding hospital. According to the World Health Organization (WHO) standard the patients' gender, age, histology grade, and so on were obtained from surgical and pathological records. All specimens were routinely processed for fixation with formalin and embedding with paraffin.

DNA extraction and polmerase chain reaction (PCR)

5–10 slides were deparaffinized in xylene, rehydrated in graded alcohol and digested by lysis buffer (300 mmol/l NaCl; 50 mmol/l Tris.hCl pH 8.0; 0.2% SDS) with proteinase K (200 mg/l), and then incubated at 55 °C overnight. DNA was extracted using phenol/chloroform, precipitated with cold alcohol and dissolved in ion-free water. The concentration was determined from its optical density (OD) at 260 nm. A set of specific primers (P1: 5′ CGG AAT TCA TGC ACC AAA AGA GAA CTG CA 3′, P2: 5′ CCC AAG CTT ACA GCT GGG TTT CTC TAC G 3′) were designed to amplify HPV-16 E6 .The PCR was performed as followed: initial denature at 94 °C for 5 min followed by denaturing at 94 °C for 50 sec, annealing at 55 °C for 50 sec and extending at 72 °C for 1 min with 30 cycles, finally prolonging at 72 °C for 10 min. The amplicon was about 494 bp long. The plasmid pBR322/hpv16 containing whole HPV-16 genome was used as positive control template and plasmid PUC19 as negative control template. The amplicons were detected by electrophoresis in 1% agarose compared with DL2000 DNA molecular weight marker.

Immunohistochemistry

Immunoperoxidase staining of formalin-fixed, paraffin-embedded tissue sections was performed using Streptavidin-Peroxidase (SP) method. After sections had been deparaffinized, rehydrated in descending alcohol dilution, they were heated in an 800 W microwave oven at maximum power for 5 min in 0.01 M citrate buffer (pH 6.0) for antigen retrieval and cooled to room temperature (RT). The activity of endogenous peroxidase in sections was

blocked by 0.3% hydrogen peroxide in methanol for 10 min at RT. After treated with 5% normal mouse serum in PBS for 10 min to block non-specific sites, the sections were incubated with anti-CD44v6 (MS-1093 Neomarkers 1:50 dilution) and anti-nm23H1 (ZM-0200, Beijing Zhongshan Biotechnology Co Ltd, China) monoclonal antibodies overnight at 4 °C. In subsequent steps, we used an ultrasensitive SP kit (Maixin Biological, Fuzhou, China) and DAB (diaminobenzidine) as a chromogen. The sections were then counterstained with hematoxylin and mounted with neutral balsam.

Evaluation of immunostaining

The standard as followed was used to determine immunohistochemistry positive signals degree: the staining of CD44v6 and nm23H1 was confined to the cell membrane/cytoplasm and cytoplasm respectively. Squamous cells in each microscope field were counted through selecting 10 microscope fields randomly. The ratio of cells with CD44v6 or nm23H1 positive to all ones counted less than 10% was negative (−), between 10% and 50% was positive (+), more than 50% was strong positive (++).

Statistical analysis

Statistical significance was determined by the chi-squared and Fisher's exact tests. Analysis was done by SPSS 11.5 software for windows. 95% confidence intervals were calculated to examine statistical significance.

Results

HPV16 E6 gene in ESCCs and NEMs from Shaanxi province

HPV16 E6 gene were detected in 24 of 40 ESCCs (60%) but 4 of 15 (26.67%) NEMs by PCR method (Fig. 1). The infection rate of HPV16 in ESCCs was higher than that in NEMs ($P = 0.028$) (Table 1).

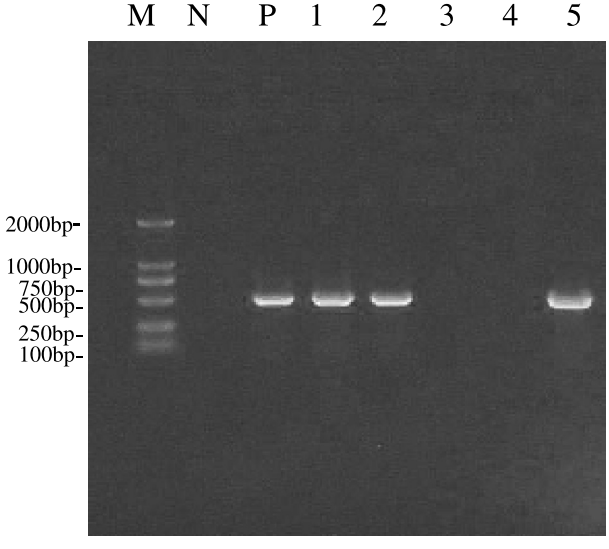
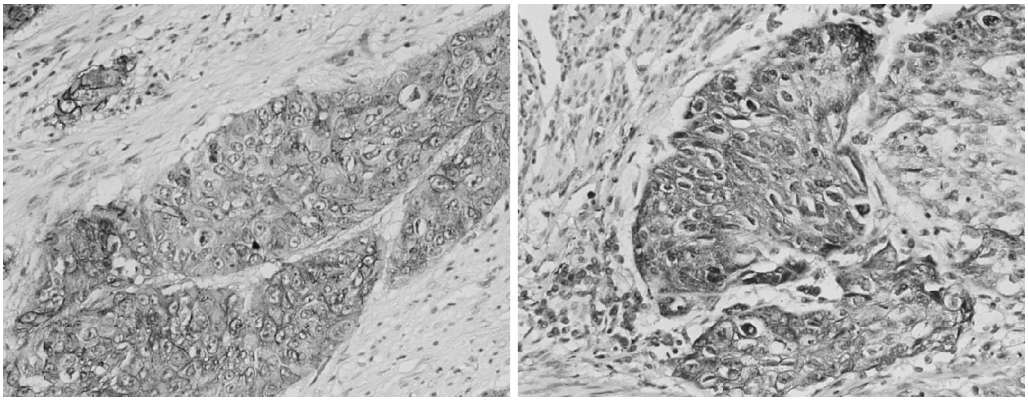


Fig. 1. PCR results of esophageal cancer samples from Shaanxi province in China. *M* DNA molecular weight standard DL2000 marker, *N* PCR of negative control, *P* PCR of positive control; *1*, *2*, *5* positive samples in ESCCs; *3*, *4* negative samples in ESCCs

Table 1. HPV16, CD44v6 and nm23H1 in normal esophageal mucosa (NEM) and ESCC

Tissue	n	HPV16		P value	CD44v6		P value	nm23H1		P value
		+ (%)	− (%)		+ (%)	− (%)		+ (%)	− (%)	
NEM	15	4 (26.7)	11 (73.3)		5 (33.3)	10 (66.7)		13 (86.7)	2 (13.3)	
ESCC	40	24 (60.0)	16 (40.0)	*0.028*	26 (65.0)	14 (35.0)	*0.035*	18 (45.0)	22 (55.0)	*0.006*
Total	55	28	27		31	24		31	24	



A **B**

Fig. 2. Example staining of CD44v6 and nm23H1 in ESCC (original magnification ×200). **A** Expression of CD44v6 in ESCC infected by HPV16, **B** expression of nm23H1 in ESCC

The expression of CD44v6 and nm23H1 in ESCCs

The expression of CD44v6 were determined in 26 of 40 ESCCs (65%) and 18 of those (45%) had nm23H1 positive staining, however the expression rates of CD44v6 and nm23H1 were 33.33 (5/15) and 86.67% (13/15) respectively in 15 NEMs (Fig. 2). The expression rate of CD44v6 in ESCCs was higher than that in NEMs ($p = 0.035$) but the expression rate of nm23H1 in ESCCs was lower than that in NEMs ($p = 0.006$) (Table 1).

The relationship between HPV16 and nm23H1, CD44V6 in ESCCs and NEMs

As far as CD44v6 expression was concerned, there existed statistical difference between ESCCs infected by HPV16 and others not infected by HPV16 ($P = 0.021$), but no statistical difference was found in respect to the expression of nm23H1 ($P = 0.436$) as Table 2 shown. In NEMs there wasn't statistical correlation between HPV16 and CD44v6, nm23H1 ($p > 0.05$) as Table 2 indicated.

Table 2. The relationship between HPV16 and nm23H1, CD44v6 in ESCCs, different grade of ESCCs and normal esophageal mucosa (NEM)

HPV16	n	CD44v6		P value	nm23H1		P value
		+ (%)	− (%)		+ (%)	− (%)	
ESCC							
+	24	19 (79.2)	5 (20.8)		12 (50.0)	12 (50.0)	
−	16	7 (43.8)	9 (56.2)	*0.021*	6 (37.5)	10 (62.5)	*0.436*
Total	40	26	14		18	22	
I grade							
+	9	5 (55.6)	4 (44.4)		6 (71.4)	3 (28.6)	
−	6	0 (0.0)	6 (100.0)	*0.044*	5 (83.3)	1 (16.7)	*0.175*
II grade							
+	8	8 (100.0)	0 (0.0)		2 (25.0)	6 (75.0)	
−	6	4 (66.7)	2 (33.3)	*0.165*	2 (33.3)	4 (66.7)	*0.773*
III grade							
+	7	6 (85.7)	1 (14.3)		2 (28.6)	5 (71.4)	
−	4	3 (75.0)	1 (25.0)	*0.658*	1 (25)	3 (75.0)	*0.898*
Total	40	26	14		18	22	
NEM							
+	4	1 (25.0)	3 (75.0)		4 (100.0)	0 (0.0)	
−	11	4 (36.4)	7 (63.6)	*0.436*	9 (81.8)	2 (18.2)	*0.100*
Total	15	5	10		13	2	

The relationship between HPV16 and nm23H1, CD44V6 in different grade of ESCCs

The expression rates of nm23H1 had no significant difference in all grades of ESCCs between ESCCs infected by HPV16 and others not infected by HPV16 ($p = 0.175, 0.775, 0.898$). In ESCCs with grade I, as to the expression of CD44v6 statistical difference was found between ESCCs infected by HPV16 and others not infected by HPV16 ($p = 0.044$), but in those with grade II and grade III there exited no significant difference respectively ($p = 0.165, 0.658$) as Table 2 indicated.

HPV16, CD44V6 and nm23H1 in ESCCs with clinic pathological data

No statistical correlation was observed between the infection of HPV16 and pathological features in ESCCs. The CD44v6 and nm23H1 had no statistical correlations with gender ($p = 0.193, 0.110$) and age ($p = 0.071, 0.972$) respectively (Table 3). However, the expression of CD44v6 had significant correlation with invasion ($p = 0.001$) and lymph node metastasis ($p = 0.014$). In addition the expression

Table 3. pathological features for HPV16, CD44v6 and nm23H1 in ESCCs

Features	n	HPV16		p value	CD44v6		p value	nm23H1		p value
		+ (%)	− (%)		+ (%)	− (%)		+ (%)	− (%)	
Gender										
Male	28	15 (53.6)	13 (46.4)		20 (71.4)	8 (28.6)		10 (35.7)	18 (64.3)	
Female	12	9 (75.0)	3 (25.0)	*0.205*	6 (50.0)	6 (50.0)	*0.193*	8 (66.7)	4 (33.3)	*0.071*
Age										
≤50	11	8 (72.7)	3 (27.3)		5 (45.5)	6 (54.5)		5 (45.5)	6 (54.5)	
>50	29	16 (55.2)	13 (44.8)	*0.312*	21 (72.4)	8 (27.8)	*0.110*	13 (44.8)	16 (55.2)	*0.972*
Invasion										
T1–T2	12	5 (41.7)	7 (58.3)		3 (25.0)	9 (75.0)		9 (75.0)	3 (25.0)	
T3–T4	28	19 (67.9)	9 (32.1)	*0.121*	23 (82.1)	5 (17.9)	*0.001*	9 (32.1)	19 (67.9)	*0.013*
Metastasis										
yes	22	10 (45.5)	12 (54.5)		18 (81.8)	4 (18.2)		5 (22.7)	17 (77.3)	
no	18	6 (33.3)	12 (66.7)	*0.243*	8 (44.4)	10 (55.6)	*0.014*	13 (72.2)	5 (27.8)	*0.002*
Grade										
I	15	9 (60.0)	6 (40.0)	*0.876*	5 (33.3)	10 (66.7)	*0.004*	11 (73.3)	4 (26.7)	*0.016*
II	14	8 (57.1)	6 (42.9)	*0.742*	12 (85.7)	2 (14.3)	*0.014*	4 (28.6)	10 (71.4)	*0.020*
III	11	7 (63.6)	4 (36.4)	*0.851*	9 (81.8)	2 (18.2)	*0.792*	3 (27.3)	8 (72.7)	*0.943*

of nm23H1 had statistical correlation with invasion ($p = 0.013$) and lymph node metastasis ($p = 0.002$) (Table 3). The expression rate of CD44v6 in ESCCs with grade II was higher than that in ESCCs with grade I ($p = 0.004$), so was the expression rate of CD44v6 in ESCCs with grade III ($p = 0.014$), but the expression rate of CD44v6 was not statistically different between ESCCs with grade II and those with grade III ($p = 0.792$). On the contrary, the expression rate of nm23H1 in ESCCs with grade II was lower than that in ESCCs with grade I ($p = 0.016$), so was the expression rate of nm23H1 in ESCCs with grade III ($p = 0.020$), however no statistical difference was found between ESCCs with grade II and those with grade III ($p = 0.943$) (Table 3).

Discussion

Some previous studies have implicated genital-mucosal strains of human papillomaviruses (HPVs) in the etiology of ESCC. The possible mechanism of high-risk HPV in transforming esophageal epithelial cells was inferred for instance increased activity of telomerase induced by HPV [27], interaction between E6 and P53 [40], the association of HPV-16 E6 with nuclear matrix [5] and others [25, 41]. Therefore HPV became the hotspot of research on etiology of ESCC. We designed a set of specific primer targeting HPV-16 E6 gene as before because E6 gene always exited in host cells in spite of viral physical state [17, 29].

In our study, 24 of 40 (60%) ESCCs from Shaanxi Province were detected to have the infection of HPV16 through PCR method. The infection rate of HPV16 was statistically different ($p < 0.05$) compared with NEMs. From this study, it was demonstrated that HPV-16 infection in esophageal epithelium was very common in patients with ESCC from Shaanxi Province and showed that HPV16 had correlation with occurrence of ESCC. Some precious reports on HPV16 in ESCC from different countries and regions indicated that the infection rate of HPV16 varied from 0–83%, which gave rise to the controversy about the role of HPV16 in ESCC. However in some Western countries such as France [2], Slovenia [23], Sweden [14], Belgium [15] and Finland [3], the HPV infection rate was close to zero. Through carefully reviewing the literature we shared the opinion with previous study [23, 29] that HPV infection played a much more significant role in ESCC in regions with a high prevalence of disease compared with that in regions with a moderate or low risk [16, 18, 20]. According to the researches in the past two years, the infection rate of HPV16 in ESCC variant from 54.5% to 83.3% seemed to increase in some regions of China [8, 17, 19, 26, 37–39]. On the one hand this may be due to the increased contamination of environment resulting in worse pollution of water, in addition the diet habits weren't changed completely. On the other hand PCR, the method we used to detect HPV16 was more sensitive than immunohistochemistry (IHC) and in situ hybridization (ISH) and the target selected was different from L1 gene because HPV16 E6 gene always exited in ESCC during the occurrence and development of ESCC but nor did L1 gene. For example, consensus L1 primers were frequently used as the proper PCR primers for its ability to detect a wide spectrum of HPV types [13, 16, 22]. But during virus integration into the host genome, L1 and L2 were often lost. Therefore, the detection using consensus primers against L1 gene might lead to a lower rate [17, 29]. Our results also showed that HPV16 had no statistical significance with gender, age, lymph node metastasis and histological grade in ESCCs ($P > 0.05$), which suggested that the infection of HPV16 might be a biological promoter during carcinogenesis of ESCC.

The nm23 gene was first found in murine melanoma cell lines and is known to have anti-metastatic function in animal studies [28]. nm23 has two isotypes, nm23-H1 and nm23-H2 which have identical amino acid sequence in 92% and also the two isotypes have identical amino acid sequence with nucleoside diphosphate kinase (NDPK) A and NDPK B. NDPK acts as the provider of nucleoside diphosphate to the cells and is related to intracellular signal transduction [10, 21, 34]. Our results suggested that nm23H1 might be involved in invasion and metastasis of ESCC but had no statistical correlation with HPV16.

CD44 has two isoforms, CD44 standard (CD44s) and CD44 variant (CD44v). The mRNA of CD44s contains no variant exons, while CD44v may contain one or more variant regions, such as CD44v6 or CD44v3-v7. CD44v6, a highly glycosylated cell surface protein now is considered to be involved in cell–cell and cell-matrix interactions and in cell motility, tumor growth, and invasion [9, 12, 24]. Our study showed that CD44v6 might be involved in invasion and metastasis of ESCC. Moreover, we firstly found that the expression of CD44v6 had

statistical difference between ESCCs infected by HPV16 and those not infected by HPV16 ($P < 0.05$). Our results also suggested that the infection of HPV16 might result in the expression of CD44v6 in the early stage of ESCC. However with the development of ESCC it seemed that other factors might be involved in the expression of CD44v6 in ESCCs especially in those not infected by HPV16 (Table 2). Dally et al. firstly found that most cervical carcinomas with expression of CD44v7/v8 were infected by HPV16, so they speculated that the infection of HPV might take place before the expression of CD44v and that HPV and CD44v might be involved in canceration and metastasis of tumor [7]. Our study made the similar observation that HPV might be connected to the expression of CD44v associated with invasion and metastasis of squamous cell carcinoma. Our results suggested that HPV16 might make host cell express CD44v6 at certain stage during HPV16 infecting host cell. But whether other CD44vs and other proteins bearing on invasion and metastasis of ESCC were expressed resulted from HPV16 was worth to investigate further in order to understand the overall relationship between HPV16 and CD44v in ESCC. Meanwhile our results also indicated that HPV16 seemed to be a new marker, which can predict not only the occurrence of ESCC but also prognosis of ESCC combined with CD44v6 examined.

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