

RELATIONSHIP BETWEEN HPV16/18 E6 AND *p53*, *p21^{Waf1}*, MDM2, Ki67 AND CYCLIN D1 EXPRESSION IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA: COMPARATIVE STUDY BY USING TISSUE MICROARRAY TECHNOLOGY

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Aim: To investigate the role of human papillomavirus (HPV) HPV16/18 E6 oncogene in the carcinogenesis of esophageal cell carcinoma (ESCC). **Materials and Methods:** Tissue microarray (TMA) block was constructed from 60 cases of paraffin-embedded ESCC tissues and pair-matched controls (adjacent normal epithelium). Immunohistochemistry (IHC) methods were applied to detect the expression of HPV16/18 E6, *p53*, *p21^{Waf1}*, MDM2, Ki67 and cyclin D1 proteins on TMA slides. *In situ* hybridization (ISH) targeting HPV gene was also used. **Results:** In ESCC samples, 18.3% (11/60) were revealed HPV16/18 E6 positive by IHC, while 40.0% (24/60) HPV positive by ISH; HPV16/18 E6 expression was significantly higher than that of control samples. In ESCC samples, the expressions of *p53*, *p21^{Waf1}*, Cyclin D1, MDM2 and Ki67 were recorded in 60.0% (36/60), 40.0% (24/60), 51.7% (31/60), 65.0% (39/60) and 88.3% (53/60) cases respectively. In ESCC samples, *p53*, MDM2 and Ki67 expression correlated with the HPV16/18 E6 expression ($p < 0.01$), *p21^{Waf1}* expression — with these of MDM2 and cyclin D1 ($p < 0.01$) whilst expression of Ki67 — with ESCC grade ($p < 0.01$). **Conclusion:** HPV might be one of etiological factor of esophageal carcinoma in Shantou, China. *p53*, MDM2 proteins may play important roles in the pathogenesis of HPV-associated ESCC.

Key Words: human papillomavirus, esophageal cell carcinoma, tissue microarray, immunohistochemistry.

Esophageal squamous cell carcinoma (ESCC) is a common cancer worldwide and has a very high mortality rate [1]. The most characteristic feature of ESCC is its distinct geographical incidence, which suggests a dominant role of environmental factors in the etiology of this disease. Furthermore, the other risk factors such as nutrition imbalance (lack or absence of vitamins and minerals), improper life style (cigarette smoking and consumption of pickled food), exposure to nitrosamines, may be implicated in the carcinogenesis of ESCC [1–2]. Nevertheless, the real causes and the mechanisms of ESCC have not been elucidated yet.

Human papillomavirus (HPV) has been reported in ESCC since the observation of condyloma-like lesions in ESCCs 24 years ago [3]. However, the reported HPV infection rates in ESCC, detected as presence of viral proteins or DNA sequences, vary from 0% to over 60% worldwide [4]. This variation may result partly from the varied disease incidence in different geographical regions [4], partly from variations in the specificity and sensitivity of the analytical techniques used [5, 6].

Shantou, located in the coastal region of Eastern Guangdong, Southern China, is one of the six high incidence regions of esophageal carcinoma in China. As we reported, the total infection rate of the high-risk HPV types 16 and 18 was very high in cancerous tissues (54.5%) by the means of polymerase chain

reaction (PCR) from surgically resected esophageal carcinoma [7, 8].

To further investigate the prevalence of HPV infection in ESCC, 60 ESCC samples and pair-matched distal normal epithelia samples were constructed into one tissue microarray block. HPV16/18 E6, a major viral oncogene of high-risk HPV type, was studied by the means of immunohistochemistry (IHC), and HPV pan probes were used for *in situ* hybridization (ISH). In the meanwhile, the expressions of *p53*, *p21*, MDM2, cyclin D1 and Ki67 proteins, which are associated with cell cycle abnormalities and cell proliferation, were also analyzed by IHC method.

MATERIALS AND METHODS

ESCC samples and tissue microarray. Sixty pairs of paraffin-embedded ESCC specimens and pair-matched adjacent “normal” tissues were chosen to create one tissue microarray (TMA) block. Among them, 29 cases were collected from the Department of Pathology at the Second Affiliated Hospital of Shantou University, 31 cases — from the Tumor Hospital of Shantou University Medical College from May 2004 to May 2005. The mean age of patients was 58.1 years (range, 43–78) and male to female ratio was 4 : 1 (48 : 12). The ESCC case's distributions among Grade 1, 2, 3 were 11, 24, 25 respectively. All hematoxylin and eosin (H&E)-stained sections were reviewed to confirm the original findings and to define representative tumor regions. Tumor stage and grade were defined according to the WHO histological classification criteria [9].

1.0 mm cores were taken from selected tissue blocks as typical of the specific diagnosis. The TMA block was created using a tissue arrayer (Becher

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Abbreviations used: ESCC – esophageal squamous cell carcinoma; HPV – human papillomavirus; IHC – immunohistochemistry; ISH – *in situ* hybridization; PCR – polymerase chain reaction; TMA – tissue microarray.

Instruments, Silver Spring, MD) in the Cybrdi Biotechnical Company in Xi'an, P.R. China. The TMA block containing 120 cores of ESCC and paired controls, was sectioned into 4 μm thick consecutive sections, then these sections were placed on slides pretreated with aminopropyltriethoxysilane (APES, Sigma), the slides were allowed to dry overnight at 42 °C, and stored at 4 °C for later use.

Immunohistochemical staining (IHC). The avidin-biotin-peroxidase complex method was used. In brief, first, the slides were deparaffinized in xylene, endogenous peroxidase was inactivated by immersing the slides in a 0.3% hydrogen peroxidase — methanol solution for 30 min at room temperature, and cross-reactivity was blocked with the normal goat serum. Next, the varied antigen retrieval method, high pressure or microwave methods, was performed in 0.1 mM citrate buffer (pH 6.0). Third, the sections were incubated overnight at 4 °C with a diluted solution of the primary antibodies (Table 1). Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated to horseradish peroxidase, and diaminobenzidine. The slides were counter-stained by hematoxylin. Negative controls were established by replacing the primary antibody with PBS and normal goat serum. Known immunostaining positive slides were used as positive controls.

The criteria of positive staining [10, 11] were partly followed in this study. Briefly, for the immunohistochemistry of p53 and p21^{Waf1}, cyclin D1, and Ki67 only nuclear staining was observed. In contrast, HPV16/18 E6 and MDM2 showed both in nuclear and cytoplasmic immunostaining. The immunoreactivity for all the proteins investigated in this study was evaluated as strongly positive (++) when more than 50% of tumor cells were stained, positive (+) when more than 25% of the cells were stained, slightly positive (\pm) when more than 10% of the tumor cells were stained, and negative (–) when less than 10% of the tumor cells were stained or when the tumor cells completely lacked immunoreactivity. All slides were observed by two independent pathologists, and the final concordant results were adopted.

In situ hybridization (ISH). *In situ* hybridization procedures were performed according to manufacturer's instruction (K1602, Dako). Briefly, the TMA slides were dewaxed in xylene, hydrated, digested in preheated pepsin-HCl at 37 °C for 7 min. After dehydration, the hybridization probes (wide spectrum HPV DNA probe cocktail: HPV 6, 11, 16, 18, 31, 33, 35, 45, 51 and 52, biotinylated, Y1404, Dako) were pipetted on the sections, which were placed under coverslips and denatured at 95 °C for 5 min. Hybridization was carried out in a humidified chamber at 37 °C for 60 min, after that each section was rinsed twice in Tris buffered saline for 3 min at room temperature, and then in preheated stringent wash solution included in the test kit at 48 °C for 30 min. Finally, the sections were incubated first with streptavidin-AP reagent at room

temperature for 20 min and then with chromogen solution (BCIP/NBT) at room temperature in the dark for 60 min. Sections of known positivity and negativity were used as internal controls.

Statistical analysis. Basic statistical calculations were performed using the statistical Package for Social Science/PC windows (SPSS). Fisher exact χ^2 test was used for statistical analysis of the results. Correlations between two parameters were performed using the Spearman's rank correlation test. Two-sided P values < 0.05 were considered to be significant.

RESULTS

Positive rate for HPV16/18 E6 expression by ICH was 18.3% (11/60) in ESCC cases, only two cases (2/60, 3.3%) showed mild expression in pair-matched adjacent normal tissues. By contrast, the positive rate by HPV ISH was 40.0% (24/60) in the same ESCC TMA tissues, 9 cases (9/60, 15.0%) showed positive staining in control tissues. There was significant difference between HPV16/18 E6 expression in tumor tissues and control tissues ($p < 0.01$, Fisher's exact test) (Table 2).

The positive staining of p53, p21^{Waf1}, cyclin D1 and Ki67 expression was observed mainly in the nuclei of cells in ESCC tissues (60.0% (36/60), 40.0% (24/60), 51.7% (31/60), and 88.3% (53/60), respectively). MDM2 was expressed both in nuclei and cytoplasm (Figure) yielding 65.0% (39/60) in ESCC samples. Compared with the paired controls, the expression of mentioned proteins was significantly different in ESCC samples (see Table 2).

Among five detected proteins, expression of p53, MDM2 and Ki67 had statistical correlations with the HPV16/18 E6 protein expression ($p < 0.05$, Table 3), and also, expression of MDM2 and Ki67 proteins seems to correlate with HPV expression (ISH) ($p < 0.01$, Table 4).

The case distribution of five stained proteins according to grade stage is shown in Table 5. There is a statistically significant correlation between Ki67 expression and ESCC grades ($p < 0.01$). The expression of other proteins seems to have no correlation with tumor's grade ($p > 0.05$).

Statistical analysis revealed a positive correlation between the expression of p21^{Waf1} and MDM2, cyclin D1 expressions in tumor tissues, and no correlation between Ki67 and p21^{Waf1} (Table 6).

DISCUSSION

During the past two decades, several studies have shown the relationship between HPV and esophageal cancer (EC), however with conflicting data [2, 4, 6, 19], which may be attributed to the geographical location with respect to either low or high incidence areas [20]. Shantou, located in southeast of China, is one of six HPV-high incidence regions of the world. It was reported that the total HPV infection rate is about 54.5% in EC tissues using PCR method [7]. In present study, IHC for HPV16/18 E6 and ISH for pan HPV methods were applied, and the results showed 11 out

Table 1. Summary of specific monoclonal antibodies used in the immunohistochemical analysis

| Antibodies | Clone | Dilution | Antigen pretreatment | Staining pattern | Positive control |
|---------------------|------------------|----------|----------------------|-------------------------|------------------|
| p53 | Monoclonal DO7 | 1 : 50 | High pressure | Nuclear | Breast cancer |
| p21 ^{Waf1} | Monoclonal 4D10 | 1 : 50 | Microwave | Nuclear | Lung cancer |
| MDM2 | Monoclonal Sup14 | 1 : 50 | Microwave | Nuclear and cytoplasmic | Osteosarcoma |
| Ki67 | Monoclonal SP6 | 1 : 100 | High pressure | Nuclear | Cervical cancer |
| Cyclin D1 | Monoclonal SP4 | 1 : 100 | Microwave | Nuclear | Cervical cancer |
| HPV16/18 E6 | Monoclonal CIP5 | 1 : 50 | High pressure | Nuclear and cytoplasmic | Cervical cancer |

All monoclonal antibodies used were purchased from Maixin_Bio (China).

Table 2. Expression of p53, p21^{Waf1}, MDM2, Ki67, Cyclin D1, HPV E6 in ESCC samples and pair-matched controls

| Sample | p53 | | p21 ^{Waf1} | | MDM2 | | Ki67 | | Cyclin D1 | | HPV E6 (IHC) | | HPV E6 (ISH) | |
|------------|--------|----|---------------------|----|--------|----|--------|----|-----------|----|--------------|----|--------------|----|
| | + | - | + | - | + | - | + | - | + | - | + | - | + | - |
| ESCC | 36 | 24 | 24 | 36 | 39 | 21 | 53 | 7 | 31 | 29 | 11 | 49 | 24 | 36 |
| Control | 9 | 51 | 14 | 46 | 8 | 52 | 13 | 47 | 14 | 46 | 2 | 58 | 9 | 51 |
| <i>p</i> * | < 0.01 | | 0.03 | | < 0.01 | | < 0.01 | | < 0.01 | | < 0.01 | | < 0.01 | |

**p*: Fisher's exact test; in this and other tables are present the number of cases.

Table 3. Relationship between expression of p53, p21^{Waf1}, MDM2, Ki67, Cyclin D1, and HPV 16/18 E6 in collected tissues

| HPV16/18 E6 | p53 | | | | p21 ^{Waf1} | | | | MDM2 | | | | Ki67 | | | | Cyclin D1 | | | |
|-------------|--------|---|----|----|---------------------|----|----|----|--------|---|----|----|--------|----|----|----|-----------|----|----|----|
| | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ |
| - | 69 | 4 | 10 | 22 | 71 | 11 | 14 | 9 | 67 | 9 | 15 | 15 | 51 | 11 | 19 | 25 | 66 | 11 | 17 | 11 |
| + | 5 | 0 | 3 | 7 | 9 | 3 | 3 | 0 | 4 | 2 | 5 | 3 | 2 | 1 | 5 | 6 | 7 | 3 | 3 | 2 |
| <i>p</i> | 0.026* | | | | 0.672 | | | | 0.019* | | | | 0.016* | | | | 0.567 | | | |

p value: Spearman correlation; in this and other tables are presented the number of cases.

Table 4. Relationship between expressions of p53, p21^{Waf1}, MDM2, Ki67, Cyclin D1, and HPV (ISH) in collected tissues

| HPV ISH | p53 | | | | p21 ^{Waf1} | | | | MDM2 | | | | Ki67 | | | | Cyclin D1 | | | |
|----------|-------|---|---|----|---------------------|---|----|----|--------|---|----|----|--------|---|----|----|-----------|---|----|----|
| | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ |
| - | 57 | 4 | 6 | 19 | 59 | 8 | 11 | 7 | 58 | 6 | 11 | 10 | 47 | 6 | 13 | 20 | 57 | 8 | 14 | 7 |
| + | 17 | 1 | 5 | 11 | 22 | 6 | 4 | 3 | 15 | 3 | 9 | 8 | 7 | 6 | 10 | 11 | 18 | 5 | 6 | 5 |
| <i>p</i> | 0.102 | | | | 0.730 | | | | 0.004* | | | | 0.004* | | | | 0.154 | | | |

p value: Spearman correlation.

Table 5. Relationship between expression of p53, p21^{Waf1}, MDM2, Ki67, Cyclin D1 and tumor grade

| Tumor Grade | p53 | | | | p21 ^{Waf1} | | | | MDM2 | | | | Ki67 | | | | Cyclin D1 | | | |
|-------------|-------|---|---|----|---------------------|---|---|----|-------|---|---|----|--------|---|----|----|-----------|---|---|----|
| | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ |
| I | 7 | 1 | 0 | 3 | 6 | 3 | 2 | 2 | 3 | 0 | 4 | 4 | 3 | 8 | 0 | 0 | 6 | 1 | 4 | 1 |
| II | 8 | 1 | 3 | 13 | 12 | 4 | 4 | 3 | 5 | 4 | 9 | 6 | 0 | 1 | 23 | 0 | 14 | 0 | 3 | 7 |
| III | 8 | 0 | 4 | 12 | 16 | 4 | 2 | 2 | 11 | 3 | 6 | 5 | 3 | 0 | 0 | 22 | 7 | 4 | 9 | 4 |
| <i>p</i> | 0.227 | | | | 0.730 | | | | 0.103 | | | | 0.001* | | | | 0.120 | | | |

p value: Spearman correlation.

Table 6. Relationship between expression of p21^{Waf1} and p53, MDM2, cyclin D1, Ki67 in tumor tissues

| p21 ^{Waf1} | p53 | | | | MDM2 | | | | Cyclin D1 | | | | Ki67 | | | |
|---------------------|-------|---|---|----|--------|---|----|----|-----------|---|---|----|-------|---|----|----|
| | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ | - | ± | + | ++ |
| - | 14 | 0 | 5 | 15 | 14 | 3 | 12 | 5 | 20 | 3 | 8 | 3 | 5 | 4 | 12 | 13 |
| ± | 1 | 1 | 1 | 7 | 4 | 1 | 3 | 3 | 4 | 1 | 3 | 2 | 1 | 2 | 4 | 4 |
| + | 5 | 0 | 1 | 2 | 0 | 2 | 2 | 2 | 2 | 1 | 2 | 4 | 1 | 2 | 3 | 2 |
| ++ | 3 | 1 | 1 | 3 | 1 | 1 | 1 | 5 | 2 | 0 | 4 | 1 | 1 | 1 | 3 | 2 |
| <i>p</i> | 0.930 | | | | 0.011* | | | | 0.008* | | | | 0.851 | | | |

p value: Spearman correlation.

of 60 positive (18.3%) by the former method, 24 out of 60 positive (40.0%) by the latter one.

The discrepancy of two HPV detecting methods in the same paraffin TMA tissues can be explained as follows: firstly, the IHC method for HPV16/18 E6 is only tested for the subtype of HPV16/18 E6 proteins, while HPV DNA cocktail probe for ISH has a wide spectrum; secondly, HPV DNA is more constant than their proteins in the paraffin tissues.

In this paper, we still find that there is statistically significant difference in HPV detection between ESCC tissues and control tissues (*p* < 0.01). So our results still support the earlier findings that HPV infection may play a role in the development of esophageal cancer [7].

It is reported that tumor suppressor proteins p53 and p21^{Waf1} play important roles in regulating G1 phase progression, which is the key modulation point in the cell cycle [10, 12, 21]. But the role of these alterations and the mechanisms underlying the interaction between various risk factors in EC are still unclear. In current study, HPV16/18 E6, p53 and associated proteins were assayed on consecutive sections from the same TMA

block. The results showed that all studied proteins were expressed significantly different in ESCC and normal tissues (*p* < 0.01), and moreover, p53, MDM2, and Ki67 expression correlated with that of HPV16/18 E6, which is consistent with result of Bahnassy et al. [22].

MDM2 (murine double minute 2) gene product can interact with both wildtype and mutant p53 and inhibit its ability to act as a transcription factor, it can also abolish the ability of p53 to induce cell cycle arrest and apoptosis [13–15, 23]. So, high frequency of MDM2 expression in our study and correlation with HPV E6 and p53, suggest that MDM2 and p53 genes may be involved in the pathogenetic cascade of HPV-associated EC.

p21^{Waf1} protein was detected in 24 of the 60 cases (40%) of ESCC in present study, which is lower than Bahnassy's report (64%) [22], but higher than Yang's report (30%) [23]. Correlation analysis showed the expression of p21^{Waf1} seemed have low correlation with these of HPV16/18 E6 and p53. This result is supported by the idea that expression of p21 is regulated negatively by p53 [24] and the reports of Natsugoe [25]. It has been reported that p21^{Waf1}, which encodes

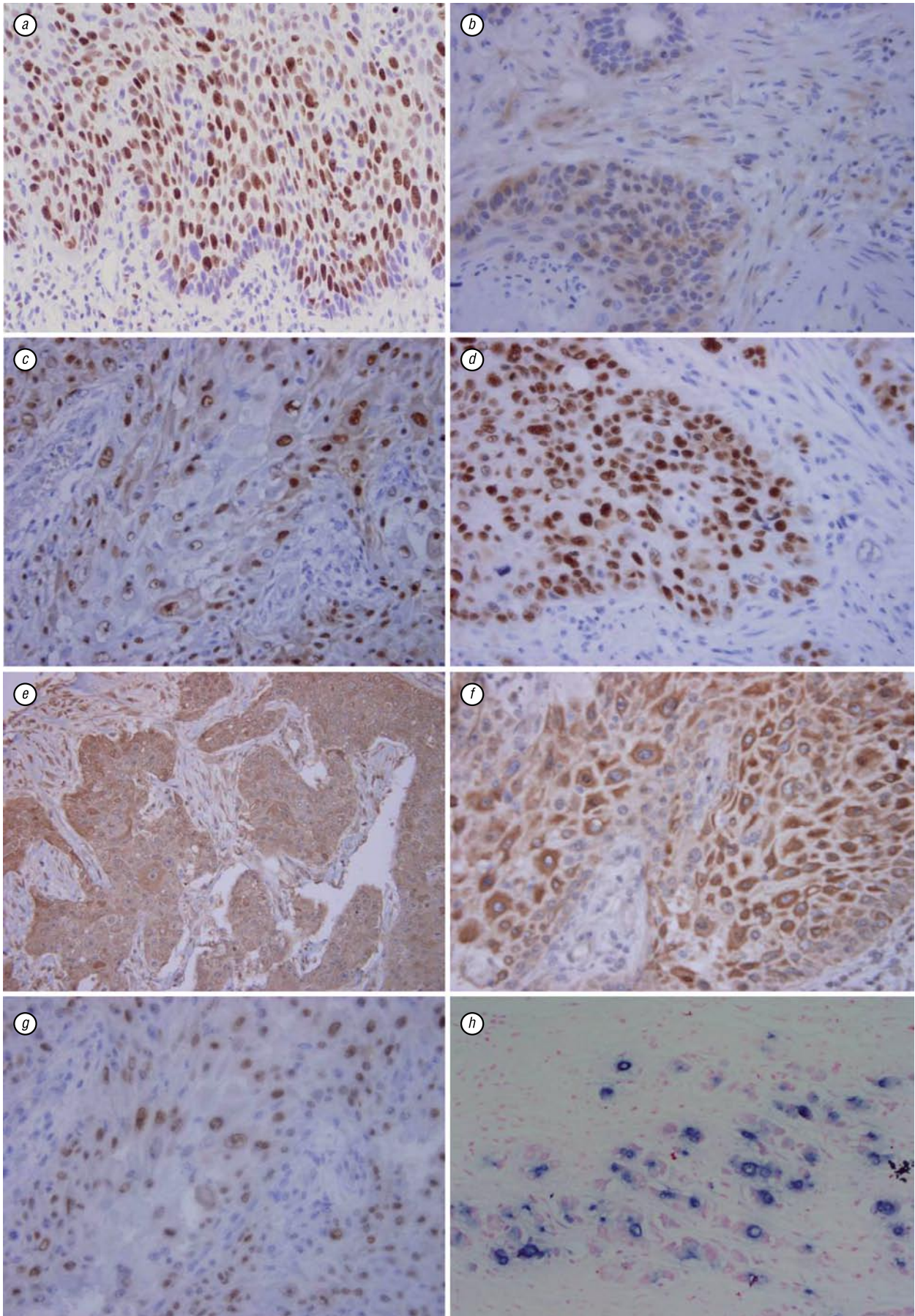


Figure. Immunohistochemical and *In Situ* Hybridization (ISH) staining of HPV and associated proteins in esophageal squamous cell carcinoma on tissue microarrays slices. a) Ki67 positive staining $\times 400$; b) HPV16/18 E6 positive staining $\times 400$; c) Cyclin D1 positive staining $\times 400$; d) p53 positive staining $\times 400$; e) MDM2 positive staining $\times 200$; f) MDM2 positive staining $\times 400$; G) p21^{Waf1} positive staining $\times 400$; h) ISH Positive staining(NBT/BCIP) $\times 400$

an inhibitor of cyclin-dependent protein kinase, is directly activated by wild type *p53* and can suppress the growth of human cells by inhibiting the activity of cyclin dependent kinases, which are required for the G1 to S transition of the cell cycle [26, 27]. All these results suggested that *p21* may progress via the *p53*-independent than the *p53*-dependent pathway in the pathogenesis of ESCC. Our results also showed that the expression of *p21^{Waf1}* has correlation with that of MDM2 ($p < 0.01$) and cyclin D1 ($p < 0.01$).

Cyclin D1 also plays an important role in enhancing EC cell transformation [18, 28]. In human ESCC, cyclin D1 overexpression is already evident in dysplasias and early cancers [29]. In current study, 31 of 60 ESCC tumor tissues (51.7%) and 14 of 60 (23.3%) of their adjacent tissues showed positive cyclin D1 expression ($p < 0.01$). Our results showed higher values than recently report (32% of EC specimens) [30, 31], and close to the other report (cyclin D1 positive expression rate was 56.5%) [32]. Cyclin D1, a protein derived from the PRAD1 on chromosome 11q13, is one of the most essential proteins for controlling the cell cycle in the p16-cyclin D1-pRb pathway [33, 34]. In our study, expression of cyclin D1 had no correlation with HPV E6 and *p53* but seemed to have correlation with that of *p21^{Waf1}* ($p < 0.01$) (Table 6). The relationship between *p21^{Waf1}* and cyclin D1 in esophageal cancer will be determined in the future studies.

Ki67 is also known as a marker for cell proliferation [18, 35]. In present study, the expression rate of Ki67 is 88.3% (53/60) in tumor tissues and 21.7% (13/60) in pair-matched controls, and Ki67 expression correlated with the tumor grade.

In conclusion, Ki67 can reflect the proliferation status of esophageal carcinoma, *p53* and MDM2 may contribute to the HPV16/18 E6 pathogenetic cascade of EC, but *p21^{Waf1}* may not play a role in the process of its development. Further studies to explore the molecular mechanisms of participation of these proteins in EC development should be performed.

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ЭКСПРЕССИЯ HPV16/18 E6, *p53*, *p21^{Waf1}*, MDM2, Ki67 И ЦИКЛИНА D1 В ТКАНИ ПЛОСКОКЛЕТОЧНОЙ КАРЦИНОМЫ ПИЩЕВОДА

Цель: исследовать роль онкогена HPV16/18 E6 вируса папилломы человека (HPV) в развитии плоскоклеточной карциномы пищевода (ESCC). **Материалы и методы:** исследованы 60 образцов ESCC и контрольные образцы нетрансформированной ткани (парафиновые блоки). Для выявления экспрессии белков HPV16/18 E6, *p53*, *p21^{Waf1}*, MDM2, Ki67 и циклина D1 в срезах для тканевых микрочипов (tissue microarray, TMA) применен метод иммуногистохимии (ИНС), для определения экспрессии генов HPV применяли гибридизацию *in situ* (ISH). **Результаты:** методами ИНС, экспрессия HPV16/18 E6 выявлена в 18,3% (11/60) случаев ESCC, в то время как по результатам метода ISH, 40,0% (24/60) образцов признаны HPV-позитивными. В образцах ESCC экспрессия *p53*, *p21^{Waf1}*, циклина D1, MDM2 и Ki67 выявлена в 60,0% (36/60), 40,0% (24/60), 51,7% (31/60), 65,0% (39/60) и 88,3% (53/60) случаев соответственно, причем экспрессия *p53*, MDM2 и Ki67 коррелировала с экспрессией HPV16/18 E6 ($p < 0,01$), экспрессия *p21^{Waf1}* — с таковой MDM2 и циклина D1, а экспрессия Ki67 — со стадией развития опухоли ($p < 0,01$). **Выводы:** HPV может являться одним из этиологических факторов развития рака пищевода в провинции Шанту, Китай. Белки *p53* и MDM2 могут принимать участие в патогенезе плоскоклеточного рака пищевода, ассоциированного с вирусом папилломатоза.

Ключевые слова: вирус папилломы человека, плоскоклеточный рак пищевода, тканевый микрочип, иммуногистохимия.