Histol Histopathol (2024) 39: 201-209

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Abnormal p16 expression and prognostic significance in esophageal squamous cell carcinoma

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Summary. Background. The purpose of this study was to analyze p16 expression status and evaluate whether abnormal p16 expression was associated with prognosis in a large-scale esophageal squamous cell carcinoma (ESCC) cohort of patients.

Methods. We retrospectively evaluated p16 expression status of 525 ESCC samples using immunohistochemistry. Associations between abnormal p16 expression and survival were analyzed.

Results. P16 negative, focal expression and overexpression were found in 87.6%, 6.9% and 5.5% of ESCC patients. No significant association was observed between abnormal p16 expression and age, sex, tumor site and location, differentiation, vessel and nerve invasion, T stage and lymph node metastasis. In all patients, the survival of p16 focal expression group tended to be better compared with negative group (disease free survival/DFS P=0.040 and overall survival/OS P=0.052) and overexpression group (DFS P=0.201 and OS P=0.258), and there was no survival difference between negative group and overexpression group. The multivariate analysis for OS and DFS found that only clinical stage was a significantly independent prognostic factor (P<0.001). When patients were divided into I-II stage (n=290) and III-IVa stage (n=235), the survival of focal expression group was better compared with negative group (DFS P=0.015 and OS P=0.019), and tended to be better compared with overexpression group (DFS P=0.405 and OS P=0.432) in I-II stage ESCC, which was not found in III-IVa stage ESCC.

Conclusion. P16 overexpression or negative expression tend to be associated with unfavorable outcomes, especially in I-II stage ESCC. Our study will help to identify a subgroup of ESCC patients with excellent prognosis after surgical therapy.

Key words: P16 focal expression, P16 overexpression, P16 negative, Prognosis, Esophageal squamous cell carcinoma (ESCC)

Introduction

Esophageal Cancer (EC) is the seventh most frequent malignancy and the sixth most common cause of cancer-associated mortalities with an estimated 572,000 new cases and 509,000 deaths worldwide in 2020 (Sung et al., 2021). Despite improvements in multidisciplinary treatments, including surgery, radiotherapy, and systemic therapy, the prognosis for EC remains poor in the "Esophageal Cancer Belt" including China (He et al., 2019). The most common histological type is esophageal squamous cell carcinoma (ESCC), which is regarded as an important public health problem in China. Recently, the major focus of cancer research is identifying the molecular changes that occur in tumorigenesis and progression (The Cancer Genome Atlas Research Network, 2017; Liu et al., 2017). A detailed search into these alterations can discover novel biomarkers, which may help classify patients at the same stage into different subgroups in terms of their prognosis and further guide surgery or adjuvant treatment. Therefore, reliable biomarkers are urgently required in ESCC.

The *p16* gene, also known as MTS-1 (major tumor

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www.hh.um.es. DOI: 10.14670/HH-18-619

Abbreviations. EC, Esophageal Cancer; ESCC, esophageal squamous cell carcinoma; MTS-1, major tumor suppressor 1; INK4a, inhibitor of CDK4a; CDKN2A, cyclin-dependent kinase inhibitor 2A; Rb, retinoblastoma protein; HPV, human papillomavirus; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; TNM, tumor, node and metastasis; TMA, tissue microarray; DFS, disease-free survival; OS, overall survival

suppressor 1), INK4a (inhibitor of CDK4a), or CDKN2A (cyclin-dependent kinase inhibitor 2A), is located on chromosome 9p21.3. It consists of 3 exons and 2 introns and encodes a tumor suppressor p16, which plays an important role in regulating the cell cycle pathway. P16 inhibits cyclinD dependent protein kinases (CDK4 and CDK6) therefore maintaining hypo-phosphorylation of the retinoblastoma protein (Rb). It ultimately inhibits the release of the transcription factor E2F, preventing cell conversion from G1 phase to S phase, and eventually suppressing cell proliferation (Lukas et al., 1995; Quelle et al., 1995). The decreased expression or inactivation of p16 attenuates the ability of Rb to inhibit cell proliferation and allows unregulated cell-cycle progression. P16 aberration is frequently observed in a wide variety of tumors (LaPak and Burd, 2014; Serra and Chetty, 2018).

P16 protein expression is frequently used as a surrogate marker for human papillomavirus (HPV) infection in many kinds of cancer, including cervical cancer (Nicolas et al., 2020) and head and neck squamous cell carcinoma (HNSCC) (Paver et al., 2020). In HPV related tumors, E7 oncoprotein integrates into the host genome and leads to inactivation of Rb, which has a negative feedback on intracellular p16 levels leading to p16 overexpression. In addition to HPV-related cancer, including lung, pancreas, colorectal, bladder, and breast tumors, p16 function is lost by gene deletions, mutations, or epigenetic silencing, which results in negative immunohistochemistry (IHC) findings (Kim and Sharpless, 2006; Mahajan, 2016). That is to say, there are two abnormal p16 expression patterns: absent and overexpressed. Many studies to date have explored the clinicopathological and prognostic significance of p16 expression in tumors (Kitamura et al., 2019; Kopetz et al., 2019; Nicolas et al., 2020).

Some studies have examined p16 IHC results in ESCC within the past few decades (Liu et al., 2007; Taghavi et al., 2010; Bai et al., 2012), however, the conclusions were doubtful. In their results, p16 expression was detected in 5.8-88.3% ESCC. There was no detailed demonstration and comparison among absent, expression and overexpression. Its influence on the prognosis of ESCC patients remains unclear. Some studies demonstrate that p16 expression was associated with favorable prognosis (Sturm et al., 2001). No prognostic significance was shown in other studies (Shimada et al., 1999; da Costa et al., 2017). The inconsistent conclusions are obtained due to several reasons such as clinical stage, sample size, IHC evaluation criteria, or ethnicity. Further studies are required to investigate the influence of p16 expression in ESCC patients. Hence, we performed this study to explore the status of p16 expression using IHC methods, and analyze the association of p16 expression with clinicopathological characteristics, and prognosis of large-scale cohort of ESCC patients.

Materials and methods

Patients and tissues

The present study included 525 patients with primary ESCC who underwent surgery at Zhongshan Hospital, Fudan University between 2007 and 2010. Eligible patients had histologically proven squamous cell carcinoma of esophagus. Exclusion criteria included patients with neoadjuvant therapy and incomplete follow-up information. Clinical data were collected from their medical records, including sex, age at diagnosis, smoking, histological grading, tumor size and location, vessel and nerve invasion. Tumor stage was determined according to the 8th edition of American Joint Committee on Cancer tumor, node and metastasis (TNM) classification system.

Informed consent was submitted by all patients. The present study was approved by the Institutional Review Broad of Zhongshan Hospital. All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration (and its subsequent updates) of the World Medical Association or comparable ethical standards.

Tissue microarray and immunohistochemistry

Tissue microarrays were constructed as described previously (Shi et al., 2013). Briefly, all donated cylinders were extracted from the most representative areas of the tumor within the paraffin block and transplanted into recipient tissue microarray (TMA) block. Available TMA sections were stained with p16 antibody (clone MX007, Maixin Biotechnology Co. Ltd, Fuzhou, China, monoclonal, 1: 400 dilution) on an automated immunostainer (Leica Biosystem) according to the manufacturer's protocol with appropriate controls. The results were evaluated by 2 pathologists who were blinded to the clinical outcome and all other data on the patients.

Evaluation of p16 expression status

p16 nuclear expression and cytoplasmic expression were scored using a histochemical or H-score-like method in which the percentage of cell staining was recorded for each intensity level (-, no staining; +, weak staining intensity; ++, moderate staining intensity; or +++, strong intensity staining). Different definitions for positive p16 expression have been used in the literature. The main definitions are summarized as follows: 1) p16 was considered positive when >10% of the cells showed both nuclear and cytoplasmic brownish staining; 2) p16 overexpression was defined as positive when nuclear or cytoplasmic expression had an intensity of 2+/3+ and distribution 70% of cancer cells (Fakhry et al., 2018). As there is little consensus in the literature about what constitutes positive staining for p16 antibodies, we

analyzed and compared our results with the above mentioned definitions.

Statistical analysis

Statistical analyses were performed with SPSS for Windows version 24 (IBM, Armonk, NY). Chi-square analysis and Fisher exact test were used as appropriate to assess the relationship between p16 expression and clinicopathological characteristics, including sex, age, smoking, histological grading, tumor size and location, vessel and nerve invasion. Disease-free survival (DFS) was calculated from the date of primary treatment to the date of recurrence, progression, or death from esophageal cancer. Overall survival (OS) was defined from the date of primary treatment to the date of death from any cause or the date of the last follow-up. The survival rates were calculated using Kaplan-Meier method and compared using a log-rank test. The univariate Cox proportional hazards model was used to analyze covariates. Factors with P value <0.05 were included in a multivariable Cox proportional hazards model using a forward stepwise procedure. All of the statistical tests performed were two-tailed, with P values <0.05 considered statistically significant.

Results

Clinicopathological Features

As reported in Table 1, which summarizes the clinical and pathological features of these 525 patients, the median age was 61 years with a wide age distribution ranging from 34 to 83 years. Four hundred and twenty-nine patients (81.7%) were males. A history of tobacco smoking was observed in 203 (38.7%) patients. As for the anatomic site, 5% cases were located in the upper esophagus, 44.8% in the middle and the other 45.5% in the lower with a mean of tumor size of 3.4 cm of the biggest axis. The histopathological diagnoses consisted of well-differentiated ESCC in 20 patients (3.8%), moderately-differentiated ESCC in 295 patients (56.2%) and poorly-differentiated ESCC in 210 patients (40.0%). The vessel and nerve invasion was observed in 22.3% and 34.5% of patients. According to the T stage, we found that 51 patients (9.7%) were in T1 stage, 116 (22.1%) patients were in T2 stage, 357 (68.0%) patients were in T3 stage and 1 (0.2%) patients were in T4 stage. Lymph node metastases were detected in 243 (46.3%) patients. According to 8th TNM stage, 290 (55.2%) patients were in the stage I-II group, and 235 (44.8%) patients were in the stage III-IVa group.

p16 expression in ESCC

The levels of p16 expression in the tumor samples of all ESCC patients are presented in Table 2. When a cut-off value of >10% was used, p16 positive was seen in 65 specimens (12.4%) and negative in 460 specimens (H-score, range 0-5, median value 0) (87.6%). When a cut-off value of >70%++ was used, p16 overexpression was seen in 29 (5.5%) specimens. Among 65 ESCC with p16 IHC positive, 29 (5.5%) cases had p16 overexpression

Table 1. Correlation between p16 expression and clinicopathologic features.

Variable	p16 IHC positive			p16 IHC overexpression	
	No	Yes	*P* value	No	Yes
Age (years)			0.125		
<60	202	22		212	12
≥60	258	43		284	17
Sex			0.080		
Female	79	17		90	6
Male	381	48		406	23
Smoking			0.095		
No	276	46		303	19
Yes	184	19		193	10
Tumor Size			0.917		
<3.4	265	37		287	15
≥3.4	195	28		209	14
Tumor Location			0.281		
Upper	23	3		24	2
Middle	199	36		219	16
Lower	214	25		229	10
Differentiation			0.743		
Well	19	1		20	0
Middle	258	37		281	14
Poor	183	27		195	15
Vessel invasion			0.153		
No	353	55		385	23
Yes	107	10		111	6
Nerve invasion			0.219		
No	297	47		323	21
Yes	163	18		173	8
pT			0.574		
T1	45	6		48	3
T2	98	18		113	3
T3	316	41		334	23
T4	1	0		1	0
Lymph node metastasis			0.611		
No	249	33		271	11
Yes	211	32		225	18
pN			0.809		
N0	249	33		271	11
N1	114	18		122	10
N2	73	12		78	7
N3	24	2		25	1
Clinical stage			0.612		
I-II	256	34		278	12
III-IVa	204	31		218	17
Disease progression			0.057		
No	204	37		227	14
Yes	256	28		269	15
Death			0.066		
No	206	37		229	14
Yes	254	28		267	15

IHC, immunohistochemistry.

with diffuse and strong staining (H-score, range 200-300, median value 250), and the other 36 (6.9%) cases showed focal and weak staining (H-score, range 10-150, median value 72.5) (Fig. 1).

p16 expression and clinicopathological parameters were analyzed. No significant association was observed between p16 positive or overexpression and age, sex, tumor size and location, histological differentiation, vessel and nerve invasion, T stage and lymph node metastasis (P>0.05, Table 2).

p16 expression and patient prognosis

The follow-up period ranged from 3 to 102 months, with a median of 31 months. Two hundred and eighty two (53.7%) patients died within a median OS time of 42.0 months (95% CI: 33.0-51.0 months). Two hundred and eighty four (54.1%) patients had tumor progression within a median DFS time of 36.0 months (95% CI: 25.8-46.2 months).

When these patients were divided into p16 positive group (n=65) and negative group (n=460) defined by a cut-off value of 10%, the positive group demonstrated a better outcome compared with the negative group, however, this did not reach statistical significance (DFS P=0.088 and OS P=0.115) (Fig. 2A,B). When these patients were divided into p16 overexpression group (n=29) and non-overexpression group (n=496) defined by a cut-off value of 70%++, there was no difference concerning DFS (P=0.888) and OS (P=0.933) of patients with p16 overexpression compared to those without overexpression (Fig. 2C,D). When these patients were divided into p16 negative group (n=460), focal expression group (n=36) and overexpression group

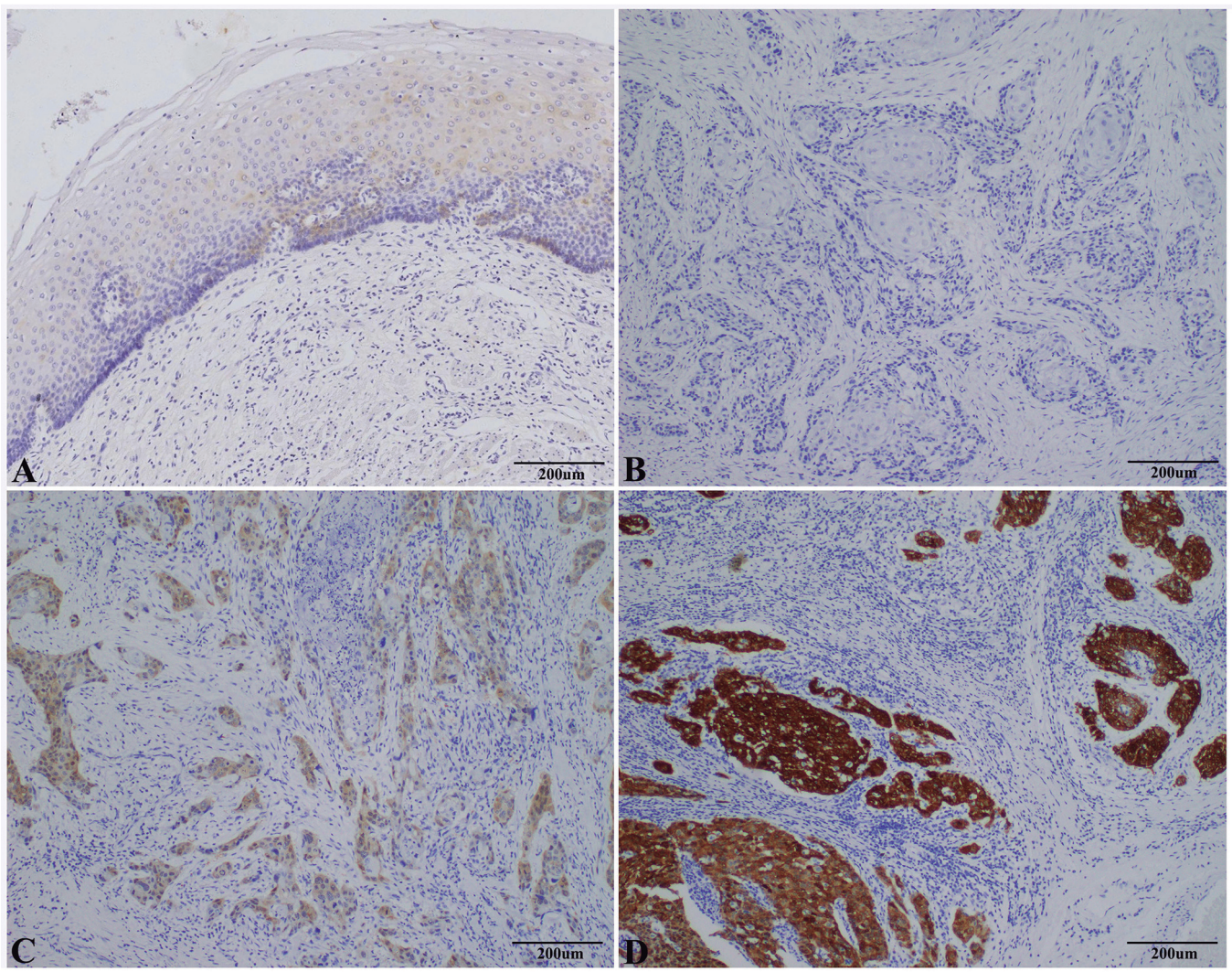

Fig. 1. The representative p16 expression patterns. **A.** p16 focal and weak expression in normal epithelium. **B.** p16 negative in ESCC. **C.** P16 focal expression in ESCC. **D.** P16 overexpression in ESCC.

(n=29) defined by the above mentioned cut-off values, the survival of focal expression group tended to be better compared with negative group (DFS *P*=0.040 and OS *P*=0.052) and overexpression group (DFS *P*=0.201 and OS *P*=0.258), and there was no survival difference between negative group and overexpression group (DFS *P*=0.780 and OS *P*=0.837) (Fig. 2E,F). The univariate analysis indicated a significant association between poor differentiation, vessel invasion, nerve invasion, higher clinical stage and poorer survival, and an association between p16 focal expression and favorable survival. Then multivariate analysis for OS and DFS was performed and included the above mentioned factors, and only clinical stage was found to be a significantly independent prognostic factor (Table 3) (Fig. 3A,B).

p16 expression and patient prognosis in I-II stage ESCC

Among 290 I-II stage patients, 110 (37.9%) patients died and 111 (38.3%) patients had tumor progression within a non-reached median OS and DFS time. When these patients were divided into p16 negative group (n=256), focal expression group (n=22) and overexpression group (n=12) defined by the above mentioned cut-off values, the survival of focal expression group was better compared with negative group (DFS *P*=0.015 and OS *P*=0.019), and tended to be better compared with overexpression group (DFS P=0.405 and OS *P*=0.432). Also, p16 overexpression group tended to have better outcome compared with the negative group, however, this did not reach statistical significance (DFS *P*=0.233 and OS *P*=0.254) (Fig. 3C,D). The univariate analysis only indicated p16 focal expression was associated with favorable DFS and OS (Table 4).

p16 expression and patient prognosis in III-IVa stage ESCC

Among 235 III-IVa stage patients, 172 (73.2%) patients died within a median OS time of 25.0 months (95% CI: 22.9-27.1 months), and 173 (73.6%) patients had tumor progression within a median DFS time of 20.0 months (95% CI: 17.8-22.2 months).

When these patients were divided into p16 negative group (n=204), focal expression group (n=14) and overexpression group (n=17) defined by the above mentioned cut-off values, there were no survival difference between focal expression group and negative group (DFS *P*=0.899 and OS *P*=0.848) or over-expression group (DFS *P*=0.976 and OS *P*=0.853) (Fig. 3E,F). In univariate statistical analysis, no prognostic factor was found in these patients (Table 4).

Discussion

P16, as an important tumor suppressor protein, plays an important role in cell cycle regulation and prevents tumor development. Serrano et al. first cloned the cDNA of its encoding gene (*CDKN2A*) in 1993 (Serrano et al.,

Table 2. Univariate and multivariate analyses of prognostic factors for survival.

	DFS		OS
	P value	HR (95% CI)	*P* value
Univariate analysis			
Sex	0.196	1.223 (0.901-1.660)	0.120
Age	0.856	0.978 (0.774-1.237)	0.754
Smoking	0.300	1.134 (0.894-1.438)	0.212
Tumor Size	0.222	1.158 (0.915-1.464)	0.169
Tumor Location	0.922	0.990 (0.809-1.211)	0.788
Differentiation	0.018	1.292 (1.045-1.597)	0.047
Vessel invasion	<0.001	1.620 (1.255-2.091)	<0.001
Nerve invasion	0.005	1.407 (1.108-1.785)	0.001
pT Stage	<0.001	1.687 (1.369-2.078)	<0.001
Lymph node metastasis	<0.001	2.789 (2.190-3.553)	<0.001
pN Stage	<0.001	1.600 (1.429-1.791)	<0.001
Clinical stage	<0.001	2.831 (2.226-3.601)	<0.001
p16 positive			
p16 focal expression	0.046	0.567 (0.325-0.991)	0.059
p16 overexpression	0.784	0.930 (0.552-1.565)	0.834
Mutivarate analysis			
Differentiation	0.346	1.110 (0.893-1.381)	0.601
Vessel invasion	0.508	1.096 (0.835-1.437)	0.631
Nerve invasion	0.303	1.137 (0.890-1.454)	0.098
Clinical stage	<0.001	2.635 (2.043-3.398)	<0.001
p16 positive	0.310		0.364
p16 focal expression	0.144	0.659 (0.376-1.153)	0.170
p16 overexpression	0.595	0.868 (0.514-1.464)	0.654

Table 3. Univariate analyses of prognostic factors for survival in stage I-II and III-IVa ESCC.

	DFS		OS
Univariate analysis	*P* value	HR (95% CI)	*P* value
I-II Stage			
Sex	0.764	0.937 (0.613-1.432)	0.850
Age	0.830	1.043 (0.712-1.528)	0.786
Smoking	0.469	0.859 (0.570-1.296)	0.626
Tumor Size	0.514	0.877 (0.591-1.301)	0.850
Tumor Location	0.677	0.935 (0.680-1.285)	0.744
Differentiation	0.104	1.321 (0.945-1.846)	0.191
Vessel invasion	0.350	1.288 (0.758-2.187)	0.488
Nerve invasion	0.916	1.023 (0.670-1.563)	0.400
p16 positive	0.045		0.052
p16 focal expression	0.025	0.270 (0.086-0.851)	0.029
p16 overexpression	0.247	0.508 (0.161-1.600)	0.266
III-IVa Stage			
Sex	0.690	1.098 (0.695-1.733)	0.796
Age	0.464	1.118 (0.829-1.508)	0.238
Smoking	0.794	1.041 (0.772-1.403)	0.816
Tumor Size	0.270	1.183 (0.878-1.594)	0.252
Tumor Location	0.105	0.807 (0.623-1.046)	0.144
Differentiation	0.932	1.012 (0.764-1.341)	0.897
Vessel invasion	0.461	1.122 (0.827-1.522)	0.519
Nerve invasion	0.142	1.251 (0.928-1.689)	0.099
p16 positive	0.977		0.974
p16 focal expression	0.899	1.042 (0.549-1.977)	0.847
p16 overexpression	0.856	1.056 (0.586-1.901)	0.890

1993). Since then it has been widely studied in the field of cancer research. Although some studies have explored the clinicopathological and prognostic significance of p16 aberration in ESCC, the results remain inconclusive because of the differences in sample sizes, methods, study populations, and evaluating criteria (Shimada et al., 1999; Sturm et al., 2001; Bai et al., 2012; da Costa et al., 2017; Ishida et al., 2021). Therefore, we conducted this study in a larger sample size to explore and validate the association between p16 status and clinicopathological factors including survival in ESCC patients.

There are two abnormal p16 expression patterns: absent and overexpression in tumors. In HPV-driven tumors such as HNSCC and cervical cancer, viral E7 oncoprotein functionally inactivates RB protein and has a negative feedback on intracellular p16, leading to its protein accumulation. Therefore, a positive p16 expression is considered when there is a diffuse block staining with strong nuclear or nuclear plus cytoplasmic staining, and focal or patchy nuclear staining and exclusive cytoplasmic staining is interpreted as negative (Fakhry et al., 2018; Nicolas et al., 2020). In non-HPV-driven tumors such as lung, breast, pancreas and colon cancer, p16 function is lost as a result of various alterations, including complete point mutation, promoter methylation, homozygous deletion and loss of heterozygosity (Kim and Sharpless, 2006; Mahajan, 2016). IHC negative for p16 protein expression is believed to be an accurate and relatively simple method for evaluating *p16* gene inactivation.

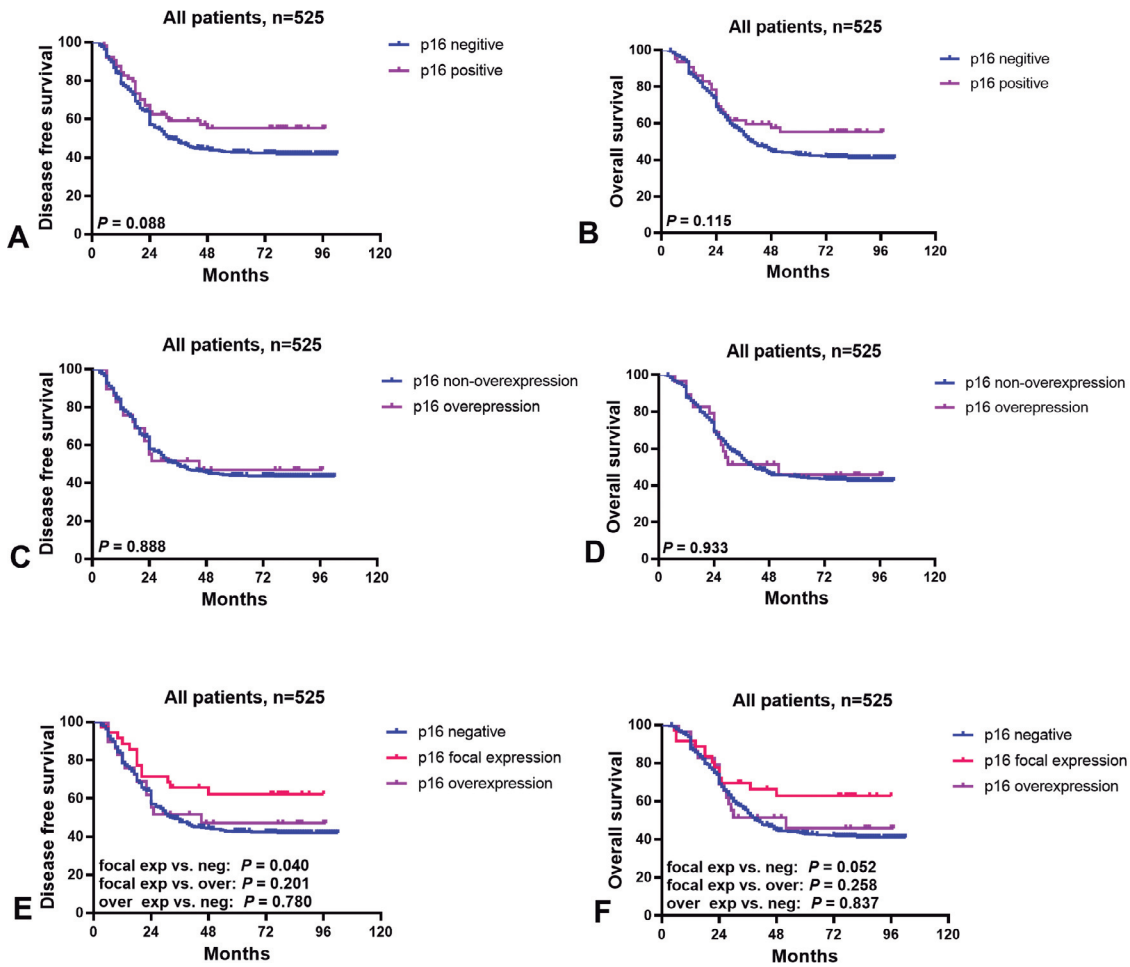

Fig. 2. P16 expression and patient prognosis in all 525 patients. **A, B.** When these patients were divided into p16 positive group (n=65) and negative group (n=460) defined by a cut-off value of 10%+, the positive group demonstrated a better outcome compared with the negative group, however, this did not reach statistical significance (DFS *P*=0.088 and OS *P*=0.115). **C, D.** When these patients were divided into p16 overexpression group (n=29) and non-overexpression group (n=496) defined by a cut-off value of 70%++, there was no difference concerning DFS (*P*=0.888) and OS (*P*=0.933) of patients with p16 overexpression compared to those without overexpression. **E, F.** when these patients were divided into p16 negative group (n=460), focal expression group (n=36) and overexpression group (n=29) defined by the cut-off values of 10%+ and 70%++, the survival of focal expression group tended to be better compared with negative group (DFS *P*=0.040 and OS *P*=0.052) and overexpression group (DFS *P*=0.201 and OS *P*=0.258), and there was no survival difference between negative group and overexpression group (DFS *P*=0.780 and OS *P*=0.837).

At present, there is little detailed discussion about the expression pattern in ESCC. Recent global data, including a Chinese study, indicate that HPV has no significant etiological role for ESCC (Koshiol et al., 2010; Teng et al., 2014; Ludmir et al., 2015; The Cancer Genome Atlas Research Group, 2017). *P16* gene inactivation through gene mutation and promoter methylation is reported in ESCC (Taghavi et al., 2010; Liu et al., 2017), and IHC expression is reported in 5.8-88.3% patients (Liu et al., 2007; Taghavi et al., 2010; Bai et al., 2012). In our study, we used cut-off values of 10%+ and 70%++, to evaluate p16 focal and diffuse expression. The former was commonly used in various tumors (Myong, 2008; Zhou and Gu, 2018). The latter was similar to the new p16 criteria, which AJCC adopted in oropharyneal cancer and included for its TNM staging (Fakhry et al., 2018). p16 overexpression was found in 5.5% of our ESCC samples. p16 overexpression using this new criteria was also reported in other non-HPV-driven tumors (Kopetz et al., 2019). The mechanisms that lead to p16 overexpression in these tumors are not well understood. Our p16 deficiency rate was 87.6%, consistent with some reports (83.1-94.2%) about ESCC (Liu et al., 2007; Lofdahl et al., 2012; da Costa et al., 2017), which demonstrate that p16 inactivation might be an important molecular event for esophagus tumorigenesis. Our negative rate was higher than some older reports (Wang et al., 2016), which tended to have small sample size (<200 cases), different cut-off values and environmental factors. Our study is one of the largest ESCC studies of p16 expression to date in China.

The prognostic effect of p16 abnormal expression on survival was explored in both HPV-driven tumors and non-HPV-driven tumors (Myong, 2008; Zhou and Gu, 2018; Nicolas et al., 2020). P16 positive has been widely identified as a prognostic factor for a better outcome.

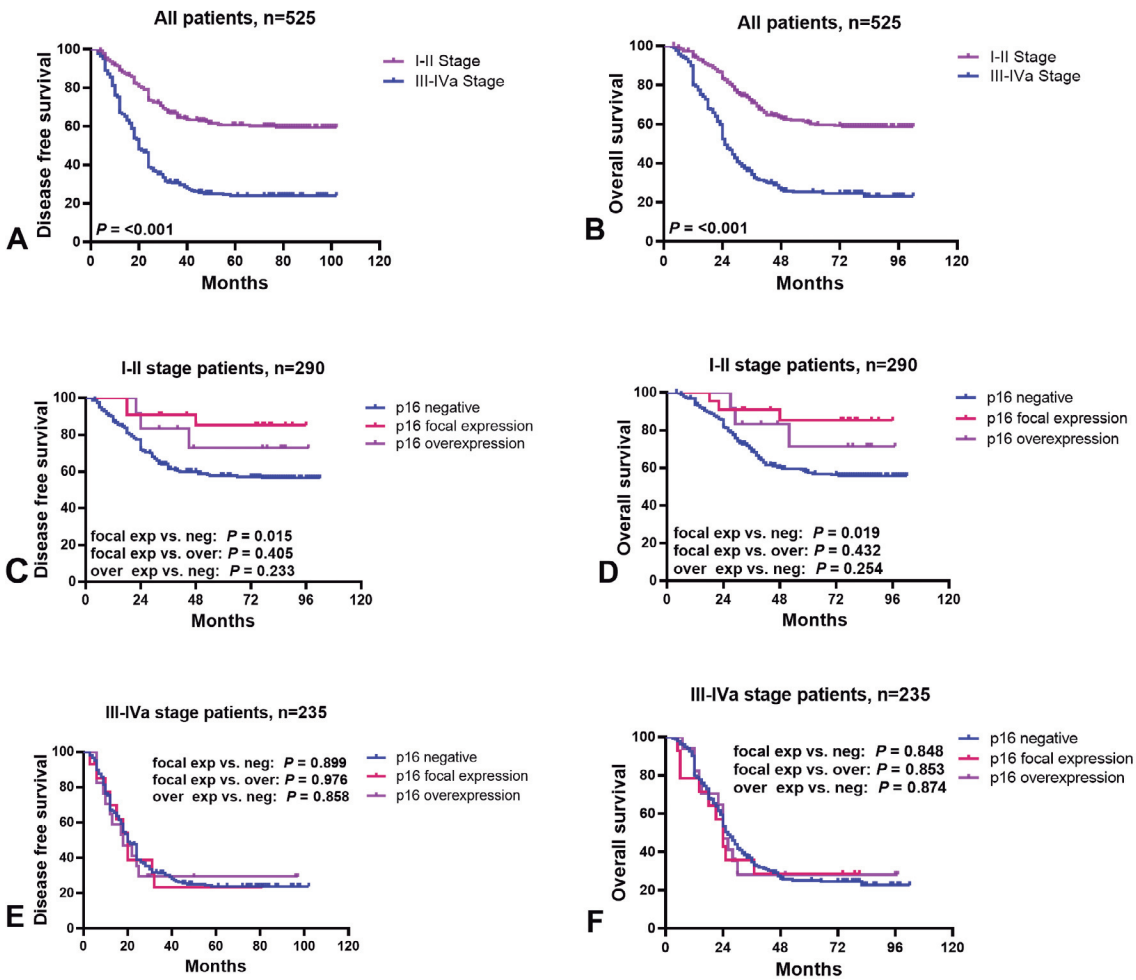

Fig. 3. P16 expression and patient prognosis in different clinical stages. **A, B.** higher clinical stage was found to be associated with poorer DFS and OS. **C, D.** in 290 stage I-II ESCC, the survival of focal expression group was better compared with negative group (DFS *P*=0.015 and OS *P*=0.019), and tended to be better compared with overexpression group (DFS *P*=0.405 and OS *P*=0.432). **E, F.** In 235 stage III-IVa ESCC, there were no survival differences between focal expression group and negative group (DFS *P*=0.899 and OS *P*=0.848) or overexpression group (DFS *P*=0.976 and OS *P*=0.853).

The higher the p16 expression, the greater the effect it is on cell-cycle arrest and survival. At present, the prognostic studies in ESCC are still limited and variable, with some controversy. High p16 expression supposedly correlated with favorable prognosis in ByIsrid's study with 53 ESCC patients (Sturm et al., 2001). However, other retrospective studies did not find p16 to function as a significant outcome predictor (Shimada et al., 1999; da Costa et al., 2017). In our study, 6.9% patients with p16 focal expression had better DFS than those without p16 expression, and 5.5% patients with p16 overexpression had no prognostic difference with those without p16 expression. What's more, 5.5% patients with p16 overexpression tended to be associated with a more unfavorable survival than 6.9% patients with p16 focal expression. That's to say, either overexpression or absence of p16 tended to be associated with unfavorable outcomes in ESCC. Some studies in ovarian cancer also showed that overexpression and silencing of p16 might predict worse outcome (Dong et al., 1997; Kudoh et al., 2002).

In our multivariate survival analysis of 525 ESCC patients, only clinical stage was found to be a significantly independent prognostic factor. Then we analyzed and verified the prognostic value of p16 expression in earlier stage (I-II stage) and later stage (III-IVa stage), separately. No significant association was observed between p16 expression and clinical stage. Among 290 I-II stage patients, p16 focal expression was associated with both favorable DFS and OS, which was not found in 235 III-IVa stage patients. In other words, reduced risk of progression and mortality in the p16-focal expression patients compared with the p16 negative was only found in earlier stage (stage I and II) ESCC patients. A similar phenomenon is also observed for other molecules (Xu et al., 2015; Song et al., 2017; Lanki et al., 2018). Abnormal p16 expression may lead to malignant, abnormal cell proliferation and accelerated tumor development in ESCC, however, many other markers may interact with p16 and also contribute to these processes, especially in III-IVa stage tumors (Meltzer, 1996; The Cancer Genome Atlas Research Group, 2017; Liu et al., 2017). These might reduce the prognostic significance of individual marker, and further studies are needed in more advanced ESCC.

Conclusion

In this investigation, we used IHC staining of TMA blocks to evaluate p16 expression patterns and their prognostic role in a large cohort of Chinese ESCC patients. To conclude, p16 focal expression was significantly associated with better DFS, especially in I-II stage ESCC, and p16 overexpression or negative expression tended to be associated with unfavorable outcomes. At present, this study is the largest sample size providing excellent power to examine differences of p16 expression patterns in Chinese ESCC. Our study will help to identify a subgroup of ESCC with excellent prognosis after surgical therapy, who might not need any further adjuvant therapy after surgery with curative intention. This, however, is only a hypothesis, and it remains to be elucidated in a prospective trial in the future.

Acknowledgements. This work was financially supported by Shanghai Municipal Health Commission (No. 20214Y0275), National Natural Science Foundation of China (No. 81702372), Shanghai Municipal Commission of Science and Technology (No. 19441904000), Shanghai Municipal Key Clinical Specialty (No. shslczdzk01302), and Shanghai Science and Technology Development Fund (No. 19MC1911000).

Competing interests. The authors declare that they have no competing interests.

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Accepted April 12, 2023