Decreased Expression of P\textsuperscript{16} Indicates the Postoperative Poor Prognosis of Esophageal Squamous Cell Carcinoma Patients

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Abstract

Background: Expression of P\textsuperscript{16} gene that is the key regulatory protein of the cell cycle has been linked with the prognosis of Esophageal squamous cell carcinoma patients.

Materials and method: By immunohistochemistry, we examined the expression status of P\textsuperscript{16} of 110 esophageal squamous cell carcinoma patients on the tissue microarrays (TMAs). The nuclear staining intensity was calculated by immunoreactivity score ranging from (0-12) and split them into two groups: No-expression & Overexpression group.

Result: Postoperatively median follow-up period of our study was 70 months. Down-regulation of P\textsuperscript{16} expression pointedly predicted decreased 5-year overall survival (P=0.001) and progression-free survival, which is statistically significant & demonstrated by Kaplan-Meier estimates using the log-rank test. Hence, P\textsuperscript{16} protein acts as an independent prognostic factor for overall survival and progression-free survival that demonstrated by multivariate Cox-regression analysis (HR=0.046 with 95% CI 0.006-0.333, P=0.002 and HR=0.064 with 95% CI 0.009-0.466, P=0.005 respectively OS & PFS).

Conclusion: P\textsuperscript{16} is a promising biomarker that is down regulated in ESCC patients and prognostic indicator for poor survival postoperatively.

Keywords: Esophageal squamous cell carcinoma; Metastasis; P\textsuperscript{16}; Overall survival; Progression-free survival

Introduction

Globally esophageal cancer effect 480,000 new cases as well as 400,000 deaths per year, which is the 6\textsuperscript{th} leading reason cancer death and suppose as 8\textsuperscript{th} most common cancer [1]. Between two main histological types (esophageal squamous cell carcinoma and esophageal adenocarcinoma), though esophageal adenocarcinoma (EAC) high incidence in western countries, yet esophageal squamous cell carcinoma (ESCC) is worst histological type of the cancer. Lack of or ignorance of early symptoms and high invasive characteristics of ESCC responsible for poor outcome. But now-a-days new and advance clinical treatment favor on increase 5-year overall survival almost 42% [2]. Consequently, to determine prognostic outcome, etiology of progression of disease & determine treatment protocol; one of the best ways is prediction of prognostic indicators. CDKN2A (P\textsuperscript{16}) is one of the significant biomarkers that regulates cell cycle as tumor suppressor gene. Locus of this protein is genetically more susceptible in many cancers [3]. Hence, P\textsuperscript{16} pondered as analyst of biological characteristics of many malignancies, for instance gastric carcinoma, cervical carcinoma, endometrial carcinoma, lung cancer, breast cancer, anal cancer, colorectal cancer, nasopharyngeal cancer; and so on [4,5]. Usually, P\textsuperscript{16} upregulated in normal cells and maintain normal cell cycle, but cancerous cell its down regulated and loss its power to control normal cell cycle. Though many researches held to observe the prognostic role of this protein regulation in ESCC in last decades, yet the accurate decisions are uncertain [6]. The aim of our study is to determine the prognostic value of P\textsuperscript{16} and effect of its expression status on patient’s survival and disease progression [7].
Materials and Method

Patient population and data collection

We allocated tissue samples of esophageal squamous cell carcinoma from 135 patients who received subtotal esophagectomy and esophagogastric anastomosis with regional lymph node dissection. These surgeries done and tissue samples collected from Qilu Hospital of Shandong University at year of 2010 and 2011. Out of 135 patients we included 110 patients as our study population because remaining 25 patients were lost to follow up. ESCC diagnosis confirmed through the pathological examination. Inclusion criteria we include patients didn’t have any chemotherapy or radiotherapy or immunotherapy preoperatively, all tumor stage, any lymph node status. We exclude younger age group, more than 25 years age group we include. From our hospital database we collected baseline clinical and investigational data, for instance age, gender, smoking, drinking habit, degree of differentiations, TNM staging, tumor stage, lymph node status, number of dissected lymph node, and so on. For TNM staging we follow “American Joint Committee on Cancer Staging Manual (7th edition, 2010).” The research design & sample collection was done according to our institutional protocols that approved from “Ethics boards of Qilu Hospital of Shandong University”. The written informed consent was acquired from all our study population.

Follow up

Our study populations were followed up till death or for at least 5 years from their date of surgery. All the patients followed up regularly; during follow-up physical examination & imaging studies performed every 3 months during 1st 2 years after receive surgery and every 6 months during the 3 years through 5 years. If necessary, routine radiological examination and esophagoscopy were performed. Remaining indicators allocated from the database for in-patients or the tumor registry for outpatients of Qilu Hospital of Shandong University.

Immunohistochemistry

All the fresh specimens were collected from the patients during surgery. We used 10% formalin for fix the fresh specimen that embedded in paraffin. The tissues collected from the Pathology Department of Qilu Hospital of Shandong University in the year of 2010-2011. All the allocated tissues were cut 4mm serial sections manner. After that, the tissue sections retrieved by 10mM citrate buffer, then de-paraffinization by Xylene & rehydration. We incubated the tissue sections in 3% H2O2 using methanol at least 20 minutes at the room temperature. After that all the samples again incubated with primary anti-CDKN2A monoclonal antibody ab108349 (1:150, Abcam, Cambridge, MA, USA) overnight in the high humid chamber at 4°C. Next morning, again incubated the slides for 30 minutes at 37°C by biotinylated secondary antibodies and streptavidin-peroxidase complex. At the end of the procedure, the slides were counterstained by 3,3’-diaminobenzidine solution with hematoxylin. Then fixed the slides with coverslip by natural balsam. We incubated our tissue sections with PBS instead of primary antibody to directed to negative controls.

Interpretation method of IHC technique

The slides were examined under a light microscope after drying off those. Scoring independently done by two investigators. The scores that were contradictory resolute by investigators steadily and scored those. For determining the staining intensity, we calculate by “Immunoreactivity score” (IRS) system. The scoring parameter as, 0=no staining; 1=weak staining; 2=moderate staining; and 3=strong staining. The final result calculated though multiplication of staining intensity by the percentage of positive cells. For count positive cell we also follow a protocol, scored as; 0=0-10% positive cell, 1=10-25 positive cells, 2=26-50% positive cells, 3=51-75% positive cells, 4=76-100% positive cells. The final score was the summation of the staining intensity and the percentage of positive cells. At the end this was further divided as negative (-), weak (+), moderate (++), and strong (+++) grade correspondingly (0-1), (2-
3), (4-5), and (6-7) (Figure 1). Depending on the expression status we ultimately divide our study group into two: non-overexpressed (- or +) group and overexpressed (++ or ++++) group.

**Study endpoints**

Our primary end point was overall survival (OS) that defined as “the time from the date of surgery to death or the last date of follow-up”. As well as secondary end point was progression-free survival (PFS) defined as “The local progression of disease from the date of surgery to local or distant progression of disease”. The local recurrences include regional lymph node metastasis or primary site recurrence.

**Statistical analysis**

All the statistical analyses were performed operating SPSS statistics version 23.0 software (SPSS Inc. Chicago, IL). Association of P16 and clinical parameters were analyzed by Chi-square test or Fisher’s exact test. To determine the prognostic value, we performed univariate analysis by Kaplan-Meier method as well as to define OS & PFS we performed log-rank test. To determine independent prognostic factor, we analyze variables by Multivariate Cox-Proportional Regression analysis. All the tests were two-side. P-values studied as significant when P<0.05.

**Result**

**Staining array**

We examined CDKN2A protein expression of ESCC by immunohistochemistry (IHC) method of tissue microarray where we include 110-FFPE tissue samples (Figure 1). After IHC it showed around 75-85% tissues were weak expressed or no expression. Within all of our study population 88 patients (80%) cancer tissue samples showed low expression or no expression.

**Clinico-pathological features of ESCC patients**

A total 135 patients met the criteria of our study. Out of that 110 patients include in our study due to 25 patients lost during follow up. The median age group is 65 years, at the time of diagnoses age ranging from 25 to 86 years, where 40 patients were female, and 70 patients were female. The median follow-up duration was 70 months; ranging from 1-120 months. We include all four tumor stage, out of that stage T1 20 cases (10.9%), stage T2 43 cases (39.09%), stage T3 35 cases (31.81%), and stage T4 20 cases (18.18%) recorded. As well as lymph node staging grouped as no positive lymph node (N0), N1 stage, N2 stage, and N3 stage that respectively included 44 cases (40%), 24 cases (21.81%), 29 cases (26.36%), and 13 cases (11.81%) in our study. Also, degree of differentiation of tumor, well differentiated tumor 32 cases (29.09%), moderately differentiated tumor 31 cases (28.18%), and poorly differentiated 47 cases (42.72%) analyzed in our study.

**Correlation between P16 expression and baseline indicators**

**Table 1**: P: Chi-square test.

| Baseline Characteristics (Numbers) | CDKN2A Overexpression | P Value |
|----------------------------------|------------------------|---------|
|                                 | No (n=88) (80%) | Yes (n=22) (20%) |
| Age                              |                     |         |
| <65 years (49)                   | 37 (75.5%) | 12 (24.5%) |
| >65 years (61)                   | 51 (83.6%) | 10 (16.4%) |
| Gender                           |                     |         |
| Female (40)                      | 36 (90%) | 4 (10%) |
| Male (70)                        | 52 (74.3%) | 18 (25.7%) |
| Smoking                          |                     |         |
| No (46)                          | 38 (82.2%) | 8 (17.4%) |
| Yes (64)                         | 50 (78.1%) | 14 (21.9%) |
| Drinking                         |                     |         |
| No (54)                          | 47 (87%) | 7 (13%) |
| Yes (56)                         | 41 (73.2%) | 15 (26.8%) |
| Differentiation                  |                     |         |
| Well (32)                        | 26 (83.3%) | 6 (18.8%) |
| Moderate (31)                    | 26 (83.9%) | 5 (16.1%) |
| Poor (47)                        | 36 (76.6%) | 11 (23.4%) |
| T stage                          |                     |         |
| T1 (12)                          | 6 (50%) | 6 (50%) |
| T2 (43)                          | 36 (83.7%) | 7 (16.3%) |
| T3 (35)                          | 27 (77.1%) | 8 (22.9%) |
| T4 (20)                          | 19 (95%) | 1 (5%) |
| N stage                          |                     |         |
| N0 (44)                          | 31 (70.5%) | 13 (29.5%) |
| N1 (24)                          | 17 (70.8%) | 7 (29.2%) |
| N2 (29)                          | 27 (93.1%) | 2 (6.9%) |
| N3 (13)                          | 13 (100%) | 0 (0%) |
| TNM stage  | I (23)          | II (33)         | III (54)        | 10 (43.5%) | 5 (9.3%)   | 0.003* |
|-----------|----------------|----------------|----------------|------------|------------|--------|
|           | 13 (56.5%)     | 26 (78.8%)     | 49 (90.7%)     |            |            |        |
| Survival  | 59 (98.3%)     | 29 (58%)       | 1 (1.7%)       | 21 (42%)   | 0.001*     |        |
| Dead (60) |                |                |                |            |            |        |
| Alive (50)|                |                |                |            |            |        |
| Disease free status | 68 (98.6%) | 20 (48.8%) | 1 (1.4%) | 21 (51.2%) | 0.001* |
| Progression (69) |            |                |                |            |            |        |
| No-progression (41) |            |                |                |            |            |        |

Abbreviation: FFPE: Formalin-Fixed Paraffin-Embedded

There were significant correlations in between CDKN2A (P16) protein expression and baseline characteristics of our study population of esophageal squamous cell carcinoma, which observed by bilateral $X^2$ test. In the 12 cases of stage T1 50% overexpressed and 50% non-overexpressed group, as well as in the stage T2, stage T3, and stage T4 respectively 36 cases (83.7%) out of 43 cases, 27 cases (77.1%) out of 35 cases, and 19 cases (95%) out of 20 cases were weakly expressed or not expressed. Also, we analyze lymph node and seen 31 (70.5%) N0 cases out of 44 cases, 17 (70.8%) N1 cases out of 24 cases, 27 (93.1%) N2 cases out of 29 cases, and 13 (100%) N3 cases out of 13 ESCC cases. And both variables are statistically significant and act as independent marker for disease diagnoses and treatment assessment. The baseline characteristics of 110 ESCC patients were summarized in Table 1.

Effect of P16 expression on survival & disease progression

![Figure 2:](image)

A. Kaplan-Meier analysis and log-rank test of P16 for OS of 110 patients. Low P16 protein expression significantly predicted decreased OS.

B. Kaplan-Meier analysis and log-rank test of P16 for PFS. Low P16 protein expression was significantly associated with decreased PFS.

C. Kaplan-Meier analysis and log-rank test of T stage, in accordance of low expression of P16 with OS. Stage T3, T4 shows poor OS with low expression of P16 in compare with stage T1, T2.

D. Kaplan-Meier analysis and log-rank test of T stage, in accordance of low expression of P16 with PFS. Stage T3, T4 shows poor PFS with low expression of P16 in compare with stage T1, T2.

E. Kaplan-Meier analysis and log-rank test of N stage, in accordance of low expression of P16 with OS. Stage N2, N3 shows poor OS with low expression of P16 in compare with stage N0, N1.

F. Kaplan-Meier analysis and log-rank test of N stage, in accordance of low expression of P16 with PFS. Stage N2, N3 shows poor PFS with low expression of P16 in compare with stage N0, N1. Abbreviations: OS: Overall Survival; PFS: Progression Free Survival.
We seen P16 lost its expression capacity in ESCC patients and it has effect on survival and recurrence of disease. From our total study population 60 patients were died during follow up, out of them 59 (98.3%) patients were lost of expression which is statistically significant (P=0.001) as well as 68 (98.6%) recurrent cases were from no expression group out of 69 cases which also statistically significant (P=0.001). The median survival month for our research population was 42 months (average 6-78 months). But within the baseline criteria's age, smoking, degree of differentiation was not significant, though gender & drinking habit were significant (P<0.05). Also, out of 22 overexpressed cases 21 (95.45%) cases were alive. To determine the correlation and prognostic importance we analyze our research data by Kaplan-Meier estimates using log-rank test to accomplish univariate analysis. Therefore, our analysis showed that low expression of P16 significantly down-regulated 5-year OS (26.4%, P=0.001) and 5-year PFS (18.2%, P=0.001) (Table 2; Figure 2A & 2B). Moreover, Regression analysis by Multivariate Cox-Regression method determined P16 down regulation act as an independent prognostic factor for OS (HR= 0.046, 95% CI=0.006-0.333; P=0.002) and PFS (HR=0.064, 95% CI=0.009-0.466; P=0.005) (Table 3).

Table 2: Univariate analysis of prognostic variables of 110 ESCC patients.

| Variables                        | 5-Year OS | 5-Year PFS |
|----------------------------------|-----------|------------|
|                                  | P value   | P value    |
| Age (<65 year vs. >65 year)      | 0.87      | 0.19       |
| Gender (Male vs. Female)         | 0.26      | 0.9        |
| Smoking (Yes vs. No)             | 0.46      | 0.6        |
| Drinking (Yes vs. No)            | 0.5       | 1.6        |
| Tumor stage (T3&T4 vs. T1&T2)    | 0.001*    | 0.001*     |
| Lymph node stage (N2&N3 vs. N0&N1) | 0.002*    | 0.001*     |
| TNM stage                        | 0.001*    | 0.001*     |
| Differentiation (well vs. moderate & poor) | 0.4       | 0.4        |
| P16 (overexpression vs. non-expression) | 0.001*    | 0.001*     |

* And bold values indicate statistically significant p value.

Table 3: Multivariate analysis of independent prognostic factors of 110 ESCC by cox regression method.

| Variables                        | 5-Year OS | 5-Year PFS |
|----------------------------------|-----------|------------|
|                                  | p value   | p value    |
| Age (<65 year vs. >65 year)      | 0.87      | 0.19       |
| Gender (Male vs. Female)         | 0.26      | 0.9        |
| Smoking (Yes vs. No)             | 0.46      | 0.6        |
| Drinking (Yes vs. No)            | 0.5       | 1.6        |
| Tumor stage (T3&T4 vs. T1&T2)    | 0.001*    | 0.001*     |
| Lymph node stage (N2&N3 vs. N0&N1) | 0.002*    | 0.001*     |
| TNM stage                        | 0.001*    | 0.001*     |
| Differentiation (well vs. moderate & poor) | 0.4       | 0.4        |
| P16 (overexpression vs. non-expression) | 0.001*    | 0.001*     |

*And bold p values indicate statistically significant values.

The Kaplan-Meier analysis also displayed that among the analyzed baseline indicators, conventional prognostic factors including tumor stage (P=0.001), lymph node (P=0.002), and degree of differentiation (P=0.001) were statistically significant in association with OS (Table 2; Figure 2C & 2E) as well as the tumor stage (P=0.001), lymph node status (P=0.001) and degree of differentiation (P=0.001) were statistically significant in association with PFS (Table 2; Figure 2D & 2F). The indicators that were statistically significant we further analyze them by multivariate cox-regression analysis due to determine the independent prognostic factor. In multivariate analysis, tumor stage in association with OS and PFS respectively Hazard ratio 1.57 with 95% confidence interval 1.19-2.06 & P value 0.001 and Hazard ratio 1.69 with 95% confidence interval 1.25-2.27 & P value 0.001. As well as the lymph node status in association with OS & PFS were respectively P=0.004 & P=0.001, which are statistically significant and act as promising prognostic indicator (Table 3).
Discussion

P16 is a cell cycle regulatory protein that encoded in CDKN2A gene in chromosome 9 (9p21.3), comprised of 3 exons, and codes for a 16 kDa protein [8]. In the cell cycle regulation this protein act as a tumor suppression gene and in the inactivated state it has association with tumorigenesis [9]. Promoter methylation habitually responsible for this inactivation. In some cancer still it is unclear of the prognostic effect of P16. This protein also known as MTSl, INK4a, CDKN2, and CDK4L. P16 resides the cycline kinase D1-CDK4/6 complex, that liable for the phosphorylation of the protein Rb, initiating arrest of cell cycle at stage G1 [10]. In the normal human cell, it is highly expressed and control the uncontrolled cell growth, while in the cancerous cell its expression is epigenetically repressed in approximately 30-40% [11-13]. As well as strong cytoplasmic staining of the protein detected in some cancers with or without HPV (human papilloma virus) infection-associated cancers, for instances cervical cancer, endometrial cancer, oral cancer, nasopharyngeal cancer, skin cancer, thyroid cancer, colorectal cancer and so on [14-17]. P16 is the major gene in the cell cycle regulation by its expression status it engaged in reverse regulation of cell proliferation and cause uncontrolled growth [18]. Several studies have exposed that down regulation of this gene expression causes the loss of its negative effects in CDK4/CDK6, as a result malignancy, abnormal cell proliferation and rapid development of tumor occur [19,20].

In many cancers we have seen this phenomenon. The prognostic and diagnostic prominence of this biomarker in the esophageal cancer has been assessed for several years, though the outcomes remain debatable. All the findings explained the importance of P16 expression status in the prognostic value determination, that the high expression of this protein shows positive clinical outcome and low expression show negative clinical outcome with decreased 5-year overall survival and recurrence. Fuj iwara et al. reported that "hypermethylation of P16 gene promoter correlates with loss of P16 expression that results in poorer prognosis in esophageal squamous cell carcinomas" [21]. So, result of this research work directed that CD133 immunoreactivity is a promising biomarker for the prognosis of ESCC. As well as, this CD133 may a major role in P27 & P16 in ESCC [22]. Another study demonstrated that P16 protein low expression associated with decreased overall survival [23]. However, one other work directed the researchers a linear relationship between up regulation of CDKN2A protein and the severity of histological transformation of mucosa ultimately causes malignant transformation. That was the controversial among many studies [24]. Yang et al. [25] demonstrated in his study that the survival status depends on P16 expression status in the patients with non-opharyngeal head & neck squamous cell carcinoma. In that study author showed the 5-year OS and PFS of P16 negative expression group respectively 57.7% & 38.6%, as well as in positive group 78.1% & 56.4%.

Past decades many studies done regarding P16 in ESCC showed different results. Taghavi et al. [26] demonstrates the P16 hypermethylation is the principal mechanism of P16 protein low expression and plays an important role in ESCC development. DNA methylation of P16 silencing the gene varies among the different group of tumors. Still the role of expression of P16 protein remains elucidated in many cancers. In Gastric cancer, we found that DNA methylation is responsible for P16 transcriptional silencing and causes neoplastic transformation [27].

To our best knowledge, our study is the first report that examining the expression status of P16 protein in esophageal squamous cell carcinoma patients to determine the postoperative prognostic status. And reveal that, low expressed CDKN2A protein in cancerous tissues had decreased 5-year overall survival and also recurrence of disease. As well as the patients with up regulated P16 are mostly alive (>95%). Hence, there is one limitation of our study that we had small study population and also lost some patient during follow up. Further large study group may be bringing more positive outcome in this study plan and more impact on diagnosis and treatment of ESCC.

Conclusion

Weak or no expression of P16 protein is significantly correlated with poor prognosis of esophageal squamous cell carcinoma patients. It has also correlation with positive lymph node and advance tumor stage. Therefore, this protein acts as a promising biomarker for postoperative prognosis and determines the further treatment protocols.

Conflict of Interests

The authors declare that they have no conflict of interest.

Informed Consent

Informed written consent taken from all patients.

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