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Review Article

Correlation of expression of Rb and P53 with short-term survival prediction and poor prognosis in esophageal carcinoma

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Abstract: Objective: To investigate the correlation of Rb and P53 with the prognosis of patients with esophageal carcinoma (EC). Methods: EC and corresponding adjacent tissues were collected from 51 patients who underwent EC surgery from February 2012 to February 2014 in Zhejiang Sian International Hospital. The expression levels of Rb and p53 in cancerous and adjacent tissues were measured by RT-PCR. Kaplan-Meier method and Log-rank test were employed to analyze the survival of patients. Cox regression model was applied to analyze the risk and prognostic factors of survival and prognosis of patients with EC. Results: EC tissues presented remarkably lower Rb and P53 levels compared with adjacent tissues ($P<0.05$). The Rb expression in EC tissues was related to differentiation degree and TNM staging ($P<0.05$). While apart from differentiation degree and TNM staging, the expression of P53 was also associated with lymph node metastasis (LNM). Kaplan-Meier revealed that the 5-year overall survival (OS) of Rb and P53 high-expression groups was dramatically higher than that of the corresponding low-expression groups ($P<0.05$). Cox regression analysis confirmed that Rb and P53 were independent risk factors affecting the prognosis of EC patients ($P<0.05$). Conclusion: Rb and P53 were lowly expressed in EC tissues and may be independent risk factors for predicting the prognosis of EC patients.

Keywords: Rb, P53, esophageal carcinoma, tumor markers, cox regression analysis

Introduction

Esophageal carcinoma (EC) is a malignant tumor worldwide, ranking the sixth cause of death from malignant tumors [1]. Among them, esophageal squamous cell carcinoma (ESCC) is the most common type of EC in China, and is the 4th leading cause of death from malignant tumors [2]. With the improvement of socio-economic status, lifestyle and medical standards, the morbidity and mortality of EC in China have been decreasing year by year, but the 5-year survival rate of patients is still overwhelmingly low, which is a treatment challenge at present [3, 4]. Therefore, finding molecular mechanisms in the development of EC is of great significance for clinical treatment and improvement of prognosis in patients [5].

The occurrence of EC is a multi-factor and multi-step process. At present, the discovery and presentation of various oncogenes and tumor suppressor genes have partially elucidated the mechanisms of tumorigenesis, and these research results are expected to point out new directions for tumor therapy [6, 7]. Located on the long arm of human chromosome 13 (13q14.2), retinoblastoma (Rb) gene is the first discovered tumor suppressor gene in human that is found to be directly or indirectly inactivated in almost all human tumor cells [8, 9]. Studies have shown that Rb functional deficiency is closely related to the development of the tumors [10]. While P53 gene is the most commonly involved tumor suppressor gene in human tumors. p53-centered regulatory network exerts marked effects on sustaining the

Table 1. Sequence of Rb, P53 and their internal reference primers

Gene	Forward primer	Reverse primer
Rb	5'-CAGGACAGCGGCCCGGAG-3'	5'-CTGC AGACGCTCCGCCGT-3'
P53	5'-TGCCAGCAGTGACAGCAGCA-3'	5'-TACGGAGGTGGAGTGCCAT-3'
GAPDH	5'-CCTCAGCATCTTATCCGAGTGG-3'	5'-TGGATGGTGGTACAGTCAGAGC-3'

normal state of cells and cell destiny, and its mediated cell signal transduction is involved in the process of tumor cell apoptosis, inhibition of angiogenesis, and arrest of cell cycle [11, 12]. Lv et al. [13] showed that MEG3 could activate p53 and predict the prognosis of ESCC, suggesting that p53 played an important role in the occurrence of EC.

As tumor suppressor genes, Rb and p53 genes are implicated in the regulation of tumor proliferation cell cycle [14, 15]. However, little research has been conducted to explore the correlation of their expression levels with survival and prognosis of EC patients. Therefore, by observing the expression levels of Rb and P53 in patients with EC, this study analyzed the correlation of their expression levels with the clinicopathological characteristics and prognosis of EC patients, so as to provide clinical references.

Materials and methods

General information

Paired EC and adjacent tissues were obtained from 51 patients who underwent EC surgery in Zhejiang Sian International Hospital from February 2012 to February 2014. Among the patients, there were 37 males and 24 females, aged 33-71 years, with an average age of 47.21±10.56 years. This study was approved by the Medical Ethics Committee of Zhejiang Sian International Hospital, and written informed consent was obtained from each patient. Inclusion criteria: All EC patients had not received chemoradiotherapy or other tumor treatment before surgery, and were confirmed as ESCC by pathological examination after surgery, with complete medical records. Exclusion criteria: Patients with autoimmune diseases, liver or kidney dysfunction; Patients with malignant tumors in other parts; Patients with trauma, surgery or severe infections within the last 3 months; Patients with a history or family history of mental illness; Patients in pregnancy or lactation; Patients with cardiac insufficiency; Patients with clinical data insufficiency.

Main instruments and reagents

ABI7500 real-time PCR instrument (ThermoFisher, 4351104); Total RNA extraction kit (Trizol method) (Chundu Biotechnology Co., Ltd., Wuhan, China, CD-13433); M-MLV reverse transcription kit (Shengke Boyuan Biotechnology Co., Ltd., Beijing, China, RTP50); UV spectrophotometer (Eppendorf, Germany, 6135000041); qReal-timePRC kit (Ziker Biological Technology Co., Ltd., Shenzhen, China, BIV-M1182-100); SYBR Green qPCR Master Mix kit (Kemin Biotechnology Co., Ltd., Shanghai, China, DXT-ST600548). The internal reference primers of Rb, P53 and GAPDH, which were detailed in **Table 1**, were synthesized by Shanghai Biotech Biotechnology Co., Ltd.

PCR detection

The tissues stored at -80°C were taken out, added with Trizol reagent, and placed at room temperature for 30 min after shaking. After full lysis, samples of EC tissues were taken out, of which 200 mg was cut and put into liquid nitrogen, and then quickly and fully ground for later use. After that, the total RNA of the tissues was extracted with Trizol reagent and dissolved in 20 μL DEPC water before it was reversely-transcribed into cDN using a reverse transcription kit. Reaction system: M-MLV: 1 μl, Olig (dT): 1 μl, RNase inhibitor: 0.5 μl, dNTPs: 1 μl, and RNAse free water was filled to make up to 15 μl. Then it was incubated at 38°C for 60 min. With cDNA as the template, quantitative PCR was performed according to the instructions of fluorescence quantitative PCR. The reaction conditions were as follows: 94°C/10 min, 94°C/30 s, 60°C/30 s, 72°C/40 s, cycling 40 times, and then it was extended at 72°C for 10 min. U6 was set as the internal reference control gene,

Table 2. Expression of Rb and P53 in esophageal and adjacent tissues (x ± sd)

Groups	n	Rb	P53
EC tissues	51	0.912±0.087	1.014±0.213
Adjacent normal tissues	51	1.337±0.092	1.146±0.134
t	-	23.97	3.746
P	-	<0.001	<0.001

and the relative expression level of Rb and P53 was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot detection of p53 and Rb protein expression in esophageal carcinoma

Proteins were extracted using MPERTM mammalian protein extraction kit (Pierce). Then 40 μg of total cell protein samples were taken for SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane. After being sealed at room temperature for 2 hours, the cells were rinsed with TBST buffer for 3 times, and then incubated with the above-mentioned p53 and p16 first antibody working solutions at 4°C overnight. Next, it was rinsed three times with TBST, added with horseradish peroxidase-labeled secondary antibody, and incubated at room temperature for 2 h. Finally, after the self-development of the chemiluminescence enhancing reagent, the X-ray film in the darkroom was photographed and imaged. Finally, the membrane was developed with chemiluminescence enhancement reagent, and the photographic image was obtained by X-ray film in the darkroom.

Follow-up

Follow-up was conducted by phone or visit. The survival period was counted from the date of diagnosis to death or February 30, 2019, which was the end of follow-up. The overall survival time was from the time of diagnosis of NSCLC to the time of death from any disease, the last follow-up, or the observation deadline. The survival period was recorded in months, and the end event was determined as death.

Statistical analysis

Data analysis was performed by SPSS22 (IBMCorp, Armonk, NY, USA). The measurement data were expressed as x ± SD and tested by two independent samples. The counting data were described by the number/percentage [n (%)] and analyzed by chi-square test. Kaplan-Meier method and Log-rank test were applied to analyze the survival of patients, and COX regression was used to analyze the relationship between patients' survival and gender, degree of differentiation, age, tumor diameter, tumor site, lymph node metastasis, TNM staging, Rb and p53 expression. The average of continuous measurement data was taken as the division point, and the dichotomous transformation was carried out. The selected factor data were quantitatively assigned according to the same standard, with the survival of patients as the dependent variable, and age, tumor diameter, tumor site, lymph node metastasis and TNM staging as the independent variables. P<0.05 was considered as a statistically significant difference.

Results

Expression of Rb and P53 in EC and adjacent tissues

As indicated by qRT-PCR, the expression levels of Rb and P53 in EC tissues were notably lower than those in adjacent tissues (P<0.05) (**Table 2**; **Figure 1**).

Rb and P53 protein expression in EC

Western blot results showed that Rb and P53 protein levels in EC tissues were significantly lower than those in adjacent tissues (P<0.05) (**Figure 2**).

Correlation of Rb and P53 with the clinicopathological characteristics of EC patients

The expression of Rb in EC was correlated with differentiation degree and TNM staging (P< 0.05), but was not associated with gender, age, tumor length or tumor diameter (cm), tumor site or LNM (P>0.05). While the expression of P53 was related to differentiation degree, LNM and TNM staging (P<0.05), but was not related to gender, age, or tumor site (P>0.05) (**Table 3**).

Survival of EC patients

According to the median value of Rb and p53, the EC patients were divided into the Rb high-expression group (P≥0.918) with 26 cases, the

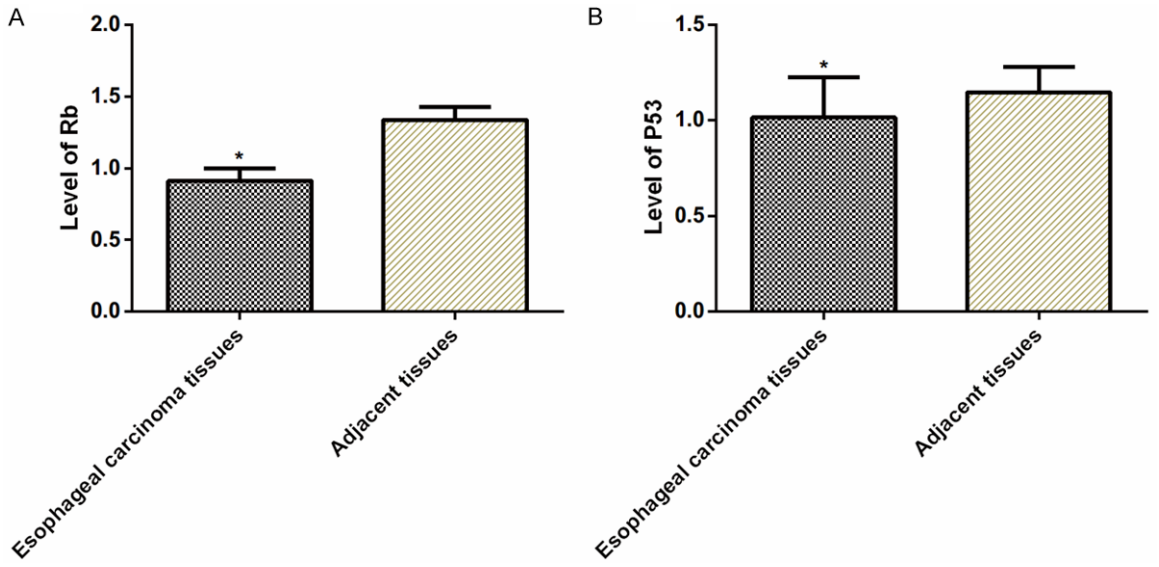

Figure 1. Expression of Rb and P53 in EC and adjacent tissues. QRT-PCR results showed that the expression levels of Rb and P53 in EC tissues were significantly lower than those in adjacent tissues (P<0.05). * indicated P<0.05 compared with the adjacent tissues.

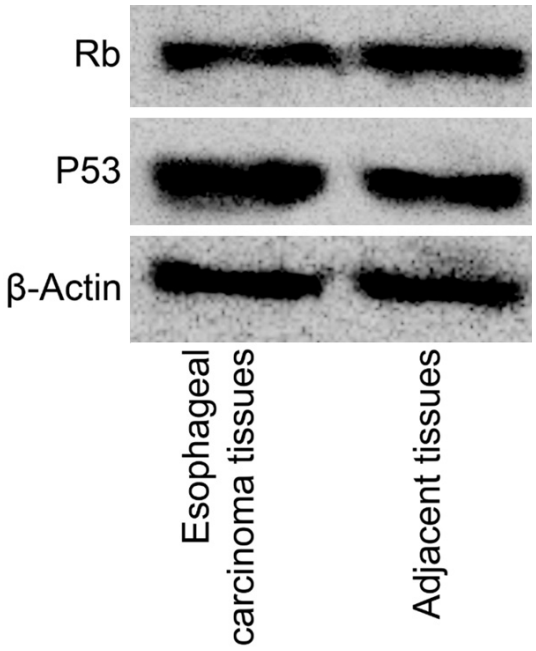

Figure 2. Protein expression of Rb and P53 in esophageal carcinoma. Western blot results showed that the protein levels of Rb and P53 in esophageal carcinoma tissues were significantly lower than those in adjacent tissues (P<0.05).

Rb low-expression group with 25 cases (P< 0.918), the P53 high-expression group with 26 cases (P≥1.041), and the P53 low-expression group with 25 cases (P<1.041). The patients were followed up by telephone, review and letter for 5 years. It was found that the 5-year OS of the Rb high-expression group was 38.46% (10/26), while that of the Rb low-expression group was 16.00% (4/25). The 5-year OS in the P53 high-expression group was 34.62% (9/26), while that in the P53 low-expression group was 20.00% (5/25). From the above data, it was obvious that the 5-year OS of the Rb and P53 high expression groups was dramatically superior to that of the corresponding low expression groups (P<0.05) (**Figure 3**).

Univariate analysis of factors influencing the prognosis of EC patients

Univariate analysis revealed that differentiation degree, tumor diameter, LNM, TNM staging, Rb and p53 were related to the survival time of EC patients (**Table 4**).

Multivariate analysis of factors influencing the prognosis of EC patients

We included the indexes with differences in univariate analysis into the assignment (see **Table 5** for the assignment table). Multivariate Cox proportional hazard regression model analysis demonstrated that LNM, TNM staging, Rb and p53 expression were independent risk factors influencing the prognosis of EC patients (P< 0.05) (**Table 6**).

Table 3. Corrrelation between Rb and P53 and the clinicopathological characteristics of EC patients (x ± sd)

Clinicopathological characteristics	n	Rb (n=51)	t/F value	*P* value	P53 (n=51)	t/F value	*P* value
Gender						0.725	0.4711
Male	37	0.902±0.065	1.392	0.169	0.987±0.249		
Female	24	0.931±0.098			1.032±0.216		
Age			1.373	0.176		0.504	0.6165
<60	34	0.895±0.069			1.007±0.251		
≥60	17	0.927±0.095			1.043±0.217		
Differentiation degree			3.579	0.036		17.030	<0.01
High differentiation	9	0.953±0.062			1.306±0.216		
Moderate differentiation	19	0.944±0.107			1.257±0.192		
Low differentiation	23	0.873±0.102			0.944±0.203		
Tumor diameter (cm)			0.846	0.436		1.708	0.1921
≤3	23	0.904±0.068			1.005±0.191		
3-7	25	0.931±0.082			1.118±0.237		
≥7	3	0.899±0.083			1.012±0.23		
Tumor site			0.829	0.443		2.27	0.114
Upper	12	0.917±0.084			0.988±0.208		
Middle	24	0.923±0.118			1.105±0.174		
Lower	15	0.882±0.074			0.994±0.201		
LNM			1.983	0.053			<0.01
Yes	11	0.879±0.08			0.96±0.273		
No	40	0.939±0.091			1.171±0.204		
TNM staging			4.383	<0.01		3.383	<0.01
I+II	37	0.957±0.068			1.186±0.211		
III	14	0.862±0.072			0.902±0.173		

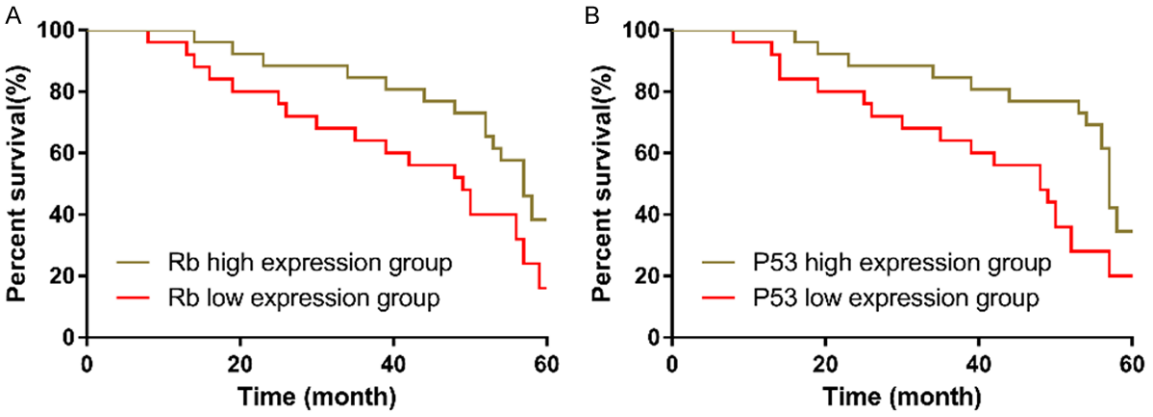

Figure 3. Relationship between the expression levels of Rb and P53 in EC and the survival time of patients.

Discussion

Esophageal carcinoma (EC) is one of the six most prevalent malignant tumors in the world. Its occurrence and development is a multi-step, multi-factor and multi-gene process, which involves the acquisition or deletion of multiple alleles, activation of proto-oncogenes, loss of function of tumor suppressor genes and changes in the expression of downstream protein products [16-18]. However, its specific pathogenesis remains poorly understood [19].

Table 4. Univariate analysis of factors influencing the prognosis of EC patients (x ± SD)

Clinicopathological features	n	Median survival time (months)	t value	*P* value
Gender			1.509	0.137
Male	37	38.23±4.51		
Female	24	39.92±3.87		
Age			0.776	0.442
<60	34	39.67±5.42		
≥60	17	38.45±5.02		
Differentiation degree			3.524	0.037
High differentiation	9	41.13±3.52		
Moderate differentiation	19	39.41±3.18		
Low differentiation	23	37.54±3.97		
Tumor diameter (cm)			10.560	<0.01
≤3	23	41.35±3.64		
3-7	25	38.26±3.45		
≥7	3	32.13±5.21		
Tumor site			1.355	0.268
Upper	12	41.24±3.21		
Middle	24	39.45±3.06		
Lower	15	39.23±4.23		
LNM			3.518	<0.01
Yes	11	36.42±2.89		
No	40	40.19±3.21		
TNM staging			4.990	<0.01
I+II	37	41.59±4.17		
III	14	34.26±5.87		
Rb			2.216	0.031
Rb high expression	26	41.27±5.16		
Rb lower expression	25	38.25±4.54		
P53			2.069	0.044
P53 high expression	26	41.77±4.62		
P53 low expression	25	39.27±3.97		

Initially found in retinoblastoma, RB gene is also closely related to EC, soft tissue sarcoma, small cell lung cancer and so on [20-22]. In mammalian cell lines, Rb gene acts as the central regulator in the process of cell cycle. The encoded RB protein, together with the structurally and functionally related P107 and P130 proteins, constitute the lactoprotein family, which can inhibit the activity of E2F transcription factor and thus stop cells in G1 phase, thereby playing a tumor inhibition role [23]. While P53 is a newly discovered multiple tumor suppressor gene located on the short arm of human chromosome 17 and is a typical tumor suppressor gene. It participates in the process of cell proliferation and differentiation, which can block the cell cycle in G1/G2 phase, regulate and induce cell apoptosis, and function as a tumor suppressor gene. Its expression product is P53 protein, which can inhibit the division of cells with DNA damage and chromosome aberration, thereby preventing the transmission of aberrations to daughter cells [24]. In current study, we comparatively analyzed Rb and P53 expression levels in EC and adjacent tissues. The results exhibited that the expression levels of Rb and P53 in EC tissues were markedly lower than those in adjacent tissues (P<0.05), indicating that Rb and P53 may be related to the occurrence and development of EC. Previous studies have demonstrated that Rb and P53 are related to EC. For example, Davelaa et al. [25] revealed that phosphorylation of S795 residues in RB protein was enhanced. In the study of Wang et al. [26], it was found that p53 was activated and down-regulated by long non-coding RNA maternal gene 3 in ESCC. Another study conducted by Kochi et al. [27] exhibited that the p53 (S-P53-AB) titer of serum antibodies was of great significance in monitoring locally advanced ESCC after surgery. All these suggest that RB and P53 may participate in the occurrence and progression of EC, but they had not explored the relationship between the two and the prognosis of EC.

In addition to the common clinical indicators such as LNM and clinical stage, molecular pathological studies demonstrated that the disorder and abnormal expression of various genes and proteins, such as p27 and HIF, were closely associated with the occurrence, proliferation, invasion and metastasis of EC, and were important factors affecting the prognosis of EC patients [28, 29]. Therefore, we discussed the correlation of the expression levels of P53 and RB with the prognosis of patients and other

Table 5. Assignment table

Factors	Assignments
Differentiation degree	Low differentiation =0, moderate differentiation =1, high differentiation =2
Tumor diameter	≤3=0, 3-7=1, ≥7=2
LNM	Yes =1, no =0
TNM staging	I+II =1, III =0
Rb	High expression =1, low expression =0
P53	High expression =1, low expression =0
Survival	Death =1, survival =0

Table 6. Multivariate analysis of factors influencing the prognosis of EC patients

Variables	β	SE	Wald χ^2	OR (95% confidence interval)	*P* value
TNM staging	1.314	0.418	7.512	3.42 (1.39-8.43)	0.008
LNM	1.283	0.269	6.343	4.13 (3.46-6.52)	0.009
Rb	1.578	0.208	12.745	4.65 (2.78-8.51)	<0.01
P53	1.602	0.315	14.127	5.25 (3.87-7.92)	<0.01

factors affecting their prognosis. A five-year OS study of EC patients revealed that the 5-year OS of the Rb high expression group was 38.46% (10/26), and that of the Rb low expression group was 16.00% (4/25), which indicated that the 5-year OS in the Rb high expression group was markedly higher than that of the Rb low expression group (P<0.05). In addition, the 5-year OS in the P53 high expression group (34.62%, 9/26) was remarkably higher than that of the P53 low expression group (20.00%, 5/25) (P<0.05). The above results indicated that the expression levels of Rb and P53 were related to the survival of patients. Then univariate analysis displayed that differentiation degree, tumor diameter, LNM, TNM staging, Rb and p53 were related to the survival time of EC patients. Further, the indicators with differences in univariate analysis were included in the assignment for multivariate Cox proportional hazard regression model analysis, which validated that TNM staging, LNM, Rb and p53 expression were indeed the independent risk factors influencing the prognosis of EC patients. All the above findings confirmed that Rb and p53 can be used as clinical indexes to evaluate the prognosis of patients with EC. Therefore, we can preliminarily conclude that Rb and p53 may play an important part in the occurrence, development and prognosis of EC.

Although the present study comparatively analyzed the expression levels of RB and P53 in EC and adjacent tissues, confirming their role in prognosis, but there are still some deficiencies. First of all, the role of serum RB and p53 in the efficacy and prognosis of EC patients remains to be explored. Second, the regulatory mechanism of the two still needs to be investigated. These deficiencies will be addressed in future studies.

In summary, RB and P53 present low expression in EC tissues and are expected to be independent risk factors for predicting the prognosis of EC patients.

Disclosure of conflict of interest

None.

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