Original Article
Prognostic significance of p27Kip1, PLK1 aberrant expression in esophageal squamous cell carcinoma

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Abstract: Objectives: To investigate the correlation between expression of p27Kip1 (p27) and Polo-like kinase1 (PLK1) in the cancerous and adjacent non-cancerous tissues of esophageal squamous cell carcinoma (ESCC) which had the clinicopathologic staging of ESCC and prognosis of patients. Methods: Quantitative real-time polymerase chain reaction and immunohistochemical detection for the expression of p27 and PLK1 (mRNA and protein) were performed on 70 paired cancerous and adjacent non-cancerous esophageal specimens. P27 and PLK1 protein expression was performed on 20 paired cancerous and adjacent non-cancerous esophageal specimens by western blot assays. Results: The relative expression of p27 was markedly lower in 46 of the 70 cases of cancerous tissues compared with the adjacent non-cancerous tissues (66%), with the relative expression higher in the other 24 cases (34%) (P<0.01), P27 relative protein expression decreased in 20 cases of cancerous tissues compared with adjacent non-cancerous tissues (85%, P<0.01), while the relative expression of PLK1 was markedly higher in 63 of the 70 cases of carcinoma tissues compared with the adjacent non-cancerous tissues (90%), with the relative expression lower in the other 7 cases (10%) (P<0.01). PLK1 relative protein expression increased in 20 cases of cancerous tissues compared with adjacent non-cancerous tissues (90%, P<0.01). Moreover, the relatively high PLK1 expression and the relatively low p27 expression in the cancerous tissues were correlated with pathological progression, lymph node metastasis and clinical staging (P<0.05). The patients with PLK1 combined with p27 positive expression in the cytoplasm of cancerous cells suffered a remarkably shortened post-operative survival compared with the patients who had PLK1 combined with p27 positive expression in the nuclei of cancerous cells (P<0.01). Conclusions: p27 down-regulation and PLK1 up-regulation in ESCC carcinoma tissues are closely related to upgrading of clinicopathologic staging. PLK1 combined with p27 positive expression in the cytoplasm of cancerous cells indicates poorer prognosis compared with that in the nuclei of cancerous cells.

Keywords: Esophageal squamous cell carcinoma, prognosis, gene, protein, clinicopathologic staging

Introduction

Esophageal cancer is one of the common malignancies of the digestive tract, which has a development concerning with the activation of oncogenes and the inactivation of cancer suppressor genes. The pathogenesis on the genetic and molecular level has been enthusiastically studied. The mutation and methylation of p27Kip1 (p27) gene have been rarely reported in tumor cells [1, 2]. p27, as a member of CDK1, negatively regulates the cell cycle, while it regulates tumor cells predominantly via the degradation of p27 protein as well as the subcellular localization and activity regulation of proteins [3]. Polo-like kinase1 (PLK1) is very important in mitosis, playing multiple roles in centrosome maturation, spindle body formation, chromosome and cytoplasm separation [4, 5], etc. PLK1 overexpression has now been detected in multiple solid tumors, such as esophageus cancer, gastric cancer, liver cancer, colorectal cancer, etc. [6-10] and correlates to poor prognosis of patients.

Seventy paired cancerous and adjacent non-cancerous esophageal specimens of ESCC cut off by surgery were collected. The gene and pro-
tein expression of p27 and PLK1 in these tissues and the subcellular localization of p27 protein were detected. The correlation between the expression of PLK1 and p27 and prognosis of patients was analyzed.

**Materials and methods**

**Materials**

*Case data:* Seventy cases of ESCC specimens (including cancerous and adjacent non-cancerous tissues) cut off by surgery in Yancheng Tumor Hospital from January, 2007 to December, 2012 were collected. The patients, aged from 35 to 80 years with an average age of 57.03 years, had not received radiotherapy or chemotherapy before radical surgery. The cancerous and adjacent non-cancerous tissues (>5 cm from tumor) were stored in liquid nitrogen immediately after surgery and then transferred to a -80°C refrigerator. The 70 cases of ESCC tissues confirmed pathologically were subjected to clinical staging according to the TNM staging system for esophage cancer, the 7th edition (2009). The post-operative follow-up visit continued until December 31, 2012, and two cases lost follow-up. The protocol was approved by the Ethics Committee of this hospital and the informed consent was signed.

*Reagents and instruments:* GAPDH, p27, PLK1 primers, Trizol (Invitrogen, USA); qRT-PCR kit and RNA RT-PCR kit (DDR047A) (TaKaRa, China); real-time fluorescence quantification PCR instrument (ABI7500) (AB, USA); p27, PLK1 McAb (Abcam, USA).

**Methods**

*RNA extraction and real-time fluorescence quantification PCR detection:* Cryopreserved tissues (30~80 mg) were pulverized with 1 mL Trizol, and then RNA was extracted. Using a spectrophotometer, RNA concentration was measured and RNA purity was qualified when the value of A260/A280 ranged from 1.8 to 2.1. Based on the instructions of RNA RT-PCR kit (Prime-Script TM), cDNA was synthesized by the two-step method. QRT-PCR (SYBR Green kit) was performed according to the instructions and three repetitions were set for each sample. For p27, the upstream primer: 5’-TGCAA-CGGAGGTCCCT-3’ and the downstream primer: 5’-CAAGCAGTGATGTATCGATAA ACAAGG-3’; for PLK1, the upstream primer: 5’-AAGAGATCCCGAGGTCCCT-3’ and the down-stream primer: 5’-TCATCTAGGAAAGGCCTG-3’; for GAPDH, the internal reference, the upstream primer: 5’-GTCAGTGAGCTGAGCT-3’ and the downstream primer: 5’-AGGGGCTCATA-TGCACTG-3’. The relative expression of p27 and PLK1 in the cancerous and adjacent non-cancerous tissues was calculated by 2-ΔΔct method. The relative expression <1 indicated down-regulation in the cancerous tissues, or otherwise, up-regulation.

*Immunohistochemical detection on p27 and PLK1 in the esophagus tissues:* Immuno-histochemical detection (S-P method) was performed to detect the protein expression of p27 and PLK1 in the cancerous and adjacent non-cancerous tissues by the Department of Pathology strictly according to operating instructions. Brown-stained nuclei or cytoplasm indicated positive. Section reading was conducted under the same condition. Two hundred cells/visual fields were counted and a total of 1000 cells were selected. Positive determination: positive cells/total cells <10%; (−); 10~19%; (±); 20~50%; (+); ≥50%; (++).

*Western blot assayed P27, PLK1 protein expression of cancerous tissues and adjacent non-cancerous tissues in patients of esophageal squamous carcinoma*

The proteins were extracted by reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland) and PMSF (Roche). The concentration of total protein was quantitated by BCA Protein Assay Kit (Beyotime). Then protein was electrophoresed by 4-12% SDS-PAGE, transferred onto nitrocellulose membranes (Sigma), and incubated with 5% defatted milk including specific primary antibody. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad, Hercules, California). β-actin antibody was used as control. In addition, rabbit anti-bcl2 (1:1,000) was purchased from Abcam; anti-P27, anti-PLK1 (1:1,000) were from Sigma.

**Statistics**

The data were analyzed with SPSS 19.0 and P<0.05 indicated statistical significance. The correlation between the differential mRNA expression of p27 and PLK1 and clinicopathologic data was analyzed with Kruskal-Wallis
Clinical significance of p27Kip1, PLK1 expression

Table 1. Correlation between PLK1 relative expression in matched cancerous tissues and adjacent noncancerous tissues and clinicopathological characteristics of esophageal squamous cell carcinoma (ESCC)

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(Kruskal-Wallis Test), *P<0.05.

Table 2. Correlation between P27 relative expression in matched cancerous tissues and adjacent noncancerous tissues and clinicopathological characteristics of esophageal squamous cell carcinoma (ESCC)

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(Kruskal-Wallis Test), *P<0.05.

Test. Categorical data were analyzed with Chi-square test or Fisher’s exact test. Kaplan-Meier method was employed to analyze the post-operative 5-year survival difference of the patients with both PLK1 and p27 positive expression in the cytoplasm of cancerous cells or with that in the nuclei of cancerous cells.

Results

Correlation between the mRNA expression of p27 and PLK1 in the cancerous and adjacent non-cancerous tissues of ESCC and clinicopathologic data

The relative mRNA expression of PLK1 was markedly higher in 63 of the 70 cases of carcinoma tissues compared with the adjacent non-cancerous tissues (90%) with the relative expression lower in the other 7 cases (10%) (Wilcoxon test, P<0.01), while the relative mRNA expression of p27 was markedly higher in 24 of the 70 cases of cancerous tissues compared with the adjacent non-cancerous tissues (34%) with the relative expression lower in the other 46 cases (66%) (Wilcoxon test, P<0.01). The relative expression values of p27 and PLK1 among the 70 cases were divided into three categories by tertiles: high, medium and low expression, and then compared with the clinicopathologic data. The result indicated that the expression levels of p27 and PLK1 did not significantly correlate with age, gender or tumor differentiation (P<0.05) but correlated with the tumor clinicopathologic staging (TNM staging), lymph node metastasis and tumor infiltration. The relative down-regulation of p27 and the relative up-regulation of PLK1 coincided with clinicopathologic staging of ESCC and lymph node metastasis (See Tables 1 and 2).
Clinical significance of p27^Kip1, PLK1 expression

Protein expression of p27 and PLK1 in the ESCC tissues

In 70 cases of ESCC and paracancerous tissues, PLK1 was expressed in cancer and adjacent tissues (Figure 1), with 62 cancerous tissues showing a strongly positive expression (Figure 1B); p27 expression to varying degree was detected mainly in the nuclei of nearly all the adjacent non-cancerous tissues (Figure 1C), and 44 cancerous tissues showed a mainly weakly positive expression in the nuclei with the expression of another 24 cases mainly in the cytoplasm (Figure 1D). The expression differences between the cancerous and the adjacent non-cancerous tissues were all statistically significant (p27, χ²=4.581, P=0.045; PLK1, χ²=18.923, P=0.005).

Figure 1. The patients of PLK1 with P27 positive expression of cytoplasm was compared with that of PLK1 with P27 positive expression of nucleus in cancerous tissue. A. Protein expression of PLK1 almost were in cell nucleus of adjacent noncancerous (*×20); B. Protein expression of PLK1 were strongly in cell nucleus of cancerous tissues (*×20); C. Protein expression of P27 were in cell nucleus of adjacent noncancerous (*×20); D. Protein expression of P27 were almost in cytoplasm of cancerous tissues (*×20); E. 24 cases with PLK1 combined with P27 positive expression of cytoplasm were compared with 20 cases with PLK1 combined with P27 positive expression of nucleus in 40 cases of PLK1 and P27 positive expression totally and Kaplane-Meier curves indicate survival significantly shortened with P27 positive expression of cytoplasm after surgery (P=0.002, log-rank test).
Clinical significance of p27<sup>kip1</sup>, PLK1 expression

**Correlation between the protein expression of p27 and PLK1 in the cancerous tissues of ESCC and the subcellular localization of p27 with post-operative survival**

Among the 44 cases of cancerous tissues showing positive protein expression of both p27 and PLK1, 24 cases showed positive expression of PLK1 and p27 in the cytoplasm of cancerous cells and 20 cases showed positive expression of PLK1 and p27 in the nuclei of cancerous cells. The Kaplan-Meier analysis indicated that the patients with positive expression of PLK1 and p27 in the cytoplasm of cancerous cells suffered a remarkably shortened post-operative survival (P=0.002, log-rank test) (See Figure 1E).

The expression levels of P27, PLK1 were analyzed by western-blot analysis and β-actin was used as control

P27 relative protein expression decreased in 20 cases of cancerous tissues compared with adjacent non-cancerous tissues (85%, P< 0.01). PLK1 relative protein expression increased in 20 cases of cancerous tissues compared with adjacent non-cancerous tissues (90%, P<0.01) (Figure 2).

**Discussion**

p27 is a heat-stable protein with a molecular weight of 27×103, discovered by Polyak, etc. in 1994 and entitled p27<sup>kip1</sup> (kinase inhibit protein 1). Human p27 gene is located at the 12p13 region, containing two introns and two exons. P27 mRNA is 2.5 kb and the cDNA is 594 bp encoding 198 amino acids, which is highly conserved in evolution [11, 12]. The premise of p27 protein displaying biological activities is its entry into the nucleus which is mediated by the nuclear localization signal (153–166). P27 can bind with cyclin and cyclin-dependent kinase (CDK), inhibiting the kinase activity of nearly all CDK-cyclin complexes, so as to negatively regulate the cell cycle [2]. The genetic mutation or deletion of p27 is rarely reported, but the changes of the expressive abundance and the subcellular distribution of p27 protein closely relate to oncogenesis. P27 abundance in cytoplasm positively correlates with adhesive growth of cells. The change of the subcellular localization of p27 (from nuclei to cytoplasm) promotes dissemination and invasion of tumor cells, indicating the relationship between high p27 expression in cytoplasm and the promotion of cellular metastasis. High p27 expression can be detected in the cytoplasm of multiple metastatic tumors, such as breast cancer, non-small cell carcinoma, etc. [1, 13, 14].

In this study, a lower mRNA expression level of p27 was found in the cancerous tissues of ESCC than that in the paired adjacent non-cancerous tissues, which correlated to clinicopathologic staging and lymph node metastasis. Immunohistochemical analysis indicated that p27 protein was located in the cytoplasm of several cancerous cases, but located mainly in the nuclei of the adjacent non-cancerous tissues. The malposition and down-regulated expression of p27 in ESCC cancer cells are consistent with the reported high p27 expression in the cytoplasm of multiple tumors. Furthermore, the mRNA expression of PLK1 was remarkably up-regulated in the cancerous tissues than that in the adjacent non-cancerous tissues, which was associated with clinicopathologic staging and lymph node metastasis. The expressive difference between p27 and PLK1 mRNA in the cancerous tissues does not significantly correlate to distant metastasis, but possibly correlates to the loss of the opportunity to receive surgery for patients with distant metastasis. There is uncertainty about the data due to only one case of distant metastasis. Immunohistochemical analysis indicated positive protein expression of PLK1 in nearly all the cancerous and adjacent non-cancerous tissues, with the nuclei of the cancerous tissues showing strongly positive expression. The samples with PLK1 combined with p27 positive expression in the cytoplasm of cancerous cells and the samples with positive expression of PLK1 and p27 in the nuclei of cancerous cells...
Clinical significance of p27Kip1, PLK1 expression

were screened out, and the corresponding 5-year follow-up data were then analyzed. It was found that positive expression of PLK1 and p27 in the cytoplasm of cancerous indicated a remarkably shortened post-operative survival and poorer prognosis.

Many intracellular proteins and factors engage in the transcription, translation and degradation of p27 protein. With the synergistic effects of multiple carcinogenic agents, intracellular p27 is down-regulated, which removes p27 inhibition on Rb protein phosphorylation resulted from CDKs, leading to cell cycle disorder. The cells then turn from G1 phase to S phase, with enhanced synthesis of DNA and excessive cellular hyperplasia, causing oncogenesis. For mice with p27 allelic deletion, the susceptibility to cancerogen-induced tumors increases and intracellular cyclinD1 is up-regulated, assisting cells to pass G1-S checkpoint and promoting malignant proliferation [15, 16]. According to Feng YB et al. [17], PLK1 regulates β-catenin expression with the assistance of proteasomes. PLK1 up-regulation inhibits the ubiquitination of β-catenin, while PLK1 down-regulation promotes the ubiquitination of β-catenin. PLK1 overexpression impacts the mutual combination among GSK-3β, β-catenin and β-TrCP, which impedes the ubiquitination of β-catenin, and promotes β-catenin accumulation. The increasing interaction between β-catenin and TCF/LEF up-regulates the downstream expression of cyclinD1 and the oncogene c-Myc, promoting cellular proliferation and oncogenesis.

In this study, p27 down-regulation and PLK1 up-regulation simultaneously promoted the expression of cyclinD1 and the activation of c-Myc in various pathways, which promoted PLK1 up-regulation and exacerbated tumor cell division and malignant proliferation through feedback [18, 19]. The malposition of p27 in cytoplasm which decreased p27 inhibition on the negative regulation of cellular proliferation, combined with PLK1 up-regulation, further promoted the malignant proliferation of tumor cells, probably causing poor prognosis. The prognosis of patients can be determined more accurately with histological detection of the gene expression and the protein expression as well as distribution of p27 and PLK1, further laying theoretical foundation for the gene target therapy of esophagus cancer.

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Disclosure of conflict of interest

None.

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Clinical significance of p27<sup>Kip1</sup>, PLK1 expression


