

# Promoter methylation-associated silencing of p27kip1 gene with metastasis in esophageal squamous cell carcinoma

YANG LING<sup>1\*</sup>, CHANGSONG ZHANG<sup>1\*</sup>, YUN XU<sup>2\*</sup>, JING ZHU<sup>1</sup>, CHANGTAI ZHU<sup>1</sup>,  
MINGZHU LU<sup>1</sup>, YONGPING LIU<sup>1</sup> and TIANBAO ZHOU<sup>3</sup>

<sup>1</sup>Clinical Oncology Laboratory, Changzhou Tumor Hospital, Medical College of Soochow University, Changzhou, Jiangsu 213001; <sup>2</sup>Department of Oncology, Nanyang Center Hospital, Nanyang, Henan 473000;

<sup>3</sup>The Hepatobiliary Surgery Center, The Affiliated Ningbo No. 2 Hospital, Ningbo University School of Medicine, Ningbo, Zhejiang 315010, P.R. China

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**Abstract.** The aim of the present study was to determine the frequency of p27kip1 promoter methylation in esophageal squamous cell carcinoma (ESCC). The methylation status of the p27kip1 promoter was analyzed by methylation-specific polymerase chain reaction (MSP) in 50 ESCC and matched non-tumor tissues. Cell lines were treated with the demethylation agent 5-aza-2'-deoxycytidine (5-Aza-CdR) and p27kip1 mRNA expression was detected by quantitative polymerase chain reaction. p27kip1 methylation was found in 36% (18/50) of ESCC patients, but only in 12% (6/50) of the corresponding non-tumor tissues (P=0.005). There were statistically significant associations between the presence of methylation and tumor metastasis (P=0.002). The p27kip1 mRNA was lower in ESCC compared with non-tumor tissues (mean  $\pm$  standard deviation,  $-0.886 \pm 3.298$  vs.  $0.988 \pm 0.257$ ; P=0.0033). Furthermore, a significant association was identified between the methylation status of the p27kip1 promoter and p27kip1 mRNA expression in the tissue (P<0.01). Thus, demethylation by 5-Aza-CdR was capable of inducing p27kip1 mRNA expression in esophageal cancer cell lines. The high promoter methylation of p27kip1 is a common phenomenon in ESCC,

which may be an important mechanism of silencing p27kip1 mRNA expression.

## Introduction

Esophageal squamous cell carcinoma (ESCC) was once the dominant type of esophageal malignancy in Western and Asian countries and China has the highest incidence of esophageal carcinoma in the world (1). The molecular mechanisms leading to neoplastic progression in esophageal carcinoma have not yet been fully understood.

A group of protein kinases known as cyclin-dependent kinases (CDKs) regulate progression through the cell cycle. Two classes of proteins, CDK inhibitors, negatively regulate the cell cycle by binding to and inhibiting CDKs. INK4 proteins, p15, p16, p18 and p19, specifically inhibit the CDK4/6 kinases, whereas Cip/Kip proteins, p21cip1, p27kip1 and p57, target the majority of cyclin-CDK complexes (2). The p27kip1 protein promotes proliferation or blocks cell cycle progression by inhibiting the activity of CDK, most notably CDK2. p27kip1 binds and inhibits cyclin E- or cyclin A-associated CDKs, and negatively regulates G1-G2 cell cycle progression (3). p27kip1 may be a potential tumor suppressor gene, which is involved in the regulation of apoptosis and cell motility (4). p27kip1 contributes to the repression of Sox2 during embryonic stem cell differentiation (5). It is hypothesized that combined deficiency in p21 and p27kip1 proteins in mice is linked to more aggressive spontaneous tumorigenesis, resulting in a decreased lifespan (6).

Alterations in the expression of p27kip1 cause deregulation of cell growth and differentiation, and promote the development of a variety of human tumors (7). A reduction in the level of p27kip1 protein contributes to tumor development by allowing an increase in CDK2 activity and cell proliferation (8). Reduced p27kip1 expression has been associated with the development of human epithelial tumors originating from the majority of human organs, including the esophagus (9). In gastric adenocarcinoma, low p27kip1 protein expression is associated with poorly differentiated and advanced tumors, and is a negative prognostic factor of potential clinical value (10,11). The developing data in human

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*Correspondence to:* Dr Tianbao Zhou, The Hepatobiliary Surgery Center, The Affiliated Ningbo No. 2 Hospital, Ningbo University School of Medicine, 328 Wangjing Road, Ningbo, Zhejiang 315010, P.R. China  
E-mail: hbzcs@126.com

\*Contributed equally

*Abbreviations:* ESCC, esophageal squamous cell carcinoma; MSP, methylation-specific PCR; 5-Aza-CdR, 5-aza-2'-deoxycytidine

*Key words:* 5-aza-2'-deoxycytidine, p27kip1, esophageal carcinoma, methylation

tumors suggest that p27kip1 may prove to be a useful clinical tool even before the mechanisms of p27kip1 inactivation are completely understood.

The altered methylation of the promoter region in tumor-associated genes may lead to transcriptional silencing, which has increasingly been cited in a number of human cancers (12). However, promoter hypermethylation has been reported as a common mechanism of p27kip1 inactivation occurring at varying frequencies. The hypermethylation of p27 may lead to the loss of p27kip1 mRNA transcription. The reduction or loss of p27kip1 protein and mRNA is potentially involved in hepatocarcinogenesis (13). Silencing of the p27kip1 gene resulting from increased methylation of the promoter region has also been observed in rodent pituitary tumor cells (14). Previous studies suggest that the evaluation of p27kip1 expression is likely to aid in improving cancer diagnosis, prevention and treatment. Therefore, clarification of the mechanisms and agents that regulate p27kip1 expression and activity in tumor cells is an area of high priority in cancer research (15,16). The aim of the current study was to analyze the promoter methylation status of the p27kip1 and mRNA expression in ESCC patients in China.

## Patients and methods

**Patients and samples.** Tumor samples from 50 esophageal cancer patients who underwent surgical resection between 2004 and 2012 were investigated, together with their adjacent non-tumor tissues. These specimens were collected from Changzhou Tumor Hospital (Changzhou, China). The present study was conducted in accordance with the ethics standards of the Committee on Human Experimentation of Soochow University (Changzhou, China). All samples were obtained during treatment procedures under curative intent. The samples were frozen in liquid nitrogen immediately following surgical resection and were maintained in liquid nitrogen for long-term storage until processing for RNA/DNA extraction.

**Cell treatments.** The ECa-9706 and TE-1 esophageal cancer cell lines (Shanghai Institutes for Biological Sciences, Shanghai, China) were maintained in the Clinical Oncology Laboratory of Changzhou Tumor Hospital. Cells were cultured in RPMI medium (Invitrogen China Ltd., Beijing, China) containing 10% fetal bovine serum. Cells were cultured in a humidified 37°C incubator containing 5% CO<sub>2</sub>. Cells were plated (3x10<sup>5</sup> cells/100 mm dish) and treated 24 h later with 5x10<sup>-6</sup> M 5-aza-2'-deoxycytidine (5-Aza-CdR) (Sigma-Aldrich, St. Louis, MO, USA). The medium was changed 24 h following drug treatment, and RNA and DNA were isolated six days after treatment.

**Bisulfite modification and methylation-specific polymerase chain reaction (MSP).** DNA was prepared from tissues and cells. Following microdissection, the tissue samples were placed into Eppendorf tubes and were incubated with proteinase K (Promega, Madison, WI, USA) at 37°C overnight. The tissue was extracted twice in phenol and twice in chloroform, followed by ethanol precipitation. Genomic DNA (3 µg) from tissue and cells was denatured with 0.3 M NaOH at 37°C for 10 min. Next, freshly prepared (208 µl) 3 M sodium bisulphate (pH 5.0) and

12 µl fresh 100 mM hydroquinone (Sigma-Aldrich Shanghai Trading Co., Shanghai, China) solutions were added. Bisulfite treatment, during which methylated DNA is protected and unmethylated cytosine is converted to uracil, was performed as described previously (17). Bisulphite-treated DNA was used for amplification of the p27kip1 promoter. Primers (Sangon Biotech Co., Shanghai, China) specific for unmethylated p27kip1, 5'-ATGGAAGAGGTGAGTTAGT-3' (sense) and 5'-AAAACCCCAATTAAAAACA-3' (antisense); or methylated p27kip1, 5'-AAGAGGCGAGTTAGCGT-3' (sense) and 5'-AAAACGCCGCCGAACGA-3' (antisense) were used, which amplify a 212 and 195 bp product, respectively (18). MSP was performed in a 25-µl reaction volume with ~25 ng bisulfite-modified DNA. Reactions were hot-started at 95°C for 5 min. This step was followed by 38 cycles at 95°C for 45 sec, 57°C for 30 sec and 72°C for 30 sec, followed by a 10-min extension at 72°C in DNA thermocycler (Agilent Technologies, Inc., Santa Clara, CA, USA). The amplification products were separated on a 2% agarose gel and visualized by ethidium bromide staining and UV transillumination. Methylation (M) was defined as  $M/(M + U) \geq 0.5$  and unmethylation (U) was defined as  $M/(M + U) < 0.5$ .

**Quantitative polymerase chain reaction (qPCR) analysis for p27kip1.** RNA was isolated from 50 ESCC tissues, adjacent normal tissues and cultured cells. The first-strand cDNA was synthesized from 2 µg total RNA. Primer sequences (Sangon Biotech Co.) of p27kip1 for qPCR were 5'-TCCGGCTAACTCTGAGGACAC-3' (sense) and 5'-TGTTTTGAGTAGAGAATCGTCGGT-3' (antisense) for the amplification of p27 mRNA (19). qPCR was performed using the Mx3000P qPCR system (Stratagene, La Jolla, CA, USA). The cDNA was then used for qPCR in a 20 µl SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd., Dailan, China). qPCR for p27kip1 mRNA expression was performed under the following conditions: 5 min at 95°C, 40 cycles of 30 sec at 95°C, 30 sec at 60°C and 1 min at 72°C. As an internal control for qPCR, β-actin mRNA expression was amplified from the same cDNA samples. All results were normalized to β-actin amplification. CT values for triplicate reactions were averaged and relative p27kip1 expression was determined with the comparative CT method, using average CT values for p27kip1 and β-actin.

**Statistical analysis.** All data were generated without knowledge of the clinical status of the samples analyzed with SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons were performed with a t-test (unpaired or paired). Univariate analyses of the interaction between p27kip1 methylation and clinical parameters were performed with Pearson's  $\chi^2$  test or Fisher's exact test. All P-values presented were two-tailed. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Methylation status of the p27kip1 promoter region in esophageal cancer and its adjacent tissue.** The methylation status of the p27kip1 promoter region was analyzed by MSP. Methylation analysis of 50 ESCC tissues and their adjacent non-tumor tissues was performed. The p27kip1 promoter was

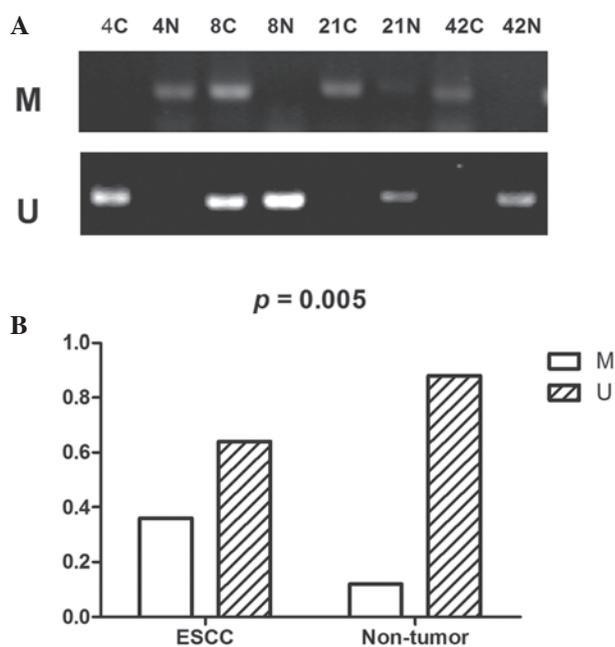


Figure 1. The methylation of p27kip1 promoter by MSP. (A) Representative patterns of methylation of the p27kip1 promoter. M, reaction specific for methylated DNA. U, reaction specific for unmethylated DNA. C, tumor tissue; N, non-tumor tissue. (B) Methylation frequency of p27kip1 in esophageal carcinoma tissue and corresponding non-tumor tissues. M, methylation; U, unmethylation. MSP, methylation-specific polymerase chain reaction.

methylated in 36.0% (18/50) of esophageal cancers and in 3.0% (6/50) of non-tumor samples. The difference in p27kip1 methylation between the tumor and non-tumor group was statistically significant ( $P=0.005$ ; Fig. 1).

**Methylation status of the p27kip1 promoter with clinicopathologic parameters in ESCC.** The association of p27kip1 methylation with clinicopathologic parameters in esophageal cancer patients is presented in Table I. The methylation status of the p27kip1 promoter was associated with tumor metastasis. Furthermore, the methylation of p27kip1 was markedly increased in patients with metastasis (59.09%, 13/22) compared with patients without metastasis (17.86%, 5/28). A significant difference was identified between methylation of the p27kip1 promoter and metastasis status in ESCC ( $P=0.002$ ). Methylation of p27kip1 was not associated with the remaining clinicopathological parameters evaluated, including gender, tumor differentiation and other features.

**Association of p27kip1 methylation with mRNA expression in tissue.** To determine whether loss of p27kip1 mRNA expression was associated with promoter methylation, p27kip1 mRNA was analyzed in 50 ESCC tissues using qPCR (Fig. 2). The expression of p27kip1 mRNA was lower (mean  $\pm$  SD,  $-0.886\pm 3.298$ ) in ESCC tissues compared with non-tumor tissues ( $0.988\pm 0.257$ ;  $P=0.0033$ ). Notably, the expression of p27kip1 mRNA was decreased in ESCC patients with methylation ( $-3.259\pm 2.439$ ) compared with unmethylation ( $0.449\pm 2.970$ ;  $P<0.0001$ ). Furthermore, p27kip1 mRNA was also decreased in non-tumor tissues with methylation ( $0.1678\pm 0.545$ ) compared with unmethylation ( $1.351\pm 2.523$ ;  $P=0.0055$ ). There was a statistically significant association

Table I. Correlation of p27kip1 methylation with clinicopathological parameters of the ESCC patients.

Variable	No.	M, n=18	U, n=32	P-value <sup>a</sup>
Gender				
Male	43	14	29	0.209
Female	7	4	3	
Depth of invasion				
T <sub>1-2</sub>	32	10	22	0.351
T <sub>3-4</sub>	18	8	10	
Lymph node metastasis				
N <sub>0-1</sub>	23	9	14	0.582
N <sub>2-3</sub>	27	9	18	
Distant metastasis				
M <sub>0</sub>	28	5	24	0.002
M <sub>1</sub>	22	13	9	
AJCC stage				
I-II	20	8	12	0.630
III-IV	30	10	20	
Differentiation				
G <sub>1</sub>	10	4	6	0.768
G <sub>2-3</sub>	40	14	26	

<sup>a</sup>Comparison was performed with Pearson's  $\chi^2$  test or Fisher's exact test. ESCC, esophageal squamous cell carcinoma; M, methylation; U, unmethylation.

between the methylation status of the p27kip1 promoter and p27kip1 mRNA expression ( $P<0.001$ ) in cancer and non-tumor samples.

**Demethylation by 5-Aza-CdR.** The ECa-9706 and TE-1 esophageal cancer lines showed methylation of the p27kip1 promoter (Fig. 3). To determine the effects of 5-Aza-CdR on p27kip1 gene expression, qPCR analyses were performed using esophageal cancer lines treated with a final concentration of 5  $\mu$ M p27kip1. However, following treatment with the demethylation reagent, the methylation of the p27kip1 promoter was demethylated (Fig. 3A). Following normalization of mRNA levels to  $\beta$ -actin, the expression of the p27kip1 gene was induced between  $-4.337\pm 0.04$  and  $-2.21\pm 0.01$  in the ECa-9706 cells ( $P=0.0011$ ); and between  $-3.513\pm 0.07$  and  $-1.337\pm 0.07$  in the TE-1 cells ( $P=0.0011$ ; Fig. 3B). These results suggested that the expression of p27kip1 may be activated by 5-Aza-CdR.

## Discussion

In the complex multistage process of esophageal cancer, the accumulation of epigenetic alterations are required for the emergence of a fully malignant tumor. Genes that inhibit cell proliferation are ideal candidates for tumor suppressor genes, which appear to be critical in the pathogenesis of a number of human malignancies (20,21).

p27kip1 is an atypical tumor suppressor that regulates G0 to S phase transitions by binding to and regulating the

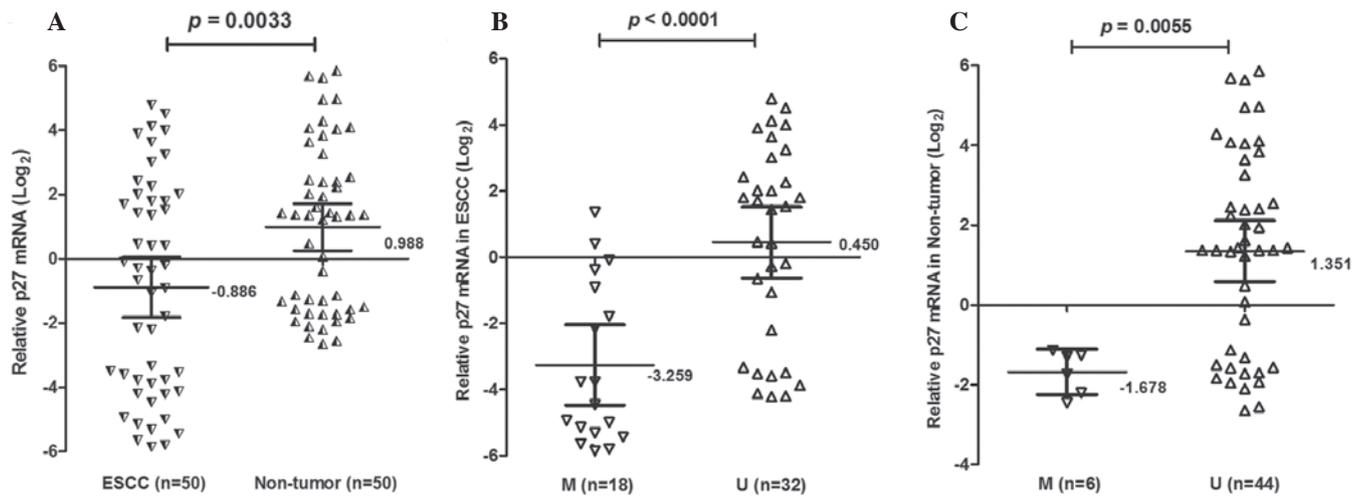


Figure 2. p27kip1 mRNA expression with methylation. (A) p27kip1 mRNA expression in ESCC and non-tumor samples. (B) p27kip1 mRNA expression with methylation in ESCC. (C) p27kip1 mRNA expression with methylation in non-tumor samples. ESCC, esophageal squamous cell carcinoma; M, methylation; U, unmethylation.

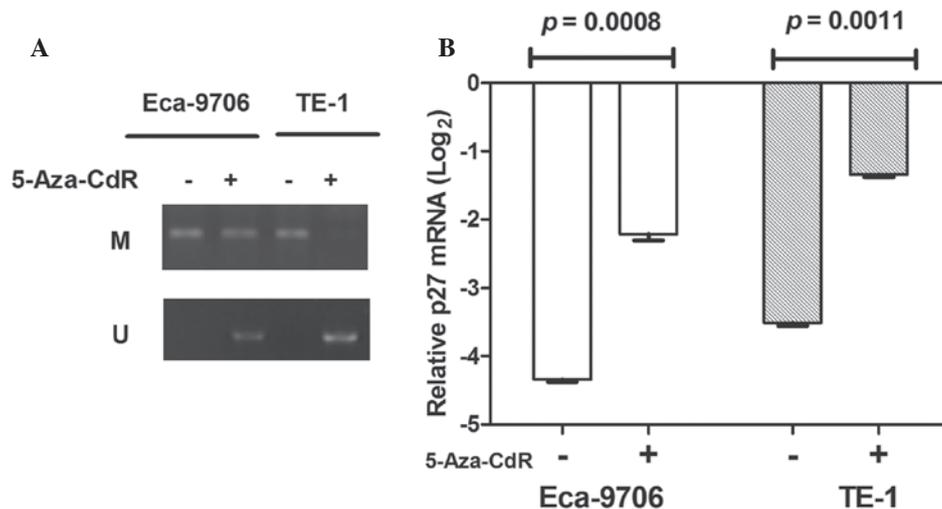


Figure 3. ECa-9706 and TE-1 esophageal cancer cell lines treated with 5-Aza-CdR. (A) Methylation status of p27kip1 promoter in esophageal cancer cell lines treated with 5-Aza-CdR. (B) p27kip1 mRNA expression is reactivated in esophageal cancer cell lines following treatment with 5-Aza-CdR. 5-Aza-CdR, 5-aza-2'-deoxycytidine; M, methylation; U, unmethylation.

activity of CDKs (22,23). Numerous antiproliferative signals lead to p27kip1 accumulation, including mitogen cytokine withdrawal, cell-cell contact and agents such as cAMP and rapamycin (24). A marked reduction in the level of p27kip1 protein, or even a complete loss is observed in ~50% of all types of human cancer (25). Downregulation of p27kip1 expression contributes to the increase in the percentage of cells entering S phase, which may lead to increased radioresistance in the established radioresistant cells (26). Although these studies are limited in their ability to provide mechanistic information, they indicate that low or absent p27kip1 protein in tumor cells is an important clinical marker of disease progression in the majority of tumor types (27).

In the present study, the frequency of hypermethylation of the p27kip1 promoter was significantly higher in esophageal cancer compared with the corresponding non-tumor tissues (36.0 and 12.0%, respectively). In addition, there was a

significant association between the p27kip1 promoter methylation status and its mRNA expression; samples with methylation exhibited a lower p27kip1 mRNA expression, whereas samples without methylation exhibited a higher expression ( $P < 0.001$ ). These findings indicate that methylation of the p27kip1 promoter may represent a common mechanism of inactivation of the tumor suppressor gene in ESCC. In the current study, p27kip1 methylation was observed to be associated with tumor metastasis in ESCC patients ( $P = 0.002$ ). These findings suggest that p27kip1 methylation may be a potential marker in association with the poor clinical outcome of ESCC patients.

However, this cell cycle inhibitor has emerged to be important in other cellular functions, including cell migration. Understanding the diverse roles of p27kip1 protein during normal cell cycle progression and tumor development may provide novel insights into tumor prognosis and aid in the development of therapeutics (28,29). 5-Aza-CdR

is incorporated into DNA during the replication process and binds DNMTs thereby causing irreversible inhibition of their enzymatic activity. The present study showed that the methylation inhibitor, 5-Aza-CdR, induced p27kip1 mRNA expression in an esophageal cancer cell line, which indicates that the epigenetic inhibitor may be useful in considering the future clinical treatment in ESCC.

In conclusion, a high frequency methylation of the p27kip1 tumor suppressor gene associated with tumor metastasis in ESCC was reported. The inactivation of p27kip1 by promoter methylation was a common phenomenon in ESCC. Additional studies are required to clarify the promoter methylation and p27kip1 expression in ESCC development.

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