

## RESEARCH ARTICLE

# Alteration of Runt-related Transcription Factor 3 Gene Expression and Biologic Behavior of Esophageal Carcinoma TE-1 Cells after 5-Azacytidine Intervention

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## Abstract

5-Azacytidine (5-azaC) was originally identified as an anticancer drug (NSC102876) which can cause hypomethylation of tumor suppressor genes. To assess its effects on runt-related transcription factor 3 (RUNX3), expression levels and the promoter methylation status of the RUNX3 gene were assessed. We also investigated alteration of biologic behavior of esophageal carcinoma TE-1 cells. MTT assays showed 5-azaC inhibited the proliferation of TE-1 cells in a time and dose-dependent way. Although other genes could be demethylated after 5-azaC intervention, we focused on RUNX3 gene in this study. The expression level of RUNX3 mRNA increased significantly in TE-1 cells after treatment with 5-azaC at hypotoxic levels. RT-PCR showed 5-azaC at 50  $\mu$ M had the highest RUNX3-induction activity. Methylation-specific PCR indicated that 5-azaC induced RUNX3 expression through demethylation. Migration and invasion of TE-1 cells were inhibited by 5-azaC, along with growth of Eca109 xenografts in nude mice. In conclusion, we demonstrate that the RUNX3 gene can be reactivated by the demethylation reagent 5-azaC, which inhibits the proliferation, migration and invasion of esophageal carcinoma TE-1 cells.

**Keywords:** 5-azacytidine - RUNX3 gene - demethylation - biologic behavior - esophageal carcinoma

*Asian Pac J Cancer Prev*, 14 (9), 5427-5433

## Introduction

During the past decades, esophageal carcinoma had been a huge burden on the health status of Chinese and people worldwide. The incidence of esophageal carcinoma continues to increase in East Asia (China, Japan and South Korea) (Kamangar et al., 2006). Many tumor suppressor genes (TSGs) have been identified in esophageal carcinoma, e.g. tetraspanin cell surface receptor uroplakin 1A (UPK1A), epidermal growth factor receptor (EGFR), transforming growth factor (TGF)- $\beta$  and esophageal cancer-related gene 1 (ECRG1) (Kong et al., 2010; Rasool et al., 2013; Shibata-Kobayashi et al., 2013). The human runt-related transcription factor 3 gene (RUNX3), localized on human chromosomes 1p36, belongs to the runt domain family of transcription factors and contains promotor 1-2, exons 1-6 and 1290 bp open reading frame (Bangsow et al., 2001).

Li et al. found inactivation of RUNX3 gene was closely related to the tumorigenesis and progression of human gastric carcinoma (Li et al., 2002). Following this breakthrough, increasing literatures report that RUNX3 gene plays an important role in both of normal

cell differentiation and carcinogenesis (Bae et al., 2004). RUNX3 gene can facilitate signal transduction in TGF- $\beta$ -Smads pathway and regulate proliferation of carcinoma cells, which is a dominant TSGs in various human malignant tumors, e.g. gastric cancer (Subramaniam et al., 2009; Zhang et al., 2013), hepatocellular carcinoma (Park et al., 2005), colorectal cancer (Ku et al., 2004) and breast cancer (Subramaniam et al., 2009).

Mountain studies have described the aberrant alternation of oncogenes and TSGs in human carcinomas. In many types of malignant carcinomas, the expression levels of RUNX3 gene are lower than normal tissues. Genic alternation of RUNX3 gene in the occurrence and development of human cancers have been identified. The expression of RUNX3 gene can be silenced by hemizygous deletion (Li et al., 2002) or mislocalization (Ito et al., 2005). However, the structure and sequence profile of RUNX3 gene are different. There are considerable conserved cytosine phosphate guanine (CpG) islands around gene promoters, which act as regulators of initiation of RUNX3 gene expression (Tang et al., 2012). Epigenetic changes are the main regulation mode of RUNX3 gene. In human carcinoma tissues, epigenetic silencing in the CpGs

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**Table 1. Primer Sequence for RT-PCR and MSP**

	Gene	Upstream primer (5'→3')	Downstream primer (5'→3')
RT-PCR	RUNX3	ACCTGTCACAACGGCCAGAAC	TTCCAGTGAGGACAGGCCAGA
	β-actin	GGCGGCACCATGTACCCT	AGGGGCCGGACTCGTCATACT
	Methylated RUNX3	AACGTTTTTCGAGAAGGCGTAGCGC	CACGATACAAACCGAAACCATTCTG
MSP	Unmethylated RUNX3	TGGGAATGTTTTTGAAGAAGGTGTAGTGT	CACAATACAAACCAAAACCATTCA

region, namely aberrant methylation of CpG islands, is one of the mechanisms responsible for deficiency of RUNX3 genes. Previous studies have shown the lack of RUNX3 expression is related to the hypermethylation of promoter in gastric cancer (Tang et al., 2012), breast cancer (Subramaniam et al., 2009) and hepatocellular cancer (Park et al., 2005). In colorectal carcinoma, 5-Aza-2'-deoxycytidine, a kind of demethylation agent, can demethylate the RUNX3 promoters and up-regulate the expression of RUNX3 gene (Deng et al., 2009).

Few scholars have studied the expression and function of RUNX3 gene in esophageal carcinoma. Eric Smith et al. had found normal esophageal epithelial tissues express RUNX3 gene highly. However, in Barrett's esophagus and esophageal carcinoma tissues, expression of RUNX3 gene was significantly silenced (Smith et al., 2008). Loss of RUNX3 gene expression occurs both in esophageal adenocarcinoma cells (Smith et al., 2008) and esophageal squamous carcinoma cells (Long et al., 2007). But CpG island methylator phenotype (CIMP) in esophageal carcinoma is unclear. The mechanism of epigenetic silence of RUNX3 gene in esophageal carcinoma is still controversial. Sequence profile of RUNX3 gene suggests that the CpG islands around promoters may be targets for epigenetic silence in esophageal carcinogenesis and progression. However, there are limited data on the methylation status of RUNX3 in various types of human esophageal carcinoma cell lines. Our study investigated alternation of expression level of RUNX3 gene induced by the 5-azaC in the TE-1 cells. Biological behaviors of esophageal squamous carcinoma TE-1 cells after treatment with 5-azaC have not been studied. So we also detected migration and invasion of TE-1 cells in vitro and growth of TE-1 cells in vivo by tumorigenesis experiment in nude mice, and further studied mechanism and effects of 5-azaC on TE-1 cells.

## Materials and Methods

### Cell culture

The human esophageal squamous carcinoma TE-1 cells (Institute of Cytobiology, Chinese Academy of Sciences, Shanghai, PRC) were maintained as monolayers in Roswell Park Memorial Institute medium (RPMI-1640) (Gibco, Rockville, MD, USA) containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. TE-1 cells in logarithmic growth phase were used for experiments. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Sigma Chemical CO., USA) was dissolved in PBS at 5 mg/mL. 5-azaC (Sigma Chemical CO., St. Louis, MO, USA) was dissolved in serum-free 1640 medium and stored at -20°C to further use.

### MTT assay

The viability of TE-1 cells was detected by MTT assay (Alvarez-Díaz et al., 2010). TE-1 cells were seeded at same concentration of 1×10<sup>4</sup> cells per well in 96-well microtiter plates. 5-azaC was added at different final concentrations (10-200 µM) in sextuplicate after 24 h pre-culture of TE-1 cells in serum-free culture medium. 50 µL MTT (Sigma Chemical CO., USA) (5 mmol/L) was added at 6, 12, 24, 48 or 72 h, respectively. Two control groups were included: one without 5-azaC treatment and one without cells. The optical density (OD) was measured at 490 nm (Spectra Max M2). The inhibition rate (IR) of cell growth was calculated as: IR = (the OD value of controls – the OD value of experimental groups) / the OD value of controls. IC<sub>50</sub> (50% inhibitory concentration) values were also calculated using Statistic Probit software.

### Intervention of 5-azaC

In three experimental groups, TE-1 cells were treated with 10, 20, 50 µM 5-azaC for 72 h based on the results of MTT analysis. While TE-1 cells of negative control group were treated without 5-azaC for 72 h.

### Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcriptase system included 2 µg RNA, oligo-dT15 primer and M-MLV reverse transcriptase (TAKARA BIO INC, Japan) in a volume of 20 µL. Gene-specific primers (Takara Shuzo Co., Kyoto, Japan) are summarized in Table 1. In the LightCycler480 (Roche, Nutley, NJ, USA), expression levels of the β-actin were used as an internal control for the normalization of RNA quantity and quality. The real-time PCR reaction conditions were 30 s at 95°C followed by 45 cycles of 30 s at 94°C, 60 s at 30°C and 30 s at 72°C. PCR reactions were terminated at 4°C, after 7 min elongation at 72°C. Measurements were performed in triplicates. The relative amount of mRNA was calculated as the calibrator normalized ratio using LightCycler480 Software 1.5. The calibrator normalized ratio was measured as the following formula:

$$RQ = 2^{-\Delta\Delta Ct}, \Delta\Delta Ct = (Ct_{\text{targeted gene}} - Ct_{\beta\text{-actin}})_{\text{targeted sample}} - (Ct_{\text{targeted gene}} - Ct_{\beta\text{-actin}})_{\text{calibration sample}}.$$

### Western blot

Protein was extracted and measured as described previously (Wang et al., 2012). Protein was transferred to nitrocellulose membranes after dissolution in 10% SDS-PAGE. Membranes were blocked for 1 h at room temperature with 5% nonfat dry milk containing 0.05% Tween 20 and 1% BSA. Then protein was incubated overnight at 4°C with primary antibodies against RUNX3 and against GAPDH (1:1,000 dilution, Santa Cruz

Biotechnology, Santa Cruz, CA, USA). After washing, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (HRP) anti-rabbit IgG (1:10,000; Santa Cruz, CA, USA) for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence (ECL) detection system (LAS-4000 MINI System, GE, Fairfield, CT, USA). The intensities of acquired bands were measured by image analysis system (Imager Reader LAS-4000, GE, Fairfield, CT, USA) and normalized to GAPDH as the endogenous control. Protein was measured by the formula:

$$\text{Relative Coefficient} = \frac{\text{Expression intensity of RUNX3}}{\text{Expression intensity of GAPDH}}$$

#### Methylation-specific PCR (MSP)

Total DNA was extracted from TE-1 cells and subjected to sodium bisulfite modification and Wizard DNA Clean-up System purification, according to manufacturer's protocol. Methylation-specific primers (Takara Shuzo Co., Kyoto, Japan) were summarized in Table 1. Briefly, the 20  $\mu$ L total reaction volume contained (2 $\mu$ L) 100ng DNA, 10 $\times$ Ex Taq Buffer (Mg<sup>2+</sup> Plus) 2  $\mu$ L, 2.5 mmol/L dNTP Mixture 1.6  $\mu$ L, TaKaRa Ex Taq (5 u/ $\mu$ L) 0.1 $\mu$ L, sense and antisense primers (each at 1  $\mu$ L) (100 ng). The reactions were hot-started at 97°C for 5min, prior to the addition of 0.75 units of Taq polymerase (Takara Shuzo Co., Kyoto, Japan). The PCR conditions were as follows: 35 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and a extension at 72°C for 15s. PCR reactions were terminated at 4°C, after 10 min elongation at 72°C. Each set of PCR reaction products was loaded onto 2.5% agarose gels, ethidium bromide-stained and visualized under UV light (AlphaImagerTM 2200, Genetic Technologies, Inc. Miami, FL, USA). The experiment was repeated three times.

#### Scrape motility assay

TE-1 cells pretreated without or with 10, 20, 50 $\mu$ M 5-azaC for 72h were plated at a density of  $1 \times 10^5$  cells/ml in 6-well plates in 1640 medium with 10% FBS. After overnight incubation, the tip of a plastic pipette was drawn across the center of the well to produce a scraped area (Zhao et al., 2008). TE-1 cells were washed twice with PBS, followed by incubation in 1640 medium. Immediately after scraping (0-hour), 48-hour as well as 72-hour incubation, TE-1 cells migrated into the scraped area was photographed with a OLYMPUS IX70 inverted microscope (Olympus Corporation, Tokyo, Japan). The migrated TE-1 cells after 48 and 72 hours were counted by an observer blinded to the study design. The time of TE-1 cells covering 6-well plates was wrote down. Each experiment was repeated four times.

#### Cell migration and invasion assay

Cell migration and invasion experiments were assayed as previously described (Shi et al., 2012) in triplication using 24-well transwell setup and polycarbonate Nucleopore filters with an 8- $\mu$ m pore size (Merck Millipore Bioscience, Germany). Prior to each experiment,

TE-1 cells were deprived of FBS during 24 hours. For invasion assays, the inserts were pre-coated with Matrigel (BD Biosciences, Erembodegem, Belgium) diluted 1:10 in serum-free Ham's F10 and the Matrigel was allowed to solidify for 1 hour at 37°C. For migration assays, the inserts were left uncoated. Each upper well was loaded with  $2.5 \times 10^5$  cells in a total volume of 200  $\mu$ L of serum-free medium. The lower wells of the chamber were loaded with 600  $\mu$ L of 1640 medium with 10% FBS. Invasion assays were allowed to proceed for 24 hours whereas migration assays were incubated for 6 hours. Any cells remaining on top of the insert were removed by scraping. Migrated cells attached to the underside were fixed for 10 min in methanol and stained with ethanol-based crystal violet solution. Cells were observed under a microscope (LEICA DM4000B, Leica, Wetzlar, Germany) and all cells in random five visual fields in the middle of the membrane were counted.

#### Nude mouse xenograft model

A total of 50 BALB/C Nude Mice (25 females and 25 males, weighting 16-18 g and aged 4 weeks) were purchased from Vital river co. [Certificate of Quality No.: SCXK (Beijing) 2012-0001] and maintained in specific pathogen-free facilities at the Experimental Animal Center of Provincial Hospital Affiliated to Shandong University. The 50 nude mice were randomly divided into five groups: (1) one black control (BC): the nude mice were injected with cell-free and serum-free culture medium; (2) one negative control (NC): the nude mice were injected with Eca109 cells of negative group; (3) three experimental groups (EG): the nude mice were injected with Eca109 cells of three experimental groups (as described in 'intervention of 5-azaC'). Mice were provided with free access to food, water and bedding at all time and were housed with a 12 h light/dark cycle in filter top cages containing a maximum of five mice per cage. A total of  $1 \times 10^6$  TE-1 cells were injected into the skin of the right shoulder of 40 nude mice in negative control group and experimental groups. Tumor volumes (V) were measured with an external caliper every 4 days and calculated by the formula:  $V = \pi/6 \times \text{width}^2 (\text{mm}^2) \times \text{length} (\text{mm})$  as described previously (Perera et al., 2008). The experiment was terminated in accordance with institutionally approved guidelines and tumors were harvested and weighted. Inhibition rate of tumor growth (IR) was calculated by the formula:  $\text{IR} = (\text{tumor weight of NC} - \text{tumor weight of EG}) / \text{tumor weight of NC} \times 100 \%$ .

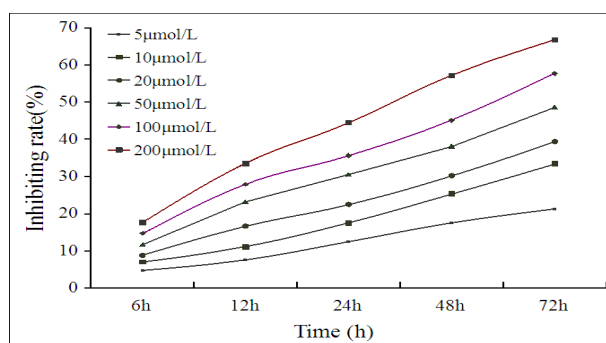
#### Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Chinese Ethics Review Committees. The protocol was approved by the the Ethics Committee of Shandong University.

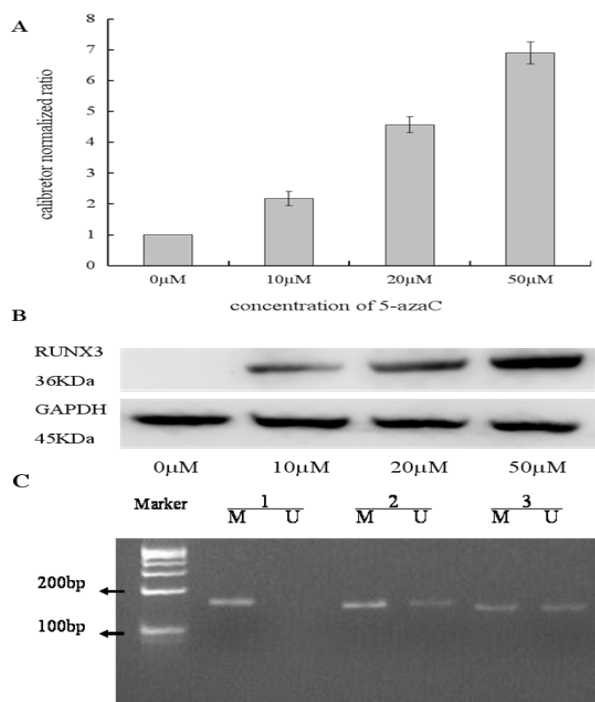
#### Statistical analyses

Statistical Package for the Social Sciences 13.0 (SPSS 13.0) was used to statistical analysis. Differences among different treatment groups were analyzed using Student's t-test or one-way analysis of variance (ANOVA). A P value < 0.05 was considered statistically significant.





**Figure 1. The Viability of TE-1 Cells Was Inhibited.** TE-1 cells were treated with increasing concentrations of 5-azaC. Cell viability was measured at various time points using the MTT assay. Results are expressed as inhibition rate (%) and are representative of three independent experiments



**Figure 2. The Expression Levels and the Methylation Degree of RUNX3 Gene Were Alternated.** 0 μM: TE-1 cells were pretreated without any drugs; 10, 20, 50 μM: TE-1 cells were pretreated with 10, 20, 50 μmol/L 5-azaC for 72h. A: Data were presented as means ( $\pm$  SD) of the calibrator normalized ratio of RT-PCR detection in triplicates. B: The gel is representative of three independent Western blot assaies. C: The gel is representative of three MSP experiments. Lanes: 1, TE-1 cells were pretreated without any drugs; 2, TE-1 cells were pretreated with 20 μmol/L 5-azaC for 72 h; 3, TE-1 cells were pretreated with 50 μmol/L 5-azaC for 72 h. M: methylated-sequence-specific PCR for RUNX3 (154bp); U: unmethylated-sequence-specific PCR for RUNX3 (158bp)

## Results

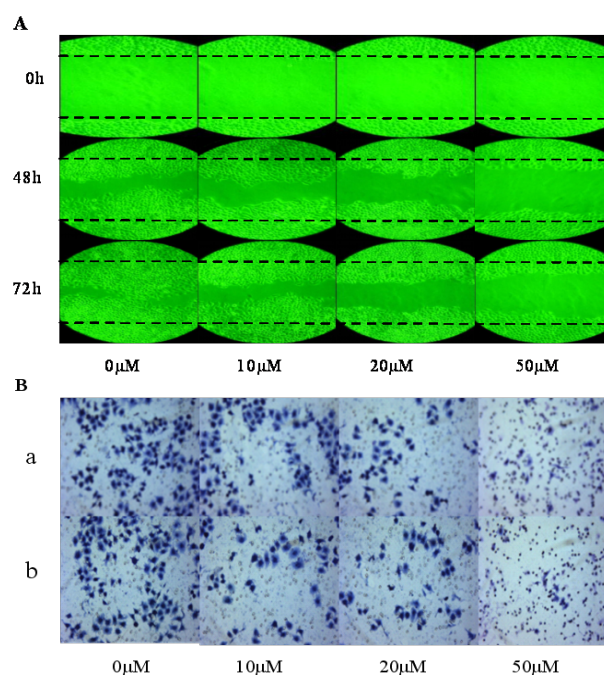
### 5-azaC inhibits the proliferation of TE-1 cell in vitro

5-azaC inhibited TE-1 cells growth, and the inhibition was dose- and time-dependent (Figure 1). The half maximal inhibitory concentration ( $IC_{50}$ ) of 5-azaC was 51.3 μM at 72h analyzed by Statistic Probit software. Thus, we chosed 10, 20, 50 μM 5-azaC in further experiments to rule out the cytotoxicity of the drug itself.

**Table 2. 5-azaC Induces RUNX3 Protein Expression in TE-1 Cells.**

	0 μM	10 μM	20 μM	50 μM
RUNX3 protein	0	0.189 $\pm$ 0.005	0.283 $\pm$ 0.015	0.407 $\pm$ 0.030

The expression levels of RUNX3 protein were presented as relative coefficient ( $\bar{x} \pm s$ , n = 3)



**Figure 3. The Migration and Invasion of TE-1 Cells Were Inhibited.** 0, 10, 20, 50 μM: TE-1 cells were pretreated with 0, 10, 20, 50 μmol/L 5-azaC for 72 h. A: Representative photographs of four separate scrape wound assaies at 48 and 72 hours (10 $\times$ 40). B: Representative photographs of the migrating (a) and invading (b) cells in five separate Transwell assaies at 72 hours (10 $\times$ 40)

### 5-azaC induces RUNX3 expression in TE-1 cells

In all three experiment groups, the mRNA levels of RUNX3 gene were significantly higher than control group (Figure 2A). The expression of RUNX3 mRNA increased along with the concentration of 5-azaC. Western blot analysis showed that RUNX3 protein expression was silenced in TE-1 cells and increased after intervention with 5-azaC for 72 h (Figure 2B, Table 2). RUNX3 protein expression increased also along with 5-azaC concentration, which is consistent with the level of mRNA. The 5-azaC at 50 μM displayed the greatest induction ability of RUNX3 gene.

### RUNX3 was demethylated by 5-azaC in TE-1 Cells

Samples presented methylated bands were scored as RUNX3 hypermethylation. Samples presented unmethylated bands were considered to be positive for RUNX3 demethylation. RUNX3 hypermethylation was significantly higher in the negative group samples compared to experiment group samples. After treatment with 5-azaC, RUNX3 gene was partially demethylated (Figure 2C).

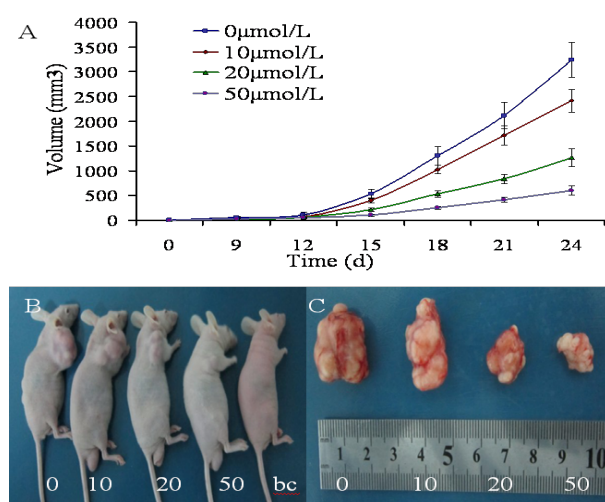
### 5-azaC inhibits the migration and invasion of TE-1 cells

The healing of scrape wound assay indicated 5-azaC

**Table 3. 5-azaC Inhibits Migration and Invasion of TE-1 Cells**

	0 $\mu$ M	10 $\mu$ M	20 $\mu$ M	50 $\mu$ M
Scrape assay				
Migration(48 h)	526 $\pm$ 18.6	321 $\pm$ 17.7	241 $\pm$ 15.6	107 $\pm$ 10.9
Migration(72 h)	715 $\pm$ 20.1	398 $\pm$ 19.3	302 $\pm$ 16.4	124 $\pm$ 13.8
Transwell assay				
Migration(72 h)	130 $\pm$ 10.6	81 $\pm$ 7.8	58 $\pm$ 6.5	14 $\pm$ 2.1
Invasion(72 h)	102 $\pm$ 9.5	60 $\pm$ 8.3	41 $\pm$ 5.2	8 $\pm$ 1.3

The number of migrating and invading TE-1 cells were calculated ( $\bar{x} \pm s$ ). Differences between groups all have statistical significance ( $P < 0.05$ )



**Figure 4. The tumorigenicity of TE-1 cells in nude mice was significantly inhibited.** A: Tumor volumes presented as a growth curve; B: Representative photographs of the nude mice at 24 days after injection; C: Representative photographs of tumors excised from mice. 0, 10, 20, 50: The nude mice were injected with TE-1 cells pretreated with 0, 10, 20, 50  $\mu$ mol/L 5-azaC for 72 h, respectively; bc (black control): The nude mice were injected with PBS

inhibited mobility of TE-1 Cells in a dose- and time-dependent way (Figure 3A). The scrape wound healing time of TE-1 cells pre-treated without or with 10, 20, 50  $\mu$ M 5-azaC for 72h was 5, 6, 8, 9 days, respectively. Transwell assay demonstrated 5-azaC attenuated the migratory capacity of TE-1 cells in a dose-dependent manner. The migratory (Figure 3Ba) and invasive (Figure 3Bb) capability of TE-1 cells were decreased effectively at the concentration of 10, 20, 50  $\mu$ M. This was evaluated quantitatively by calculation of the number of migrating and invasion cells (Table 3).

#### 5-azaC inhibits the growth of TE-1 xenografts in vivo

There was no tumorigenesis in BC group, while tumorigenesis rate was 100% in NC and EG group (Figure 4). Tumor xenografts grew rapidly from the second week. The differences in tumor volume between NC and EG group was also increasingly significant. 24 days later, the mean tumor volumes of xenografts was 3241 $\pm$ 349 mm<sup>3</sup> in the NC group and 2411 $\pm$ 241, 1259 $\pm$ 179, 610 $\pm$ 87 mm<sup>3</sup> in the 10, 20, 50  $\mu$ M of 5-azaC experiment group, respectively (Figure 4A). The weight of xenografts was 2.59 $\pm$ 0.38 g in the NC group and 1.89 $\pm$ 0.18, 1.18 $\pm$ 0.20, 0.49 $\pm$ 0.21 g ( $P < 0.05$ ) in the 10, 20, 50  $\mu$ M of 5-azaC

experiment group, respectively. IR of tumor xenografts in the 10, 20, 50  $\mu$ M of 5-azaC experiment group was 27.0%, 54.4%, 81.1%, respectively.

## Discussion

5-AzaC, a pyrimidine ring analogue, had anti-proliferation and anti-neoplasia activity in acute myeloid leukemia (Aimiuwu et al., 2012) and hepatocellular carcinoma (Acun et al., 2011). But the overall clinical utility of 5-AzaC was disappointing because of its activities of induce-neoplasia in mammalian cells. Recently, 5-AzaC was used as inducer resulting in activation of silencing genes and alternations of phenotypic expression. Ring carbon 5 of 5-azaC is replaced with nitrogen. When 5-azaC incorporate into DNA, it binds DNA methyltransferase (DNMT) in an irreversible and covalent manner (Kiziltepe et al., 2007). Consequently, 5-azaC inhibits methylation of genomic DNA and causes almost complete demethylation or hemi-demethylation. DNA methylation is the most common and important mechanism of TSGs inactivation in various human carcinoma (Grønbaek et al., 2007; Dachrut et al., 2009). It's worth noting that hypermethylation of TSGs may occur in early stage of carcinogenesis of dysplasia cells (Park et al., 2007).

In our investigation, we studied the alternation of RUNX3 gene expression and cellular biological behaviors of TE-1 cells in vivo and in vitro after 5-AzaC intervention. We first performed the MTT assay to estimate the cytotoxicity of 5-AzaC in TE-1 cells. Results showed that 5-AzaC inhibited the proliferation of TE-1 cells in a dose- and time-dependent way. The  $IC_{50}$  was 51.3  $\mu$ M at 72h. So we chose 10, 20, 50  $\mu$ M as the intervention concentration to minimize the cytotoxicity of 5-AzaC. Both PCR and West blotting assay showed that 5-AzaC induced the expression of RUNX3 gene. Furthermore, with higher concentration, the expression of RUNX3 gene was increased. West blotting assay also demonstrated that RUNX3 gene was silenced in esophageal squamous carcinoma TE-1 cells. Our results are similar to studies in hepatocellular cancer (Park et al., 2005), esophageal squamous carcinoma cells (Long et al., 2007), breast cancer (Subramaniam et al., 2009) and gastric cancer (Tang et al., 2012). But reports of Smith et al., are different. The expression of RUNX3 gene could be induced by 5-AzaC analog in esophageal adenocarcinoma OE33 cells. However, the expression levels of RUNX3 mRNA were not significantly increased after DNMT inhibitor intervention in esophageal adenocarcinoma TE-7 cells. These studies indicted the diversity and complexity of RUNX3 gene regulation in esophageal cancer cells. The different results may be due to the following reasons: a) inconsistency of RUNX3 gene expression in esophageal carcinoma cell lines; b) the difference of differentiation grade and malignancy of cell lines; c) the influence of environmental factors (eg. the concentration of DNMT inhibitor, experiment condition, culture time and serial passages); d) the different detection methods.

To study the mechanism of inactivation of RUNX3 gene, we performed MS-PCR detection on TE-1 cells

pretreated without and with 5-AzaC at concentration of 20, 50  $\mu$ M. Results showed 5-AzaC could cause demethylation of RUNX3 gene in TE-1 cells. On the other hand, MS-PCR assay indicated RUNX3 gene was hypermethylated in esophageal adenocarcinoma TE-1 cells. Previous studies had reported methylation status of RUNX3 gene in esophageal adenocarcinoma cell lines. Hypermethylation of RUNX3 gene was only found in one of fifteen cell lines (6.7%). As for esophageal squamous cell carcinoma tissues, RUNX3 promoter methylation were detected only in four of seventy tissue samples (5.7%) (Sugiura et al., 2008). But our experiment results are similar to those of the majority of studies (Long et al., 2007). We demonstrated that loss of RUNX3 gene expression may result from hypermethylation in TE-1 cells. The silence of RUNX3 gene in TE-1 cells was reversed and can be reactivated by demethylation.

To our limited knowledge, this is the first study to evaluate the relationship between 5-azaC and migration and invasion abilities of TE-1 cells. Migration and invasion is one of the important features of deterioration of esophageal carcinoma. Local and distant metastasis are also the primary cause of incomplete surgical resection and clinic treatment failure. Tumor cells metastasis is mainly involved in invasion of surrounding tissue, deformation or movement, degradation of extracellular matrix and colonization. Scrape-wounding and transwell assays both indicated movement, migration and invasion of TE-1 cells could be attenuated by 5-azaC in a dose-dependent way. The grow of TE-1 cells in vivo was detected by tumorigenesis assay of nude mice. The tumor volume and weight of the EG were significantly lower than those of the NG ( $p < 0.05$ ). The IR of 5-azaC was 81.1% at concentration of 50  $\mu$ M and 54.4% at 20  $\mu$ M. Clearly, RUNX3 expression was higher in TE-1 cells of experiment group than in control group. The mechanism of RUNX3 expression is intricate. The malignancy of TE-1 cells is also regulated by genic and epigenic mechanisms. In this study, RUNX3 gene was involved in the effects of 5-azaC on migration, invasion, growth of esophageal carcinoma in vitro and in vivo. We demonstrated that lose of RUNX3 expression is closely related to reversible hypermethylation in TE-1 cells. We also demonstrated that reactivation of RUNX3 gene was one of potential mechanisms of 5-azaC impeding proliferation, migration and invasion of TE-1 cells.

We acknowledge the limitations of this study, because 5-azaC is a nonspecific DNMT inhibitor. Promoter of other genes in TE-1 cells may be demethylated and the expression of other TSGs may be changed at the same time. In this study, we only focused on RUNX3 gene, a powerful TSG in esophageal carcinoma. The CpG islands are located at various positions, such as exons and introns, not confined in promoter region of the TSGs. Although 79% of CpG islands are located in the promoter and the first exon, others are outside of these regions (Fukasawa et al., 2006). So data of methylation status of RUNX3 gene in promoter region is limited. The lack of genome-wide epigenomic approaches (Beck et al., 2008) also is a weakness of this manuscript. Further studies are needed to establish to better understand the methylation profile

of RUNX3 gene and biologic behavior of esophageal carcinoma cells.

In accordance with previous studies, Our studies demonstrate that aberrant hypermethylation of TSGs is a common event occurred in esophageal carcinoma and suggest that RUNX3 may be specific markers for diagnosis and treatment in esophageal carcinoma with a wide prospect of clinical application in future.

## Acknowledgements

This work was supported by grants from Natural Science Foundation of China (No. 81172161). The author (s) declare that they have no competing interests.

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