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BRIEF ARTICLE

# Study on *RIZ1* gene promoter methylation status in human esophageal squamous cell carcinoma

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## Abstract

**AIM:** To investigate the promoter region methylation status of retinoblastoma protein-interacting zinc finger gene 1 (RIZ1) in the human esophageal squamous cell carcinoma (ESCC) cell lines and tissues and verify the relationship between methylation of RIZ1 and oncogenesis, tumor progression and metastasis etc of ESCC.

**METHODS:** Methylation-specific polymerase chain reaction (MSP) was used to investigate the promoter region methylation status of RIZ1 in 6 ESCC cell lines. One cell line where RIZ1 promoter region methylation was detected was selected for the next study, where the cell line was treated with 5-aza-CdR. Real-time polymerase chain reaction was used to investigate its influence on the transcription of RIZ1. Experiments using frozen pathological specimens from 47 ESCC patients were performed using the same MSP methodology.

**RESULTS:** Promoter methylation of *RIZ1* gene was detected in TE13, CaEs17 and EC109 cell lines and the cell line TE13 was chosen for further study. The expression of RIZ1 mRNA in TE-13 was up-regulated after treatment with 5-aza-CdR. The rate of methylation in carcinomas tissues was significantly higher than those in matched neighboring normal and distal ending normal tissue, and the deviation of data was statistically significant ($\chi^2$ = 24.136, $P$ < 0.01). Analysis of the gender, age familial history, tumour deviation, tumour saturation, lymph gland displacement and clinical staging of 47 samples from ESCC patients showed that the fluctuation of data was not statistically significant.

**CONCLUSION:** Promoter methylation may play an important role in the epigenetic silencing of *RIZ1* gene expression in human ESCC. RIZ1 is considered to be a potential tumor suppressor gene and may be a biological parameter for testing early stage human ESCC.



**Key words:** Retinoblastoma protein-interacting zinc finger gene 1; Tumor suppressor genes; Esophageal squamous cell carcinoma; Promoter methylation; Methylation-specific polymerase chain reaction

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## INTRODUCTION

Esophageal cancer is one of the most aggressive malignancies with poor prognosis in the world. Esophageal squamous cell carcinoma (ESCC) is a major histological form of the disease, especially in the Northern part of China[1], which is different from Europe and America. Like other types of solid tumors, the development of ESCC is due to the accumulation of the abnormal expression of oncogenes and tumor suppressor genes (*TSG*). Several genetic alterations have been associated with the development of ESCC including p53 and p16 mutations, amplification of cyclin D, c-myc, and EGFR, and allelic loss on chromosomes[2-5]. In mammalian development, DNA methylation has an essential regulatory function which suppresses gene activity by changing chromatin structure[6,7]. It has become apparent that aberrant DNA methylation of promoter region CpG islands may serve as an alternate mechanism to genetic defects in the inactivation of TSG in human malignancies[8,9].

In recent years, researchers found that the retinoblastoma protein-interacting zinc finger gene (*RIZ*) maps to the distal short arm of human chromosome 1 (1p36), a region thought to harbor TSG for a variety of human cancers. The *RIZ* gene normally produces two protein products of different length, RIZ1 and RIZ2. RIZ1 contains the positive regulatory (positive regulatory domain I binding factor 1 and RIZ) domain, but RIZ2 lacks this domain[10]. In many human cancers, RIZ1 is considered a TSG because RIZ1 can induce G2-M arrest and apoptosis. Moreover, a knockout study showed that RIZ1 is a tumor susceptibility gene in mice[11]. The expression of RIZ1 is frequently silenced in many human malignant tumours, including carcinomas of the breast, prostate, and thyroid gland[12-14]. Recently, methylation of RIZ1 promoter CpG islands has been proposed as a common mechanism in inactivating RIZ1. Increasing clinical evidence reveals a positive correlation of reduced RIZ1 expression with increased risk for metastasis, indicating that RIZ1 may be a potential new TSG[12-14]. Although RIZ1 is a putative tumor suppressor in several cancer types, for instance breast cancer[15], gastric cancer[16], lung cancer[17] and so on, the role of RIZ1 in human ESCC has not been reported. In this study, we analyzed methylation status of the RIZ1 promoter and its relationship with RIZ1 mRNA expression in human ESCC cell lines. In addition, the study examined the relationship between methylation of the *RIZ1* gene in the promoter region, oncogenesis, tumor progression, metastasis and hereditary factors etc of ESCC.

## MATERIALS AND METHODS

### Cell lines and tissues

The human ESCC cell lines KYSE150, KYSE510, TE13, EC9706, CaEs17 and EC109 were provided by the Institute of Cellula Nervosa in Tianjin Huanhu Hospital and were cultured in recommended media RPMI1640 (GIBCO, HEPES 4.76 g/$NaCO_3$ 2.0 g/RPMI-1640 10.4 g/dd$H_2O$ 1000 mL) supplemented with 10% newborn bovine serum (GIBCO), 1 × L-glutamine and 1 × penicillin-streptomycin. Cells were maintained at 37 ℃ in a humidified environment with 5% $CO_2$.

Carcinoma, matched adjacent normal (> 2 cm from the tumor) and distal ending normal (> 5 cm from the tumor) tissues were obtained in our department during surgical excision from 47 patients with ESCC. All specimens were placed in liquid nitrogen immediately after resection and stored at -80 ℃ until RNA or genomic DNA (gDNA) extraction. No patient had received chemotherapy or radiation therapy prior to surgery. All patients were confirmed to have ESCC by pathologic test.

### DNA extraction, purification and bisulfite modification

gDNA from cell lines or ESCC frozen tissues was extracted by using a Dneasy kit (Biomiga). All extracted genomic DNA was treated with sodium bisulfite (Sigma) as reported previously. Briefly, 2 μg gDNA was denatured by 5.5 μL of 3 mol fresh NaOH (final concentration 0.3 mol/L) for 10 min at 37 ℃. 30 μL of 10 mmol/L hydroquinone (Sigma) and 520 μL of 3 mol/L sodium bisulfite (pH 5.0) were added, away from light. The mixture was inverted, added to 200 μL liquid paraffin to prevent water evaporation and reagent oxidation, then incubated at 50 ℃ for 16 h. The modified DNA was purified using the Wizard DNA clean-up system (Promega). The purified DNA was treated again with NaOH and precipitated. DNA was resuspended in 20 μL of LoTE, 2 μL of which were subjected to polymerase chain reaction (PCR) amplification.

### Methylation-specific polymerase chain reaction and sequencing

Methylation-specific primers were designed to cover 23 CpG dinucleotides numbered -124--103 (forward) and 32-52 (reverse). Similarly, unmethylation-specific primers were designed to cover 23 CpG dinucleotides numbered -123--103 (forward) and 32-52 (reverse). Primers specific for methylated DNA (forward 5'-GTGGTGGTTATTGGGCGACGGC-3'; reverse 5'-GCTATTTCGCCGACCCCGACG-3') and unmethylated DNA (forward 5'-TGGTGGTTATTGGGTGATGGT-3'; reverse 5'-ACTATTTCACCAACCCCAAGA-3') were added to the reaction and expected to generate 177-bp and 175-bp products, respectively. PCR conditions were 40 cycles of denaturation at 94 ℃ for 30 s, annealing at 68 ℃ for methylation-specific amplification or at 60 ℃ for unmethylation-specific amplification for 45 s and extension at 72 ℃ for 60 s, then sequencing of PCR products.

### Reexpression of RIZ1 through 5-aza-CdR treatment

$2 \times 10^5$ TE13 cancer cells were seeded into 6-well plates and treated with 10 μmol/L special DNA methyltransferase (DNMT) 5-aza-CdR (Sigma) for 3 d. The drug liquid was replaced every day, reagent was wiped out and

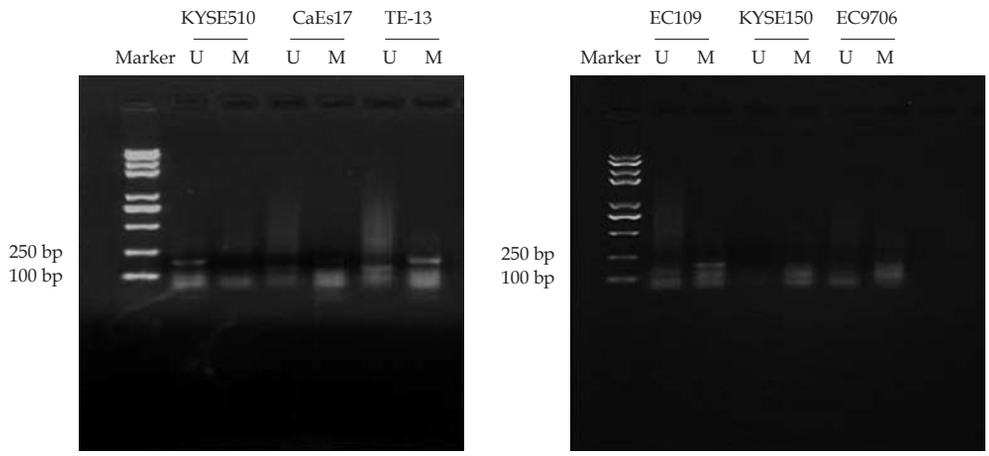


**Figure 1 Methylation-specific polymerase chain reaction analyses of retinoblastoma protein-interacting zinc finger 1 promoter methylation status using genomic DNA extracted from 6 human esophageal squamous cell carcinoma cell lines, products of 177 bp and 175 bp were expected for methylated (M) and unmethylated (U) DNA.** M: Methylation-specific amplification (177 bp); U: Unmethylation-specific amplification (175 bp).

incubation was continued routinely for 5 d. RNA was isolated, and real-time PCR was performed as described previously.

### RNA extraction and reverse transcription reaction

Total cellular and tissue RNA was isolated by Trizol (Invitrogen) reagent according to the manufacturer's recommendations. Cellular RNA was isolated from $5 \times 10^6$ to $1 \times 10^7$ cells by 1 mL Trizol decomposition and tissues samples were ground into a fine powder using a mortar and pestle, and incubated in Trizol solution (100 g/L) for 15 min. Then 1/5 volume of chloroform was added. After vigorous agitation standing for 5 min, the inorganic phase was separated by centrifugation at 12000 *g* for 15 min at 4 ℃; RNA was then precipitated in the presence of equivolume isopropanol and centrifuged at 12000 *g* for 10 min at 4 ℃. RNA pellets were washed with 1 mL 75% ice-cold ethanol [diethypyrocarbonate (DEPC) treated] and centrifuged at 8000 *g* for 5 min at 4 ℃ then dissolved in DEPC-treated $H_2O$. Total RNA was quantified and concentration determined using ultraviolet (UV) spectrophotometry (Beckman Coulter) by absorbency at 260/280 nm and 1.2% denaturing agarose gel. For real-time polymerase chain reaction (real-time quantitative PCR) analysis, 2 μg RNA was reverse transcribed using reverse transcriptase M-MLV (Takara), Ribonuclease inhibitor (Takara) and dNTP mixture (Takara), according to the manufacturer's protocol; the cDNA templates were subjected to PCR amplification.

### RT-PCR, sequencing and Real-time PCR

One μL cDNA from the TE13 cell line treated or not by 5-aza-CdR was used as the template to amplify specific fragments in 25 mL reaction mixture (10 × easy taq buffer 2.5 μL, 2 mol/L dNTP 2.5 μL, F primer 1 μL, R primer 1 μL, cDNA 1 μL, Easy taq 0.3 μL, $ddH_2O$ 16.7 μL) under the following conditions: denaturation at 94 ℃ for 3 min, 35 cycles at 94 ℃ for 30 s, at 56 ℃ for 30 s, at 72 ℃ for 20 s, then extensions at 72 ℃ for 10 min. The primer (10 μmol/L) sets were: RIZ1, forward 5'-TCTGCTGTTGACAAGACCC-3', reverse 5'-GCATCAATGCACATCCATC-3'. The RIZ1 primer set yielded a band at 167 bp. 12 mL RT-PCR reaction product was analyzed by electrophoresis on a 12 g/L agarose gel. The electrophoresis images were scanned by UV spectrophotometer (Beckman Coulter). Sequencing of 0.75 μL of the resultant cDNA from TE13, which was mixed with 2 × SYBR Premix Ex Taq™ (Takara), was then performed. The primer (10 μmol/L) sets used were: RIZ1, forward 5'-TCTGCTGTTGACAAGACCC-3', reverse 5'-GCATCAATGCACATCCATC -3'; GAPDH, forward 5'-GAAGGTGAAGGTCGGAGTC-3', reverse 5'-GGGTGGAATCATATTGGAAC -3'. The amplifications were performed in LightCycler (Roche) real-time PCR system according to the manufacturer's protocol. Each sample was run in triplicate for each gene. An initial denaturation step at 94 ℃ for 5 min was followed by 45 cycles of denaturation at 95 ℃ for 5 s, annealing at 59 ℃ for 20 s, extension at 72 ℃ for 10 s, then the solubility temperature curve assay was performed.

### Statistical analysis

*t*-test was used to compare the measurement data, for instance the RIZ1 mRNA expression levels with primary ESCC and the adjacent and distal ending normal tissues by Real-time PCR. The relative quantitative results were analyzed by comparison of $2^{-\text{average}\Delta\Delta CT} \times 100\%$. $\chi^2$ test was also used to estimate the enumeration data, for example the results. $P$ values < 0.05 were considered statistically significant.

## RESULTS

### Methylation status detecting of RIZ1 in human ESCC cell lines

MSP analyses of RIZ1 promoter methylation status using genomic DNA extracted from 6 human ESCC cell lines, products of 177 bp and 175 bp were expected for methylated (M) and unmethylated (U) DNA. Promoter methylation of *RIZ1* gene was detected in TE13, CaEs17, EC109

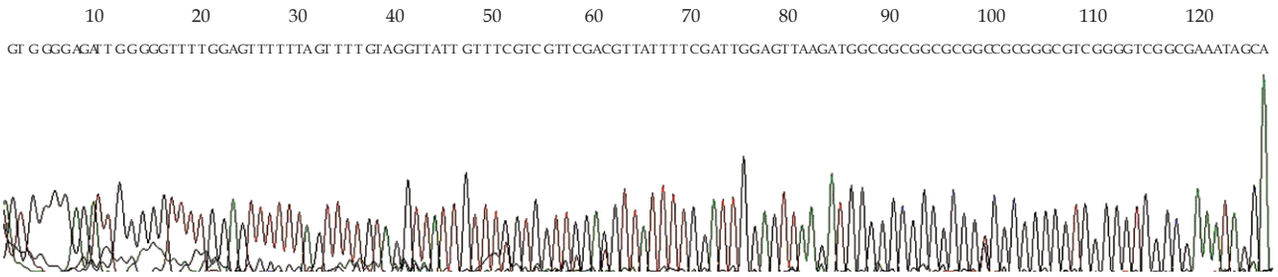


**Figure 2 Sequencing analysis of methylated (M) polymerase chain reaction products of methylation-specific polymerase chain reaction from TE13**

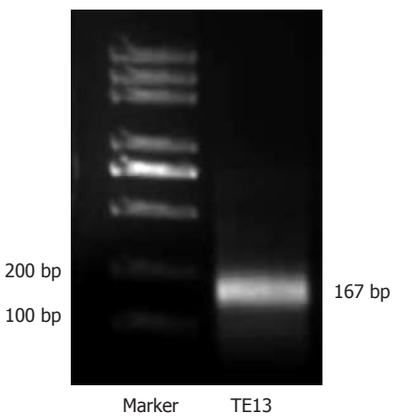


**Figure 3 Reverse transcription-polymerase chain reaction for RIZ1 expression in TE13 treated or not by 5-aza-CdR.**

but not KYSE510, KYSE150 and EC9706 (Figure 1).

All cytosines were changed to thymines, except cytosines in CpG dinucleotide in M-sequences obtained from amplification using M-primer (Figure 2).

### *RIZ1 mRNA expression of treated TE13 by DNMT compared with expression in untreated TE13*

The expression of RIZ1 was higher after use of 5-aza-CdR than before its use ($P < 0.01$) (Figures 3-5).

### *Methylation of RIZ1 gene in promoter region of ESCC tissues*

Among 47 nonselective ESCC patients and matched adjacent normal and distal ending normal esophageal tissue, 26, 3 and 0 cases, respectively, exhibited methylation in the CpG island of the RIZ1 promoter. The corresponding methylation ratios were 55.3%, 6.4% and 0.0%. The rate of methylation in carcinomas tissues was significantly higher than that in matched adjacent normal and distal ending normal tissues, and the deviation of data was statistically significant ($P < 0.01$). The difference in methylation rates between matched adjacent normal and distal ending normal tissues possesses no statistical significance ($P > 0.05$). In the 3 samples where methylation was positive in matched neighbouring normal tissues, methylation also existed in the corresponding carcinoma tissues. MSP electrophoresis of ESCC patients with RIZ1 methylation positive amplification in both carcinomas and matched normal tissues is illustrated in Figure 6A, while that with RIZ1 methylation positive amplification only in carcinomas tissues but not in matched normal tissues is shown in Figure 6B.

The relation between analysis of the methylation state of the CpG island in the *RIZ1* gene promoter by $\chi^2$ verification and the clinical information of the 47 sufferers of the ESCC is illustrated in Table 1. It can be seen that there was no correlation between the methylation state of the CpG island in the *RIZ1* gene promoter of the 47 samples and the sufferers' gender, age, familial history, tumour deviation, tumour saturation, lymph gland displacement and clinical stages, respectively. The fluctuation of data was not statistically significant ($P > 0.05$ in all groups) (Table 1 ).

## DISCUSSION

Among the numerous genes that are known to be silenced in human cancers, for example p53, Syk, APC, BRCA1, *etc.*[18-20], RIZ1 is one of the few with a proven role in causing cancer as demonstrated. Whereas previous studies demonstrate reduced *RIZ1* gene expression to be common in cancers, this study confirms that RIZ1 is commonly silenced by DNA methylation. The RIZ1 promoter has been demonstrated to have the characteristics of a CpG island, which suggests that RIZ1 is a target of inactivation by epigenetic mechanisms[21]. In prostate cancer, 42.6% of cancer cases were reported to have RIZ1 methylation, and was more frequent in patients with a high-grade malignancy[13]. In gastric adenocarcinoma, hypermethylation of RIZ1 was found in 69% of cancer tissues and in 21% of corresponding non-neoplastic mucosa[22]. In thyroid carcinoma Lal *et al*[14] reported that all of the 31 cancerous cases were methylated, and methylation was significantly frequent compared with normal thyroid tissues (33%). Du *et al*[21] reported that methylation of RIZ1 was detected in 44% (11/25) of breast cancer specimens and 62% (20/32) of liver cancer specimens. However, RIZ1 mutation has not been detected in these cancers[23]. Thus, DNA methylation may represent the preferred mechanism of RIZ1 inactivation in these cancers. Furthermore, because many types of human cancer cell lines exhibit reduced RIZ1 expression, we predict that *RIZ1* gene methylation will be commonly found in many types of human cancer tissue. In a previous study, we found that, compared with normal tissues, the expres-

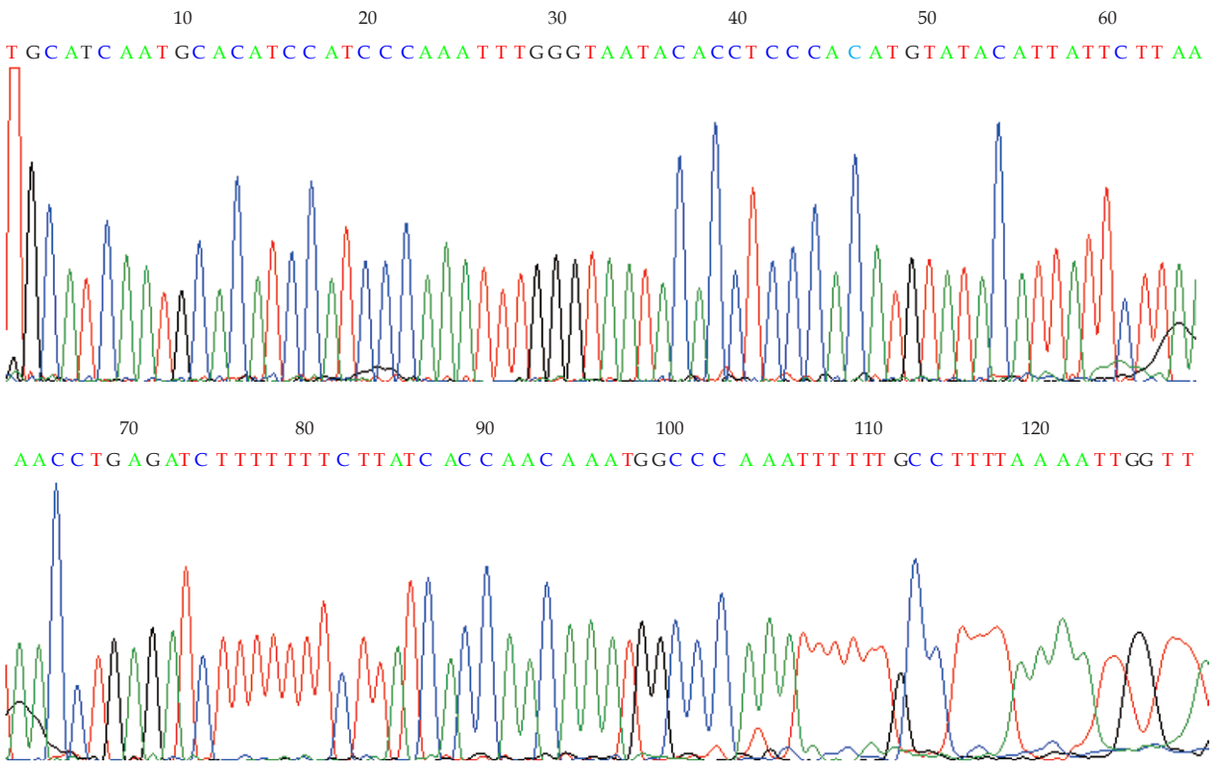


**Figure 4 Sequencing analysis of reverse transcription-polymerase chain reaction production using retinoblastoma protein-interacting zinc finger gene 1 F-primer, which is the fragment aimed at.**

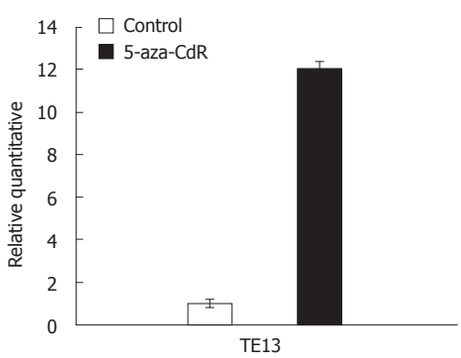


**Figure 5 The expression of retinoblastoma protein-interacting zinc finger 1 when the drug 5-aza-CdR is used.**

sion of RIZ1 mRNA was significantly lower in cancer tissues than in the adjacent non-cancerous tissues ($P < 0.01$). But in this study there was no significant difference between cancer tissues and the adjacent non-cancerous tissues ($P = 0.067$). No RIZ1 protein expression was observed in the cancer tissues, as it was 0% (0/12), while the expression level in the normal tissues was 66.67% (8/12). There was a statistically significant difference between RIZ1 mRNA and protein expression ($P < 0.05$) which indicates that RIZ1 expression may reduce the occurrence of ESCC. RIZ1 may be a candidate tumor suppressor in ESCC.

In this paper, we explored DNA methylation of RIZ1 in the promoter region among human ESCC cell lines, malignant human ESCC, its matched adjacent normal and distal ending normal tissues. MSP was used to detect

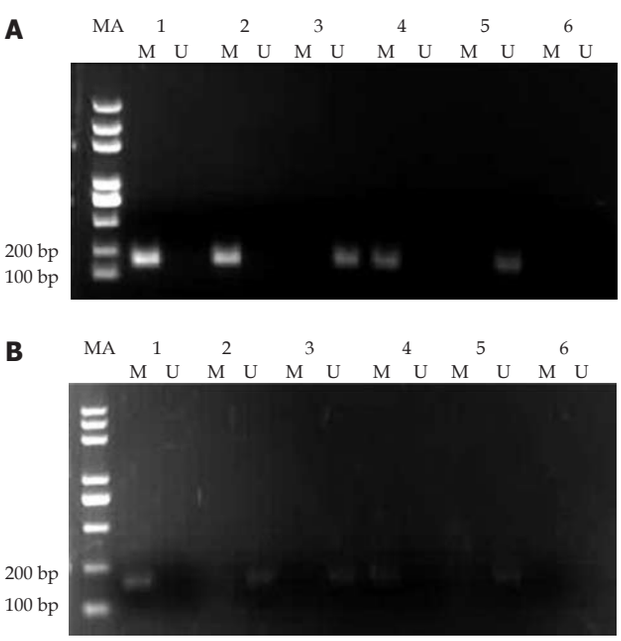


**Figure 6 Methylation-specific polymerase chain reaction electrophoresis for cancerous, neighboring and distal ending normal tissues.** A: The results of electrophoresis for the polymerase chain reaction (MSP) products of positive retinoblastoma protein-interacting zinc finger gene 1 (RIZ1) methylation amplification sufferers in both their cancerous and neighboring normal tissues; B: The results of electrophoresis for the MSP products of sufferers in whose cancerous tissue positive RIZ1 methylation amplification appears while positive unmethylation amplification is present in the corresponding neighboring normal tissues. MA: Marker; M: Methylation (177bp); U: Unmethylation (175bp);1: Carcinomas; 2: Matched neighboring normal esophageal tissues; 3: Distal ending normal esophageal tissues; 4: Postive control for methylation; 5: Postive control for unmethylation; 6: Negative control.

**Table 1 Correlation between methylation of retinoblastoma protein-interacting zinc finger gene 1 gene promoter region and clinicopathologic factors in 47 esophageal squamous cell carcinoma patients' tissues**

| Factor | Methylation | Unmethylation | *n* | $\chi^2$ | *P* value |
|---|---|---|---|---|---|
| Gender | | | | 0.340 | 0.560 |
| Male | 23 | 15 | 38 | | |
| Female | 7 | 2 | 9 | | |
| Age | | | | 0.029 | 0.865 |
| > 50 | 14 | 20 | 34 | | |
| ≤ 50 | 5 | 8 | 13 | | |
| Family history | | | | 0.151 | 0.698 |
| Present | 6 | 3 | 9 | | |
| Absent | 20 | 18 | 38 | | |
| Differentiation | | | | 0.346 | 0.841 |
| Well | 6 | 9 | 15 | | |
| Moderately | 6 | 13 | 19 | | |
| Poorly | 4 | 9 | 13 | | |
| Depth of invasion | | | | 1.420 | 0.492 |
| Mucosa and submuscosa | 4 | 5 | 9 | | |
| Muscle | 3 | 6 | 9 | | |
| Serosa | 16 | 13 | 29 | | |
| Lymph node metastasis | | | | 3.489 | 0.062 |
| Present | 10 | 12 | 22 | | |
| Absent | 5 | 20 | 25 | | |
| Stage | | | | 3.670 | 0.55 |
| Ⅰ-Ⅱ | 6 | 18 | 24 | | |
| Ⅲ-Ⅳ | 12 | 11 | 23 | | |

the promoter region methylation status of *RIZ1* gene in human ESCC cell lines including KYSE150, KYSE510, TE13, EC9706, CaEs17 and EC109. Promoter methylation of *RIZ1* gene was detected in TE13, CaEs17 and EC109. TE13 was chosen for further research and treated with 5-aza-CdR. Real-time PCR shows us that the expression of RIZ1 mRNA in TE-13 was up-regulated after treatment by the drug. The results of study using ESCC pathological frozen specimens illustrate that the R1Z1 gene promoter of the ESCC possesses a 55.3% methylation positive ratio (26/47). RIZ1 methylation positive amplification was seen in 26 sets of carcinoma tissue from 47 sufferers and unmethylation positive amplification was seen in 21. In matched neighboring normal esophageal tissues, the RIZ1 methylation positive amplification appeared in 3 cases, while unmethylation positive amplification was found in 44 cases. Furthermore, in distal ending normal esophageal tissues, the methylation positive amplification did not exist, i.e., all 47 studied cases possessed unmethylation positive amplification. Among those 3 sufferers with RIZ1 methylation positive amplification in matched neighbouring normal esophageal tissues, the corresponding carcinoma tissue also contained RIZ1 methylation positive amplification. In the 47 sufferers of ESCC, 3 possessed methylation in carcinoma tissue and matched neighbouring normal tissue but possessed unmethylation in distal ending normal tissue. Twenty three sufferers showed methylation in carcinoma tissues, but unmethylation in matched neighbouring normal tissue and distal ending normal tissue. Twenty one sufferers possessed unmethylation in carcinoma, matched neighbouring normal and distal ending normal tissues. The rate of methylation of RIZ1 promoter in ESCC was higher than that of adjacent normal tissues ($\chi^2 = 24.1$, $P < 0.01$).

DNA methylation in the promoter region may play an important role in the epigenetic silencing of *RIZ1* gene expression. However, the test to determine *RIZ1* gene promoter methylation shows there is no methylation found in 2 other human ESCC cell lines. This means that dimunition of RIZ1 expression is not always triggered by promoter methylation. One such potential mechanism is that RIZ1 silencing could be caused by a defect in a certain transcription factor that normally activates the RIZ1 promoter. Another potential mechanism is mutation in the RIZ1 promoter. However, given the prevalence of DNA methylation, these other mechanisms are not likely to be commonly involved, and further research is required to find out the true mechanism. Furthermore, we did not observe any significant correlation between RIZ1 methylation and tumour grade. This may be attributable to a relatively small sample size and the complexity of the unselected patient population. Additional detailed studies using a patient cohort should be done needed to examine the value of RIZ1 methylation as a diagnostic or prognostic marker. Another explanation is that gene methylation exists in the early stage of ESCC, but not in the middle and late stages. Hence, there is a statistically insignificant discrepancy during the development of tumour and the displacement of lymph gland. We guess that the methylation of the CpG island in RIZ1 may be an important molecular mechanism during the appearance and development of ESCC in the early stage, and may become a biological parameter for testing early stage of ESCC.

The vital importance of the epigenetic changes on the generation and development of tumours has been thoroughly realized by human beings. Nowadays, there exists an expert database of DNA methylation for researchers (www.methdb.de). The DNA methylation abnormality of malignancy characterizes significantly according to: specifics of the tumour, specifics and reversibility of genes and tissues, *etc*. Methylation testing techniques possess high sensibility and specificity. Thereinto, the MSP technique can be applied for testing some small quantity of tissues sections, phlegm and urine, *etc*., as well as for clinical follow-up through quantitative analysis of relevant genes. Therefore, further research on the mechanisms of the tumour suppressor gene *RIZ1* on esophageal cancer may show some new parameters of early diagnosis and prognosis evaluation for esophageal cancer. The DNMT, 5-aza-CdR, has been clinically applied for curing some solid tumors and some hematological diseases, such as myelodysplastic syndrome, acute myeloid leukemia, etc. A new therapy target for esophageal cancer may be found by strengthening the research on DNA methylation of the genes and the significance of application of 5-aza-CdR.

## ACKNOWLEDGMENTS

We thank Dr. Si-Cheng Zhao for verbal changes in the material.

## COMMENTS

### Background

Recently, methylation of the *retinoblastoma protein-interacting zinc finger* gene (*RIZ1*) promoter CpG islands has been proposed as a common mechanism in inactivating RIZ1. Increasing clinical evidence reveals a positive correlation of reduced RIZ1 expression with increased risk for metastasis, indicating that RIZ1 may be a potential new tumour suppressor gene (*TSG*). However, although RIZ1 is a putative tumour suppressor in several cancer types, for instance breast cancer, gastric cancer, lung cancer, the role of RIZ1 in human esophageal squamous cell carcinoma (ESCC) has not been reported.

### Research frontiers

In mammalian development, DNA methylation has an essential regulatory function of suppressing gene activity by changing chromatin structure. It has become apparent that aberrant DNA methylation of promoter region CpG islands may serve as an alternate mechanism to genetic defects in the inactivation of TSG in human malignancies.

### Innovations and breakthroughs

China is a country with a high incidence of ESCC, and the pathological type is mainly squamous cell carcinoma, which is different from adenocarcinoma reported in other countries. The present study aimed to discover the effect and mechanism of the anti-cancer gene *RIZ1* on ESCC. The results illustrate that low expression of RIZ1 in ESCC is relevant to promoter methylation. Methylation takes place in the early stage of carcinogenesis, and it may become a molecular biological parameter for early diagnosis.

### Applications

The methylation of RIZ1 is the major reason for the low expression in ESCC. This could take place in early stages of ESCC, and is expected to be a molecular biological parameter for early diagnosis.

### Peer review

This paper reports RIZ1 promoter methylation in esophageal cancer in a usual way.

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