Forkhead box E1, frequently methylated in colorectal cancer, inhibits cancer cell growth and metastasis by changing the cellular actin skeleton

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Abstract

Forkhead box E1 (FOXE1), also known as thyroid transcription factor 2 (TTF-2), belongs to a large family of forkhead transcription factors. It plays important roles in embryogenesis, cell growth, and differentiation. Cancer-specific FOXE1 hypermethylation events have been identified in several cancers. However, the expression and function of FOXE1 in the tumorigenesis of colorectal cancer remain still unknown. In this study, we examined FOXE1 expression and methylation in normal colon mucosa, CRC cell lines, and primary tumors by immunohistochemistry, semi-quantitative RT-PCR, methylation-specific PCR, and bisulfite genomic sequencing. We found that FOXE1 was frequently methylated and silenced in CRC cell lines and was downregulated in CRC tissues compared with paired adjacent non-tumor tissues. Meanwhile, low FOXE1 expression was significantly correlated with lymph node metastasis and advanced TNM stages, indicating its potential as a tumor marker. Subsequently, we established colon cancer cell lines with stable FOXE1 expression to observe the biological effect on colorectal cancer, including cell growth, migration, actin cytoskeleton, and growth of human colorectal xenografts, in nude mice. Ectopic expression of FOXE1 could suppress tumor cell growth and migration and affect the organization of the actin cytoskeleton together with suppressing tumorigenicity in vivo. FOXE1 methylation was frequently seen in association with a complete absence of or downregulated gene expression, and FOXE1 plays a suppressive role in the development and progression of colorectal cancer.

Introduction

Colorectal cancer (CRC) is the third-most common malignant tumor in the world, with more than 1.2 million new cases reported each year. While the incidence of CRC has stabilized in developed countries [1, 2], it has increased in China in recent decades [3]. Although certain progress has been made in the treatment of CRC in recent years, especially new targeted drugs that have improved the survival time of patients with advanced CRC [4], the overall five-year survival rate is still only 30%–60% [5]. The main factor affecting the prognosis of CRC patients is that most patients are already in an advanced stage when diagnosed. Therefore, early diagnosis and treatment are keys to improving the survival rate of CRC patients.

Lifestyle, environment, and diet factors may cause epigenetic changes in disease-related genetic information, which plays an important role in the initiation and progression of CRC [6]. There are many mechanisms of epigenetic modification, including DNA methylation, histone modification, and non-coding RNA regulation [7]. Abnormal DNA methylation is characterized by widespread genome-wide hypomethylation and hypermethylation of CpG islands in the gene-promoter region [8]. Hypomethylation can promote chromosome instability and aneuploidy abnormalities. Hypermethylation of promoters of some tumor suppressor genes often leads to gene silencing, which can lead to the activation or inhibition of various signaling pathways, thus affecting various stages of tumor initiation and progression [9, 10].

Hypermethylation of CpG islands in the promoter regions of genes expressed in normal colon mucosa leads to transcriptional repression in CRC [11, 12]. We can facilitate early diagnosis and prognosis
analysis of tumors by analyzing the DNA methylation status of patients [13].

*FOXE1*, also known as thyroid transcription factor 2, is a member of the FOX transcription factor superfamily. The human *FOXE1* gene, located on chromosome 9q22, is 3.5 kb in length and has a single exon [14,15]. Its complementary DNA encodes a protein consisting of 376 amino acid residues. The FOXE1 protein consists of a FOX domain with nuclear localization signals at both ends and a poly-alanine channel [15] whose length varies in the population from 11–19 amino acid residues, with 14 amino acid residues being the most common [16–18].

*FOXE1* is a thyroid-specific transcription factor with an important role in the early stage of thyroid embryo development that regulates the correct migration of thyroid primordia and the normal differentiation and proliferation of thyroid cells. Recent studies have found that FOXE1 is closely related to the occurrence and development of many human tumors, including thyroid cancer [19, 20], pancreatic cancer [21], skin cancer [22], and breast cancer [23]. Furthermore, FOXE1 expression can be restored following treatment with demethylation reagent 5-Aza [24].

Previously, it has been reported that *FOXE1* has significantly different methylation statuses in normal colon mucosal tissues and CRC tissues, but its biological function and mechanism in the occurrence and development of CRC remain unclear. Therefore, in this study, we examined the frequency of *FOXE1* inactivation and explored its functions and mechanisms in CRC.

**Materials And Methods**

**Cell lines, tumor samples, and normal control tissues**

Eleven colorectal cell lines (Colo320, DLD-1, HCE8693, HCT-116, HT-29, RKO, SW620, SW480, LOVO, Colo205, and HCT-8) were used. The cell lines were maintained at 37°C in a humidified 5% CO₂ incubator in RPMI 1640, Dulbecco's modified Eagle's medium, or McCoy's5A medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS).

Human CRC tissue was collected from patients with adenocarcinoma confirmed pathologically without direct surgical resection of neoadjuvant therapy between December 2000 and April 2007 in Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, and obtained with informed consent from patients.

FOXE1 immunohistochemical analysis was performed on 10 normal colonic mucosa specimens, 128 primary CRC specimens, 27 metastatic lymph node specimens, and 29 colorectal adenomas (including nine tubular adenomas, eight serrated adenomas, and 12 villous adenomas). Methylation-specific PCR MSP testing was performed on the tumor tissue and normal adjacent tissue samples of 35 CRC patients stored in a tissue bank. TNM staging for each patient was based on the seventh edition of tumor TNM staging issued by the American Cancer Federation (AJCC, 2010). In terms of the degree of differentiation, we divided tumors into a highly differentiated group (including highly differentiated
tubular adenocarcinoma and papillary adenocarcinoma cases), moderately differentiated group (including moderately differentiated tubular adenocarcinoma cases), and poorly differentiated group (including poorly differentiated adenocarcinoma, undifferentiated adenocarcinoma, mucinous adenocarcinoma, and signed-ring cell carcinoma cases).

**RNA extraction and semi-quantitative RT-PCR**

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), as described by the manufacturer. Reverse transcription of the reaction mixture (20 μL) containing total RNA (1 μg) to cDNA was done with M-MLV (Promega Corporation, Madison, WI, USA). The mRNA expression levels of *FOXE1* were determined by semi-quantitative reverse-transcription PCR (RT-PCR) with GoTaq polymerase (Promega Corporation, Madison, WI, USA). The transcription of the principal gene *GAPDH* was used as the internal control. Specific primers were designed according to the *FOXE1* sequence. All sequences of primers used were showed in table1.

**Bisulfite treatment and promoter methylation analysis**

Genome DNA was extracted from tissues using a Tiangen DNA mini kit (Tiangen, Beijing, China), following the manufacturer’s instructions. Bisulfite modification of DNA was performed as previously described [25]. MSP was performed using AmpliTaq-Gold DNA polymerase (Applied Biosystems, Waltham, MA, USA). The PCR products were identified on 1.5% agarose gels.

**Demethylation treatment using 5-aza-2′-deoxycytidine and trichostatin A**

*FOXE1*-silenced cell lines were cultured in a 10-cm dish with 1×10^6^ cells each. After overnight culturing, the cells were treated with 5-aza (10 μmol/L) for 72 h and TSA (300 nmol/L) for 24 h. Finally, we harvested the treated cells and extracted DNA and RNA for use.

**Immunohistochemistry**

IHC was performed using the ChemMate EnVision detection kit (Dako, Carpinteria, CA, USA) as described by the manufacturer. Briefly, the selected sections were incubated with primary *FOXE1* antibody (1:250 Abcam), GAPDH antibody (1:200 Invitrogen) overnight at 4 °C, and then incubated with ChemMate EnVision/HRP rabbit/mouse reagent as a secondary antibody. Afterward, the sections were developed using ChemMate DAB+ chromogen and counterstained with hematoxylin. The percentage of positive cells was evaluated and scored as 0 (< 5%, negative), 1 (5%–25%, sporadic), 2 (26%–50%, local), 3 (51%–75%, diffuse), or 4 (> 75%, positive) points. The intensity of staining was evaluated and scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining) points. Then, both scores were multiplied to produce an immunoreactivity score (IRS) value ranging from 0 to 12 points to evaluate the association of *FOXE1* expression with clinicopathological parameters in a manner corresponding to four expression intensities: 0–1 points, negative; 2–4 points, weakly positive (+); 5–8 points, moderately positive (++); and 9–12 points, strongly positive, (+++). Patients were then grouped into two categories.
based on expression intensity: low expression (negative or weakly positive) and high expression (moderately or strongly positive).

**Immunofluorescence**

For immunofluorescence, cells (2×10⁵ cells/well) were seeded in the coverslip in a six-well plate with a coverslip inside. After 24 h of culture, cells were fixed in 3.7% paraformaldehyde for 10 min and incubated in PBS with 0.1% Triton X-100 for 4 min on ice, then blocked in 5% FBS for 20 min. Coverslips were moved to a slide and cells were washed three times with PBS; then, we added 200 μL rhodamine phalloidin (Invitrogen, Carlsbad, CA, USA) of 100 nM and additionally incubated cells at room temperature shielded from light for 30 min. Nuclei were stained with PBS with 2 μg/mL of DAPI (Roche, CH) for 10 min. Staining was photographed by an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). All of the experiments were repeated three times.

**Construction of FOXE1 expression vector**

The DLD-1 CRC cell line was transfected with control or FOXE1-expressing plasmid (pCMV6 Entry; Origene, Rockville, MD, USA) using the MegaTran 1.0 transfection reagent (Origene). Stable FOXE1-expressing clones were selected for further study.

**Monolayer and soft agar colony formation assays**

1000 FOXE1 expression vector stable transfected and parental cells were plated in 10 cm dishes, respectively, and allowed to grow for two weeks at 37°C in 5% CO₂. Surviving colonies (≥50 cells/colony) were counted under a microscope after gentian violet staining. The experiments were performed in triplicate.

For the soft agar assay, transfected cells were suspended in a growth medium containing 0.3% agar and seeded into a six-well plate overlaid with 0.5% low-melt agar. Surviving colonies (≥50 cells) were photographed and counted after 14–25 days at 37°C in 5% CO₂. The experiments were performed in three wells in triplicate.

**Wound-healing assay**

Cell motility was assessed using a scratch wound assay. The transfected cells and controls were cultured in six-well dishes until confluent. The cell layers were carefully wounded using sterile tips and washed twice with phosphate-buffered saline. Cells were incubated with fresh medium and photographed under a phase contrast microscope at 0, 12, 24, 36, and 48 h after wounding. The experiments were performed in triplicate.

**Cell migration assay**
For the Transwell assay, cells were trypsinized and resuspended in a corresponding medium containing 1% FBS at a density of 1×10^6 cells/mL. One hundred microliters of cell suspension was added to the upper chamber of a Transwell system (Corning, Corning, NY, USA) consisting of inserts containing 8-mm pore-size PET membranes. Six hundred microliters of medium containing 2.5% FBS was placed in the lower chamber. After the indicated amount of incubation time at 37°C in 5% CO₂, cells remaining in the upper chamber were removed carefully by cotton swab, and those on the bottom side of the chamber membrane were fixed, stained with 0.25% crystal violet, photographed, and counted under a light microscope. The experiments were performed in triplicate.

**In vivo subcutaneous tumor model**

All of the *in vivo* experimental protocols were approved by the animal care committee of Sir Run Run Shaw Hospital, Zhejiang University. Viable *FOXE1*-transfected cells and controls (5×10^6 cells in 0.1 mL of PBS) were injected subcutaneously into the right dorsal flank of six-week-old male BALB/c nude mice (six mice per group). Tumor volume was assessed every two days for five weeks. Tumor volume was calculated with the following formula: (short diameter)^2 × (long diameter)/2.

**Statistical analysis**

Statistical calculations were performed using SPSS version 18.0 for Windows (IBM Corporation, Armonk, NY, USA). Pearson's chi-squared test was used to analyze the association between FOXE1 expression level and clinicopathological parameters. Results are presented as mean ± standard deviation values, and comparisons between groups were completed by analysis of variance. *P* < 0.05 was considered statistically significant.

**Results**

*Methylation and silencing of FOXE1 in CRC cell lines*

First, we tested FOXE1 expression in CRC cell lines by semi-quantitative RT-PCR. Our results showed that FOXE1 was expressed in only two cell lines while being silenced in the other nine. *FOXE1* methylation status was tested by MSP to elucidate the effect of promoter methylation in the downregulation of *FOXE1*. Methylation of *FOXE1* was detected in all of the cell lines with silenced *FOXE1* expression (Figure 1A). We treated the CRC cell lines with FOXE1 expression silencing (DLD-1 and RKO) with 5-Aza and TSA and found that FOXE1 expression could be restored. Representative results are shown in Figure 1B.

**Figure 1.** FOXE1 expression and its promoter CGI methylation in CRC cell lines and primary tumors. (A) FOXE1 was greatly reduced or silenced in most colorectal cancer cell lines (9/11). (B) Pharmacological demethylation with 5-Aza and histone deacetylase inhibitor TSA restored FOXE1 expression in methylated and silenced cell lines and demethylation was confirmed by MSP. (C) Sequence of promoter region CpG island. (D) Typical BGS results. Each short vertical line is one CpG site. FOXE1 methylation was analyzed by BGS analysis. One row of circles represents an individual allele of the FOXE1 promoter.
analyzed. Each circle represents one CpG site and filled circles are methylated CpG sites. (E) Representative MSP results of primary tumor tissues (T) and their paired normal tissues (N) and MSP results of normal colorectal mucosa biopsy tissues.

We further examined the detailed methylation profiles of *FOXE1* CGI by bisulfite genomic sequencing analysis, including those CpG sites analyzed by MSP (Figure 1C). The results showed that methylated CpG sites could not be found in the Colo 320 cell line with high FOXE1 expression, while it was markedly increased in the HCE8693 cell line with low FOXE1 expression and densely detected in the DLD-1 cell line with *FOXE1* silencing. The representative results are shown in Figure 1D.

Meanwhile, both MSP and bisulfite genomic sequencing showed that *FOXE1* CGI was remarkably demethylated after treatment with 5-Aza and TSA (Figure 1B and D). These results suggest a direct link between CGI methylation and FOXE1 expression.

We also analyzed *FOXE1* methylation status in 10 normal colorectal tissues, 35 primary colorectal tumors, and paired adjacent non-tumor tissues. *FOXE1* methylation was not detected in normal mucosal tissues but was detected in 85.7% (30/35) of tumors and only 20.0% (7/35) of adjacent non-tumor tissues. Representative results are shown in Figure 1E. These results suggested that *FOXE1* gene methylation may be closely related to the development of CRC.

**FOXE1 was downregulated in CRC tissues**

We investigated FOXE1 expression in a total of 128 primary CRC tissues, 27 metastatic lymph node tissues, 29 colorectal adenomas (tubular adenoma, serrated adenoma, and villous adenoma) tissues, and 10 normal colorectal mucosal tissues by immunohistochemistry. High FOXE1 expression was shown in all of the normal mucosal epithelium (10/10) tissues but only 25% (32/128) of primary CRC tissues and 7.4% (2/27) of metastatic lymph node tissues. This trend suggests that the expression of FOXE1 in CRC is significantly decreased, and decreased expression of FOXE1 may be associated with lymph node metastasis of CRC (Table 1).

**Table 1. FOXE1 expression in normal colon mucosa and colorectal cancer tissues**
<table>
<thead>
<tr>
<th>Tissue samples</th>
<th>n</th>
<th>Low (%)</th>
<th>High (%)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Normal colonic mucosa samples</td>
<td>10</td>
<td>0 (0)</td>
<td>10 (100)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Primary colorectal cancer</td>
<td>128</td>
<td>96 (75.0)</td>
<td>32 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Metastatic lymph nodes</td>
<td>27</td>
<td>25 (92.6)</td>
<td>2 (7.4)</td>
<td></td>
</tr>
<tr>
<td>Tubular colorectal adenoma</td>
<td>9</td>
<td>2 (22.2)</td>
<td>7 (77.8)</td>
<td>0.006*</td>
</tr>
<tr>
<td>Serrated colorectal adenoma</td>
<td>8</td>
<td>0 (0)</td>
<td>8 (100)</td>
<td></td>
</tr>
<tr>
<td>Villous colorectal adenoma</td>
<td>12</td>
<td>8 (66.7)</td>
<td>4 (33.3)</td>
<td></td>
</tr>
</tbody>
</table>

Interestingly, the high expression rate of FOXE1 in tubular adenoma and serrated adenoma was like that found in the normal colon mucosa, while FOXE1 expression in villous adenoma was significantly decreased, close to the level of that associated with primary CRC. Representative immunohistochemical staining results are shown in Figure 2.

Figure 2. Representative immunohistochemical staining of FOXE1, which was strongly expressed in the cytoplasm and nucleus of normal epithelial and some stromal cells but silenced or weakly expressed in tumor cells and lymph nodes, moreover, FOXE1 was highly expressed in colorectal tubular adenoma and serrated adenoma but weakly expressed in villous adenoma: normal colon mucosa (a), primary colorectal cancer (b), metastatic lymph node (c), colorectal tubular adenoma (d), serrated adenoma (e), and villous adenoma (f).

Correlation between FOXE1 expression and clinicopathological features of CRC patients

We analyzed the correlation between FOXE1 protein expression and the clinicopathological features of CRC patients. The tissues were separated into two groups by immunohistochemistry result: low expression (negative or weakly positive) and high expression (moderately or strongly positive). The correlation between FOXE1 protein-expression levels and clinicopathological features of CRC patients, including sex, age, tumor location, histological differentiation, depth of invasion, lymph node and distant metastasis, and TNM stage, is shown in Table 3. The results suggest that the expression of FOXE1 protein in primary CRC is significantly correlated with lymph node metastasis and TNM stage.

Interestingly, the high expression rate of FOXE1 in tubular adenoma and serrated adenoma was like that measured in normal colon mucosal tissues, while the FOXE1 expression level in villous adenoma was significantly decreased, being closer to the level in primary CRC (Table 2).

Table 2. Correlation of FOXE1 protein expression and clinicopathological features in 128 cases of primary colorectal cancer
<table>
<thead>
<tr>
<th></th>
<th>FOXE1 immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64</td>
</tr>
<tr>
<td>Female</td>
<td>64</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>60.15</td>
</tr>
<tr>
<td>≥60.15</td>
<td>62</td>
</tr>
<tr>
<td>60.15</td>
<td>66</td>
</tr>
<tr>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>78</td>
</tr>
<tr>
<td>Left colon</td>
<td>29</td>
</tr>
<tr>
<td>Right colon</td>
<td>21</td>
</tr>
<tr>
<td>Histopathological grade</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>46</td>
</tr>
<tr>
<td>moderately</td>
<td>47</td>
</tr>
<tr>
<td>Poorly</td>
<td>35</td>
</tr>
<tr>
<td>pT</td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>3</td>
</tr>
<tr>
<td>pT2</td>
<td>29</td>
</tr>
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<td>pT3</td>
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<td>pT4</td>
<td>4</td>
</tr>
<tr>
<td>pN</td>
<td></td>
</tr>
<tr>
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<td>60</td>
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<td>pN1/2</td>
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<tr>
<td>pM</td>
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<td>pM0</td>
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</tr>
<tr>
<td>pM1</td>
<td>15</td>
</tr>
</tbody>
</table>
**FOXE1 expression inhibited CRC cell proliferation and migration**

To investigate the biological function of FOXE1, a FOXE1-expressing plasmid was stably transfected into DLD-1 and RKO cells without FOXE1 expression. Then, ectopic expression of FOXE1 was confirmed by RT-PCR and western blotting (Figure 3A).

**Figure 3.** (A) FOXE1 expression in stably transfected cells as confirmed by RT–PCR and western blotting. (B) Monolayer culture and quantitative analysis of colony number; values are presented as the mean ± SD. (C) Soft agar assay and quantitative analysis of colony number; values are presented as the mean ± SD. (D) Images of xenograft tumors formed in nude mice injected with FOXE1-Transfected DLD-1 cells (Left) and empty vector-transfected DLD-1 cells(right).

To evaluate the effects of FOXE1 expression on the migration of colorectal tumor cells, wound-healing and Transwell migration assays were performed. Wound healing was prolonged in the FOXE1-transfected cells compared with that in the control cells (Figure 4A). FOXE1-transfected cells also showed significant suppression of migration compared with that in control cells after 24 h of incubation during Transwell assay (Figure 4B).

Moreover, both monolayer culture and soft agar assays showed that the colonies formed among the FOXE1-transfected cells were significantly fewer in number and smaller in size than those of the control cells ($P < 0.05$, Figure 3B and C).

To observe the morphology of DLD-1 cells, cells transfected with FOXE1 or empty vector and parental cells were stained with rhodamine–phalloidin to observe the organization of actin fibers. Cells transfected with FOXE1 displayed diffuse cytoplasmic actin arranged irregularly and significantly fewer thin stress fibers, whereas control groups of cells showed better arrangement of actin fibers, supporting a possible regulatory role for FOXE1 in actin cytoskeleton reorganization (Figure 4C).

These results indicate that FOXE1 possesses growth and migration-inhibitory activities in CRC cells and might function as a tumor suppressor.

**Figure 4.** (A) Representative results of wound-healing assay. (B) Transwell migration assay and quantitative analysis of relative cell count shown; values are presented as mean ± SD (*$P < 0.05)$ (C) Effect of FOXE1 transfection on morphology of DLD-1 and RKO cells, rhodamine-labeled Phalloidin was used to indicate actin polymerization.

**FOXE1 inhibited CRC cell growth in nude mice**
Finally, we investigated whether FOXE1 expression had a suppressive effect on tumor cell growth in vivo. We modeled xenografts in nude mice using DLD-1/vector-transfected and DLD-1/FOXE1-transfected cells. All six of the xenografts of DLD-1/vector-transfected cells showed tumor growth. Overall, the tumor formation rate was 100%, and the tumor volume reached $709 \pm 95 \text{ mm}^3$ after five weeks. The growth curve of a transplanted tumor is shown in Figure 3D. However, there was no obvious subcutaneous tumor found among the six nude mice injected with DLD-1/FOXE1-transfected cells, and the tumor-formation rate was 0%. Our results suggest that FOXE1 can significantly inhibit tumorigenesis in vivo.

**Discussion**

FOXE1 has been widely studied as a thyroid-specific transcription factor in thyroid cancer, and its polymorphisms (rs965513 and rs1867277) are thought to increase susceptibility to thyroid cancer [26]. In recent years, the promoter region of FOXE1 has been found to be methylated in various tumors. Venza et al. reported that the loss of or down-regulation of FOXE1 expression in cutaneous squamous cell carcinoma is related to hypermethylation of the promoter region of FOXE1 [24]. This suggests that epigenetic mechanisms such as DNA methylation may be the main reason for the silencing of FOXE1 expression in tumors. FOXE1, as a tumor-specific methylation marker, can be detected in the pancreatic juice of pancreatic cancer patients [21] and serum of breast cancer patients [23]. Melotte et al. discovered that high expression of FOXE1 can reduce the number of clones in CRC cell lines [27]. CRC arising on the background of inflammatory bowel disease (IBD), colitis-associated cancer (CAC), is often more aggressive than sporadic CRC [28]. Low expression of FOXE1 has been shown to be positively associated with inflammatory bowel disease (IBD) [29]. This suggests its potential value as a tumor marker in clinical diagnosis.

In this study, we found that FOXE1 mRNA was not expressed in most CRC cell lines by RT-PCR. However, FOXE1 gene-promoter hypermethylation was confirmed in these tumor cell lines. After imbuing tumor cells with silenced or reduced expression of FOXE1 using demethylation reagent 5-AZA and histone deacetylase inhibitor TSA, we found that FOXE1 expression could be restored, indicating that FOXE1 methylation is the main cause of FOXE1 gene-silencing. FOXE1 is methylated in most CRC tissues but not in normal adult colorectal mucosal tissues. Unlike in CRC cell lines, non-methylated mRNA fragments were also detected in primary CRC tissues, which may be related to the presence of non-tumor cells in primary tumor tissues. Similar results were also found in the study of Zhang et al., who found that the methylation rate was 63.2% in 19 stage I CRC specimens, while no methylation was found in normal intestinal mucosal epithelium samples.

These results suggest that methylation of the FOXE1 promoter region is a frequent and tumor-specific molecular event in CRC. Immunohistochemistry was used to detect the expression of FOXE1 protein in CRC tissues. The results showed that the expression of FOXE1 protein in primary CRC tissues was significantly lower than that in normal colon mucosa tissues, and the expression of FOXE1 protein was highly expressed in all 10 of the normal colon mucosal samples. The expression of FOXE1 in metastatic lymph nodes was also significantly lower than that in primary CRC tissues. Correlation analysis between
FOXE1 expression and clinicopathological features showed that loss of FOXE1 expression is significantly associated with lymph node metastasis and poor TNM stage in CRC patients. These results suggest that the downregulation of FOXE1 expression may be associated with the progression of CRC.

In the 1970s, Morson et al. proposed the classic sequential theory of normal mucosa, benign adenoma, and adenocarcinoma in CRC [30]. The evolution of adenoma to adenocarcinoma is associated with genomic and epigenetic alterations that accumulate progressively during the development of CRC. It has been reported that more than 90% of colorectal tumors are derived from adenomas through this classical pathway [31]. Colorectal adenoma is considered a precancerous lesion preceding CRC. In terms of pathological features of colorectal adenoma, villous colorectal adenoma has the highest malignant tendency, while tubular colorectal adenoma and serrated colorectal adenoma are significantly less likely to develop into cancer. Our immunohistochemical results showed that the positive rate of FOXE1 protein in villous colorectal adenomas of the large intestine was significantly lower than that in tubular colorectal adenomas and serrated colorectal adenomas. The difference in FOXE1 protein expression in different types of colorectal adenomas may be related to tumor heterogeneity. Further studies are needed to investigate the role of FOXE1 in the evolution of colorectal adenoma to adenocarcinoma in the future.

Through cell viability assay, monolayer, and soft agar colony formation assays, we found that the growth rate and proliferation ability of tumor cell lines were significantly decreased after being transfected with FOXE1. Although FOXE1 did not promote the apoptosis of tumor cells, it may inhibit the growth of tumor cells by arresting the cell cycle. Consistent with the results of FOXE1 inhibition of tumor cell proliferation in vitro, FOXE1 transfection could significantly inhibit the tumorigenesis of DLD-1 cells in vivo. We also found that the FOXE1 gene had a certain inhibitory effect on migration compared relative to the trend in the control group, which is also consistent with the immunohistochemical results.

The cellular actin skeleton plays an important role in maintaining cell morphology and cell motility. Actin comes in monomeric and polymeric forms, with the monomeric form of actin, known as G-actin, being globular, and the polymeric form of actin, called F-actin, being fibrous. The polymerization and reorganization of the cellular actin skeleton are the first and key steps of cell migration. During cell migration, the content of F-actin is often increased (actin polymerization), which enhances cell motility [32]. In our experiment, we labeled F-actin with rhodamine–phalloidin and carried out cellular immunofluorescence assays. The results suggested that FOXE1 could prevent the recombination of F-actin and significantly reduce the number of stress fibers, which may be a reason why FOXE1 weakens the migration ability of tumor cells.

In conclusion, our results suggest that the silencing or downregulation of FOXE1 in CRC is mainly regulated by methylation of the promoter region, and FOXE1 may play a role as a tumor suppressor gene. Although the specific molecular mechanism of FOXE1 in the tumorigenesis and progression of CRC is still unclear, the hypermethylation of the FOXE1 gene-promoter region may have potential value for the clinical diagnosis of CRC.
Declarations

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

Ethics approval and consent to participate

All animal experiments were approved by the animal care committee of Sir Run Run Shaw Hospital, Zhejiang University.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References


Figures
Figure 1

FOXE1 expression and its promoter CGI methylation in CRC cell lines and primary tumors. (A) FOXE1 was greatly reduced or silenced in most colorectal cancer cell lines (9/11). (B) Pharmacological demethylation with 5-Aza and histone deacetylase inhibitor TSA restored FOXE1 expression in methylated and silenced cell lines and demethylation was confirmed by MSP. (C) Sequence of promoter region CpG island. (D) Typical BGS results. Each short vertical line is one CpG site. FOXE1 methylation was analyzed by BGS analysis. One row of circles represents an individual allele of the FOXE1 promoter analyzed. Each circle represents one CpG site and filled circles are methylated CpG sites. (E) Representative MSP results of primary tumor tissues (T) and their paired normal tissues (N) and MSP results of normal colorectal mucosa biopsy tissues.
Figure 2

Representative immunohistochemical staining of FOXE1, which was strongly expressed in the cytoplasm and nucleus of normal epithelial and some stromal cells but silenced or weakly expressed in tumor cells and lymph nodes, moreover, FOXE1 was highly expressed in colorectal tubular adenoma and serrated adenoma but weakly expressed in villous adenoma: normal colon mucosa (a), primary colorectal cancer (b), metastatic lymph node (c), colorectal tubular adenoma (d), serrated adenoma (e), and villous adenoma (f).
Figure 3

(A) FOXE1 expression in stably transfected cells as confirmed by RT-PCR and western blotting. (B) Monolayer culture and quantitative analysis of colony number; values are presented as the mean ± SD. (C) Soft agar assay and quantitative analysis of colony number; values are presented as the mean ± SD. (D) Images of xenograft tumors formed in nude mice injected with FOXE1-Transfected DLD-1 cells (Left) and empty vector-transfected DLD-1 cells (right).
Figure 4

(A) Representative results of wound-healing assay. (B) Transwell migration assay and quantitative analysis of relative cell count shown; values are presented as mean ± SD (*P < 0.05) (C) Effect of FOXE1 transfection on morphology of DLD-1 and RKO cells, rhodamine-labeled Phalloidin was used to indicate actin polymerization.

Supplementary Files

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- TableS1.docx