Downregulation of RBMS3 Is Associated with Poor Prognosis in Esophageal Squamous Cell Carcinoma

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Abstract

Deletions on chromosome 3p occur often in many solid tumors, including esophageal squamous cell carcinoma (ESCC), suggesting the existence at this location of one or more tumor suppressor genes (TSG). In this study, we characterized RBMS3 gene encoding an RNA-binding protein as a candidate TSG located at 3p24. Downregulation of RBMS3 mRNA and protein levels was documented in approximately 50% of the primary ESCCs examined. Clinical association studies determined that RBMS3 downregulation was associated with poor clinical outcomes. RBMS3 expression effectively suppressed the tumorigenicity of ESCC cells in vitro and in vivo, including by inhibition of cell growth rate, foci formation, soft agar colony formation, and tumor formation in nude mice. Molecular analyses revealed that RBMS3 downregulated c-Myc and CDK4, leading to subsequent inhibition of Rb phosphorylation. Together, our findings suggest a tumor suppression function for the human RBMS3 gene in ESCC, acting through c-Myc downregulation, with genetic loss of this gene in ESCC contributing to poor outcomes in this deadly disease. Cancer Res; 71(19): 6106–15. ©2011 AACR.

Introduction

Esophageal cancer is one of the most common fatal cancers worldwide (1). It ranks as the ninth most frequent cancer in the world, with a remarkable geographic distribution between and within countries. High-risk areas include northern Iran, South Africa, and northern China, where esophageal squamous cell carcinoma (ESCC) is still the main cancer burden and the fourth most common cause of death (2). Although epidemiologic studies indicate that tobacco and alcohol consumption are the major risk factors for squamous esophageal cancer in the low-risk regions of Europe and North America, the etiologic agents in high-risk regions have yet to be convincingly identified. Previous studies in high-risk regions showed a strong tendency toward familial aggregation (3, 4), suggesting that genetic susceptibility may play a role in the pathogenesis of ESCC.

Chromosomal deletion is a major cause of the inactivation of tumor suppressor genes (TSG). Loss of 3p is one of the most frequent genetic alterations in ESCC detected by comparative genomic hybridization and LOH (5–8), suggesting the existence of one or more TSGs at frequently deleted regions. In our recent study, single-nucleotide polymorphism (SNP) mass array was applied to investigate LOH on 3p in 100 primary ESCC cases (9). Four commonly deleted regions (CDR) at 3p26.3, 3p22, 3p21.3, and 3p14.2 were identified. Two candidate TSGs within these CDRs, including PLCD1 (10) and PCAF (11), have been investigated. In this study, another candidate TSG, RBMS3 at 3p24, was investigated. RBMS3 was first identified by screening a human fibroblast cDNA library with a labeled DNA fragment derived from α2(1) collagen promoter Box 5A (12). The protein contains 2 RNA-binding domains and it can bind strongly to synthetic poly-U and poly-A oligoribonucleotides, suggesting that it is an RNA-binding protein (12). RBMS3 belongs to the family of c-Myc gene single-strand binding proteins (MSSP), which has 2 additional members, RBMS1 and RBMS2 (13, 14). RBMS1 was reported to be involved in binding to c-Myc protein (14), but no such function has been described for RBMS3. It was reported that RBMS3 expressed in activated hepatic stellate cells and increased expression of transcription factor Prx1 (15). Nevertheless, there was no report investigating the functions of RBMS3 in tumor development and/or progression.

In this study, expression of RBMS3 in primary ESCCs and ESCC cell lines was investigated by real time-PCR and immunohistochemistry (IHC) staining. To examine the possible tumor-suppressive activity of RBMS3 in ESCC, RBMS3 was transfected into 2 ESCC cell lines, KYSE30 and KYSE180. The tumor-suppressive effects of RBMS3 were characterized by both in vitro and in vivo assays. The tumor-suppressing mechanisms of RBMS3, as well as its clinical significance, were also investigated.
Materials and Methods

Cell lines and primary tumor specimens
Three esophageal cancer cell lines—KYSE30, KYSE140, and KYSE180—were obtained from DSMZ, the German Resource Center for Biological Material (16, 17) in 2006. Chinese ESCC cell lines EC18, EC109, and HKErC1 were kindly provided by our colleagues at the University of Hong Kong (Professors G. S. Srivastava and G. S. Tsao; ref. 18). The cells were confirmed by cytogenetics as being of human origin in 2009. Forty pairs of primary ESCCs and adjacent nontumorous tissues were collected immediately after surgical resection at Linzhou Cancer Hospital (Henan, China). Tissue samples used in the study were approved by the Committees for Ethical Review of Research Involving Human Subjects at Zhengzhou University (Zhengzhou, China).

SNP detection in tissue samples
Genomic DNA was extracted from tumor samples using TIANamp genomic DNA Kit (Tiangen) according to the manufacturer’s instructions. SNP site (rs987693) within RBMS3 gene was PCR amplified and sequencing analyzed with designed primers (Supplementary Table S1).

5-AZA-2′-deoxycytidine treatment
To study whether demethylation could restore RBMS3 expression in ESCC cell lines, 3 × 10^5 cells were treated with the DNA demethylating agent 5-AZA (Sigma-Aldrich) at 0.1, 1, 10, 50, and 100 μmol/L for 3 days. Drugs and culture medium were refreshed every day during the treatment.

Establishment of RBMS3-expressing cell lines
Omiclink-RBMS3 was purchased from GeneCopoeia Inc. and subcloned into pcDNA3.1 (+) (Invitrogen) according to the manufacturer’s protocol. RBMS3 was then stably transfected into ESCC cell line KYSE30 (RBMS3-30) using Lipofectamine 2000 (Invitrogen). Blank vector pcDNA3.1 (+) transfected cells (Vec-30) were set as controls. RBMS3 was also transiently transfected into ESCC cell lines KYSE140, KYSE180, and HKErC1 cells.

Quantitative real-time PCR
Total RNA from cultured cells and tumor tissues was extracted using Trizol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription of total RNA was done using SuperScript Kit (Invitrogen), and cDNA was subjected to quantitative reverse transcriptase PCR (qRT-PCR) for RBMS3 with 2 pairs of primers (Supplementary Table S1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18s rRNA was used as internal control. qRT-PCR was done using the SYBR Green Supermix and ABI7900HT Fast Real-Time PCR system (Applied Biosystems). The assays were done in triplicate and values were normalized using the internal control. Quantitative data were exported and RBMS3 expression was normalized by internal control. PCR products were subjected to dissociation curve analysis using the light cycler system to exclude amplification of nonspecific products. Quantitative RT-PCR data were processed using the ΔC_T method as described previously (19).

Tissue microarray and immunohistochemistry
Archives of paraffin blocks from 183 patients with ESCC were obtained from Linzhou Cancer Hospital (Henan, China). None of the patients in this study had received follow-up radiation or chemotherapy. The age of these ESCC patients ranged from 40 to 80 years at the time of surgery (median age, 59 years) and the male:female ratio was 1.3:1. The tissue microarray (TMA) blocks were constructed according to a method described previously (20). Multiple sections (5-μm thick) were used for IHC. In brief, paraffin-embedded TMA sections were deparaffinized and blocked with goat serum for 30 minutes at room temperature, followed by incubation with Mouse anti-RBMS3 (1:40; Abnova) overnight at 4°C. After incubation with horseradish peroxidase–linked secondary antibody (Real Envision Detection Kit; Gene Tech) for 30 minutes, TMA sections were counterstained with Mayer’s hematoxylin. The degree of immunostaining was viewed and scored separately by 2 independent investigators without any prior knowledge of clinicopathologic data. Expression of RBMS3 was scored as absent (total absence of staining), very weak (faint staining in <25% of tumor cells), moderate (moderate staining in ≥25% to <75%, or strong staining in <25% of tumor cells), and strong (moderate staining in ≥75%, or strong staining in ≥25% of tumor cells). In this study, moderate/strong nuclear staining was defined as positive staining and absent/very weak staining as downregulation.

Tumor-suppressive function of RBMS3
To test the tumor-suppressive function of RBMS3, XTT assay, foci formation assay, and colony formation in soft agar were done on cells that overexpressed RBMS3 and vector control cells. Cell proliferation rates of RBMS3-overexpressing cell colonies and vector control cells were compared by XTT assay. Cells were seeded into 96-well plates at a density of 2 × 10^3 cells per well. The cell growth rate was measured using CCK-8 Kit (Tojindo) according to the manufacturer’s instructions. Three independent assays were conducted. For foci formation assay, 2 × 10^3 cells were plated into 6-well plates. Two weeks later, the surviving colonies were fixed, stained with crystal violet, and counted. The result was represented as means ± SD of 3 independent assays. Colony formation in soft agar was done by growing 2 × 10^3 cells in 0.4% bacto-agar on a bottom layer of solidified 0.6% bacto-agar in 6-well plates. After 2 weeks, colonies consisting of more than 50 cells were counted and the result was represented as means ± SD of triplicate independent assays.

In vivo tumor formation assay
The in vivo tumor-suppressive effect of RBMS3 was investigated by xenograft mouse experiments as previously described (10). Animal experiments were done in compliance with the guidelines for the Welfare of Experimental Animals in Sun Yat-sen University. RBMS3-30 and Vec-30 cells (2 × 10^6) were injected s.c. into left and right flanks of
4-week-old male nude BALB/c mice (n = 8), respectively. Mice were then examined for tumor growth by measuring tumor size with calipers every 3 days for 20 days. Tumor volume was calculated \( V = 0.5 \times L \times W^2 \). Following sacrifice, tumors were excised, fixed in 10% formalin, and embedded in paraffin block for IHC study.

**Western blot analysis**

Western blot analysis was done according to the standard protocol with antibodies for RBMS3 (Abnova), GAPDH, c-Myc, Phospho-Rb (Ser780, Ser795, and Ser807/811), CDK2, CDK4, cyclin D1, and cyclin E (Cell Signaling Technology).

**Chromatin immunoprecipitation PCR**

The chromatin immunoprecipitation (ChIP) experiment was conducted using an EZ-Magna ChIP G Kit (Upstate Biotechnology) according to the manufacturer’s instructions. RBMS3-binding DNA fragments were pulled down by an anti-RBMS3 antibody (Abnova) or pooled IgG from mouse (Santa Cruz Biotechnology) as a negative control. The primers used...
PCR amplification of the precipitated DNA fragments are listed in Supplementary Table S1.

**Electrophoretic mobility shift assay**
Biotin end-labeled probe (Supplementary Table S1) was synthesized by Invitrogen. Nuclear extract was extracted using NucBuster Extraction Kit (Novagen). Electrophoretic mobility shift assay (EMSA) was done as the procedure of LightShift Chemiluminescent EMSA Kit (Thermo Scientific). Briefly, biotin-labeled probe (100 fM or 200 fM) and 15-mg nuclear extract were incubated for 20 minutes at room temperature. Free probe was separated from DNA–protein complexes by electrophoresis on a native 6% polyacrylamide gel in 0.5 TBE buffer. After electrophoresis, the DNA was transferred to positively charged nylon membrane, cross-linked, and detected by chemiluminescence.

**RNA interference**
RBMS3 expression was silenced by double-stranded siRNA targeting RBMS3 (siRNA-RBMS3; Supplementary Table S1) and scramble siRNA, which were obtained from Ambion’s predesigned siRNA database (Ambion, Inc.). RBMS3-30 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Gene silencing effect was evaluated by qRT-PCR 48 hours after siRNA treatment (21). Scramble siRNA was used as negative control. The effect of RNA interference (RNAi) was evaluated by foci formation assay 48 hours after siRNA treatment.

**Statistical analysis**
Statistical calculations were carried out with the SPSS statistical software package (Version 16.0; SPSS, Inc.). All data were expressed as means ± SD. The Pearson $\chi^2$ test was used to analyze the relationship between RBMS3 expression and clinicopathologic features. Survival curves were generated according to the Kaplan–Meier method and the statistical analysis was done by log-rank test. Student $t$ test was used to analyze data from cell growth, foci formation, soft agar assays, and tumor formation in nude mice. The value of $P < 0.05$ was considered statistically significant.

**Results**

**RBMS3 is frequently downregulated in ESCC**
To determine the expression pattern of RBMS3 in ESCC tissues, qRT-PCR was done in 40 pairs of tumor tissues and their corresponding nontumorous esophageal tissues. RBMS3 expression was normalized by internal control GAPDH. The result showed that downregulation of RBMS3 was detected in 18 of 40 (45%) of ESCC tissues compared with their paired nontumorous tissues (Fig. 1A). The expression of RBMS3 in 6 ESCC cell lines was also tested by RT-PCR, and the result showed that RBMS3 was downregulated in 3 cell lines (HKESC1, KYSE30, and KYSE140; Fig. 1B). Expression of RBMS3 protein was also compared between tumor and paired nontumor samples using a TMA containing 183 pairs of ESCCs by IHC. Informative TMA cases were observed in 161 normal tissues and 119 tumor cases. The noninformative samples included lost samples and samples with too few cells or with inappropriate staining. Moderate and strong nuclear staining of RBMS3 was detected in 115 of 161 (71.43%) informative normal tissues. Absent and very weak expression of RBMS3 was detected in 63 of 119 (52.9%) tumor tissues (Fig. 1C).

**Downregulation of RBMS3 is associated with DNA copy number loss**
Because no CpG island was found in the promoter region of RBMS3, demethylation agent 5-Aza-2'-deoxycytidine was used for PCR amplification of the precipitated DNA fragments listed in Supplementary Table S1.

**Table 1. Association of RBMS3 downregulation with clinicopathologic characteristics of ESCC patients**

<table>
<thead>
<tr>
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<th>Informative cases</th>
<th>RBMS3 downregulation</th>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>≤57 y</td>
<td>58</td>
<td>35 (60.3%)</td>
<td>0.115</td>
</tr>
<tr>
<td>&gt;57 y</td>
<td>61</td>
<td>28 (45.9%)</td>
<td></td>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>52</td>
<td>27 (51.9%)</td>
<td>0.845</td>
</tr>
<tr>
<td>Female</td>
<td>67</td>
<td>36 (53.7%)</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>7 (41.2%)</td>
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<tr>
<td>2</td>
<td>78</td>
<td>44 (56.4%)</td>
<td></td>
</tr>
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<td>3</td>
<td>24</td>
<td>12 (50%)</td>
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<tr>
<td>T1–2</td>
<td>55</td>
<td>27 (49.1%)</td>
<td>0.435</td>
</tr>
<tr>
<td>T3–4</td>
<td>64</td>
<td>36 (56.2%)</td>
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<tr>
<td>N0</td>
<td>103</td>
<td>55 (53.4%)</td>
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</tr>
<tr>
<td>N1</td>
<td>16</td>
<td>8 (50%)</td>
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</table>

$^a$Pearson $\chi^2$.  

Figure 2. Kaplan–Meier analysis shows that expression of RBMS3 was significantly associated with better overall survival in 119 ESCC cases ($P = 0.034$, log-rank test).
to treat 3 ESCC cells (HKESC1, KYSE30, and KYSE140) with downregulated RBMS3 to test whether promoter methylation affects the expression of RBMS3. The result showed that demethylation treatment could not restore the expression of RBMS3 (data not shown). In our previous study, LOH at the SNP site rs987693 within the RBMS3 gene was detected in 31 of 51 (61%) of primary ESCCs (9). LOH at rs987693 site was tested in the cohort of 40 ESCC samples used in this study. LOH was detected in 11 of 17 (64.7%) of the informative cases (Fig. 1D). Downregulation of RBMS3 was observed in 8 of 11 (72.7%) of the ESCCs with LOH at RBMS3 site, which was significantly higher than those cases without LOH (1/6, 16.7%, \( P = 0.0498 \)).

**Clinical significance of RBMS3 downregulation**

To examine the clinical significance of RBMS3 downregulation in ESCC, the correlation of RBMS3 downregulation with clinicopathologic features was investigated. The association study showed that downregulation of RBMS3 was not significantly associated with age, gender, tumor differentiation, or TNM stage of the patient (Table 1). Interestingly, Kaplan–Meier analysis revealed that the downregulation of RBMS3 was significantly (log-rank test, \( P = 0.034 \)) correlated with poorer outcome of patients with ESCC (Fig. 2).

**RBMS3 has tumor-suppressive ability**

To investigate whether RBMS3 has tumor-suppressive ability, RBMS3 was stably transfected into KYSE30 cells (RBMS3-30) and transiently transfected into KYSE180 cells (RBMS3-180). Empty vector–transfected cells were used as control (Vec-30 or Vec-180). Expression of RBMS3 in RBMS3-30 and RBMS3-180 cells was confirmed by RT-PCR (Fig. 3A and B). The tumor-suppressive function of RBMS3 was tested by both in vitro and in vivo assays. Cell growth assay showed that the growth rates were significantly decreased in RBMS3-30 colonies (\( P < 0.05 \), Student \( t \) test) and RBMS3-180 cells (\( P < 0.05 \), Student \( t \) test), respectively, compared with control cells (Fig. 3C). Soft agar assay showed that RBMS3 could significantly inhibit colony formation in soft agar in RBMS3-30 (\( P < 0.01 \), Student \( t \) test) and RBMS3-180 cells (\( P < 0.05 \), Student \( t \) test) compared with control cells.
Similarly, RBMS3 could also significantly inhibit foci formation ability in RBMS3-overexpressed cells (RBMS3-30 and RBMS3-180) compared with vector control cells (P < 0.01). The results are expressed as mean ± SD of 3 independent experiments. B, summary of tumor growth curves in nude mice induced by RBMS3-30 and Vec-30 cells. The average tumor volume was expressed as mean ± SD in 8 inoculated sites for each group (**, P < 0.001). C, representative samples of tumor formation in nude mice. Tumors induced by RBMS3-30 (left) and Vec-30 cells (right) are indicated by red and black arrows, respectively. Excised tumors are shown in the bottom insets. D, representative samples of RBMS3 expression detected by IHC in xenograft tumors induced by RBMS3-30 (left) and Vec-30 cells (right).

The tumor-suppressive potential of RBMS3 was also evaluated by xenograft tumor formation in athymic nude mice. Subcutaneous visible tumor was observed in the right flank (Vec-30) in all 8 tested animals on day 8 after injection. However, visible tumor in the left flank (RBMS3-30) was only observed in 4 nude mice on day 8. Xenograft tumor growth curve showed that tumor induced by RBMS3-30 cells grew much more slowly than did the Vec-30 cells (P < 0.001; Fig. 4B). Twenty days after injection, tested mice were sacrificed and the tumors were excised for further analysis. The average volume of tumors induced by RBMS3-30 cells (91.9 ± 68.7 mm³) was significantly decreased compared with tumors induced by Vec-30 cells (651.97 ± 120.65 mm³, P < 0.001; Fig. 4C). RBMS3 expression in xenograft tumors was studied by IHC, and the result showed that RBMS3 expression was only detected in tumors induced by RBMS3-30 cells but not in tumors induced by Vec-30 cells (Fig. 4D).

RBMS3 downregulates c-Myc and CDK4

Because RBMS3 belongs to the family of MSSP, the effect of RBMS3 on c-Myc expression was investigated by qRT-PCR and Western blot analysis. The results showed that the expression of c-Myc was downregulated in RBMS3-30 cells compared with that of Vec-30 cells (Fig. 5A). Expression of other cell-cycle–related proteins, including CDK2, CDK4, cyclin D1, and cyclin E, was also studied by Western blot analysis, and the results showed that only CDK4 was downregulated (Fig. 5A). The downregulation effect of RBMS3 on c-Myc and CDK4 in KYSE30 cells was confirmed by qRT-PCR (Fig. 5B). The downregulation effect of RBMS3 on c-Myc and CDK4 was further confirmed in 2 other ESCC cell lines, KYSE140 and HKESC1, by qRT-PCR. The result showed that transient transfection of RBMS3 into these 2 cell lines could effectively downregulate expression of c-Myc and CDK4 (Fig. 5C).
RBMS3 binds to the putative DNA replication origin of c-Myc

A sequence of 21 bp about 2 kb upstream of the human c-Myc gene has been identified as putative DNA replication origin and a transcriptional enhancer, which can be bound by MSSPs (22). ChIP assay was done in EC18 as described previously (23) to confirm the interaction of RBMS3 with the fragments (F1: nt -2588/-2421; F2: nt -2313/-2085; F3: nt -1202/-1020; and F4: nt -717/-503) from the 5' flanking region of c-Myc (Fig. 5D). The results showed that only DNA F2 containing the putative DNA replication origin sequence of c-Myc, but not F1, 3, and 4, could be detected in RBMS3-ChiPed DNA fragments by PCR (Fig. 5D).

To further confirm that RBMS3 could bind to the putative DNA replication origin sequence of c-Myc, EMSA was done using a 60-bp probe containing the binding sequence. As shown in Fig. 5E, the EC18 nuclear extract bound to the probe and formed DNA–protein complex. These results showed that RBMS3 is able to bind directly to the putative DNA replication origin of c-Myc.

Silencing RBMS3 by RNAi inhibits its tumor-suppressive ability

To further show that the tumor-suppressing function of RBMS3 was through downregulation of c-Myc expression, RNAi assay was applied to silence RBMS3 expression. qRT-PCR results showed that the expression of RBMS3 in RBMS3-30

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Figure 5. RBMS3 downregulates c-Myc and CDK4 expression. A, expression of RBMS3, c-Myc, CDK2, CDK4, cyclin D1, and cyclin E was compared between RBMS3-30 and control Vec-30 cells by Western blot analysis. GAPDH was used as loading control. B, qRT-PCR analysis showed that ectopic expression of RBMS3 could downregulate c-Myc and CDK4 expression in KYSE30 cells. Results are expressed as mean ± SD of 3 independent experiments (**, P < 0.01). C, when RBMS3 was transiently transfected into KYSE140 and HKESC1 cells, c-Myc and CDK4 expression was decreased significantly (*, P < 0.05). D, schematic diagram representing the distribution of RBMS3-binding loci in the regulatory region of c-Myc (top). Four individual fragments representing 5' upstream sequences of c-Myc (F1: nt -2588/-2421; F2: nt -2313/-2085; F3: nt -1202/-1020; and F4: nt -717/-503). PCR was used to detect the existence of fragments 1, 2, 3, and 4 in DNA fragments pulled down by an anti-RBMS3 antibody. IgG was used as a negative control. E, EMSA was used to detect the interaction between RBMS3 and c-Myc double-stranded DNA probes (100 fm or 200 fm). Supershift band could be detected when nuclear extract (15 µg) from EC18 cells was added.
cells could be effectively silenced by siRNA against RBMS3, which subsequently upregulated c-Myc and CDK4 expression, compared with RBMS3-30 cells treated with scramble siRNA (Fig. 6A). Foci formation assay showed that silencing RBMS3 expression could increase foci formation ability compared with control cells (Fig. 6B).

**RBMS3 reduces CDK4 and phosphorylated Rb expression**

Because CDK4 plays a critical role via the inactivation of Rb, the level of inactive form of Rb (phosphorylated Rb) was then compared between RBMS3-30 and Vec-30 cells by Western blot analysis. The result showed that no obvious change was detected in total Rb protein between RBMS3-30 and Vec-30 cells. However, inactive forms of Rb (phosphorylated at Ser807/811 and Ser780) were reduced in RBMS3-30 cells compared with those in Vec-30 cells (Fig. 6C). Phosphorylated Rb (ser780) in cells increased significantly when RBMS3 was silenced by RNAi compared with scramble control.

**Discussion**

Deletion of 3p is one of the most frequent alterations in primary ESCC. Here we report the characterization of one candidate tumor suppressor gene, RBMS3, at 3p24. Downregulation of RBMS3 in mRNA and protein level was detected in 45% and 52.9% of primary ESCCs, respectively. LOH study showed that DNA copy number loss was detected in 64% (11/17) of ESCC cases. Interestingly, downregulation of RBMS3 was significantly associated with poor outcome of ESCC patients (P = 0.034), suggesting that RBMS3 might play an important role in ESCC development and progression. The tumor-suppressive function of RBMS3 was characterized in 2 ESCC cell lines by either stable transfection (KYSE30) or transient transfection (KYSE180). Both in vitro and in vivo assays were used to investigate the tumor-suppressive potential of RBMS3. In addition, silencing RBMS3 expression by RNAi could inhibit its tumor-suppressive ability. The results showed that RBMS3 could effectively suppress cell growth, decrease foci formation and colony formation in anchorage-dependent and anchorage-independent assays, and inhibit tumor formation in nude mice.

RBMS3 belongs to a family of MSSPs including 3 members. MSSPs are believed to regulate DNA replication, transcription, apoptosis, and cell-cycle progression by interacting with a sequence of 21 bp about 2 kb upstream of the human c-Myc gene (22). Moreover, MSSP-1 and MSSP-2 can bind directly to the C-terminal portion of c-Myc, which, along with Max, forms...
a ternary complex, which loses the binding activity to the recognition sequence of c-Myc/Max complex, thereby abrogating the E-box–dependent transcription activity of c-Myc (14). In another study, MSSP has been reported to be released from a putative DNA replication origin of the c-Myc gene after it complexed with a catalytic subunit of a DNA polymerase alpha (24). Although RBMS3 contains 2 consensus motifs of an RNA-binding protein, like other MSSP family members, which could artificially bind to DNA through chimeric GAL4 hybrid system (12), its role in the regulation of c-Myc expression has not been shown.

In this study, we found that RBMS3 could directly bind to the putative DNA replication origin sequence about 2 kb upstream of the c-Myc gene and, subsequently, downregulated c-Myc expression in both mRNA and protein levels. This result suggests that RBMS3 could also abrogate the transcription activity of c-Myc like MSSP-1 and MSSP-2. In addition, ectopic expression of RBMS3 could downregulate CDK4 expression in ESCC cells. It is not clear whether RBMS3 could directly downregulate CDK4 expression. c-Myc is a proto-oncogene that plays an important role in tumorigenesis in many malignances through stimulating expression of cyclin A, D, E, CDK2, and CDK4 (25, 26). CDK4 is a master integrator that initiates the phosphorylation of the central oncosuppressor Rb and inactivates its blocking activity of E2F transcription (27, 28). Therefore, we further studied the phosphorylation status of Rb and the result showed that expression of RBMS3 could reduce the phosphorylation form of Rb. These results suggest that the tumor-suppressive mechanism of RBMS3 might be through the inhibition of c-Myc and CDK4 expression, and sequentially downregulates Rb phosphorylation and prevents E2F activity, which is required for cell-cycle progression, and further downregulates c-Myc expression. Taken together, our data show that RBMS3 is a novel tumor suppressor gene in ESCC, and its downregulation is associated with poor prognosis in ESCC. Further characterization of the tumor-suppressive function and mechanism of RBMS3 will not only greatly facilitate our understanding of ESCC development and progression but will also provide novel therapeutic targets in ESCC treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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