**RBMS3 at 3p24 Inhibits Nasopharyngeal Carcinoma Development via Inhibiting Cell Proliferation, Angiogenesis, and Inducing Apoptosis**

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**Abstract**

Deletion of the short arm of chromosome 3 is one of the most frequent genetic alterations in many solid tumors including nasopharyngeal carcinoma (NPC), suggesting the existence of one or more tumor suppressor genes (TSGs) within the frequently deleted region. A putative TSG RBMS3 (RNA binding motif, single stranded interacting protein 3), located at 3p24-p23, has been identified in our previous study. Here, we reported that downregulation of RBMS3 was detected in 3/3 NPC cell lines and 13/15 (86.7%) primary NPC tissues. Functional studies using both overexpression and suppression systems demonstrated that RBMS3 has a strong tumor suppressive role in NPC. The tumor suppressive mechanism of RBMS3 was associated with its role in cell cycle arrest at the G1/S checkpoint by upregulating p53 and p21, downregulating cyclin E and CDK2, and the subsequent inhibition of Rb-ser780. Further analysis demonstrated that RBMS3 had a pro-apoptotic role in a mitochondrial-dependent manner via activation of caspase-9 and PARP. Finally, RBMS3 inhibited microvessel formation, which may be mediated by down-regulation of MMP2 and β-catenin and inactivation of its downstream targets, including cyclin-D1, c-Myc, MMP7, and MMP9. Taken together, our findings define a function for RBMS3 as an important tumor suppressor gene in NPC.

**Introduction**

Nasopharyngeal carcinoma (NPC) is a distinct and geographically important disease [1], which accounts for 80,000 new cases and 50,000 deaths per year [2]. The majority (75–90%) of newly diagnosed NPC patients have loco-regionally advanced disease, commonly with cervical nodal metastases [3]. Currently, the standard of care for these patients consists of concurrent chemoradiotherapy with cisplatin-based regimens, generally followed by adjuvant chemotherapy. The cause for NPC development is complex, including viral, genetic and environmental factors[4–6]. It is widely accepted that infection by the Epstein-Barr virus (EBV) plays a vital role in the pathogenesis of NPC; however, the molecular pathogenesis is also associated with the inactivation of tumor suppressor genes (TSGs). To date, the exact cellular and molecular mechanisms leading to NPC have not been systematically evaluated.

The 3p chromosomal region is frequently deleted in multiple solid tumors [9], suggesting the existence of one or more TSGs contributing to the risk of developing NPC. Through massive expression profiling and epigenetic characterization, we and others have identified several interesting 3p targets genes in human cancers, including BLU [10], RBMS7 [11] and two closely located 3p22 genes, DLEC1 [12] and PLCD1 [13,14]. RBMS3, a gene located at 3p24-p23, encodes an RNA-binding protein that belongs to the c-Myc gene single-strand binding protein (MSSPs) family [15]. The MSSP family members are single strand DNA-binding proteins that cooperate with the Myc protein to regulate DNA replication, gene transcription, cell cycle progression, and induction of apoptosis. C-Myc is over-expressed in most cancers and executes its diverse functions through transcriptional regulation of its target genes [16]. Over-expression of c-Myc was also found as a frequent genetic abnormality in NPC [17,18]. The RBMS3 protein was isolated through its binding to an upstream element of the alpha2 [4] collagen promoter [19]. The RBMS3 protein localizes in the cytoplasm, suggesting it may be involved in a cytoplasmic function such as controlling RNA metabolism, rather than transcription. Multiple alternatively spliced transcript variants encoding various isoforms of RBMS3 have been found. Although RBMS3 could be a potential cooperator of the Myc...
protein, its role in the pathogenesis of NPC remains unclear. In the present study, the expression pattern of RBMS3 in primary NPCs and NPC cell lines was investigated. The tumor suppressive effects and corresponding mechanisms of RBMS3 were characterized.

**Results**

**RBMS3 is Frequently Down-regulated in NPC**

Quantitative real-time PCR (qRT-PCR) was performed to evaluate the expression levels of RBMS3 in 15 pairs of primary NPCs and their corresponding non-tumor samples. Down-regulation of RBMS3 was detected in 13/15 (86.7%) NPC tissues compared to their normal counterparts (Fig. 1A). Furthermore, the box plot showed a highly significant difference in the mean expression levels of RBMS3 between NPC tumors and non-tumor samples (p<0.001; Fig. 1B). We next examined RBMS3 expression in NPC cell lines. The result showed that RBMS3 was downregulated in all three tested NPC cell lines (C666, CNE2 and SUNE1) compared to the immortalized nasopharyngeal (NP) cell line, NP460 (Fig. 1C). The protein expression level of RBMS3 was also evaluated in 30 pairs of primary NPCs and non-tumor samples by immunohistochemical staining (IHC). Moderate or strong nuclear staining of RBMS3 was detected in 30 non-tumor samples (p=0.0001; Fig. 1D).

**RBMS3 has Tumor Suppressive Ability**

To investigate whether RBMS3 has tumor suppressive ability, RBMS3 was stably transfected into 2 NPC cell lines (SUNE1 and CNE2), and 4 clones (SUNE1-R4, SUNE1-R5, CNE2-R1 and CNE2-R2) were selected for functional studies. Empty vector-transfected cells were used as control (SUNE1-V1 and CNE2-V1). Expression of RBMS3 in SUNE1-R4, SUNE1-R5, CNE2-R1 and CNE2-R2 cells was confirmed by qPCR (Fig. 2A) and Western blot analysis (Fig. 2B). Tumor suppressive function of RBMS3 was studied by cell growth assay, foci formation assay, and tumor xenograft experiment. Cell growth assay showed that the growth rates were significantly decreased in SUNE1-R4, SUNE1-R5, CNE2-R1 and CNE2-R2 cells (p<0.05, Student’s t-test) compared to SUNE1-V1 cells and CNE2-V1 cells, respectively (Fig. 2B). Focus formation assay showed that RBMS3 could significantly inhibit foci formation (p<0.001, Student’s t-test) in SUNE1-R4, SUNE1-R5, CNE2-R1 and CNE2-R2 cells compared to control cells (Fig. 2C).

The tumor suppressive potential of RBMS3 was also evaluated by xenograft tumor formation in athymic nude mice. Subcutaneous tumor formation was observed in all nude mice injected with SUNE1-V1 (n=10) and CNE2-V1 (n=10) cells 28 days post-injection. Xenograft tumor growth curve showed that tumors induced by SUNE1-R4 and SUNE1-R5 cells grew significantly slower than the SUNE1-V1 cells (p<0.05) (Fig. 2D). The average volume of the tumors induced by SUNE1-R4 (630.00±135.18 mm³) and SUNE1-R5 (664.00±144.68 mm³) cells were significantly smaller compared to the tumors induced by SUNE1-V1 cells (1687.80±270.37 mm³, p<0.05) (Fig. 2D). Similarly, the average volume of the tumors induced by CNE2-R1 (501.90±73.12 mm³) and CNE2-R2 (522.13±74.19 mm³) were significantly reduced compared to the tumors induced by CNE2-V1 cells (770.46±187.07 mm³, p<0.05) (Fig. 2D).

**RBMS3 Arrests Cell Cycle at G1-S Checkpoint**

To understand the tumor suppressive mechanism of RBMS3, flow cytometry was performed to compare the DNA content between the SUNE1-Vec and SUNE1-RBMS3 cells. The results showed that the proportions of SUNE1-RBMS3 cells in the G1-phase and S-phase were significantly increased and decreased, respectively, in the SUNE1-RBMS3 cells (p<0.05) compared with SUNE1-Vec cells, suggesting that RBMS3 was able to arrest cell cycle at G1/S checkpoint (Fig. 3A). Western blot analysis found that the G1/S checkpoint promoting factors (CDK2, cyclin E and cyclin D1) and inhibiting factors (p53 and p21) were downregulated and upregulated in RBMS3-transfected NPC cells, respectively, compared to control cells (Fig. 3B). Since CDK2 plays a critical role in the inactivation of Rb, the level of the inactive form (phosphorylated) of Rb was also compared between RBMS3-transfected and vector transfected NPC cells by Western blotting. The result showed that inactive form of Rb (Ser780) was reduced in RBMS3-transfected cells compared to control cells. However, the total amount of Rb protein did not change significantly (Fig. 3B).

**Ectopic Expression of RBMS3 Induces Apoptosis**

To explore whether RBMS3 has a pro-apoptotic effect, the apoptotic index was compared between SUNE1-Vec and SUNE1-RBMS3 cells by TUNEL staining. Prior to staurosporine (STS) treatment, TUNEL analysis revealed that the apoptotic index in SUNE1-Vec cells (0.5%±0.9%) was lower than SUNE1-RBMS3 cells (7.1%±6.0%; p=0.057). After STS treatment, the apoptotic index in SUNE1-Vec (14.8%±4.1%) was significantly lower than that of SUNE1-RBMS3 (45.3%±4.5%; p<0.05), confirming that RBMS3 had a pro-apoptotic ability (Fig. 4A). To elucidate the molecular basis of apoptosis, we examined the potential for pro-apoptotic mitochondrial permeability transition by measuring the loss of mitochondrial ΔΨm using JC-1 dye. Red or orange fluorescence indicates intact mitochondria, whereas green fluorescence indicates a collapse in mitochondrial ΔΨm. The results showed that the mitochondrial permeability and apoptotic index in RBMS3-transfected cells were significantly higher than those of control cells even prior to STS treatment (Fig. 4B). Western blot analysis also indicated that the cleavage of caspase-9 and PARP was dramatically increased in SUNE1-RBMS3, however, no change was observed for caspase-8 (Fig. 4C).

**RBMS3 Inhibits Angiogenesis**

To study the potential effect of RBMS3 on angiogenesis, the development of microvessel in tumor sections of mouse xenografts was examined by IHC staining with a vascular endothelial cell marker CD34. As shown in Fig. 5A, a robust angiogenic response, as determined by high CD34 positivity, was observed in the empty vector-induced tumors. Conversely, CD34-positive vessels were rarely found within the RBMS3-induced tumors. The number of vessels counted in RBMS3-induced and empty vector-induced tumors was summarized in Table 1. Furthermore, the mRNA expression level of VEGF was also compared between RBMS3-transfected and vector-transfected NPC cells by qPCR analysis. As shown in Fig. 5B, the expression of VEGF was dramatically decreased in RBMS3-transfected NPC cells compared to control cells (p<0.05, Fig. 5B).

A recent study found that silencing β-catenin expression by RNA interference could inhibit angiogenesis-related gene expression (e.g., MMP9, MMP2, and VEGF) in hepatocellular carcinoma cells [20]. We next studied whether RBMS3 could intercept the expression of β-catenin in NPC cells. As shown in Fig. 5C and Fig. 5D, β-catenin from both whole cell extracts and nuclear fractionation was downregulated in RBMS3-transfected NPC cells compared to control cells. The downstream targets of β-catenin including C-Myc, MMP7, MMP9, and MMP2 (the latter two are angiogenesis-related proteins) were also detected in
RBMS3-transfected NPC cells. As expected, all the β-catenin downstream targets including the two angiogenesis-related proteins MMP9 and MMP2 were significantly downregulated in RBMS3 transfected NPC cells, suggesting that RBMS3 has a strong angiogenesis inhibiting role in NPC (Fig. 5E).

Figure 1. Downregulation of RBMS3 in nasopharyngeal carcinoma (NPC). (A) Expression of RBMS3 in 15 primary NPC cases was compared using qPCR between tumor tissues (T) and their paired normal tissue (N). GAPDH was set as an internal control. (B) RBMS3 expression was normalized by internal control GAPDH. Statistical analysis confirmed the qPCR results (p<0.001). (C) qPCR analysis of RBMS3 expression in three NPC cell lines (C666, CNE2 and SUNE1). The fold changes of RBMS3 expression were compared with the immortalized NP cell line, NP460. (D) Immunohistochemical detection of RBMS3 protein in NPC tissue samples and non-tumor nasopharyngeal tissues. Normal: strong positive staining for RBMS3 protein in normal nasopharyngeal epitheliums (arrowhead). NPC1: weak positive staining for RBMS3 protein in NPC tissues (arrowhead). NPC2: negative staining for RBMS3 protein in NPC tissues (arrowhead).

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Figure 2. Tumor suppressor function of RBMS3 in NPC cells. Expression of RBMS3 in RBMS3-transfected SUNE1 (SUNE1-R4 and R5) and CNE2 cells (CNE2-R1 and R2) was confirmed by quantitative PCR (A) and Western blot (B). Empty vector-transfected cells (SUNE1-V1, CNE2-V1) were used as control. (C) An MTT assay was used to compare cell growth rate between RBMS3- and empty vector-transfected NPC cells. The results found that cell
To further demonstrate the tumor-suppressive function of RBMS3, RNAi was used to knockdown endogenous RBMS3 expression in NP460 cells. The silencing effect was confirmed by both qPCR and western blotting (Fig. 6A). The function of RBMS3 in RBMS3 knockdown NP460 cells was studied by cell growth assay, foci formation assay, and cell cycle analysis. The results showed that knockdown of RBMS3 in NP460 cells could increase cell growth rate (Fig. 6B), enhance foci formation ability (Fig. 6C), and promote cell cycle (Fig. 6D) compared to control cells. Moreover, we found that the expression of VEGF was significantly upregulated in RBMS3 knockdown NP460 cells by qPCR analysis (Fig. 6E), whereas β-catenin was upregulated and p53 was downregulated in RBMS3 knockdown NP460 cells by western blotting (Fig. 6F), as compared to control cells. These observations further support that RBMS3 is an important tumor suppressor with anti-proliferation and anti-angiogenesis abilities in NPC.

Discussion

The etiology of NPC is complex, and includes viral, genetic and environmental factors [4–6]. The earliest and most common genetic change observed in NPC patients maps to the short arm of chromosome 3 where deletions are observed frequently. One candidate tumor suppressor gene, RBMS3, which resides on human chromosome 3p24-3p23, is widely expressed in the embryo as well as in the adult organisms. In the present study, downregulation of RBMS3 was frequently detected in NPCs at both the mRNA and protein levels, suggesting that RBMS3 might play a pivotal role in the NPC development and progression. The observation that this protein localizes in the nuclear suggests that it may be involved in a nuclear function such as controlling RNA transcription. The tumor-suppressive function of RBMS3 was characterized in two NPC cell lines (SUNE1 and CNE2). Both in vitro (cell growth rate, colony formation in soft agar and foci formation) and in vivo (tumor formation in nude mouse) assays
Figure 4. Ectopic expression of RBMS3 induces apoptosis. (A) Representative images of TUNEL staining. After STS treatment, more apoptotic cells (green) were detected in SUNE1-RBMS3 cells compared to SUNE1-V1 cells (arrowhead). The apoptotic indexes of SUNE1-RBMS3 cells and SUNE1-V1 cells (before and after STS treatment) were summarized in the right panel (*p < 0.05). (B) This event was measured by loss of mitochondrial ΔΨm using JC-1 dye. Red or orange fluorescence indicates intact mitochondria, whereas green fluorescence indicated a collapse in mitochondrial ΔΨm. Prior to STS treatment the SUNE1-RBMS3 cells reveal a significant ΔΨm loss (green fluorescence, ×400) and higher apoptotic index compared to SUNE1-V1 cells (red/orange fluorescence; left panel) (*p < 0.05). (C) The cleavages of caspase-9, caspase-8, and PARP were compared between RBMS3-transfected and empty vector-transfected SUNE1 cells. β-actin was used as loading control.

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**Figure 5. RBMS3 inhibits angiogenesis.** (A) The tumor sections were stained for the endothelial cell marker CD34 (x200). The RBMS3-induced tumors had a lower degree of microvessel densities compared to empty vector-induced tumors. (B) Expression of VEGF in RBMS3-transfected SUNE1 and CNE2 cell lines. (C) β-catenin expression in RBMS3-transfected SUNE1 and CNE2 cell lines. (D) Western blot analysis of β-catenin (total) and β-actin in SUNE1 and CNE2 cell lines. (E) Western blot analysis of c-myc, MMP7, MMP9, and MMP2 in SUNE1 and CNE2 cell lines.
demonstrated that RBMS3 had a strong tumor suppressive potential in NPC cells.

Molecular analysis found that the tumor-suppressive effect of RBMS3 was closely associated with its role in cell cycle arrest at G1/S checkpoint via upregulation of p53 and p21, and downregulation of cyclin D1, cyclin E/CDK2 and Rb-ser780. The increased expression of p53, which is a DNA-binding protein and functions as a tumor suppressor, can induce the expression of p21. p21 has a pivotal role in the G1/S transition through inhibiting the cyclin E/CDK2 complex [21]. Activation of the cyclin E/CDK2 complex can destruct Rb-E2F binding, which contributes to the reduction of phosphorylation form of Rb, and finally activates the transcription of genes necessary for S-phase entry and cell cycle progression [22,23]. These findings indicate that RBMS3 could be necessary for an orderly G1/S transition in NPC cells.

Another data supporting that RBMS3 is a tumor suppressor in NPC was its role in inducing apoptosis. Notably, the apoptotic index between RBMS3 transfected NPC cells and control cells showed significant difference after STS treatment, indicating a unique mechanism underlying the strong pro-apoptotic effect of RBMS3 in NPC cancer cells. It has been reported that RBMS3 increased the expression of Prx1 transcription factor [19], which is a tumor suppressor in esophageal squamous cell carcinoma [24]. Prx1, as a novel interaction partner for the lifespan regulator protein p66Shc, binds to the N-terminal domain of p66Shc [25,26], and subsequently induces changes in the permeability transition of mitochondrial membranes and results in the release of cytochrome C and apoptosome activation [27,28]. Interestingly, our results also found that the pro-apoptotic role of RBMS3 was mediated through the intrinsic mitochondrial pathway, as caspase-9 and mitochondrial permeability, but not caspase-8, was increased. However, whether RBMS3 could induce the expression of Prx1 and lead to the activation of a similar apoptotic pathway need to be further investigated.

Whether or not the growth of vessels occurs in a tissue depends on the equilibrium between the relative amounts of molecules that induce and inhibit angiogenesis [29]. A growing body of evidence illustrates that the inactivation of TSG and activation of oncogenes play an important role in this phenotypic switch. For instance, oncogenic stimuli such as ras, v-src and c-jun [30–33] and inactivation of p53, VHL, and RB2/P130 results in an increased VEGF production [30,34,35]. Our data demonstrate that RBMS3 can modulate the angiogenic balance and inhibit the microvessel formation potentially through increasing the expression of p53 and decreasing the expression of MMP9 and MMP2 [36]. β-catenin is maintained at low levels in quiescent cells by interaction with protein kinases, such as adenomatous polyposis coli, casein kinase 1, and glycogen synthase kinase 3 [37]. The aberrant cytoplasmic accumulation of β-catenin induces Tcf/Lef-mediated transcriptional activity, and enhances the invasion and migration of oral squamous cell carcinoma [38]. The translocation of β-catenin into the nucleus can initiate carcinogenesis when Wnt is present [39]. β-catenin levels in nucleus are increased in 92% of NPC tumors, making β-catenin an important component in NPC development [40,41]. Our data showed that β-catenin from both whole cell extracts and nuclear fractionation was significantly downregulated in RBMS3 transfected NPC cells compared to control cells, suggesting that RBMS3 could inhibit the nuclear translocation of β-catenin in NPC cells. The inhibition of β-catenin nuclear translocation in RBMS3-transfected cells was further confirmed by detecting downregulation of its targets including c-Myc, MMP7, MMP9 and MMP2. Overexpression of c-Myc has been detected in about 90% of NPCs which was associated with poor survival rate in NPC patients [42,43]. MMP7 is one of the well-known targets of β-catenin. It has been reported that MMP7 plays an essential role in cancer invasion and metastases [44,45]. In this study, MMP7 expression could be effectively downregulated when RBMS3 was introduced into NPC cells.

Moreover, our group recently revealed that RBMS3 could directly bind to the promoter region of c-Myc [46], suggesting that RBMS3 also possesses the DNA binding activity. Based on our findings, we hypothesize that RBMS3 may regulate certain key proteins involved in cell cycle, apoptosis and angiogenesis. The potential molecular mechanisms underlying the RBMS3-mediated tumor suppression in NPC are summarized in Fig. 7. In conclusion, our data provide a foundation to explore the role of RBMS3 in the NPC pathogenesis. More comprehensive understanding of the tumor suppressive mechanisms of RBMS3 in NPC would provide much more effective therapeutic strategy for the management of NPC patients.

Materials and Methods

Ethics Statement

Tumor specimens used in this study were approved by the Committees for Ethical Review of Research involving Human Subjects at University of Hong Kong. Written informed consent was obtained from the patients involved. Animal operation was carried out according to the protocols approved by the Committee on the Use of Live Animals in Teaching and Research (CULATRA). Licence which could conduct live animal experiments was approved by Hong Kong Department of Health (licence no. (09–770) in DH/HA&P/8/2/3 Pt.17).

Cell Lines and Primary Tumor Specimens

EBV-positive NPC cell line C666 was derived from NPC tissue harbored latent EBV infection [47,48], while EBV-negative NPC cell lines CNE2 and SUNE1 were derived from poorly differen-

Table 1. Quantitation of microvessels formed in empty vector and RBMS3-transfected NPC cells induced mouse tumor xenografts (n = 10 tumors/group).

| Cell lines | *Vessel count | p    |
|------------|---------------|------|
|            | Vec           | RBMS3|      |
| SUNE1      | 78.6±16.9     | 23.4±8.5 | <0.001** |
| CNE2       | 87.9±27.1     | 29.6±6.7 | <0.001** |

*Values represent mean±standard error.
**Statistically significant as compared to control cells.

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Figure 6. RBMS3-silenced NP460 cell showed malignant features. (A) siRNA against RBMS3 effectively reduced the mRNA and protein expression of RBMS3 in NP460 cells compared to siScramble-transfected cells. (B) An MTT assay was used to compare cell growth rates between
sirRBMS3- and siScramble-transfected NP460 cells (* p<0.05; ** p<0.001, Student’s t-test). Values were expressed as mean ± SD of three independent experiments. (C) Foci formation assay showed that the number of foci was significantly increased in sirRBMS3-transfected NP460 cells compared to control cells (* p<0.05, Student’s t-test). The results were expressed as mean ± SD of three independent experiments. (D) Representative and summary of DNA content detected by flow cytometry showed that the percentage of cells in the S phase was higher while the percentage of cells in the G1 phase was lower in sirRBMS3-transfected NP460 cells than that in control cells. (* p<0.05, Student’s t-test). (E) Expression of VEGF in sirRBMS3- and siScramble-transfected NP460 cells was confirmed by qPCR. (* p<0.05, Student’s t-test). The results were expressed as mean ± SD of three independent experiments. (F) β-catenin was significantly up-regulated while p53 was significantly down-regulated in sirRBMS3-transfected NP460 cells compared to control cells by Western blot analysis. β-actin was used as a loading control.

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**Figure 7. A schematic diagram showing the role of RBMS3 in malignant transformation of nasopharyngeal carcinoma cell.** Activation →, inhibition ←.
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were transfected with siRNA using LipofectamineTM 2000 signed siRNA database (Ambion, Inc., Austin, TX). NP460 cells scramble siRNA, which were obtained from Ambion’s prede-3 xenograft experiment. Approximately 2 \times 10^6 RBMS3-expressing cells and control cells were injected subcutaneously (s.c.) into the right and left hind legs of 4-week-old nude mice (n = 10 for each group), respectively. Tumor formation in nude mice was monitored over a 4-week period. The tumor volume was calculated by the formula 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.

Small Interfering RNA Transfection

RBMS3 expression was silenced by double-stranded siRNA of targeting of RBMS3 (sense 5'-CCAACCGCAUGAUUGCCACAt-3'; antisense 5'-UUGGGAUCAUGGC- GGUGUGGt-3') and scramble siRNA, which were obtained from Ambion’s predesigned siRNA database (Ambion, Inc., Austin, TX). NP460 cells were transfected with siRNA using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) according to the manufacture’s instruction. Forty-eight hours after transfection, the effect of targeting of RBMS3 (sense 5'-CCAACCGCAUGAUUGCCACAt-3'; antisense 5'-UUGGGAUCAUGGC- GGUGUGGt-3') and scramble siRNA, which were obtained from Ambion’s predesigned siRNA database (Ambion, Inc., Austin, TX). NP460 cells were transfected with siRNA using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) according to the manufacture’s instruction. Forty-eight hours after transfection, the effect of gene-silencing was measured by qPCR and Western blot analysis. Scramble siRNA was used as a negative control.

Cell Cycle Analysis

RBMS3 and vector-transfected SUNE1 cells (1 \times 10^6) were cultured in RPMI medium containing 10% fetal bovine serum (FBS), while si-RBMS3 and si-scramble transfected NPC cells were cultured in defined keratinocyte serum-free medium. Serum was withdrawn from culture medium when cells were 70% confluent. After 72 h, 10% FBS was added in the medium for an additional 12 h. Cells were fixed in 70% ethanol, stained with propidium iodide, and DNA content was analyzed using Cytomics FC (Beckman Coulter).

Immunohistochemistry (IHC)

Tumor sections (5 \mu m thick) from primary NPCs or mouse xenografts were used for immunohistochemical analysis. Briefly, paraffin-embedded sections were deparaffinized, blocked with goat serum, followed by incubation with mouse anti- RBMS3 (1:100) or anti-CD34 (1:100) overnight at 4°C. After incubation with horseradish peroxidase linked secondary antibody for 30 min, the sections were counterstained with Mayer's hematoxylin. For RBMS3 staining, the scores were determined by the intensity of staining: positive staining (moderate and strong nuclear staining) and downregulation (absent and weak staining). Microvascular Density Analysis

Tumor xenograft sections were stained for CD34, and four random medium-power fields (\times 200) were photographed for each tumor. Vessels were counted by a blinded observer using standard criteria [52].

Immunofluorescence (IF)

RBMS3 and vector-transfected SUNE1 cells were grown on gelatin-coated cover slips, fixed with 4% paraformaldehyde, permeabilized in PBS, which contain 0.1% Triton-X 100, and blocked with 1% bovine serum albumin. The cells were then treated with antibodies targeting β-catenin (Cell Signaling Technology, Danvers, MA) at 4°C overnight. After washing with PBS, cells were incubated with FITC-conjugated anti-mouse secondary antibody and Texas red-conjugated anti-rabbit secondary antibody at room temperature for 1 hour. Antiﬁde 4', 6-diamidino-2-phenylindole (DAPI) solution was added, and images were captured.

Transferase-mediated dUTP Nick-end Labeling (TUNEL) Assay

RBMS3 and vector-transfected SUNE1 cells were treated with straurosporine (STS; 1 \mu M) for 2 hours. Morphological changes in the nuclear chromatin undergoing apoptosis were detected by terminal deoxynucleotidyl TUNEL assay according to the manufacturer’s protocol (Roche, Mannheim, Germany). Images were captured using a Leica DMRA fluorescence microscope (Rueil-Malmaison, France). Triplicate independent experiments were performed.

Mitochondrial Membrane Potential Assay

A MitoPTM JC-1 Detection kit (Immunochemistry Technologies, Bloomingtom, MN) was used to detect the loss of mitochondrial membrane potential (∆Ψm). Briefly, cells were cultured on cover slips until 80% confluence was attained in a 6-well plate. Before and after STS treatment, cells were washed twice with phosphate-buffered saline (PBS) and incubated with the ∆Ψm-sensitive dyes JC-1 at 37°C for 15 min, and a fluorescence microscope was used to capture the image with a Leica DMRA.

Cell Fractionation

Separation of nuclear and cytosolic fractions was performed using the NucBuster Protein Extraction kit (Novagen Inc, Madison, WI) according to the manufacturer’s instructions.

Western Blot Analysis

Western blot was performed according to the standard protocol with antibodies for cyclin D1, β-actin, E-cadherin, MMP7, and MMP9 (Santa Cruz Biotechnology, Santa Cruz, CA); p21, p27, cyclin E, p53 caspase-8, and caspase-9 (Cell Signaling Technology, Danver, MA); and RBMS3 (Abnova, Taiwan).

Statistical Analysis

Statistical analysis was performed with the SPSS standard version 13.0. Results expressed as mean ± SD were analyzed using Student’s t test. Differences were considered significant when P value was <0.05.

Author Contributions

Conceived and designed the experiments: JC LF DLK XG. Performed the experiments: JC. Analyzed the data: CZ JC. Contributed reagents/materials/analysis tools: LC SD LZ JT CQ AMGW KK YL ML. Wrote the paper: JC TC XG.
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