

Alpha gene upregulates TFEB expression in renal cell carcinoma with t(6;11) translocation, which promotes cell canceration

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Abstract. Renal cell carcinoma (RCC) with t(6;11) translocation has been characterized by the fusion of the Alpha gene with the TFEB gene. However, the underlying molecular mechanisms remain greatly uncharacterized and effective targeted therapy has yet to be identified. In this study, we examined the role of the Alpha gene in this tumor entity and the function of the fusion gene Alpha-TFEB product *in vitro* and *in vivo*. Our results revealed that the luciferase activity of Alpha1, Alpha2, Alpha3, Alpha4 and Alpha5 significantly increased compared with that of the pGL3-Basic group ($P < 0.01$). The luciferase activity also increased significantly in the Alpha1, Alpha2 and Alpha5 groups compared with that of the normal TFEB gene group ($P < 0.01$). In addition, the luciferase activity of Alpha5 was the strongest located in the 643-693 base sequence. The stable transfection of Alpha-TFEB into HK-2 and CaKi-2 cells promoted the expression of Alpha-TFEB mRNA and TFEB protein. Furthermore, the overexpression of TFEB increased cell proliferation and enhanced the cell invasive ability, and decreased cell apoptosis in the Alpha-TFEB stably transfected cells *in vitro*. *In vivo* experiments revealed that the overexpression of TFEB promoted tumorigenicity in nude mice, which was consistent with our *in vitro* results. On the whole, these data indicate that the overexpression of TFEB confers a potent oncogenic signal and may thus be a novel therapeutic target in RCC with t(6;11) translocation.

Introduction

Renal cell carcinoma (RCC) with t(6;11) translocation is a new subtype of RCC, which was first reported by Argani *et al* in 2001 (1) and was officially recognized in the 2013 International Society of Urological Pathology

Vancouver Classification of Renal Neoplasia (2). In a previous study, we first reported a Chinese case of RCC with t(6;11) translocation with a novel Alpha-TFEB fusion point (3). To the best of our knowledge, approximately 63 cases have been described in the literature (1,3-29). The reported age range is from 3 to 77.6 years (mean age, 33.9 years; median age, 34 years), predominantly occurring in young adults with a slight male predominance. RCC with t(6;11) translocation has a broad morphological appearance without a distinctive gross appearance; thus, it can be easily misdiagnosed as another type of renal neoplasm. Immunohistochemical and molecular genetic analyses are essential for the accurate diagnosis of RCC with t(6;11) translocation.

RCC with t(6;11) translocation has been characterized by the fusion of the Alpha gene on 11q12 or q13 with the TFEB gene on 6p21, resulting in the overexpression of TFEB protein. The Alpha gene, also known as MALAT1, encodes a ~7.5 kb or ~8.5 kb transcript (4,30). There are no indications for RNA splicing in the Alpha gene and open reading frames are shorter than 55 amino acids within the transcript. It is assumed that the Alpha gene does not encode a functional protein (4). Therefore, from these data, it is doubtful whether Alpha gene is a strong promoter that upregulates TFEB expression in RCC with t(6;11) translocation, and whether the product of fusion gene Alpha-TFEB promotes cell canceration.

In this study, we examined the role of the Alpha gene in this rare tumor entity and the function of the fusion gene Alpha-TFEB product *in vitro* and *in vivo*. Our results revealed that the Alpha gene was a strong promoter. The stable transfection of Alpha-TFEB into HK-2 and CaKi-2 cells promoted the expression of Alpha-TFEB mRNA and TFEB protein. Furthermore, the overexpression of TFEB increased cell proliferation and enhanced the cell invasive ability, and decreased cell apoptosis in Alpha-TFEB stably transfected cell lines *in vitro*. The results of *in vivo* experiments revealed that the overexpression of TFEB promoted tumorigenicity in nude mice, which indicated that the overexpression of TFEB confers a potent oncogenic signal and may thus be a novel therapeutic target in RCC with t(6;11) translocation.

Materials and methods

Promoter prediction and primers synthesis. As our research group previously reported, the break point of the Alpha gene was at nucleotide 1810 and fell in the 1205 bp breakpoint

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Table I. Sequences of primers used for PCR amplification and plasmid construction.

Groups	Primer pairs	Primer sequence (5'→3')	Genomic position (5'→3')	Size of PCR products (bp)
Alpha1	F1 (<i>SacI</i>)	<u>GAGCTC</u> GATCAGAGTGGGCCACTGCCA	1-21	1,854
	R1 (<i>NcoI</i>)	CCATGGGCAGGGGGAGGCCAGAATGA	1854-1829	
Alpha2	F2 (<i>HindIII</i>)	<u>AAGCTT</u> TTGTGAGGTGTTTGATGACC	396-415	1,459
	R2 (<i>NcoI</i>)	CCATGGGCAGGGGGAGGCCAGAATGA	1854-1829	
Alpha3	F3 (<i>KpnI</i>)	<u>GGTACC</u> GCTAAGGGCAAAATGTACAAACT	1451-1473	404
	R3 (<i>NcoI</i>)	CCATGGGCAGGGGGAGGCCAGAATGA	1854-1829	
Alpha4	F4 (<i>SacI</i>)	<u>GAGCTC</u> AGTAAAGCCCTGAACTATCA	278-297	256
	R4 (<i>NcoI</i>)	CCATGGCAGCTTATGGAACCTTGAAT	533-515	
Alpha5	F5 (<i>SacI</i>)	<u>GAGCTC</u> GTGATCGAATTCGGTGATGCGAGT	617-642	139
	R5 (<i>NcoI</i>)	CCATGGACTTATCTGCGGTTTCCT	755-738	
pTFEB	F (<i>SacI</i>)	<u>GAGCTC</u> GACTCTGGACTTTCTCTAATAATAA		600
	R (<i>NcoI</i>)	CCATGGCCTGAGCTTGCTGTCATGTT		

The underlined sequences represent restriction sites.

cluster region (3). Based on this, the promoter region prediction for the Alpha gene (GenBank Accession no. AF203815) was performed using online promoter prediction software (www.genomatix.de), and 5 pairs of primers with restrictive sites were designed for different lengths of Alpha. In addition, the promoter sequences of the normal TFEB gene (named pTFEB) were searched from the eukaryotic promoter database and the corresponding primers were designed (Table I). The primers were synthesized by Invitrogen (Shanghai, China).

Ethics approval and consent. The ethics approval and consent for the use of human tissue was confirmed by the Ethics Committee of Anhui Medical University.

DNA extraction and PCR. Genomic DNA, extracted from formalin-fixed, paraffin-embedded tumor tissue samples obtained at surgery from a patient (26-year-old male) with RCC with t(6;11) translocation, as previously described (3), served as templates for PCR according to the manufacturer's instructions of Phusion High-Fidelity PCR kit (Thermo Fisher Scientific, Carlsbad, CA, USA). The PCR conditions were as follows: Pre-denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C (Alpha1, Alpha2 and Alpha4), 52°C (Alpha3), 58°C (Alpha5) for 30 sec, extension at 72°C for 2 min (Alpha1), 90 sec (Alpha2), 30 sec (Alpha3, Alpha4, Alpha5), followed by a final extension at 72°C for 10 min. PCR amplification for pTFEB was performed with the DNA from one healthy individual as templates. All PCR products were confirmed by 1.5% agarose gel electrophoresis and purified using the MiniBEST DNA Extraction kit (Takara, Dalian, China) according to the manufacturer's recommendations.

Construction of recombinant reporter plasmids. The purified PCR products were cloned into pGEM-T vectors (Promega, Madison, WI, USA) with T4 ligase (Takara) at 16°C. The

constructs were then transformed into the freshly prepared competent cells DH5α (Tiangen Biotech Co., Ltd, Beijing, China). Following cell culture for 16 h at 37°C, Ampicillin-resistant bacterial colonies were selected randomly and amplified. The selected cloning plasmids, named Alpha1-T, Alpha2-T, Alpha3-T, Alpha4-T, Alpha5-T and pTFEB-T were identified by double enzyme digestion and sequencing. The collected DNA fragments were incorporated into pGL3-Enhancer vectors to construct recombinant reporter plasmids named pGL3-Enhancer-Alpha1, pGL3-Enhancer-Alpha2, pGL3-Enhancer-Alpha3, pGL3-Enhancer-Alpha4, pGL3-Enhancer-Alpha5 and pGL3-Enhancer-pTFEB.

Luciferase assay. The 293T cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco, Scoresby, VIC, Australia). The recombinant plasmids, pGL3-Enhancer-Alpha1, 2, 3, 4, 5, pTFEB, were transfected into 293T cells with the pRL-TK vector (Promega, Madison) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The pGL3-Control, pGL3-Basic and pRL-TK Vector were used as positive, negative and internal controls, respectively. Luciferase activity was assessed at 48 h following transfection using the Bright-Glo™ Luciferase Assay system (Promega). The ratio of Firefly/*Renilla* luciferase was calculated. Each plasmid experiment was replicated with 6 duplication wells.

Overlap PCR for Alpha-TFEB fusion gene amplification. The previously constructed Alpha1-T and TFEB cDNA plasmids (Generay Biotech Co., Ltd., Shanghai, China) were used as templates for overlap PCR cloning. Four primers (Alpha-F, Alpha-R, TFEB-F and TFEB-R) were designed (Table II). The PCR conditions were as follows: Alpha gene (1,854 bp): 98°C for 3 min, then 35 cycles of 98°C for 5 sec, 54°C for 30 sec,

Table II. Sequences of primers used for Alpha-TFEB fusion gene amplification.

	Primer sequence (5'→3')	Genome position (5'→3')	Size of PCR products (bp)
Alpha	F: <u>ATCGAT</u> GATCAGAGTGGGCCACTGCCA	1-21	1,854
	R: TTTTAGTAGCTTTTT GATGTGATTTTAAACCAACTTCC	1854-1829	
TFEB	F: AAAGCTACTAAAA ATGGCGTCACGCATAGGGTT	1-20	915
	R: <u>GGATCCT</u> CACAGCACATCGCCCTCCTCCATG	890-915	

The underlined sequences represent *Bsp*DI and *Bam*HI restriction sites; the bold sequences represent the required joint part to connect the Alpha and TFEB fragments. F, forward; R, reverse.

Table III. Sequences of primers used for Alpha-TFEB mRNA.

Groups	Primer sequence (5'→3')	Size of PCR products (bp)
Alpha-TFEB	F: AGAAGATGAGGGTGTTTACG	407
	R: TTGTTCCCATAGGTCTCG	
β-actin	F: CTCCATCCTGGCCTCGCTGT	268
	R: GCTGTCACCTTCACCGTTCC	

F, forward; R, reverse.

72°C for 120 sec, followed by a final extension at 72°C for 10 min; TFEB gene (915 bp): 98°C for 3 min, then 35 cycles of 98°C for 5 sec, 57°C for 30 sec, 72°C for 55 sec, followed by a final extension at 72°C for 10 min. The PCR products were confirmed by 1% agarose gel electrophoresis and purified. The Alpha and TFEB products (100 nmol/ml) were then arranged in annealing buffer solution (0.01 M Tris-HCl pH 7.5, 0.001 M EDTA, 0.1 M NaCl), water-bathed at 95°C for 10 min, and cooled down for 1-2 h at room temperature. The amplification of the Alpha-TFEB fusion gene (2,769 bp) was performed with the primers Alpha-F and TFEB-R according to the instructions provided with the Phusion High-Fidelity PCR kit (Thermo Fisher Scientific). The amplification condition was identical with that of Alpha gene.

Construction of recombinant lentiviral vector. The purified PCR product of Alpha-TFEB was subcloned into the pGEM-T vector with T4 ligase and then transformed into DH5α as described above. The cloning plasmids were screened with Ampicillin, and plasmid DNA was extracted and sequenced. The selected cloning plasmids named Alpha-TFEB-T and pLVX-Puro vector (Clontech, Kusatsu, Japan) were digested with *Bsp*DI and *Bam*HI (New England Biolabs, Ipswich, MA, USA). The digested products were purified, and the target gene and the linearized pLVX-Puro vector were then ligated by T4 ligase. The constructed plasmid, named pLVX-Puro-Alpha-TFEB, was transformed into DH5α competent cells. The constructed vector was screened and purified as described above. Double enzyme digestion was performed to confirm the ligation and the products were observed by 1% agarose gel electrophoresis.

Lentiviral packaging and titer determination. The 293FT cells (a kind gift from Professor Jason Chen, Columbia University, New York, NY, USA) were cultured in DMEM containing 10% FBS in a 37°C incubator with 5% CO₂. The pLVX-Puro-Alpha-TFEB plasmid and its packaging plasmid containing 4.5 μg psPAX2 and 4.5 μg pMD2.G (Clontech) were co-transfected into 293FT cells using Lipofectamine 2000 (Life Technologies). After 72 h, supernatants were collected from these cells and passed through a 0.45 μm filter (EMD Millipore, Billerica, MA, USA). Lentivirus titer determination was performed using the Lenti-X p24 Rapid Titer kit (Clontech), and the viral titer of this package was 8.0x10⁶ TU/ml. The ultimate titer was 8.0x10⁸ TU/ml using Lenti-X Concentrator (Clontech). The concentrated vector was stored at -80°C until use.

Cell culture and stable transfection. The CaKi-2 (human papillary renal cell carcinoma cell line) (31) and HK-2 (normal human renal epithelial cell line) cells were obtained from Vinhak Biological Technology Co., Ltd. (Shanghai, China). The cells were seeded at a density of 1x10⁵ cells/ml on 24-well plates in complete medium without antibiotics for 14 h, and the culture medium was then replaced with 0.3 ml fresh medium with 4 μg/ml retronectin (Takara). The HK-2 and CaKi-2 cells were infected with pLVX-Puro-Alpha-TFEB at a multiplicity of infection (MOI) of 20 according to the pre-experimental result. The positive cell clones containing the Alpha-TFEB fusion gene were screened out by puromycin (Thermo Fisher Scientific) (1.5 μg/ml for the HK-2 cell line and 2.0 μg/ml for the CaKi-2 cell line) and named HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB.

Reverse transcription PCR (RT-PCR). The 4 groups of cells, HK-2, CaKi-2, HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB, were digested and collected. Primers were designed using Primer 5.0 software (Table III) and synthesized by Invitrogen. RT-PCR of the Alpha-TFEB fusion gene was performed with the PrimeScript™ One Step kit (Takara) following the manufacturer's instructions. β-actin was used as an internal control. The one-step RT-PCR conditions were 50°C for 30 min, 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 10 min. The PCR products were confirmed by 1.5% agarose gel electrophoresis. The experiments were run 3 times in independent conditions.

Western blot analysis. Total protein was extracted with lysis buffer (pH 7.4, containing 0.1% SDS, 100 mM NaCl, 1% Triton-X 100, 10 mM Tris, 1 mM EDTA, 0.5% sodium deoxycholate, 1 mM PMSF, 60 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 μ g/ml pepstatin) at 24 h after transfection and protein concentrations were determined using a BCA protein assay kit (23227; Pierce Biotechnology, Rockford, IL, USA). The extracted proteins were subjected to 10% SDS-PAGE and electrotransferred onto PVDF membranes (Millipore, Molsheim, France). The membranes were then incubated with primary antibodies including TFEB (1:500, sc-11005, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β -actin (1:1,000, sc-376421, Santa Cruz Biotechnology) overnight at 4°C followed by incubation with donkey anti-goat IgG-HRP (1:5,000, sc-2020, Santa Cruz Biotechnology) and goat anti-mouse IgG-HRP (1:5,000, sc-2005, Santa Cruz Biotechnology) at room temperature for 1 h, respectively. Immunoreactive proteins were detected using the SuperSignal West Femto kit (Pierce Biotechnology) and images were captured using the Tanon-5500 Multi Image System (Tanon Science & Technology Ltd., Shanghai, China). β -actin was used as the internal reference.

Immunofluorescence assay. The HK-2, CaKi-2, HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB cells were plated at 1×10^4 cells/ml in 24-well chamber slides. The cells were washed, fixed, permeated and blocked as previously described (32). The cells were then incubated with the primary antibody TFEB (1:250, sc-11005, Santa Cruz Biotechnology) for 1 h at 37°C. After washing 3 times with PBS, the cells were incubated with the secondary fluorescence antibody (donkey anti-goat IgG-FITC, 1:1,000, sc-2024, Santa Cruz Biotechnology) for 30 min at 37°C. The cells were then mounted with 70% glycerol and observed under an inverted fluorescence microscope (Olympus IX73002; Olympus, Tokyo, Japan).

Cell proliferation assay. Cell proliferation was measured by MTT assay. The cells were plated into 96-well plates (1×10^3 cells/well) and each cell line in 8 wells. The blank group contained only medium without cells. At 24, 48, 72 and 96 h of incubation, 10 μ l MTT (5 mg/ml) were added to each well followed by incubation for an additional 4 h at 37°C. Subsequently, 100 μ l DMSO were then added for 10 min to dissolve the formazan crystals. The absorbance values were measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. All assays were repeated 3 times.

Soft agar assay. The cells were plated in 6-well plates and dispersed by slightly shaking the plates. The cell suspension (0.05 ml) was mixed with 0.7% agar and 2X DMEM culture medium and plated on top of 1.2% base agar layer in 60-mm dishes. The mixture was incubated at 37°C in a 5% CO₂ humidified incubator for 10 to 14 days until significant colony formation was observed. The number of colonies was counted using an inverted microscope (Olympus IX73002; Olympus). The colony formation efficiency was calculated using the following formula: Colony forming efficiency (%) = (colonies formed/cells incubated) \times 100%.

Invasion assay. Matrigel Transwell assays were performed to assess the cell invasive potential (BD Biosciences,

Erembodegem, Belgium). The cells were starved in DMEM without FBS overnight, and the cell suspension (200 μ l) was then loaded to the upper chamber and DMEM medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 24 h, the invading cells were methanol-fixed and stained with 0.1% crystal violet (Sigma, St. Louis, MO, USA). Five low-magnification areas (\times 100) were randomly selected and counted. All assays were performed in triplicate.

Flow cytometric analysis. Cell apoptosis was determined by flow cytometric analysis using the Annexin V-FITC/PI apoptosis kit (BD Biosciences) according to the manufacturer's instructions. The HK-2, CaKi-2, HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB cells were separately seeded into 6-well plates and incubated for 24 h at 37°C. The culture media in 3 wells were removed and the cells were washed twice with PBS, and then starved in serum-free DMEM. Following starvation for 12, 24 and 48 h, the cells were stained with 10 μ l of Annexin V-FITC and 10 μ l of PI, and analyzed using a flow cytometer (Beckman Coulter, Boulevard Brea, CA, USA).

Establishment of tumor xenografts in nude mice. The cells were trypsinized and resuspended in DMEM without FBS. A total of 15 BALB/C-nu/nu 5-week-old female nude mice with an average weight of 18–20 g were purchased from the National Rodent Laboratory Animal Resources, Shanghai, China. They were kept in an environment with a controlled temperature (25 \pm 2°C), humidity (50–70%) with free access to food and water. The mice were randomly divided into 5 groups of 3 mice in each as follows: The HK-2 group, CaKi-2 group, HK-2-Alpha-TFEB group, CaKi-2-Alpha-TFEB group and DMEM medium without FBS group. A total of 100 μ l (1×10^8) cells were injected into the armpits of the nude mice. At 4 weeks after the injection, the mice were euthanized, and the tumors were removed and weighed. Tumor volume was calculated using the following formula: Tumor volume = $0.5 \times \text{length} \times \text{width}^2$. The experiments were approved by the Institutional Animal Care and Use Committee of Anhui Medical University (Hefei, China) and were in compliance with all regulatory guidelines.

Statistical analysis. Data are presented as the means \pm standard deviation. Statistical differences between sample means were analyzed by an unpaired, two-tailed Student's t-test using SPSS 16.0 software. A two-way ANOVA was used to assess the significance between different groups. Multiple comparison analysis was performed using the Tukey method. Significance was set at $P < 0.05$.

Results

Promoter predictions and PCR products. According to the results of Alpha gene promoter predictions with score cut-off value of 0.80 (Fig. 1A), 5 pairs of primers with restrictive sites were designed for different lengths of Alpha (Fig. 1B). The Alpha1 and Alpha4 contain the 369–419 promoter prediction region. Alpha1, Alpha2 and Alpha5 all contain the 643–693 prediction region. Alpha1, Alpha2 and Alpha3 all contain the 1646–1696 prediction region. One pair of primer for pTFEB was also designed. The size of the PCR products for Alpha1, 2, 3, 4, 5 was 1,854, 1,459, 404, 256 and 139 bp,

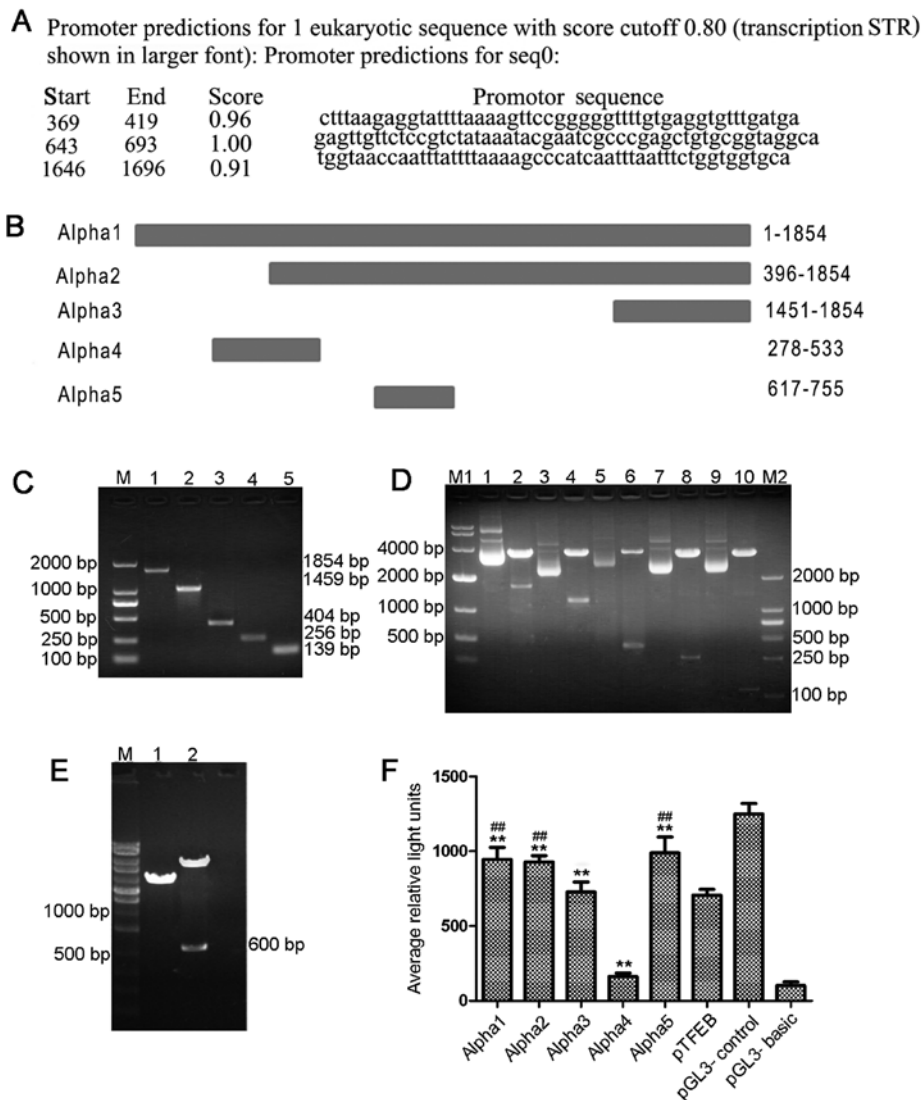


Figure 1. Alpha gene is a strong promoter. (A) The result of Alpha gene promoter predictions with a score cut-off value of 0.80. (B) The genetic positions of Alpha1, 2, 3, 4 and 5. (C) The size of PCR products for Alpha1, 2, 3, 4, 5 were 1,854, 1,459, 404, 256 and 139 bp, respectively. Lane M1, DNA marker 2000; lane 1, Alpha1; lane 2, Alpha2; lane 3, Alpha3; lane 4, Alpha4; lane 5, Alpha5. (D) Enzyme identification of recombinant reporter plasmids containing different Alpha gene segments. Lane M1, DNA marker 10000; lane 1, pGL3-Enhancer-Alpha1; lane 2, pGL3-Enhancer and Alpha1 by enzyme cutting; lane 3, pGL3-Enhancer-Alpha2; lane 4, pGL3-Enhancer and Alpha2 by enzyme cutting; lane 5, pGL3-Enhancer-Alpha3; lane 6, pGL3-Enhancer and Alpha3 by enzyme cutting; lane 7, pGL3-Enhancer-Alpha4; lane 8, pGL3-Enhancer and Alpha4 by enzyme cutting; lane 9, pGL3-Enhancer-Alpha5; lane 10, pGL3-Enhancer and Alpha5 by enzyme cutting; lane M2, DNA marker 2000. (E) Enzyme identification of recombinant reporter plasmids containing pTFEB. Lane M, DNA marker 10000; lane 1, pGL3-Enhancer-pTFEB; lane 2, pGL3-Enhancer and pTFEB by enzyme digestion. (F) Luciferase activity analysis of Alpha1, Alpha2, Alpha3, Alpha4, Alpha5 and pTFEB. **P<0.01, compared with the pGL3-Basic group. ***P<0.01, compared with the pTFEB group.

respectively (Fig. 1C). The PCR product of pTFEB was 600 bp (data not shown).

Identification of recombinant reporter plasmids. Through TA cloning, the cloning plasmids Alpha1-T, Alpha2-T, Alpha3-T, Alpha4-T, Alpha5-T and pTFEB-T were identified by double enzyme digestion and confirmed by sequencing. The recombinant reporter plasmids, pGL3-Enhancer-Alpha1, pGL3-Enhancer-Alpha2, pGL3-Enhancer-Alpha3, pGL3-Enhancer-Alpha4, pGL3-Enhancer-Alpha5 and pGL3-Enhancer-pTFEB, were identified by double enzyme digestion, and target fragments (1,854, 1,459, 404, 256, 139 and 600 bp) were shown by electrophoresis (Fig. 1D and E). The results indicated that recombinant reporter plasmids were successfully constructed containing the target segments.

Alpha gene is a strong promoter. Compared with the pGL3-Basic group, the luciferase activity of the Alpha1, Alpha2, Alpha3, Alpha4 and Alpha5 groups significantly increased (P<0.01). The luciferase activity was also markedly increased in the Alpha1, Alpha2 and Alpha5 groups compared with that of the pTFEB group, and the luciferase activity of Alpha 5 was the strongest (P<0.01) (Fig. 1F and Table IV). Alpha1, Alpha2 and Alpha5 all contain the 643-693 base sequence. The experimental data showed that the Alpha gene in RCC with t(6;11) translocation has a promoter activity, and the strongest active region is located in the 643-693 base sequence.

Successful construction of Alpha-TFEB lentiviral expression vector. The Alpha-TFEB fusion gene was amplified by overlap PCR and the expected product of Alpha-TFEB fusion,

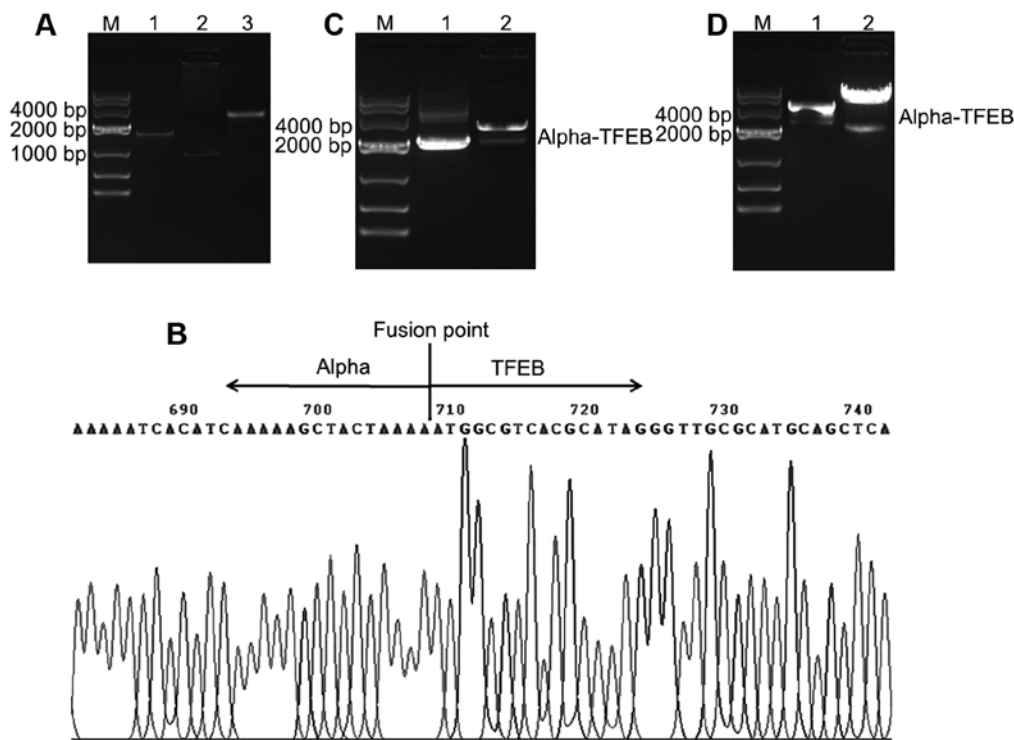


Figure 2. Construction of Alpha-TFEB lentiviral expression vector. (A) Alpha-TFEB fusion gene was amplified by overlap PCR. Lane M, DNA marker 10000; lane 1, amplified Alpha gene (1,854 bp); lane 2, amplified TFEB gene (915 bp); lane 3, amplified Alpha-TFEB fusion gene (2,769 bp). (B) The cloning plasmids containing Alpha-TFEB were sequenced. (C) Double enzyme digestion of cloning plasmid Alpha-TFEB-T. Lane M, DNA marker 10000; lane 1, Alpha-TFEB-T; lane 2, pGEM-T vector and Alpha-TFEB by enzyme cutting. (D) Enzyme identification of recombinant lentiviral vector pLVX-Puro-Alpha-TFEB. Lane M, DNA marker 10000; lane 1, pLVX-Puro-Alpha-TFEB; lane 2, pLVX-Puro vector and Alpha-TFEB by enzyme cutting

2,769 bp, was detected by 1% agarose gel electrophoresis (Fig. 2A). Sequencing analysis confirmed that the inserted Alpha-TFEB fragment in the pGEM-T vector was consistent with the target sequence (Fig. 2B). The cloning plasmid Alpha-TFEB-T was identified by double enzyme digestion and shown by 1% agarose electrophoresis (Fig. 2C). The Alpha-TFEB lentiviral expression vector, pLVX-Puro-Alpha-TFEB, was digested by restriction enzyme and distinguished by electrophoresis (Fig. 2D). The data indicated that the lentiviral expression vector containing Alpha-TFEB fusion was successfully constructed.

Stable transfection of Alpha-TFEB upregulates the expression of Alpha-TFEB mRNA and TFEB protein. The results of RT-PCR revealed that the expression of Alpha-TFEB mRNA could be detected in the HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB (407 bp) cells (lanes 1 and 2), while there was no mRNA expression of the target gene in untransfected cells HK-2 and CaKi-2 (Fig. 3A; lanes 3 and 4). The RT-PCR product of β -actin (268 bp) is shown in Fig. 3B. Sequencing analysis for the RT-PCR product of Alpha-TFEB mRNA confirmed that it was consistent with the target sequence. The results of western blot analysis revealed that the relative expression levels of TFEB protein (53 kDa) were significantly higher in the HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB groups as compared to the HK-2 and CaKi-2 groups (Fig. 3C). Immunofluorescence assay revealed that green fluorescence was also evident in the HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB cells, while there was very little fluorescence observed in the HK-2 and CaKi-2 cells (Fig. 3D).

Table IV. Promoter activity analysis of different Alpha gene segments.

Groups	Luciferase activity	t-value	P-value ^a
pTFEB	706.61±103.31		
pGL3-Basic Vector	113.00±53.05	21.68	<0.001
pGL3-Control Vector	1,206.28±162.96	10.99	<0.001
Alpha1	945.89±170.79	19.76	<0.001
Alpha2	966.56±117.35	28.12	<0.001
Alpha3	752.89±147.22	17.35	0.283
Alpha4	189.94±50.67	4.45	<0.001
Alpha5	1,042.28±166.54	22.56	<0.001

^avs. pTFEB group.

Overexpression of TFEB promotes cell proliferation, colony formation and invasion, and suppresses cell apoptosis. The stable transfection of Alpha-TFEB upregulated the expression of Alpha-TFEB mRNA and TFEB protein in the HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB cells. To assess the function of stably transfected cells overexpressing TFEB, an MTT assay, soft agar assay, Matrigel Transwell assay, and flow cytometric analysis were performed. The results of MTT assay revealed that the proliferation rate of the HK-2-Alpha-TFEB group following transfection at 24, 48, 72 and 96 h (0.4893 ± 0.1135 , 1.0505 ± 0.1103 , 1.3591 ± 0.1490

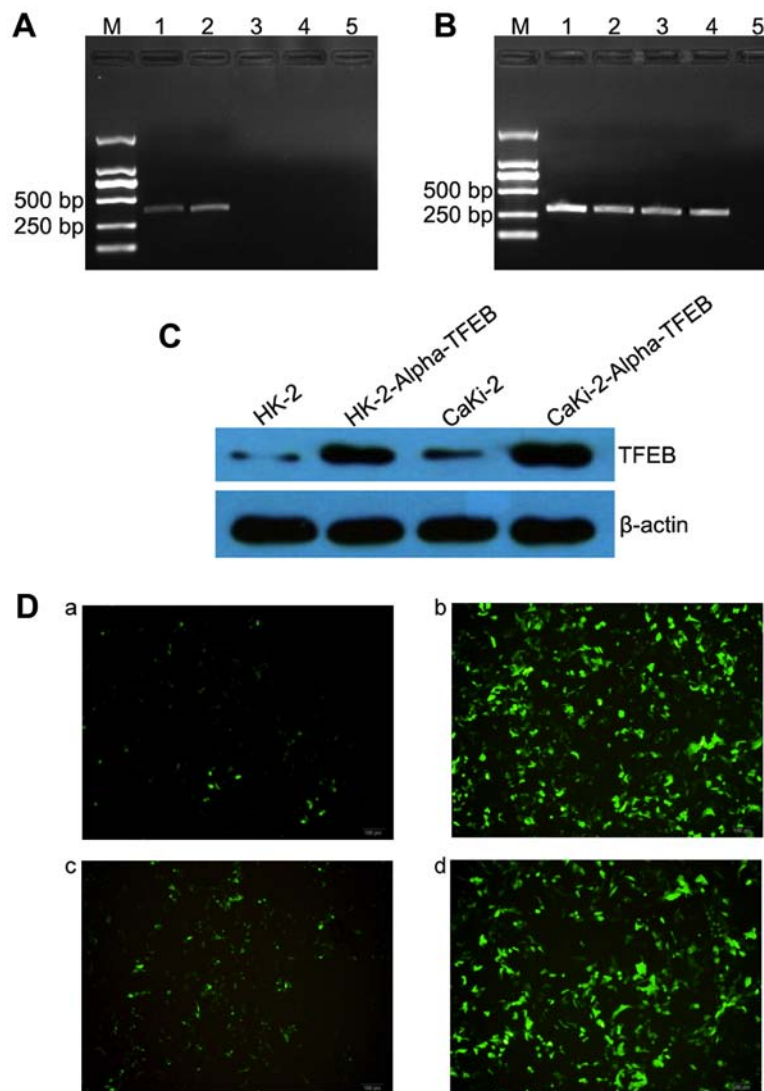


Figure 3. Overexpression of Alpha-TFEB mRNA and TFEB protein in stably transfected Alpha-TFEB cells. (A) Expression of Alpha-TFEB mRNA was analyzed by RT-PCR (407 bp). Lane M, DNA marker 2000; lane 1, HK-2-Alpha-TFEB cells; lane 2, CaKi-2-Alpha-TFEB cells; lane 3, HK-2 cells; lane 4, CaKi-2 cells; lane 5, blank control (without templates). (B) β -actin (268 bp) was used as an internal control. Lane M, DNA marker 2000; lane 1, HK-2-Alpha-TFEB cells; lane 2, CaKi-2-Alpha-TFEB cells; lane 3, HK-2 cells; lane 4, CaKi-2 cells; lane 5, blank control (without templates). (C) TFEB protein levels were examined by western blot analysis. β -actin was used as an internal control. (D) TFEB protein levels were analyzed by indirect immunofluorescence assay. Panel a, HK-2 cells; panel b, HK-2-Alpha-TFEB cells; panel c, CaKi-2 cells; panel d, CaKi-2-Alpha-TFEB cells.

and 2.7889 ± 0.1670 , respectively) was markedly higher than that of the HK-2 group (0.4885 ± 0.0602 , 0.7163 ± 0.0620 , 1.1188 ± 0.1512 and 2.2284 ± 0.2819 , respectively). Similarly, the proliferation rate of the CaKi-2-Alpha-TFEB group (0.5718 ± 0.0954 , 1.1873 ± 0.1231 , 1.5718 ± 0.1499 , 3.6584 ± 0.1597 , respectively) was significantly higher than that of the CaKi-2 group (0.5513 ± 0.0654 , 0.9993 ± 0.1223 , 1.4022 ± 0.1574 , 3.0461 ± 0.1969 , respectively) (Fig. 4A). These effects were time-dependent. A similar result was observed in the soft agar assay: There was a significant difference in the colony-forming efficiency between the HK-2-Alpha-TFEB group and HK-2 group ($t=14.20$, $P=0.0049$), and between the CaKi-2-Alpha-TFEB group and the CaKi-2 group ($t=6.145$, $P=0.0255$) (Fig. 4B and C). Matrigel Transwell assay also demonstrated that the overexpression of TFEB significantly increased the migratory and invasive capacity of the HK-2 and CaKi-2 cells (Fig. 5A and B). Flow cytometric analysis indicated that the apoptotic ratio of the HK-2-Alpha-TFEB

group following transfection at 12, 24, and 48 h (4.80 ± 1.15 , 27.03 ± 1.10 and $46.43 \pm 1.97\%$, respectively) was significantly lower than that of the HK-2 group (18.87 ± 0.35 , 37.67 ± 1.91 and $64.17 \pm 2.22\%$, respectively). Likewise, the apoptotic ratio of the CaKi-2-Alpha-TFEB group (4.37 ± 1.11 , 23.60 ± 1.85 and $41.00 \pm 0.61\%$, respectively) was significantly lower than that of the CaKi-2 group (9.77 ± 0.55 , 26.77 ± 2.41 and $45.20 \pm 2.61\%$, respectively) (Fig. 5C). These data indicated that the overexpression of TFEB promoted cell proliferation, colony formation and invasion, and inhibited cell apoptosis.

Overexpression of TFEB promotes tumorigenicity in nude mice. To examine the effects on tumorigenesis of the overexpression of TFEB in nude mice, *in vivo* tumor formation assay was carried out. The results of the *in vivo* experiments revealed that a tumor mass could be observed in the CaKi-2 group, HK-2-Alpha-TFEB group and CaKi-2-Alpha-TFEB group, while no mass was observed in the HK-2 group and

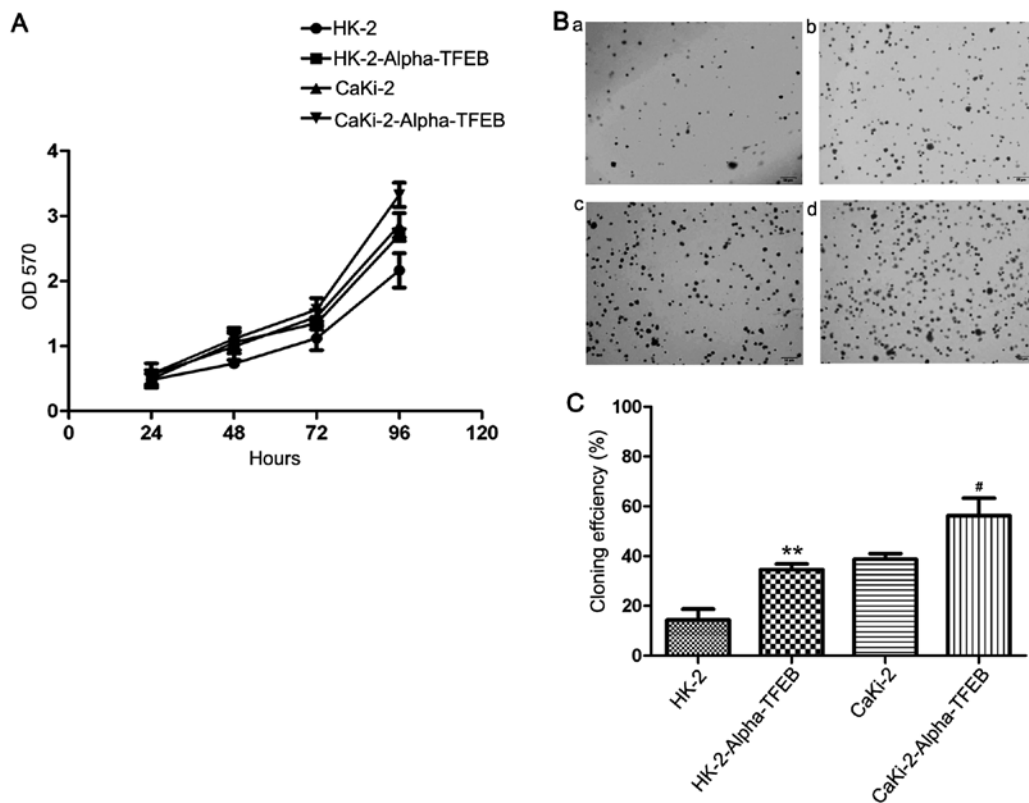


Figure 4. Overexpression of TFEB promotes cell proliferation and colony formation. (A) The proliferation rate of stably transfected and untransfected cells examined by MTT assay. (B and C) The colony-forming efficiency of stably transfected and untransfected cells determined by soft agar assay. Panel a, HK-2 cells; panel b, HK-2-Alpha-TFEB cells; panel c, CaKi-2 cells; panel d, CaKi-2-Alpha-TFEB cells. ** $P < 0.01$, compared with the HK-2 group. # $P < 0.05$, compared with the CaKi-2 group.

DMEM medium without FBS group. In the HK-2-Alpha-TFEB group and CaKi-2-Alpha-TFEB group, a significant increase in tumor volume and weight, compared to the HK-2 group and CaKi-2 group was observed (Fig. 6A and B). The maximum diameter of the largest tumor was 11.0 mm and the maximum tumor volume was 198.0 mm³. The weight of the mice upon sacrifice was 24.87 ± 0.74 , 21.17 ± 1.27 , 20.27 ± 1.11 , 19.90 ± 1.31 and 25.33 ± 0.85 g in the HK-2 group, CaKi-2 group, HK-2-Alpha-TFEB group, CaKi-2-Alpha-TFEB group and DMEM medium without FBS group, respectively (data not shown). No mice developed multiple tumors. The average tumor weight in the HK-2-Alpha-TFEB group (0.42 ± 0.07 g) was significantly higher than that in the HK-2 group (0.00 ± 0.00 g) ($P < 0.01$). The average tumor weight in the CaKi-2-Alpha-TFEB group (0.41 ± 0.03 g) was also higher than that in the CaKi-2 group (0.17 ± 0.02 g; $P < 0.01$) (Fig. 6C).

Discussion

RCCs constitute multiple heterogeneous cancer types that account for approximately 90% of all adult renal malignancies. The most common types are clear cell, papillary and chromophobe, each with a different histology and clinical course (18). RCC with translocation is a distinct subtype of RCCs harboring recurrent gene rearrangements (33). The majority of RCCs with translocation are with the Xp11.2 translocation which usually occurs in children and young adults, resulting in various types of gene fusions involving the TFE3 gene

located on chromosome Xp11.2 (33,34). Another less common translocation is the t(6;11) translocation in RCC, involving the fusion between the Alpha gene and TFEB gene (1). The majority of cases of RCC with t(6;11) translocation seem to be indolent (3,11,14), although recurrence occurs in 17% of patients (15) and some adult patients present with metastasis or succumb to the disease (9,13,15). It has been almost 2 decades since the first case of RCC with t(6;11) translocation reported in 2001 (1). However, its molecular biology remains greatly uncharacterized and effective targeted therapy has yet to be identified (35).

The translocation of t(6;11) (p21;q12 or q13) in RCC causes the fusion of the Alpha gene with the TFEB gene (6). To date, all Alpha-TFEB fusions were shown to fall in the 1,205 bp breakpoint cluster region of the Alpha gene, and in the 289 bp breakpoint cluster region numbered from the 5' end of exon3 of TFEB gene (35). We have previously detected a novel fusion point of Alpha-TFEB which the breakpoint was at nucleotide 1810 in the Alpha and -94 in the TFEB (3). Argani *et al* (6) demonstrated that DNA-PCR and RT-PCR products of the Alpha-TFEB fusion gene were identical due to the lack of splice signals in an intronless gene Alpha. The fusion of Alpha-TFEB results in the overexpression of a full-length wild-type TFEB protein as opposed to a chimeric protein (4). To date, no TFEB fusion partners other than Alpha have been detected in RCC with t(6;11) translocation (35). Therefore, from these data, it is doubtful as to whether the Alpha gene is a strong promoter that upregulates TFEB expression. In

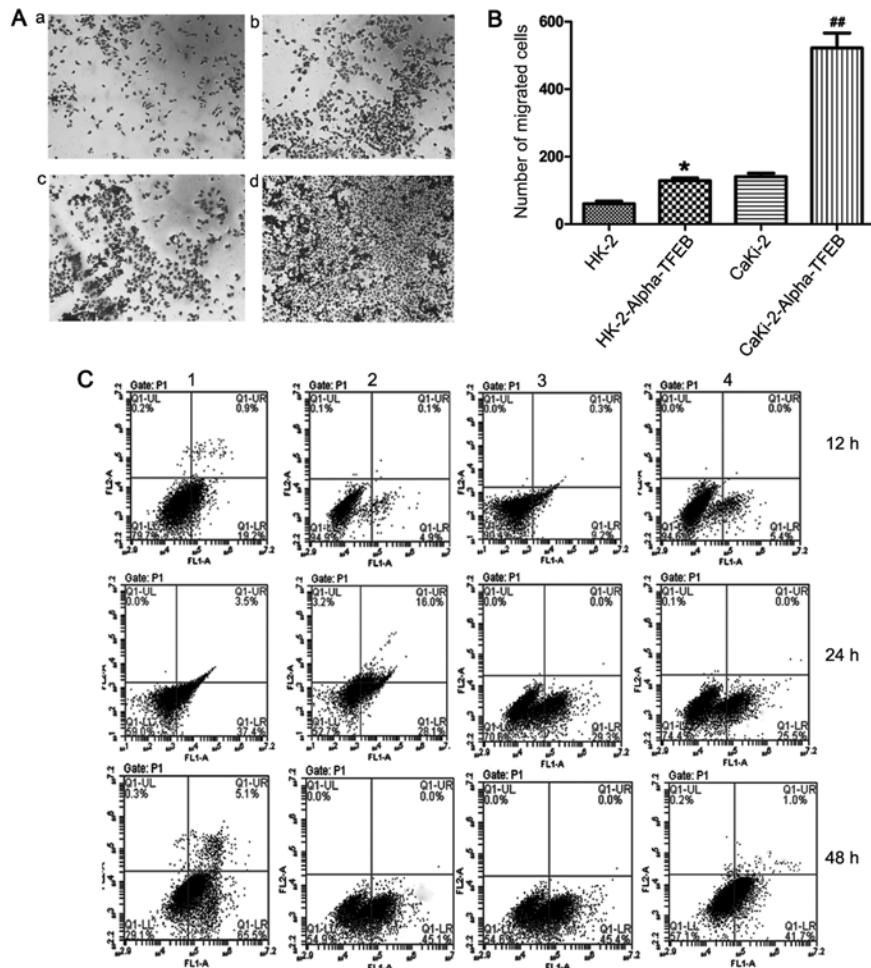


Figure 5. Overexpression of TFEB promotes the cell migration and suppresses the cell apoptosis. (A and B) The migration and invasion capacity was detected by Matrigel Transwell assay. Panel a, HK-2 cells; panel b, HK-2-Alpha-TFEB cells; panel c, CaKi-2 cells; panel d, CaKi-2-Alpha-TFEB cells. * $P < 0.05$, compared with the HK-2 group. ** $P < 0.01$, compared with the CaKi-2 group. (C) The apoptotic ratio of stably transfected and untransfected cells was analyzed by flow cytometry. Upper left quadrant, necrotic cells; bottom left quadrant, live cells; upper right quadrant, late apoptotic cells; lower right quadrant, early apoptotic cells. Panels 1, HK-2 cells; panels 2, HK-2-Alpha-TFEB cells; panels 3, CaKi-2 cells; panels 4, CaKi-2-Alpha-TFEB cells.

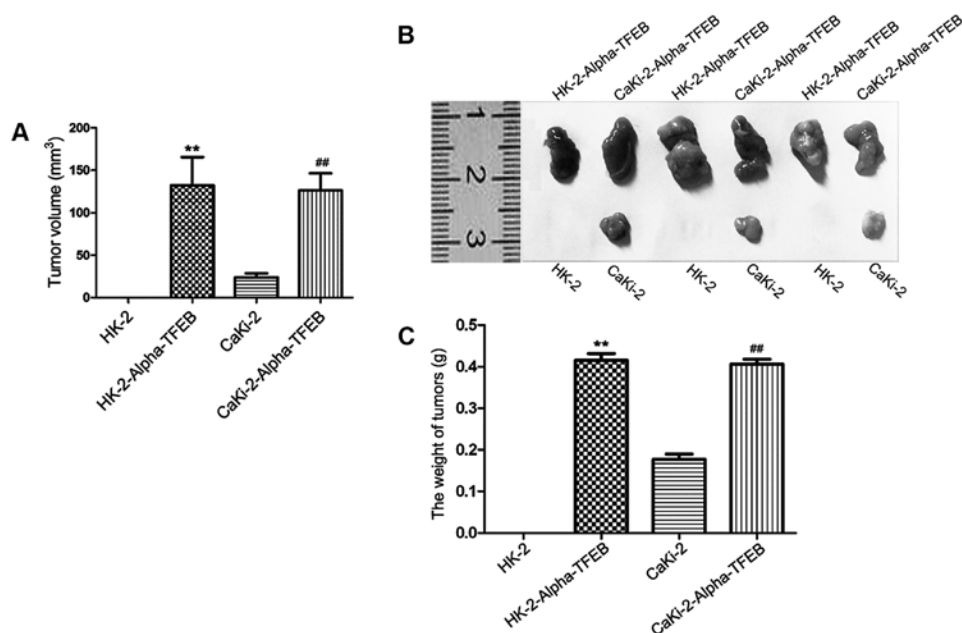


Figure 6. Tumorigenicity in nude mice. (A) Tumor volume was calculated. ** $P < 0.01$, compared with the HK-2 group. ** $P < 0.01$, compared with the CaKi-2 group. (B) The formative tumors were removed and photographed. (C) The tumors were weighed. ** $P < 0.01$, compared with the HK-2 group. ** $P < 0.01$, compared with the CaKi-2 group.

this study, in order to clarify this issue, luciferase assay for 5 different lengths of Alpha gene and normal TFEB gene was performed, and our results suggested that the Alpha gene has a strong promoter activity and the strongest promoter activity region is located in the 643-693 base sequence.

Based on the above-mentioned results, we constructed an Alpha-TFEB lentiviral expression vector and transfected this vector into HK-2 cells and CaKi-2 cells. Following the successful transfection of Alpha-TFEB, the expression of Alpha-TFEB mRNA could be detected in the HK-2-Alpha-TFEB and CaKi-2-Alpha-TFEB cells, while there was no mRNA expression of the target gene in untransfected HK-2 and CaKi-2 cells. This result was identical to that obtained from the tissue test of RCC with t(6;11) translocation and normal kidney (6). Kuiper *et al* (4) reported that Alpha-TFEB fusion transcripts were expressed in t(6;11)(p21;q13)-positive cells, but not in control human genomic DNA by northern blotting, and Alpha-TFEB mRNA was detected in tissue from RCC with t(6;11) translocation, but not in normal kidney tissue by RT-PCR analysis. Real-time RT-PCR analysis, reported by Kuiper *et al* (4), revealed that the Alpha-TFEB mRNA levels were up to 60-fold upregulated in RCC with t(6;11) translocation cells as compared to wild-type TFEB mRNA levels in normal kidney samples. A native TFEB protein, but not a chimeric protein, was overexpressed in RCC with t(6;11) translocation. Argani *et al* (6) detected strong nuclear TFEB labeling in all 7 cases of RCC with t(6;11) translocation, whereas 1,089 other unrelated neoplasms and normal tissues did not show nuclear TFEB labeling. We have previously reported a high Alpha-TFEB DNA level and strong nuclear TFEB labeling in tissue from RCC with t(6;11) translocation (3). In this study, the results of western blot analysis revealed that the expression levels of TFEB protein were significantly higher in the positively transfected cells as compared to the untransfected cells, which suggested that the fusion of Alpha-TFEB led to a high expression of TFEB protein. The role of Alpha may not be specific in RCC. However, the fusion of Alpha-TFEB is specific to RCC with t(6;11) translocation.

The TFEB gene is encoded by 51,083 bp on chromosome 6p21.1 and produces a 2,364 bp mRNA transcript consisting of a 302 bp 5' untranslated region followed by a start codon in exon 3, and a stop codon in exon 10 followed by a 621 bp 3' untranslated region (35). TFEB mRNA encodes a 476 AA protein with a 54 AA basic region and helix-loop-helix domain including a putative nuclear localization signal (35). TFEB is one of 4 members of the microphthalmia transcription factor (MiT) family, which also includes TFE3, TFEC and MiTF (33). RCCs with Xp11.2 and t(6;11) are now grouped as MiT family translocation RCC in the light of clinical, morphologic and molecular mimics. MiT members share similar DNA-binding and activation domains. Their functions are diverse, and are related to cell growth and differentiation (36). The oncogenic activity of MiT members has been reported in clear cell sarcoma (37). Giatromanolaki *et al* (38) demonstrated that the overexpression of TFEB was associated with autophagy, the migratory phenotype and a poor prognosis in non-small cell lung cancer. TFEB may promote the growth of pancreatic ductal adenocarcinoma by autophagy regulation (39). The role of TFEB in RCC with t(6;11) translocation has yet to be determined.

No cell line has yet to be derived from patients with RCC with t(6;11) translocation (35). Lentiviral vectors are useful experimental instruments for stable gene delivery. In this study, we constructed an Alpha-TFEB lentiviral expression vector. Following the successful transfection of Alpha-TFEB into HK-2 cells and CaKi-2 cells, the expression levels of Alpha-TFEB products were significantly higher in positively transfected cells as compared to untransfected cells. We also explored the role of Alpha-TFEB product by *in vitro* experiments. An MTT assay, soft agar assay, Matrigel Transwell assay, and flow cytometric analysis were performed. Our results revealed that the overexpression of TFEB promoted cell proliferation, colony formation and invasion, and inhibited cell apoptosis. Furthermore, to validate the effectiveness of the overexpression of TFEB, an *in vivo* tumor formation assay was performed. The results of the *in vivo* experiments revealed that a tumor mass was observed in the CaKi-2 group, HK-2-Alpha-TFEB group and CaKi-2-Alpha-TFEB group, but not in the HK-2 group and culture medium group. The HK-2-Alpha-TFEB group and CaKi-2-Alpha-TFEB group had a significantly increased tumor volume and weight, compared to the HK-2 group and CaKi-2 group. These findings were consistent with those of our *in vitro* experiments, which indicated that the significant transcriptional and translational overexpression of TFEB may ultimately drive renal tumorigenesis by controlling cell growth, differentiation and metabolism. Chronic myeloid leukemia (CML) is driven by BCR-ABL fusion and BCR-ABL inhibitors are effective therapies in CML (40). The anaplastic lymphoma kinase (ALK) inhibitor, crizotinib, was first approved by the US Food and Drug Administration for the treatment of ALK-rearranged non-small cell lung cancer in 2011 (41). Whether TFEB inhibitor is a potential target for the therapy of patients with RCC with t(6;11) translocation warrants further investigation.

In conclusion, Alpha is a strong promoter and the strongest promoter activity region is located in the 643-693 base sequence. The stable transfection of Alpha-TFEB into HK-2 cells and CaKi-2 cells promoted the expression of Alpha-TFEB mRNA and TFEB protein. Furthermore, the overexpression of TFEB increased cell proliferation and invasion, and decreased cell apoptosis in cells stably transfected with Alpha-TFEB expression vector *in vitro*. The *in vivo* experiments using nude mice indicated that the overexpression of TFEB promoted tumorigenicity in nude mice, suggesting that TFEB may function as a cancer oncogene. These data suggest that the effects of Alpha-TFEB are specific in this type of tumor, which results in the overexpression of a native TFEB protein and then promotes cell canceration. The overexpression of TFEB confers a potent oncogenic signal and may be a novel therapeutic target in RCC with t(6;11) translocation.

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