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Original Article

TFEB promotes BCL-2 expression by upregulating its promoter activity in the t(6;11) translocation renal cell carcinomas

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Abstract: t(6;11) translocation renal cell carcinoma (RCC) is classified as a subset of the MiT family translocation RCCs and characterized by harboring the Alpha-TFEB fusion gene. However, the development mechanism of this tumor and its effective treatment have not been fully identified yet. The purpose of this study was to explore the relationship between TFEB and BCL-2 in Alpha-TFEB stably transfected cell lines and in t(6;11) RCC tumor tissue. An Alpha-TFEB eukaryotic expression vector was constructed and stably transfected into CaKi-2 and HK-2 cells. RT-PCR and real-time RT-PCR were used to measure the mRNA expressions of TFEB and BCL-2, and immunohistochemistry, Western blot and dual immunofluorescence assays were used to evaluate the TFEB and BCL-2 protein expressions. MTT proliferation assays and flow cytometry were also performed. Furthermore, luciferase reporter assays were used to evaluate the BCL-2 promoter activity. An Alpha-TFEB eukaryotic expression vector was successfully constructed and stably transfected into CaKi-2 and HK-2 cells (named CaKi-2-TFEB and HK-2-TFEB cells). Compared with the CaKi-2 and HK-2 groups, the TFEB and BCL-2 mRNA expression levels were significantly upregulated in the CaKi-2-TFEB and HK-2-TFEB groups respectively. The TFEB and BCL-2 protein expressions showed a similar result. The overexpression of TFEB and BCL-2 promoted cell proliferation and inhibited cell apoptosis. Moreover, the overexpression of TFEB upregulated the promoter activity of BCL-2. Our data suggest that the overexpression of TFEB promotes BCL-2 expression by upregulating its promoter activity and ultimately results in the development of t(6;11) translocation RCC. BCL-2 inhibitors may serve as potential therapeutic targets for t(6;11) translocation RCC.

Keywords: TFEB, BCL-2, promoter activity, t(6;11) translocation, renal cell carcinoma

Introduction

T(6;11) translocation renal cell carcinoma (RCC) was first reported in 2001 [1] and is classified as a subset of the microphthalmia (MiT) family translocation RCCs, including another Xp11 translocation RCC in the 2016 World Health Organization (WHO) tumor classification [2]. The first Chinese patient suffering from t(6;11) translocation RCC with a new Alpha-TFEB fusion point was reported by our research group [3]. The Alpha gene (also called MALAT1), an intronless gene, encodes a transcript of 7.5 kb or 8.5 kb but doesn't encode a functional protein [4]. The TFEB gene encodes a 2364 bp transcript and a 476 amino acid protein [5]. This neoplasm is characterized by harboring an Alpha-TFEB fusion gene composed of an Alpha gene and a TFEB gene on 11q12 (or q13) and 6p21, which causes high native TFEB protein expressions [6]. It frequently occurs in children and adolescents, but rarely in adults. However, the frequency of translocation RCC may be widely underestimated in adults because of misdiagnoses with other renal cell tumors due to inadequate workups or morphological similarities, such as clear cell RCC, papillary RCC, epithelioid angiomyolipoma, and so on [7]. It's essential for the accurate diagnosis of this tumor to combine the morphology, the immunohistochemistry, and the genetic analysis. Recently, more cases of t(6;11) translocation RCC have been diagnosed by combining the immunohistochemistry with the anti-TFEB anti-

Table 1. The overlap PCR primers used in this study

	Primer sense	Sequences (5'→3')	PCR products (bp)
Alpha	Forward	*TCGCGA*GTGATCGAATTCCGGTGATGCGAGT	1219
	Reverse	**CCTATAGTGAGTCGTATTA**TTTTAGTAGCTTTTTGATGTGATTT	
TFEB	Forward	**TAATACGACTCACTATAGG**ATGGCGTCACGCATAGGGTT	1451
	Reverse	*TCTAGA*GCACATCGCCCTCCTCCATGCTGAA	

Bold sequences indicate the connection of Alpha and TFEB fragments; the italic sequences represent NruI and XbaI restriction sites.

bodies and fluorescence *in situ h*ybridization with TFEB probes in paraffin-embedded tumor tissues [8-14]. However, the development mechanism of this tumor is still unclear, and an effective treatment has not been identified [15].

It is now widely known that apoptotic resistance is an important step in tumorigenesis [16]. The BCL-2 family proteins play a crucial role in the regulation of cell apoptosis and caspase activation, and the BCL-2 protein is the main anti-apoptotic factor of the BCL-2 family of proteins [17, 18]. The human BCL-2 gene, located on 18q21.33, was first identified as a proto-oncogene and activated by translocation between chromosome 14 and 18 in follicular B cell lymphoma, encoding a 26 kDa protein consisting of 239 amino acids localized in the endoplasmic reticulum, mitochondria, and perinuclear membrane, and widely expressed in embryonic tissues [19-21]. The aberrant expression of the BCL-2 protein has been proved to contribute to many kinds of human malignant neoplasms, including cancers, leukemias, and lymphomas [22]. McGill et al. [23] showed that the endogenous BCL-2 promoter is occupied by the MiTF protein in melanoma cells, and the endogenous expression of BCL-2 is modulated through the up-regulation or down-regulation of MiTF in melanoma cells, melanocytes, and osteoclasts, suggesting that BCL-2 is a target of MiTF at the transcription level. TFEB, MiTF, TFE3 and TFEC, the transcription factor members of the MiT family, have identical DNA binding domains, which indicates that TFEB may have target gene repertoire overlap with the other three transcription factors [24]. In clear cell sarcoma, by co-transfecting the encoding TFEB plasmids with MiTF shRNA, TFEB can save the lethal effect of MiTF gene knockdown and completely rescue cell vigor in a dose-dependent manner [25].

Our previous studies revealed that high expressions of TFEB enhance cell invasion, promote cell proliferation, and reduce cell apoptosis in cell lines stably transfected with Alpha-TFEB, and it also promotes tumorigenicity in nude mice [26]. At present, the specific TFEB overexpression mechanism in the tumorigenesis of t(6;11) translocation RCC is unclear. Overexpressed TFEB acts as an aberrant transcription factor that activates the expression of multiple downstream targets, including targets normally activated by MiTF [2]. BCL-2 is a target of MiTF at the transcription level. Pecciarini et al. [27] demonstrated that there were TFEB and BCL-2 protein overexpressions in the tumor tissue of t(6;11) RCC compared to the normal tissue. However, it is doubtful whether TFEB promotes tumorigenesis by upregulating BCL-2 expression. Thus, we aimed to explore the mRNA and protein expressions of BCL-2 and the TFEB and BCL-2 correlations in Alpha-TFEB stably transfected cell lines and in t(6;11) RCC tumor tissue.

Materials and methods

Primer synthesis and DNA extraction

Primer 5.0 software was used to design the primers for Alpha and TFEB (**Table 1**), and they were synthesized by the Shanghai Invitrogen Company (China). The genomic DNA was derived from a case of t(6;11) translocation RCC and extracted from tumor tissue samples fixed with formalin and embedded with paraffin as described before [3].

Overlap PCR for the Alpha-TFEB fusion gene

Alpha1-T plasmids were constructed as described [26]. The TFEB cDNA plasmids were obtained from the Shanghai Generay Biotech Company (China). The overlap PCR reactions were performed as described [26] using the

Table 2. The RT-PCR primers used in this study

	Primer sense	Sequences (5'→3')	PCR products (bp)
TFEB	Forward	GGAGATGACCAACAAGCAGC	451
	Reverse	GGACATGGTGGACAGAAGTG	
BCL-2	Forward	ATGGCAAATGACCAGCAGA	345
	Reverse	GCAGGATAGCAGCACAGGA	
β-actin	Forward	CTCCATCCTGGCCTCGCTGT	268
	Reverse	GCTGTCACCTTCACCGTTCC	

Alpha1-T plasmids and TFEB cDNA plasmids as templates. The forward and reverse primers of TFEB and Alpha are displayed in **Table 1**. The amplification products of the Alpha gene, the TFEB gene, and the Alpha-TFEB fusion gene were electrophoresed on a 1% agarose gel. Then the amplification products were purified according to the instructions using a Takara MiniBEST DNA Kit (Dalian, China).

Construction of a eukaryotic vector of Alpha-TFEB

The purified PCR product of Alpha-TFEB was connected to the clone vector pGEM-T (Promega, WI) by T4 ligase (Takara, China), and then the constructed plasmid was transformed into competent DH5α (Tiangen, Beijing, China). The transformed bacteria were cultured at 37°C overnight and screened with ampicillin. The recombinant plasmids, named Alpha-TFEB-T, were extracted and characterized using 1% agarose gel electrophoresis, and then sent to Invitrogen for sequencing. Both the Alpha-TFEB-T and pcDNA3.1 (+) plasmids were digested with NruI and XbaI restriction enzymes (Takara), electrophoresed, purified, and then connected with T4 ligase. The recombinant plasmid was called pcDNA3.1-Alpha-TFEB, and transformed in DH5α, screened, and purified as described above. The constructed eukaryotic expression vector was confirmed using double enzyme digestion and observed on 1% agarose gel electrophoresis.

Cell culture and screening of the stable cell lines

The CaKi-2 (human RCC cell line) and the HK-2 cells (normal human renal cell line) (Vinhaket, Shanghai, China) were cultured in a 37°C, 5% CO_2 incubator with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA) and added to DMEM (Gibco, Carlsbad, CA). The CaKi-2 and HK-2 cells were counted and inoculated on 24-well culture plates for 24 h with a concentration of 1×10^5 cells/ml, and then transfected with recombinant plasmids (pcDNA3.1-Alpha-TFEB) using liposome 2000 purchased from Life Technologies (Carlsbad, CA) according to the guidelines. 24 h after the transfection, the cells were incubated in a complete medium for 48 h before exposure to G418 (Life Technologies) for selection. Then the complete medium was replaced with one containing 0.1 mg/ml G418 for about a week. If the cells were overgrown, the culture medium containing G418 was used for the culturing, and the G418 concentration was increased to 0.8 mg/ml after a week, and then the screening was continued for 2 weeks. Positive clones were selected with 0.2 mg/ml G418, and the screened cells were labeled respectively (the CaKi-2-TFEB and the HK-2-TFEB cells).

Reverse transcription PCR (RT-PCR)

The total RNAs of the CaKi-2, CaKi-2-TFEB, HK-2 and HK-2-TFEB cells were extracted using the Trizol method. The primers were synthesized by Invitrogen, and their sequences are shown in **Table 2**. The RT-PCRs for TFEB, BCL-2, and β-actin were performed using One Step RT-PCR kits (Takara) according to the manufacturer's instructions under the following conditions: one cycle at 50°C × 30 min, 94°C × 4 min, 35 cycles at 94°C × 30 s, 55°C × 30 s, 72°C × 30 s, and one cycle at 72°C × 10 min. The lengths of the PCR products were identified using electrophoresis on 1.5% agarose gel.

Real-time RT-PCR

The total RNAs for the real-time RT-PCR and the RT-PCR were the same, and the primers used are shown in **Table 3**. The real-time RT-PCR was carried out using a One Step SYBR RT-PCR Kit (Takara) on an ABI 7500 real-time PCR instrument (ABI, Carlsbad, CA) in accordance with the manufacturer's methods. The cycle conditions included one cycle of 42°C × 5 min, 95°C × 5 min, 40 cycles at 95°C × 5 s and 60°C × 20 s. GAPDH served as an internal reference to normalize the mRNA expression levels among the samples. The relative gene expression levels were analyzed by using the $2^{-\Delta\Delta CT}$ method.

Table 3. The teal-time RT-PCR primers used in this study

	Primer sense	Sequences (5'→3')	PCR products (bp)
TFEB	Forward	ACCTGTCCGAGACCTATGGG	222
	Reverse	CGTCCAGACGCATAATGTTGTC	
BCL-2	Forward	GGTGGGGTCATGTGTGTGG	89
	Reverse	CGGTTCAGGTACTCAGTCATCC	
GAPDH	Forward	ACAACTTTGGTATCGTGGAAGG	101
	Reverse	GCCATCACGCCACAGTTTC	

Western blot assays

The Western blot assays were carried out as described before [26]. The proteins extracted from cells were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Millipore, France). Then, the blots were incubated overnight at 4°C separately with various primary TFEB antibodies (1:1000, ab270604, Abcam, Cambridge, UK), BCL-2 (1:1,000, 4H8C6, Proteintech, Chicago, USA), Caspase-9 (1:1,000, ab32539, Abcam), Cleaved Caspase-3 (1:500, ab32042, Abcam), and GAPDH (1:2,000, D16H11, Cell Signaling Technology, Danvers, MA), and then incubated for 1 h at room temperature separately with the appropriate secondary antibodies, including IgG-HRP (anti-rabbit) (1:1,000, A0208, Beyotime Biotechnology, Shanghai, China) for TFEB, Cleaved Caspase-3, Caspase-9 and GAPDH, and IgG-HRP (anti-mouse) (1:1,000, A0216, Beyotime Biotechnology) for BCL-2. The proteins were observed using a SuperSignal detection kit (Pierce, Rockford, IL) on a Type 5500 Multi Imaging Detection System (Tanon, Shanghai, China). GAPDH served as an internal control. The relative protein expression levels were quantified using Image J software (v. 1.53, National Institutes of Health, USA) and normalized to GAPDH.

Dual immunofluorescence

The cells were inoculated in 24-well chamber slides for 24 h with a concentration of 1×10^4 cells/ml, fixed for 15 min using 4% paraformaldehyde, permeated for 10 min using 0.5% Triton X-100, incubated for 1 h with 1% bovine serum albumin sealing fluid, and then incubated with primary antibodies against TFEB (1:500, ab270604, Abcam) and BCL-2 (1:500, 4H8C6, Proteintech) at 4°C overnight. Next, they were incubated for 1 h at room temperature with their respective secondary antibodies including Alexa Fluor 555 IgG (donkey anti-rabbit) (1:1,000, A0453, Beyotime Biotechnology) and Alexa Fluor 488 IgG (goat anti-mouse) (1:1,000, A0428, Beyotime Biotechnology). DAPI (1:500, C1002, Beyotime Biotechnology) was used to stain the nuclei and a fluorescence microscope (Olympus IX73002, Tokyo, Japan) was used to capture the images. For the quantification, the integrated optical density (IOD) values of the images were analyzed using Image J software (v. 1.53, National Institutes of Health).

Immunohistochemistry

Immunohistochemical analyses were used to examine the protein expressions in the tumor tissue and normal renal tissue on 4 μm tissue sections fixed with formalin, and embedded with paraffin using the EnVision two-step method as described before [28]. Briefly, the slices were incubated at 4°C overnight with the primary antibodies of TFEB (1:300, sc-166736, Santa Cruz, CA), BCL-2 (ready-to-use, Beijing Zhongshan Jinqiao Biotechnology, Beijing, China) followed by incubation with a general-type EnVision reagent (Beijing Zhongshan Jinqiao Biotechnology) for 30 min at 37°C. Then, 3',3'-diamino-benzidine was used for chromogen. The staining was read using with an Olympus BX43 lighted microscope (Tokyo, Japan). Appropriate negative and positive controls were included. The TFEB nuclear immunoreactivity was evaluated microscopically by estimating the proportion (%) of positive cells. Staining of <10%, 10-50% or >50% of the tumor cells or the normal renal cells was considered negative, focally positive, or diffusely positive, respectively as previously described [29]. The BCL-2 expression was judged to be positive when >5% of tumor cells or the normal renal cells showed cytoplasmic staining and sections with 5% or less of the staining were considered negative as described [30]. For the quantification, five randomly-selected HPFs (400×) were analyzed, and the average positive rate was calculated.

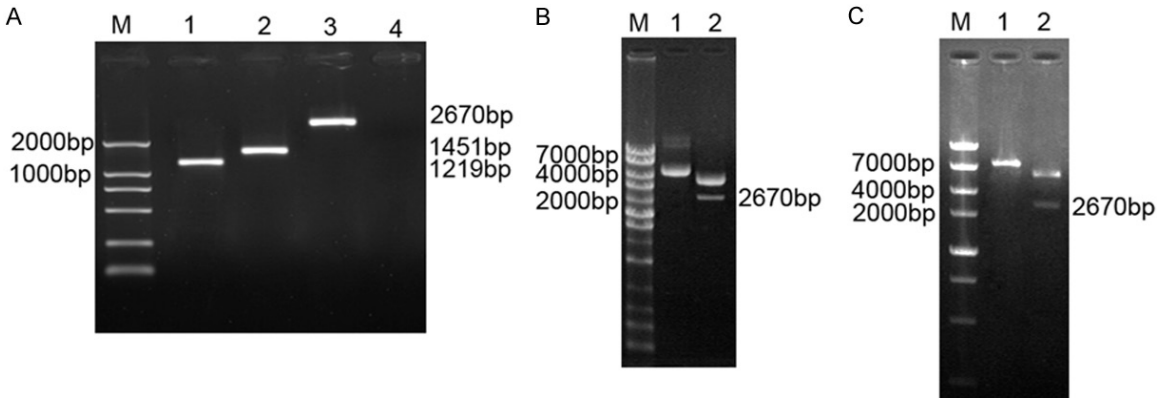


Figure 1. An Alpha-TFEB eukaryotic vector was successfully constructed. A. Alpha, TFEB, and Alpha-TFEB were measured using 1% agarose gel eletrophoresis. The M, 1, 2, 3, and 4 lanes represent DNA marker 2,000, the Alpha fragment, the TFEB fragment, the Alpha-TFEB fragment, and no DNA. B. The enzyme cutting of the cloning plasmid Alpha-TFEB-T was characterized using 1% agarose gel electrophoresis. The M, 1, and 2 lanes represent DNA marker 10,000, the Alpha-TFEB-T fragment, and the Alpha-TFEB, and the pGEM-T fragments. C. The enzyme cutting of the pcDNA3.1-Alpha-TFEB plasmid. The M, 1, and 2 lanes represent DNA marker 10,000, pcDNA3.1-Alpha-TFEB, and Alpha-TFEB and pcDNA3.1 (+).

MTT cell proliferation assays

The cells with a concentration of 1×10^4 cells per ml were seeded in replicate wells for each cell line into 96-well plates. The other two wells contained culture medium, but no cells were used, so they were blank correction wells. After 24, 48, 72, 96 h of culture, 10 μl MTT and 100 μl DMSO were added to each well. A microplate reader (Bio-Rad, Hercules, CA) was used to measure the absorbance values at 570 nm.

Apoptotic analysis

Flow cytometry was used to measure the cell apoptosis with an Annexin V-FITC/PI Apoptosis Detection Kit (C1062, Beyotime Biotechnology) following the manufacturer's guidelines. In brief, the cells were plated in 6-well plates, and allowed to adhere at 37°C. After 24 h, the complete medium was removed and DMEM without FBS was added. After 12, 24, 48 h of starvation, 10 μl Annexin V-FITC and 10 μl PI in dark conditions were added to the cells for 15 min, and a Beckman Coulter flow cytometer (Brea, CA) was used to analyze the quantities of the apoptotic cells.

Luciferase reporter assays

The BCL-2 reporter plasmid (Addgene #15381) contains the BCL-2 promoter region from ATG to -3934 obtained from Linda Boxer. The Luciferase reporter assay system (Promega) was used to determine the luciferase activity of the BCL-2 promoter. The cells with a concentration of 5×10^5 cells per ml were inoculated for 24 h in 96-well plates before their co-transfection with the pRL-TK and BCL-2 reporter plasmids with liposome 2000 (Life Technologies). pRL-TK, pGL3-Control, and pGL3-Basic vectors were used as internal, positive, and negative controls, respectively. After 48 h of transfection, the normalized luciferase activities were evaluated by the ratios of the firefly to renilla luciferase activities for each sample.

Statistical analysis

The data are shown as the mean ± standard deviation (SD) and analyzed using GraphPad Prism 7.0. The means of two independent groups were compared using unpaired, two-tailed t-tests, and the differences among the different groups were analyzed using a two-way ANOVA, followed by Sidak's multiple comparison post hoc test. A value of $P<0.05$ was considered statistically significant.

Results

The successful construction of an eukaryotic expression vector of Alpha-TFEB

The PCR products of Alpha, TFEB, and Alpha-TFEB, 1219 bp, 1451 bp, 2670 bp, respectively, were determined using 1% agarose gel eletrophoresis and were as expected (**Figure 1A**).

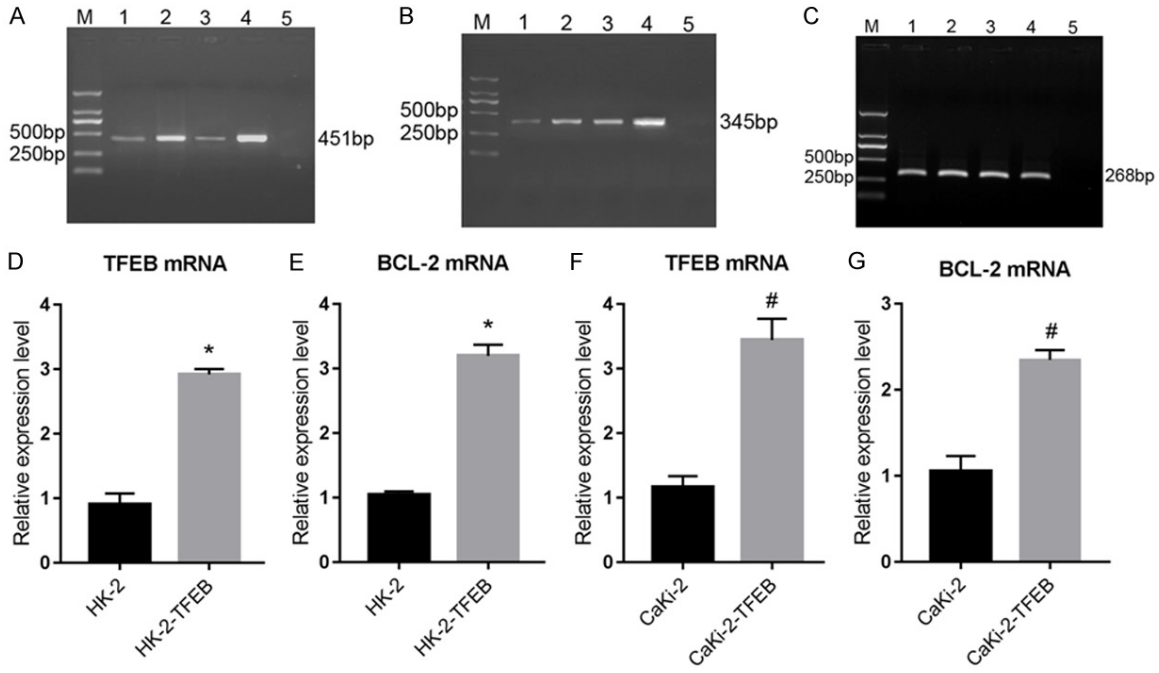


Figure 2. TFEB mRNA and BCL-2 mRNA were both upregulated in stable transfected cells with Alpha-TFEB. Our RT-PCR results revealed that the expressions of TFEB mRNA (A) and BCL-2 mRNA (B) were upregulated in the stable transfected cells. β-actin served as an internal reference (C). The M, 1, 2, 3, 4, and 5 lanes represent DNA marker 2,000, HK-2, HK-2-TFEB, CaKi-2, and CaKi-2-TFEB cells, and no DNA. Our real-time RT-PCR experiment showed the relative expression levels of TFEB mRNA (D and F) and BCL-2 mRNA (E and G). The data are shown as the means ± SD (n=3). * vs. the HK-2 group, $P<0.05$, # vs. the CaKi-2 group, $P<0.05$.

The Alpha-TFEB PCR fragment was connected to pGEM-T and then inserted into DH5α cells. The cloning plasmids Alpha-TFEB-T were characterized using 1% agarose gel electrophoresis (**Figure 1B**). The inserted Alpha-TFEB fragment was confirmed to be consistent with the target sequence using a DNA sequence analysis. The Vector Alpha-TFEB-T was successfully recombined with plasmid pcDNA3.1 (+). The digested products of the recombinant plasmid, pcDNA3.1-Alpha-TFEB, were visualized on 1% agarose gel eletrophoresis, which showed that the plasmid pcDNA3.1-Alpha-TFEB was digested by NruI and XbaI to 5428 bp and 2670 bp DNA fragments, and the two fragments respectively corresponded to pcDNA3.1 (+) (5428 bp) and the target gene Alpha-TFEB (2670 bp) as expected (**Figure 1C**).

TFEB mRNA and BCL-2 mRNA were both upregulated in the stable transfection of the Alpha-TFEB cells

RT-PCR and real-time RT-PCR were used to determine the TFEB and BCL-2 mRNA in the CaKi-2-TFEB cells and the HK-2-TFEB cells (the stable transfection cells), and in the CaKi-2 cells and HK-2 cells (the untransfected cells). The RT-PCR results revealed that the mRNA expressions of TFEB and BCL-2 were upregulated in the HK-2-TFEB group compared with the HK-2 group, as well as in the CaKi-2-TFEB group when compared with the CaKi-2 group (**Figure 2A** and **2B**). β-actin served as an internal reference (**Figure 2C**). There was a similar result observed in the real-time RT-PCR experiment. The relative mRNA expression levels of TFEB and BCL-2 were significantly higher in the HK-2-TFEB group when compared with the HK-2 group (**Figure 2D** and **2E**), as well as in the CaKi-2-TFEB group when compared with the CaKi-2 group, respectively (**Figure 2F** and **2G**).

The TFEB and the BCL-2 protein levels were both increased but the cleaved caspase-9 and the cleaved caspase-3 protein levels were decreased in the stable transfection of the Alpha-TFEB cells

The relative expression levels of the TFEB protein (66 kDa) and the BCL-2 protein (26 kDa) were both significantly increased but the

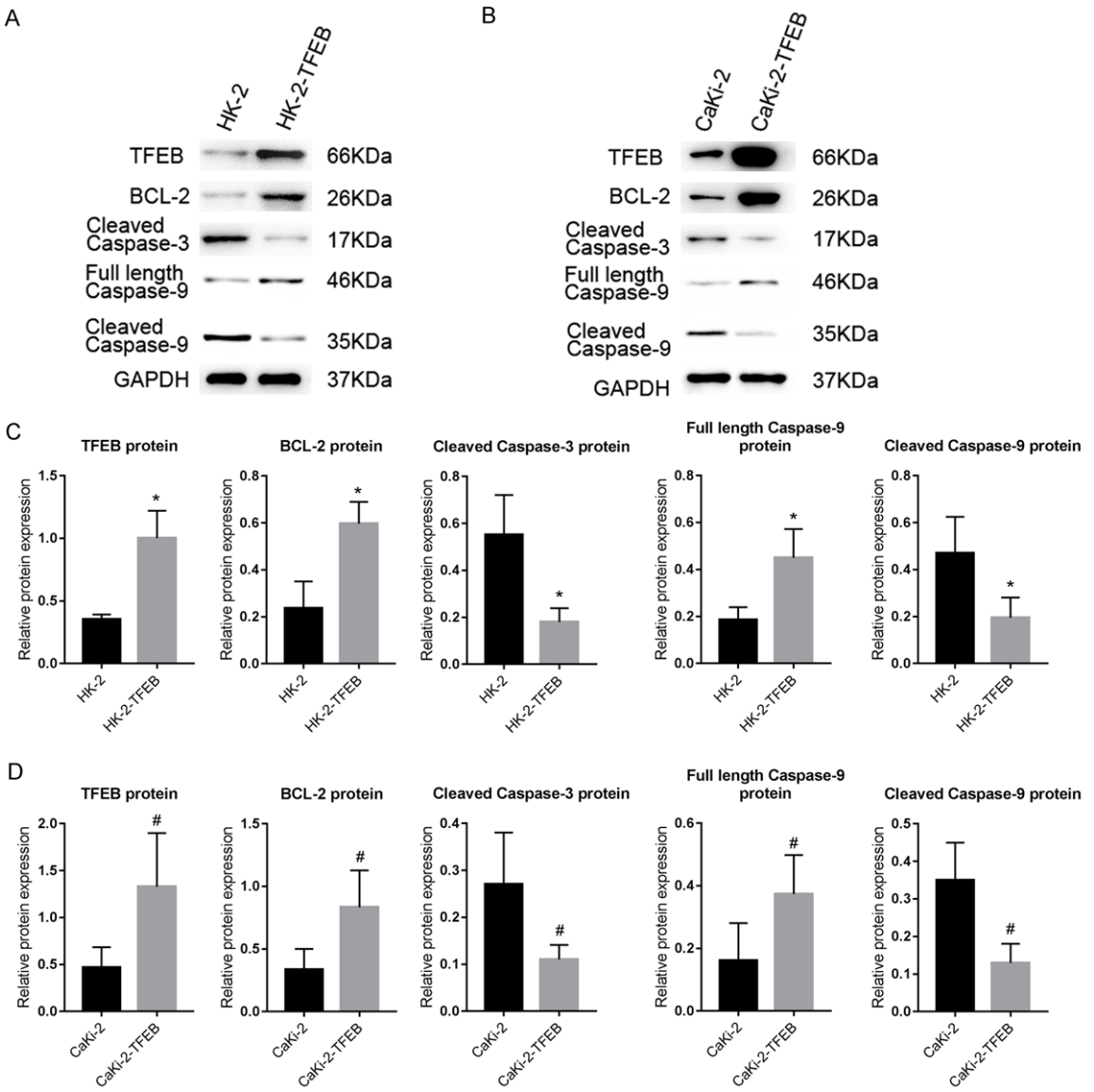


Figure 3. The expressions of the different proteins in the stable transfection of the Alpha-TFEB cells and the untransfected cells. The relative expression levels of the TFEB proteins and the BCL-2 proteins were both increased while the Cleaved Caspase-9 protein and the Cleaved Caspase-3 protein were both decreased in the HK-2-TFEB group when compared to the HK-2 group (A and C), as well as in the CaKi-2-TFEB group when compared to the CaKi-2 group (B and D) in the western blot experiment. The data are shown as the means ± SD (n=4). * vs. the HK-2 group, $P<0.05$, # vs. the CaKi-2 group, $P<0.05$.

Cleaved Caspase-9 protein (35 kDa) and Cleaved Caspase-3 protein (17 kDa) expression levels were both markedly decreased in the HK-2-TFEB group when compared with the HK-2 group (**Figure 3A** and **3C**), as well as in the CaKi-2-TFEB group when compared with the CaKi-2 group in the Western blot experiment (**Figure 3B** and **3D**). In the double immunofluorescence assay, the average IOD values of the TFEB and BCL-2 proteins were significantly higher in the CaKi-2-TFEB group and in the HK-2-TFEB group than they were in the CaKi-2 group and the HK-2 group, respectively (**Figure 4A-E**).

The overexpression of the TFEB and BCL-2 proteins in the t(6;11) RCC tumor tissues

The hematoxylin and eosin staining indicated that the normal renal tubules consisted of regular flat and cubic epithelial cells (**Figure 5A**), but the cytoplasms of the tumor cells in the t(6;11) RCC tumor tissues were clear or rich in eosinophilic granules, with round nuclei and small nucleoli (**Figure 5B**). In the immunohistochemical assay, the normal renal tubular cells showed negative expressions of TFEB and low expressions of BCL-2 (**Figure 5C** and **5E**), but the

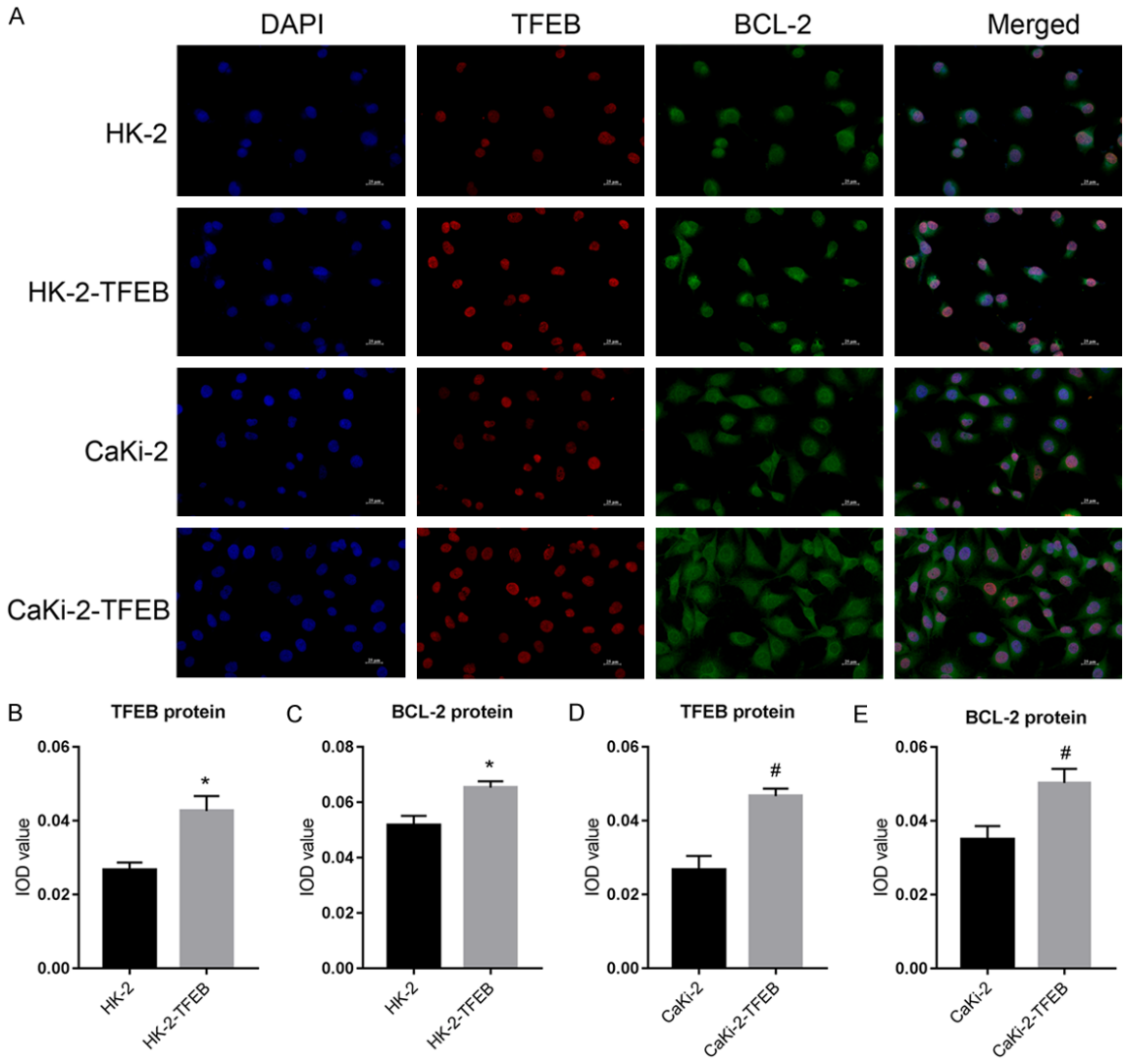


Figure 4. A double immunofluorescence assay showed that the average IOD values of the TFEB and BCL-2 proteins were significantly higher in the CaKi-2-TFEB group and the HK-2-TFEB group than they were in the CaKi-2 group and the HK-2 group, respectively (A-E) (400×). The scale bars represent 25 μm. The data are shown as the mean ± SD (n=3). * vs. the HK-2 group, $P<0.05$, # vs. the CaKi-2 group, $P<0.05$.

tumor cells showed diffuse nuclear positivity of TFEB and cytoplasmic positivity of BCL-2 (**Figure 5D** and **5F**). The average positive rates of the TFEB and BCL-2 proteins were significantly higher in the t(6;11) RCC tumor tissues than they were in the normal renal tubules (**Figure 5G** and **5H**).

The overexpression of TFEB and BCL-2 promoted cell proliferation and inhibited cell apoptosis

The TFEB and BCL-2 expressions were upregulated in the CaKi-2-TFEB cells and the HK-2-TFEB cells with the stable transfection of Alpha-TFEB. The MTT test results showed that the proliferation rate of the HK-2-TFEB cells was higher than the proliferation rate of the HK-2 cells after 48, 72, and 96 h of transfection, and the proliferation rate of the CaKi-2-TFEB cells was higher than the proliferation rate of the CaKi-2 cells (**Figure 6A-C**). These effects were time-dependent. The flow cytometry revealed that the apoptotic ratio of the HK-2-TFEB cells was more decreased than the apoptotic ratio of the HK-2 cells after 12, 24, and 48 h of transfection, and the apoptotic rate of the CaKi-2-TFEB cells was lower than the apoptotic rate of

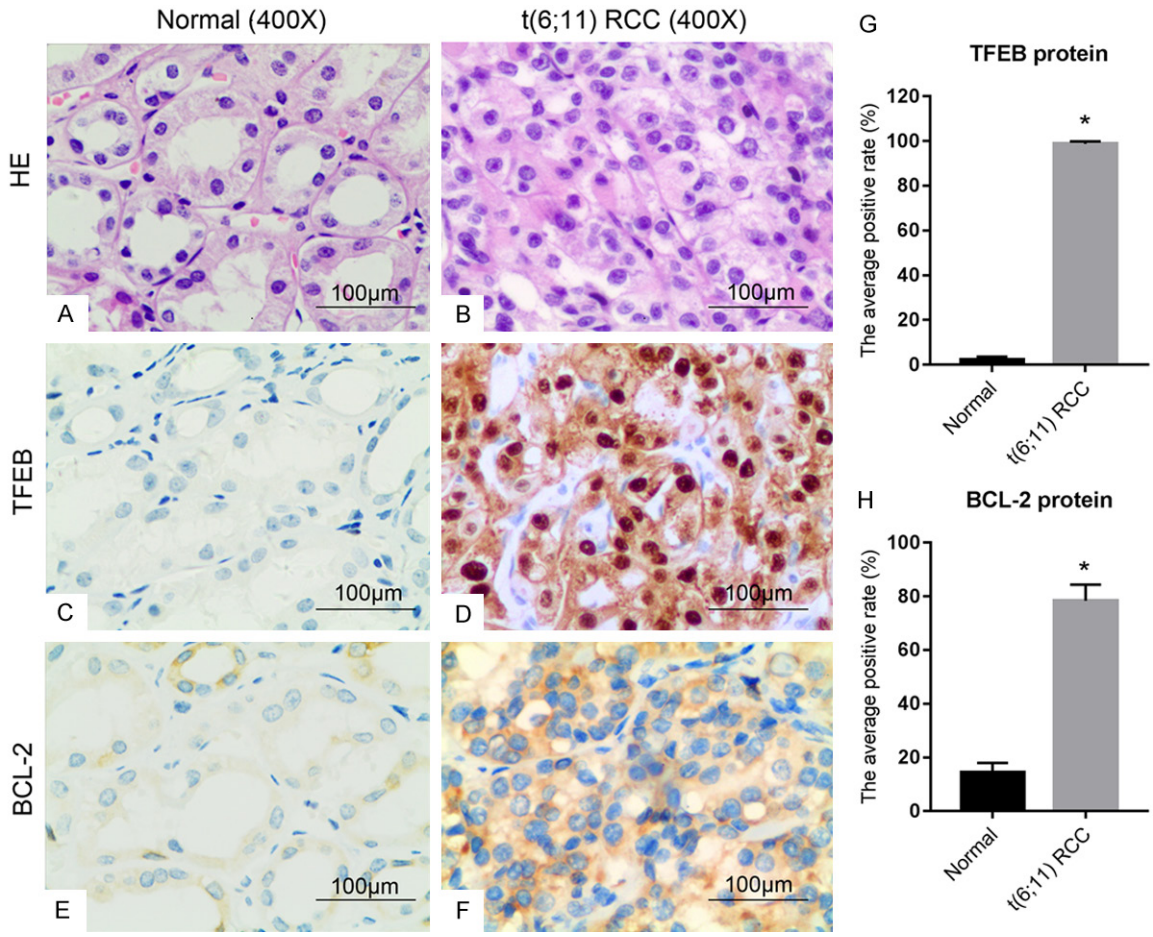


Figure 5. The immunohistochemical results of the TFEB and BCL-2 protein expressions between the t(6;11) RCC tumor tissues and the normal renal tubules. Hematoxylin and eosin staining of the normal renal tubules (A) and t(6;11) RCC tumor tissues (B) (400×). Negative expression of TFEB (C) and a low expression of BCL-2 (E) in the normal renal tubules (400×). Strong nuclear expressions of TFEB (D) and the cytoplasmic staining of BCL-2 (F) in the t(6;11) RCC tumor cells (400×). The scale bars represent 100 μm. The average positive rates of the TFEB and BCL-2 proteins were significantly higher in t(6;11) RCC tumor tissues than they were in the normal renal tubules (G and H). The data are shown as the means ± SD (n=5). * vs. the normal renal tubules, $P<0.05$.

the CaKi-2 cells after 24 and 48 h of transfection (**Figure 6D-F**).

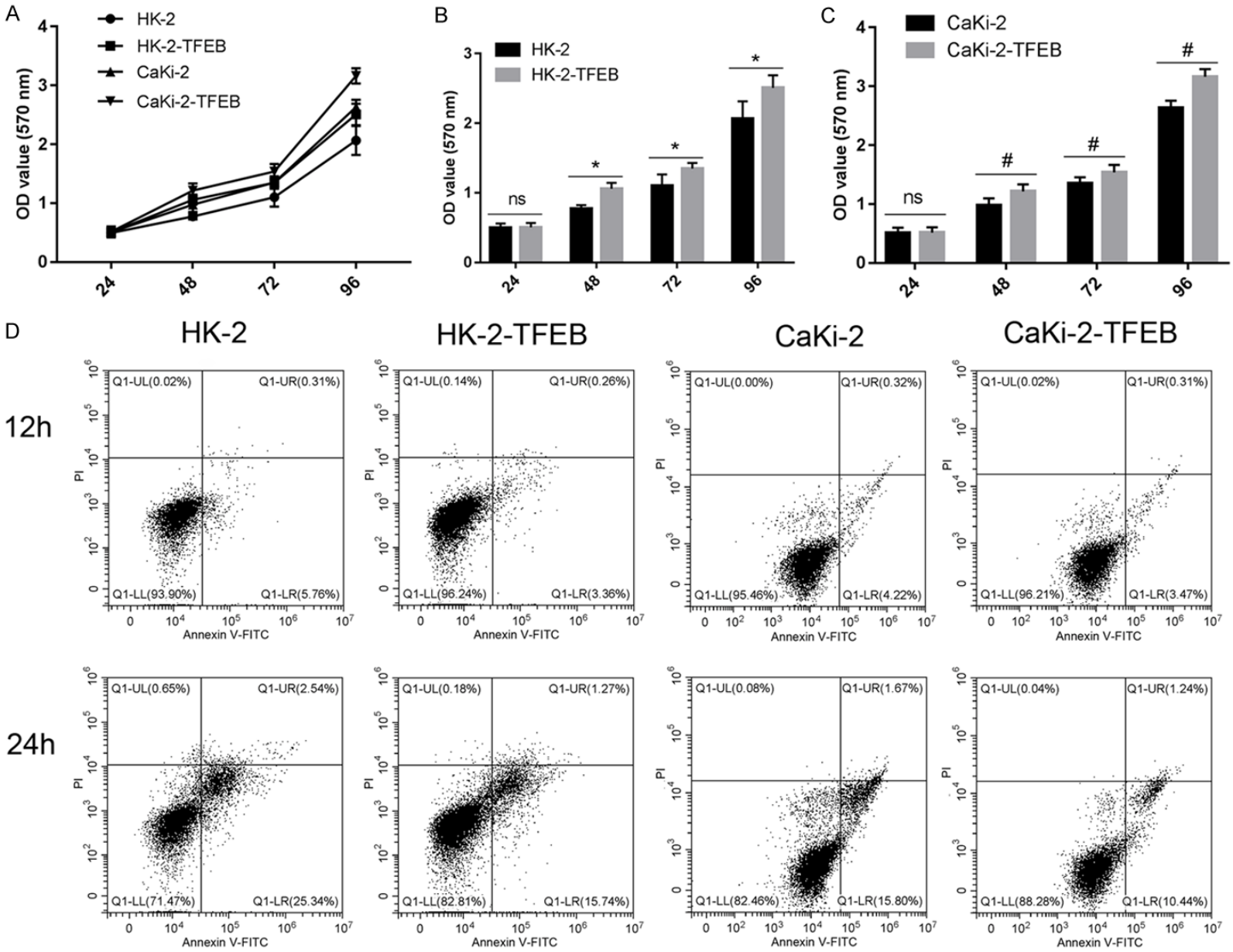
Overexpression of the TFEB upregulated BCL-2 promoter activity

The overexpressions of TFEB and BCL-2 were demonstrated in the stably transfected CaKi-2-TFEB and HK-2-TFEB cells. In order to judge whether the BCL-2 transcriptional activity is regulated by the expression of TFEB, the BCL-2 reporter plasmid, containing the BCL-2 promoter region from ATG to -3934, was utilized, and the luciferase reporter assay was used to determine the BCL-2 promoter activity. The results showed that the luciferase activities of the CaKi-2-TFEB cells and the HK-2-TFEB cells were significantly increased when compared with the CaKi-2 cells and the HK-2 cells, respectively (**Figure 6G**).

These results indicate that the overexpression of TFEB increases the BCL-2 expression by upregulating its promoter activity, which promotes cell proliferation and inhibits the apoptosis of RCC cells (**Figure 6H**).

Discussion

Currently, there is no cell line derived from t(6;11) translocation RCC patients [5]. In order to clarify the relationship between TFEB and BCL-2, a eukaryotic expression vector with Alpha-TFEB fusion gene was constructed, then


A
HK-2
HK-2-TFEB
CaKi-2
CaKi-2-TFEB
OD value (570 nm)
B
C
ns
*
#
D
HK-2
HK-2-TFEB
CaKi-2
CaKi-2-TFEB
12h
24h
Q1-UL(0.02%)
Q1-UR(0.31%)
Q1-LL(93.90%)
Q1-LR(5.76%)
Q1-UL(0.14%)
Q1-UR(0.26%)
Q1-LL(96.24%)
Q1-LR(3.36%)
Q1-UL(0.00%)
Q1-UR(0.32%)
Q1-LL(95.46%)
Q1-LR(4.22%)
Q1-UL(0.02%)
Q1-UR(0.31%)
Q1-LL(96.21%)
Q1-LR(3.47%)
Q1-UL(0.65%)
Q1-UR(2.54%)
Q1-LL(71.47%)
Q1-LR(25.34%)
Q1-UL(0.18%)
Q1-UR(1.27%)
Q1-LL(82.81%)
Q1-LR(15.74%)
Q1-UL(0.08%)
Q1-UR(1.67%)
Q1-LL(82.46%)
Q1-LR(15.80%)
Q1-UL(0.04%)
Q1-UR(1.24%)
Q1-LL(88.28%)
Q1-LR(10.44%)
PI
Annexin V-FITC

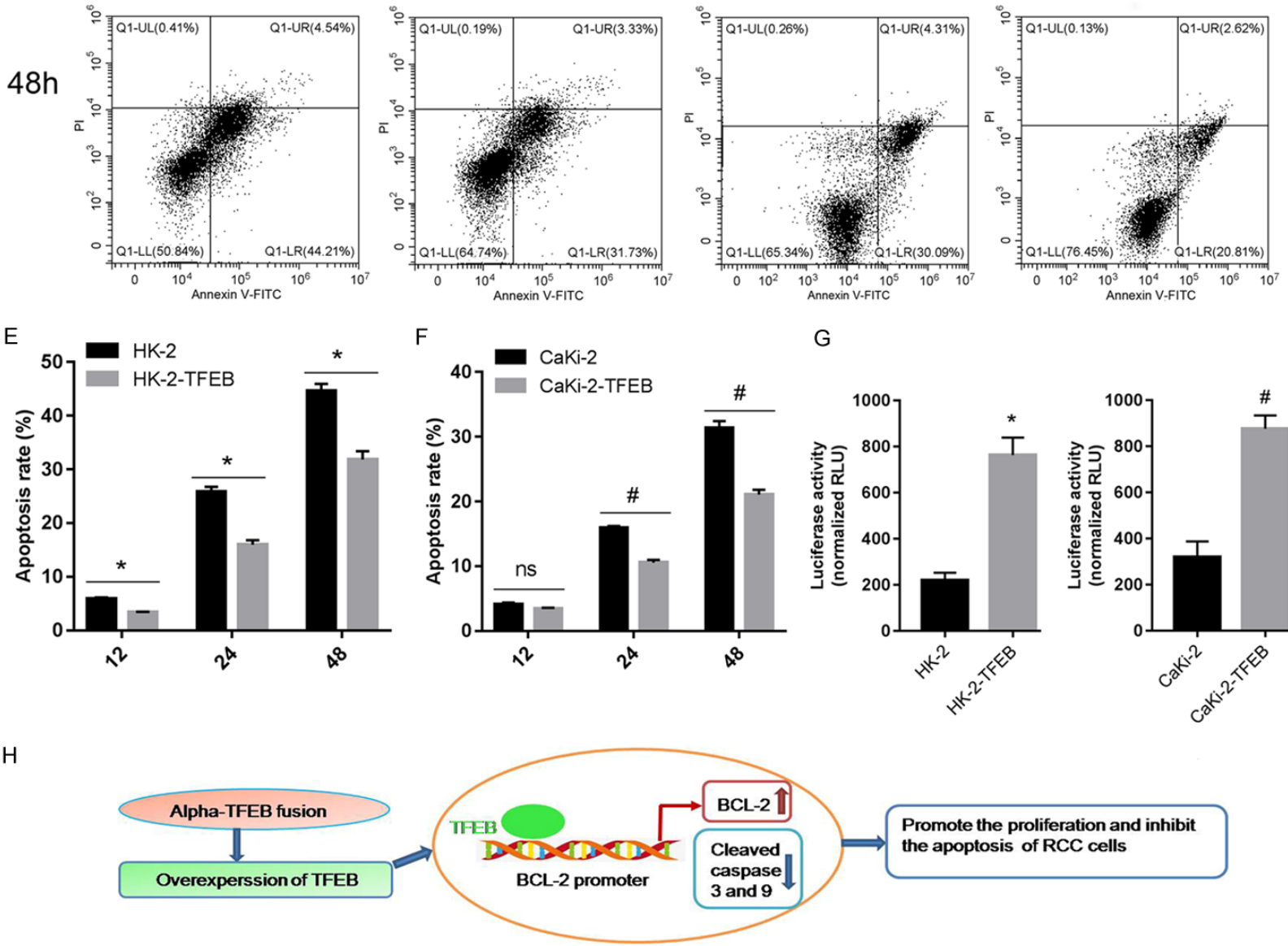

48h
Q1-UL(0.41%)
Q1-UR(4.54%)
Q1-LL(50.84%)
Q1-LR(44.21%)
Q1-UL(0.19%)
Q1-UR(3.33%)
Q1-LL(64.74%)
Q1-LR(31.73%)
Q1-UL(0.26%)
Q1-UR(4.31%)
Q1-LL(65.34%)
Q1-LR(30.09%)
Q1-UL(0.13%)
Q1-UR(2.62%)
Q1-LL(76.45%)
Q1-LR(20.81%)
PI
Annexin V-FITC
E
HK-2
HK-2-TFEB
Apoptosis rate (%)
F
CaKi-2
CaKi-2-TFEB
ns
G
Luciferase activity (normalized RLU)
HK-2
HK-2-TFEB
CaKi-2
CaKi-2-TFEB
H
Alpha-TFEB fusion
Overexperssion of TFEB
TFEB
BCL-2 promoter
BCL-2
Cleaved caspase 3 and 9
Promote the proliferation and inhibit the apoptosis of RCC cells

Figure 6. The overexpressions of TFEB and BCL-2 promoted cell proliferation and inhibited cell apoptosis . Furthermore, the TFEB overexpression upregulated the BCL-2 promoter activity. A-C. The MTT test results showed that the proliferation rates of the CaKi-2-TFEB cells and HK-2-TFEB cells after their transfection for 48, 72, and 96 h were higher than the proliferation rates of the CaKi-2 cells and the HK-2 cells, respectively. The effects were time-dependent. The data are shown as the means ± SD (n=8). * vs. the HK-2 group, $P<0.05$, # vs. the CaKi-2 group, $P<0.05$. ns, not significant. D-F. Flow cytometry revealed that the apoptotic ratio of the HK-2-TFEB cells was lower than the apoptotic ratio of the HK-2 cells after 12, 24, 48 h of transfection, and the apoptotic rates of the CaKi-2-TFEB cells were lower than the apoptotic rates of the CaKi-2 cells after 24, 48 h of transfection. The data are shown as the means ± SD (n=3). * vs. the HK-2 group, $P<0.05$, # vs. the CaKi-2 group, $P<0.05$. ns, not significant. G. The luciferase activities of the CaKi-2-TFEB cells and the HK-2-TFEB cells were significantly increased when compared with the CaKi-2 cells and the HK-2 cells, respectively. The data are shown as the mean ± SD (n=6). * vs. the HK-2 group, $P<0.05$, # vs. the CaKi-2 group, $P<0.05$. H. The schematic diagram shows that the TFEB overexpression increases the BCL-2 expression by upregulating its promoter activity, which promotes the proliferation and inhibits the apoptosis of the RCC cells.

transfected into CaKi-2 and HK-2 cells, and finally the stable transfection cells (the CaKi-2-TFEB cells and the HK-2-TFEB cells) were successfully screened. In this study, the RT-PCR assays and real-time RT-PCR experiments both demonstrated that the TFEB and BCL-2 mRNA were both upregulated in the CaKi-2-TFEB and HK-2-TFEB group when compared with the untransfected CaKi-2 and HK-2 group, respectively. Pecciarini et al. [27] also demonstrated that TFEB mRNA is amplified in t(6;11) RCC tumor tissues, with higher expression levels in the RCC tumor tissues compared to the normal samples.

In the current study, we investigated TFEB and BCL-2 protein expressions not only in stably transfected CaKi-2-TFEB and HK-2-TFEB cells, but also in untransfected CaKi-2 and HK-2 cells using Western blot, immunohistochemistry, and dual immunofluorescence assays. Our Western blot analysis revealed that the relative protein expression levels of TFEB and BCL-2 were both increased but the Cleaved Caspase-9 protein and the Cleaved Caspase-3 protein were both decreased in the CaKi-2-TFEB and HK-2-TFEB group when compared to the CaKi-2 and HK-2 group respectively. The result of the Western blot was similar to what was found in the t(6;11) RCC tumor tissues and the normal samples by Pecciarini et al. [27]. Our imunohistochemistry assay also confirmed that the average positive rates of the TFEB and BCL-2 proteins were significantly higher in the t(6;11) RCC tumor tissues than they were in the normal renal tubules. Similarly, our double immunofluorescence assay showed that the average IOD values of the TFEB and BCL-2 proteins were significantly increased in the CaKi-2-TFEB group and the HK-2-TFEB group compared to the CaKi-2 group and the HK-2 group, respectively. Overexpressions of TFEB and BCL-2 were measured in the stably transfected CaKi-2-TFEB cells and in the HK-2-TFEB cells when compared with the untransfected CaKi-2 cells and the HK-2 cells. We also investigated the cell apoptosis using flow cytometry and the cell proliferation using MTT assays in these cells, which demonstrated that the proliferation rates were higher but the apoptotic ratios were lower in the CaKi-2-TFEB group and the HK-2-TFEB group when compared with the CaKi-2 group and the HK-2 group. Moreover, the Cleaved Caspase-9 protein and Cleaved Caspase-3 protein expressions showed an opposite trend to that of BCL-2 in the stable transfection cells and the untransfected cells. Almarzoug et al. [31] also indicated that the expression of Caspase-3 was an opposite trend to that of BCL-2 in the human normal and cancer liver cells. The above results showed that the TFEB and BCL-2 overexpressions enhanced the cell proliferation and reduced the cell apoptosis.

In order to judge whether the BCL-2 transcriptional activity is regulated by the expression of TFEB, luciferase reporter assays were used to determine the BCL-2 promoter activity. Our results indicated that the luciferase activities were markedly high in the stably transfected CaKi-2-TFEB cells and in the HK-2-TFEB cells, but they were very low in the untransfected CaKi-2 cells and the HK-2 cells. Based on the all above-mentioned results, we speculate that the overexpression of TFEB increases the BCL-2 expression by upregulating its promoter activity, which promotes cell proliferation and inhibits cell apoptosis and ultimately results in the development of t(6;11) translocation RCC. Venetoclax is an oral biologically active BCL-2 inhibitor that is highly effective for patients with chronic lymphocytic leukemia [32, 33].

BM-1197 is a novel BCL-2 inhibitor which exerts strong anti-tumor effects in the treatment of malignant lymphomas [34]. Whether BCL-2 inhibitors are potential therapeutic targets for patients with t(6;11) translocation RCC deserves further study.

Limitations: The current study focused on the relationship between TFEB and BCL-2 in the Alpha-TFEB stably transfected cell lines and in t(6;11) RCC tumor tissue. BCL-2 inhibitors are effective in the treatment of some malignant tumors. However, we did not elucidate the effects of BCL-2 inhibitors on t(6;11) translocation RCC. Next, we need to clarify whether BCL-2 inhibitors have high application prospects for the treatment of t(6;11) translocation RCC patients.

Conclusion

Our results confirmed that the stable transfection of Alpha-TFEB into CaKi-2 cells and HK-2 cells results in the overexpression of TFEB and BCL-2 at the transcriptional and translational levels, and inhibits cell apoptosis and promotes cell proliferation. Moreover, TFEB overexpression upregulates the promoter activity of BCL-2. The above data suggest that TFEB overexpression promotes BCL-2 expression by upregulating its promoter activity and ultimately results in the development of t(6;11) translocation RCC. BCL-2 inhibitors may serve as potential therapeutic targets for t(6;11) translocation RCC patients in the future.

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Disclosure of conflict of interest

None.

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