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Effects of neuroblastoma breakpoint family member 1 (NBPF1) gene on Akt-p53-Cyclin D pathway and growth of cutaneous squamous carcinoma cells

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Neuroblastoma breakpoint family member 1 (NBPF1) is involved in the occurrence and development of tumors. However, only a limited number of studies were conducted on NBPF1 and cutaneous squamous cell carcinoma (SCC). This study mainly explored the expression and mechanism of NBPF1 in SCC. SCC tissue and adjacent tissues samples were randomly selected. NBPF1 gene was overexpressed in A431 cell line using plasmid transfection technique. Cell viability was tested by cell counting kit-8 (CCK-8) assay. Flow cytometry was used to determine cell cycle and apoptosis. Western blot and RT-qPCR were performed to determine the expression levels of proteins and mRNAs. The NBPF1 gene was lowly expressed in SCC tissues. The expression level of NBPF1 gene was the lowest in A431 cell line. The cell viability of A431 was reduced after transfection. Overexpression of NBPF1 not only arrested A431 cells in G1 phase and promoted apoptosis, but also upregulated the expressions of Bax and p53 mRNA and protein, while the expressions of Bcl-2, Survivin and Cyclin D1 were downregulated. Akt-p53-Cyclin pathway was inhibited when NBPF1 gene expression was upregulated. Upregulation of NBPF1 might promote apoptosis of A431 cells and block cell cycle via inhibiting the activation of Akt-p53-Cyclin signaling pathway.

Key words: squamous cell carcinoma, neuroblastoma breakpoint family member 1, apoptosis, cell cycle

Incidence of skin cancer is increasing annually as living environment deteriorates [1]. On the basis of risk rank from low to high, there are three main types of skin cancer: basal cell carcinoma, squamous cell carcinoma and melanoma [2]. Cutaneous squamous cell carcinoma (SCC) originates from deterioration of keratinocytes in epithelial tissues and is characterized by a rapid development and an easy spread [3, 4].

At present, the method for treating SCC is mainly surgical treatment [5]. For SCC patients with a low risk of metastasis, especially for primary SCC patients with a diameter smaller than 1 cm and a clear boundary, surgical resection can achieve better results [5]. However, for SCC patients with a high recurrence and metastatic risk, about 10% of patients who received a standardized topical treatment showed no obvious effect. For patients in middle-late stage SCC, radiotherapy and photodynamic therapy could provide certain effects. However, systemic chemotherapy is still a main approach for treating metastatic SCC. Noticeably, such an approach is highly toxic and accompanied with side effects [6–8]. Therefore, it is important to study the pathogenesis of SCC and find new therapeutic targets for safer treatment.

Studies have suggested that the occurrence of SCC may be related to ultraviolet radiation, chemical factors and trauma. However, the pathogenesis of SCC remains partially known. Neuroblastoma breakpoint family member 1 (NBPF1) is located on chromosome 1p36 where many tumor suppressor genes have been found [9, 10]. NBPF1 was first discovered in neuroblastoma (NB) and it was found to be involved in the occurrence and development of NB [9]. In recent years, studies have found that NBPF1 is involved in the occurrence of multiple tumors. It has been found that NBPF1 is significantly downregulated in neuroblastoma cell lines [11], and that NBPF1 has growth inhibitory effects on cervical cancer cells [12]. However, relation between NBPF1 and SCC is not currently identified. This article explored the role of NBPF1 gene in SCC by detecting the expression characteristics of NBPF1 gene in SCC tissues and the effect of NBPF1 overexpression on SCC cells. Our findings will provide new targets for the discovery of SCC drugs.

Materials and methods

SCC samples. A total of 52 SCC samples and adjacent tissues (34 males and 18 females, aged between 48 and 80 years old, an average age of 57.84±5.75 years old) were collected from Jiangyin Hospital of Traditional Chinese Medicine from September 2016 to September 2017. RT-qPCR was performed to determine NBPFl mRNA levels in each SCC tissue sample. 4 cases were randomly selected to detect NBPFl protein expressions in SCC tissues and adjacent tissues by performing western blot. All patients signed informed consent. The experiment has been approved by the Ethics Committee of Jiangyin Hospital of Traditional Chinese Medicine.

Cells culture. Human immortalized keratinocytes cell HaCaT was from Thermofisher Scientific. Human skin squamous cell carcinoma A431 was purchased from ATCC (USA). Human skin squamous cell carcinoma HSC-5 was obtained from Japanese Collection of Research Bioresources Cell Bank. Human skin sarcoma cells HS1-CLS was purchased from Cell Lines Service. Cells were cultured in DMEM medium containing 10% fetal bovine serum at 37 °C in an incubator with 5% CO_2. Culture-related reagents were purchased from GIBCO Invitrogen (USA).

Construction and transfection of NBPF1 expression plasmid. Wild-type NBPF1 coding sequence was sub-cloned into pcDNA3.1 (Sangon Biotech, China) in order to construct a pcDNA3.1-NBPF1 expression vector. NBPF1 transfections were performed using Lipofectamine 2000 (Invitrogen, USA) and treated as NBPF1 group. The empty pcDNA3.1 plasmid was used as the empty vector group. The protein expression of NBPF1 was determined by western blot after transfection. The following experiments were performed at 24 h after transfection.

Cell counting kit-8 (CCK-8) assay. CCK-8 assay was performed to test cell viability at 12 h, 24 h and 48 h after transfection. Kits were purchased from Tongren (Japan). The cells were digested using trypsin and then diluted to a concentration of 3×10^4 cells/ml. Next, 100 µl transfected cells were inoculated into 96-well plate (3×10^3 cells/well) and pre-incubated in an incubator. CCK-8 reagent was then added and cultured for 4 h. Absorbance at 450 nm were measured using a microplate reader (ELX 800, Bio-Teck, USA) and cell viability was calculated.

Flow cytometry. Flow cytometry was used to detect apoptosis and cell cycle. Kits were purchased from BD Pharmingen (USA). The cell apoptosis of A431 cells was determined by Annexin-V-FITC stain. 1×10^6 cells were washed with PBS at 4 °C and then resuspended to a concentration of 4×10^5 cells/ml. Five µl Annexin-V-FITC was added into 200 µl cell solution and 5 µl propidium iodide was then added. The samples were incubated at room temperature in the dark for 15 min. After incubation, 300 µl binding buffer was added and the sample was stored on ice. The cell cycle of A431 cells was tested by PI/RNase staining. The cells were fixed by pre-cold with 70% ethanol at 4 °C overnight. The cells were washed with 1 ml pre-cold PBS, resuspended and then PI/RNase was added (BD Pharmingen) for staining at room temperature in the dark for 30 min. Flow cytometry (Becton Dickerson, San Jose, CA, USA) was performed to determine cell apoptosis and cell cycle.

RT-qPCR. RT-qPCR was applied to test mRNA expression levels of NBPF1, Bax, Bcl-2, Survivin, p53 and Cyclin D1. The cells were triturated and lysed using Trizol (TaKaRa, Japan) at 0 °C for 5 min. RNAs were extracted by $CHCl_3$ (Aladdin, China) and dissolved in DEPC water (Sigma aliquots). RNA concentration was measured by UV spectrophotometer (NanoDrop One Microvolume UV-Vis spectrophotometer, Thermofisher, USA). Reverse transcription assays were performed on RNA samples using reverse transcription kits (TaKaRa, Japan) to synthesize cDNA. Reverse transcription reaction condition was set at 37 °C for 15 min, while reverse transcriptase inactivation condition was set at 85 °C for 15 s. RT-qPCR experiments were performed with SYBR Prellix Ex TaqTM Real-Time PCR Kit (TaKaRa, Japan). PCR was performed by activating DNA polymerase at 95 °C for 5 min, followed by 40 cycles of two-step PCR (at 95 °C for 10 s and at 60 °C for 30 s) and a final extension at 75 °C for 10 min and then held at 4 °C. DNase and RNase-free water were used as the templates of negative control experiences. All primers were obtained from Genewiz (Suzhou, Jiangsu China) and listed in Table 1. The formula $2^{-\Delta\Delta CT}$ was applied to analyze the mRNA expression.

Western blot. NBPF1, Bax, Bcl-2, Survivin, p53, Cyclin D1, Akt and p-Akt proteins were detected by western blot. Cells were lysed with NP40 lysis buffer (Beyotime, China). A standard curve was drawn using the BCA protein assay kit (Thermo Fisher) to determine the protein concentration. The proteins were separated by 10% SDS-PAGE gel and then transferred onto PVDF membranes (Bio-Rad, USA), which were blocked with 5% non-fat milk at room temperature for

Table 1. The sequences of primers.

Primer name	Sequence (5'-3')	Product size (bp)
NBPF1-Forward	GCGAGGCTGCCCGAGCTTCT	286
NBPF1-Reverse	GACTTCGCGTAACTTCCCATTCA	
Bax-Forward	TCCACCAAGAAGCTGAGCGAG	345
Bax-Reverse	TTCTTTGAGTTCGGTGGGGTC	
Bcl-2-Forward	CTGGTGGACAACATCGC	317
Bcl-2-Reverse	GGAGAAATCAAACAGAGGC	
Survivin-Forward	CCCTTTCTCAAGGACCACCGCATC	133
Survivin -Reverse	GCCAAGTCTGGCTCGTTCTCAGT	
p53-Forward	CTGAGGTCGGCTCCGACTATACCACTATCC	360
p53-Reverse	CTGATTCAGCTCTCGGAACATCTCGAAGCG	
Cyclin D1-Forward	CTGGCCATGAACTACCTGGA	245
Cyclin D1-Reverse	GTCACACTTGATCACTCTGG	
β-actin-Forward	GCTGCGTGTGGCCCCTGAG	252
β-actin-Reverse	ACGCAGGATGGCATGAGGGA	

2 h. Primary antibodies (anti-NBPF1, Abcam, ab 196803, dilution: 1:800; anti-Bax, Abcam, ab32503, dilution: 1:700; anti-Bcl-2, Abcam, ab32124, dilution: 1:600; anti-Survivin, Abcam, ab76424, dilution: 1:700; anti-p53, Abcam, ab26, dilution: 1:600; anti-Cyclin D1, Abcam, ab134175, dilution: 1:800; anti-Akt, Abcam, ab8805, dilution: 1:800; anti-p-Akt, Abcam, ab38449, dilution: 1:800) were incubated with the membranes at 4 °C overnight. Next, secondary antibodies (goat anti-mouse IgG, Abcam, ab6785, 1:8000; rabbit anti-mouse IgG, Abcam, ab99697, dilution: 1:9000; mouse anti-rabbit IgG, Invitrogen, BA1034, 1:7000; donkey anti-rabbit IgG, R&D, NL004, 1:5000; Rabbit Anti-Human IgG, Abcam, ab6759, dilution:1:10000, mouse anti-human, Abcam, ab1927, 1:8000) were incubated with the membranes at room temperature for 1.5 h. Chemiluminescence detection was carried out using enhanced chemiluminescent (ECL) reagents (EMD Millipore). The density of the blots was measured using Quantity One Analysis software version 4.6.2 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data were presented as mean±standard deviation (SD). SPSS 20 (SPSS, Inc., Chicago, IL, USA) was used to analyze data. One-way analysis of variance (ANOVA) following Turkey's multiple comparison was carried out to compare differences among experimental groups. The statistical significance was determined as $p<0.05$.

Results

NBPF1 expression in SCC tissues. RT-qPCR and western blot were performed to test the expression levels of NBPF1 in SCC tissues and adjacent tissues. We found that approximately 58% (30/52) NBPF1 mRNA in SCC tissues were lower than those in adjacent tissues (Figure 1A), and that SCC protein in SCC tissues were also significantly lower than that in adjacent tissues ($p<0.05$) (Figures 1B, 1C). This showed that NBPF1 gene was lowly expressed in SCC.

NBPF1 expression in different cells. RT-qPCR and western blot were performed to detect the expression levels of

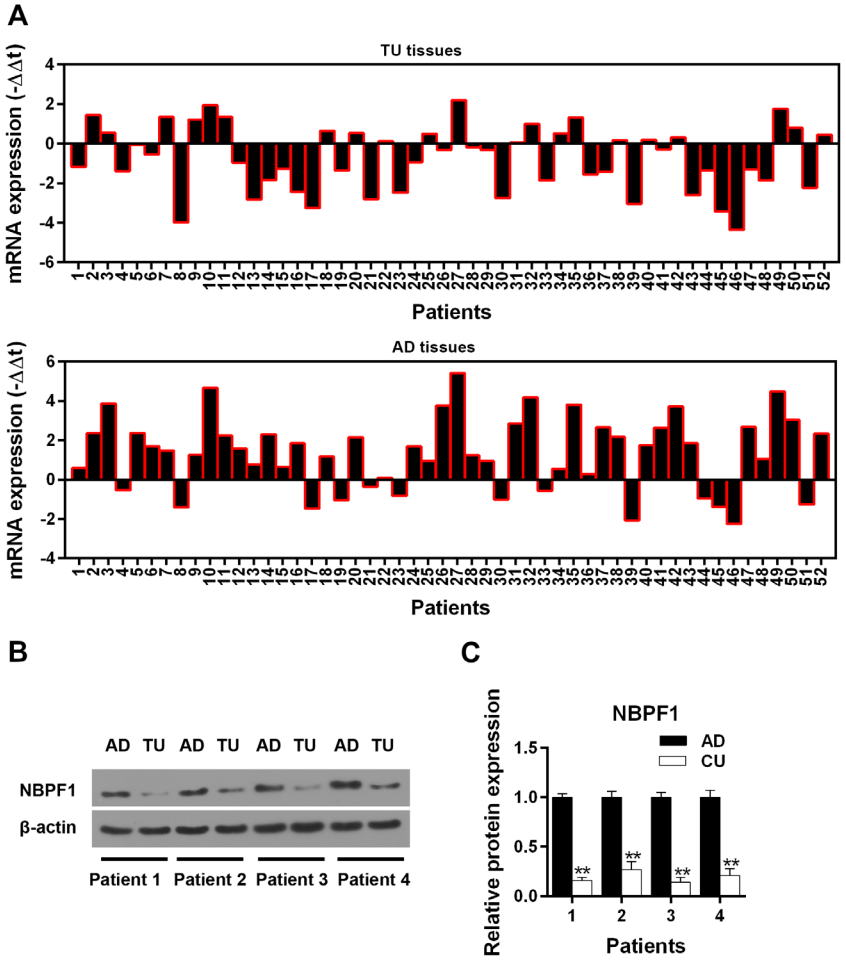

Figure 1. The expression of NBPF1 in CSCC tissues. A) The NBPF1 mRNA in CSCC tissues and adjacent tissues of 52 patients were detected by RT-qPCR. B, C) The NBPF1 protein in CSCC tissues and adjacent tissues of 4 patients were tested using western blot. *p<0.05, **p<0.01, versus AD. Adjacent tissues – AD; Tumor tissues – TU.

NBPF1 in HaCaT, A431, HSC-5 and HS1-CLS cells. NBPF1 mRNA and protein levels in A431, HSC-5 and HS1-CLS cells were significantly lower than those in HaCaT cells. Noticeably, its expression in A431 cells was the lowest (Figures 2A, 2B, 2C). Therefore, the subsequent cell experiments were performed using A431 cells.

NBPF1 expression after transfection. The protein expression of NBPF1 was determined by western blot after plasmid transfection in control group, empty vector group and NBPF1 group. The expression levels of NBPF1 protein in NBPF1 group were higher than those in the other two groups ($p<0.01$) (Figure 3). This showed that overexpression

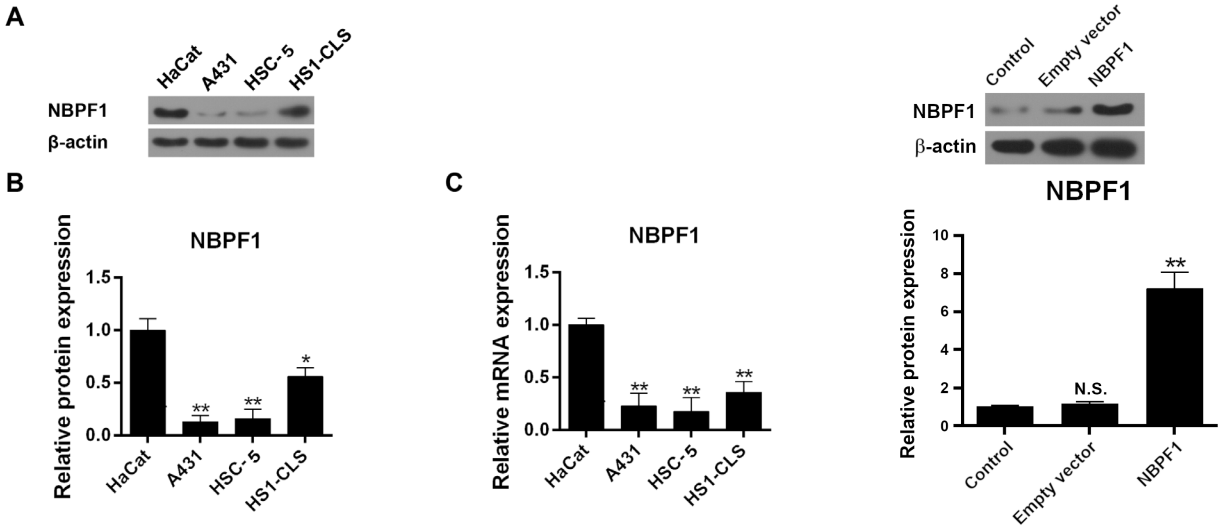

Figure 2. The expression of NBPF1 in different cells. The NBPF1 protein (A, B) and mRNA (C) in A431, HSC-5 and HS1-CLS cells and human immortalized keratinocytes cell HaCaT were examined by western blot and RT-qPCR, respectively. *p<0.05, **p<0.01, versus HaCaT. Three replicates were performed independently.

Figure 3. Effects of overexpression plasmid transfection on NBPF1 expression. Western blot was applied to test the NBPF1 protein levels in control group, empty vector group and NBPF1 group. *p<0.05, **p<0.01, versus empty vector group. N.S. showed no significant difference from the control group. Three replicates were performed independently.

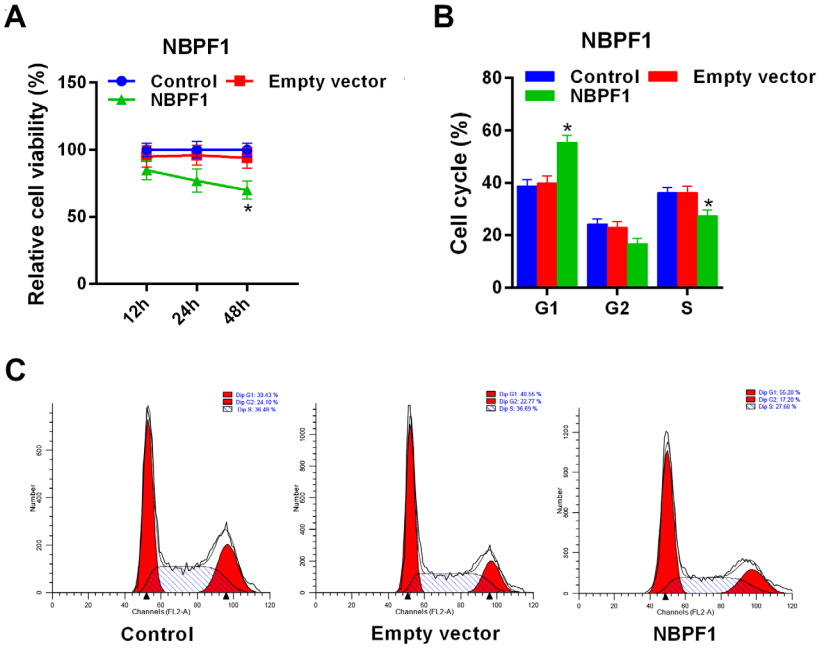

Figure 4. Effects of NBPF1 overexpression on cell viability and cell cycle. A) CCK-8 assay was performed to determine cell viability of A431 cells after 12, 24 and 48 hours of plasmid transfection. B) Flow cytometry assay was applied to detect the cell cycle after transfection. C) Representative cell cycle histograms. *p<0.05, **p<0.01, versus empty vector group. Three replicates were performed independently.

plasmid transfection upregulated the expression of NBPF1 in A431 cells.

Effects of NBPF1 overexpression on cell viability and cell cycle. To investigate the effects of NBPF1 overexpression on A431 cells, cell viability and cell cycle were examined 24 h after transfection by carrying out CCK-8 assay and flow cytometry, respectively. The results showed that the cell viability of A431 cells was reduced 48 h after transfection ($p<0.05$) (Figure 4A). The experimental results also showed that the proportion of cells in the NBPF1 group in G1 phase was higher than those in the empty group and control group, while the proportion of cells in S phase was decreased ($p<0.05$) (Figure 4B–C), indicating that overexpression of NBPF1 could reduce cell viability and arrest A431 cells in G1 phase.

Effects of NBPF1 overexpression on apoptosis. Apoptosis was detected by flow cytometry 24 h after transfection to help investigate why NBPF1 overexpression reduced cell viability. The results showed that the apoptosis rate in the NBPF1 group was higher than those in the empty group and control group ($p<0.01$) (Figures 5A, 5B). To explore the effects of NBPF1 overexpression on the apoptosis of A431 cells, RT-qPCR and western blot were performed to detect the expressions of apoptosis-related proteins and mRNA in three groups. The results showed that Bax protein and mRNA levels of NBPF1 group were significantly upregu-

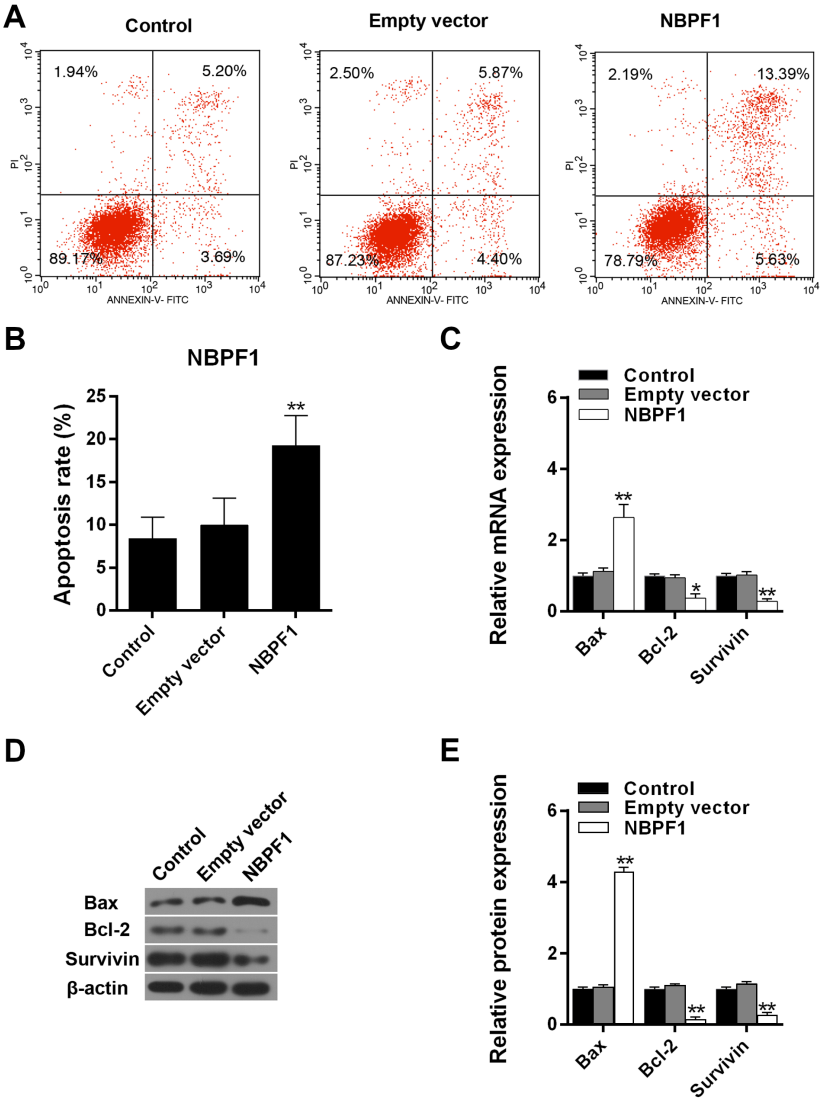

Figure 5. Effects of NBPF1 overexpression on cell viability and cell cycle. A) Representative dot plots and B) apoptosis rates in control group, empty vector group and NBPF1 group were detected using flow cytometry. The apoptosis-related genes Bax, Bcl-2 and Survivin mRNAs (C) and proteins (D, E) in three groups were detected using RT-qPCR and western blot, respectively. *p<0.05, **p<0.01, versus empty vector group. Three replicates were performed independently.

lated, while the levels of Bcl-2 and Survivin were downregulated (Figures 5C–5E). This showed that NBPF1 overexpression could not only induce apoptosis of A431 cells, but also promote the expression of apoptotic protein and inhibit the expression of anti-apoptotic protein.

Effects of NBPF1 overexpression on Akt-p53-Cyclin pathway. The expressions of Akt-p53-Cyclin pathway-related genes were examined in order to study the mechanism by which NBPF1 gene affected A431 cell cycle and apoptosis. The results showed that overexpression of NBPF1 promoted protein and mRNA expressions of p53 while downregulated protein and mRNA expressions of Cyclin D1 (Figures 6A–6C). In addition, p-Akt levels were also significantly downregulated by NBPF1 overexpression (Figures 6D, 6E). This showed that overexpression of NBPF1 activated Akt-p53 pathway and inhibited Cyclin D1 expression.

Effects of NBPF1 knockdown on viability of A431 cells and p-Akt/Akt expression. To validate the role of NBPF1 in A431 cells, we detected the effects of NBPF1 knockdown on cell viability of A431 by CCK-8 assay. Our data revealed that under the effect of NBPF1 knockdown, the cell viability was promoted (Figure 7A) and the expression of p-Akt was upregulated (Figure 7B).

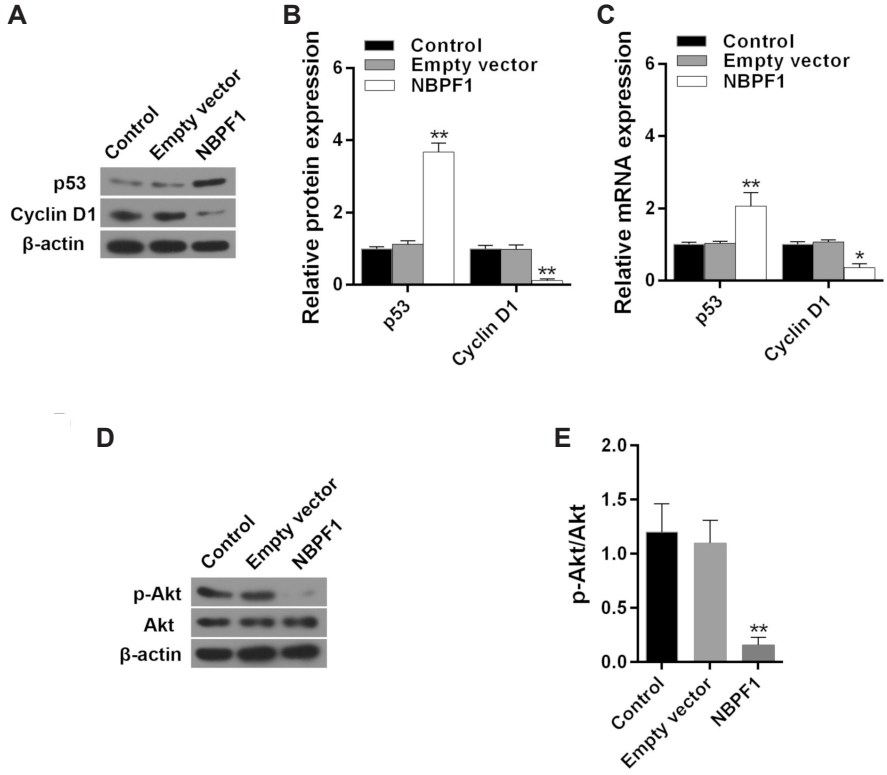

Figure 6. Effects of NBPF1 overexpression on Akt-p53-Cyclin pathway. A–E) Western blot and RT-qPCR were carried out to detect Akt, p-Akt, p53 and Cyclin D1 proteins and mRNA expression levels in control group, empty vector group and NBPF1 group, respectively. *p<0.05, **p<0.01, versus empty vector group. Three replicates were performed independently.

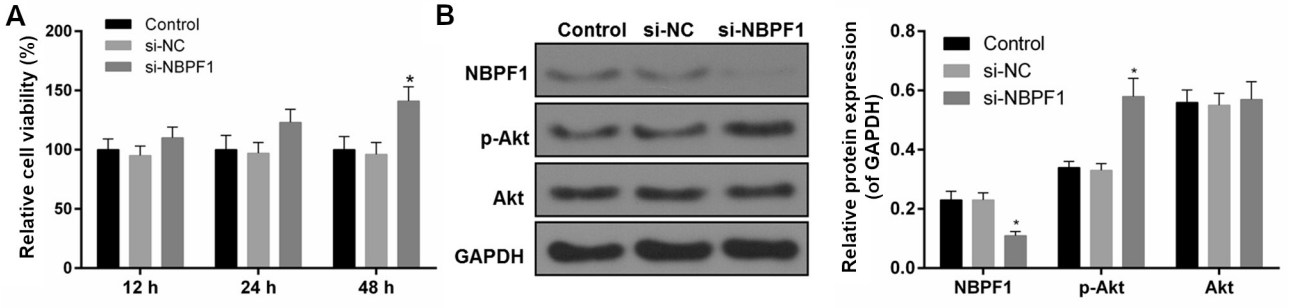

Figure 7. Effects of NBPF1 gene knockdown on cell viability and p-Akt/Akt expression. A) CCK-8 assay was performed to detect cell viability. B) Western blot was performed to detect the protein expression of NBPF1, Akt and p-Akt. *p<0.05 versus si-NC group. Three replicates were performed independently.

Discussion

The NBPF gene is one of minority human gene families and compared with other primates, human NBPF copies are significantly higher [13]. However, a limited number of studies were conducted on the function of the NBPF gene. Previous studies have shown that overexpression of NBPF1 led to a decrease in cell growth by soft agar test and that NBPF1 had a suppressive effect on tumor [14]. As a gene that inhibits cell proliferation, NBPF1 is downregulated in cervical cancer and breast cancer and is involved in the occurrence and development of tumors [11, 12, 15]. Noticeably, to the best of our knowledge, no research has been done on NBPF1 in SCC.

To study the expression characteristics of NBPF1 in SCC tissues, we detected 52 cases of SCC specimens, which had been collected clinically. The results showed that the expression level of NBPF1 mRNA decreased in majority of SCC tissues. 4 cases were randomly selected to examine SCC tissues and adjacent tissues and the result showed that the protein and mRNA expression levels of NBPF1 in SCC tissues were significantly lower than those in the adjacent tissues. This suggested that NBPF1 might have a low expression in SCC. Previous researches also demonstrated that NBPF1 was downregulated in different tumor cell lines [11, 12]. Therefore, we detected the expression levels of NBPF1 gene in three skin cancer cell lines including A431, HSC-5 and HS1-CLS cells. The levels of NBPF1 mRNA and protein were significantly lower than those in the sense of HaCaT cells and they were the lowest in A431 cells. Therefore, A431 cell line was selected to be used in follow-up experiments.

As the expression level of NBPF1 was low in A431 cell line, we investigated the effect of NBPF1 overexpression in A431 cell line. Plasmid transfection technology was applied to upregulate the expression of NBPF1 [16]. We used transient transfection technology to analyze the effect of overexpressed NBPF1 on CSCC cells, which may have some limitations, stable transfection may better explain this result. The results showed that increased expression of NBPF1 led to a significant decrease in cell viability and increased NBPF1 expression also allowed the A431 cell cycle to be organized in the G1 phase. Meanwhile, apoptosis experiments also showed that increased NBPF1 expression level promoted A431 cell apoptosis. To further explore the mechanism by which NBPF1 overexpression affected cell viability and apoptosis, RT-qPCR and western blot were performed to detect apoptosis-related proteins. The results showed that upregulation of NBPF1 expression promoted Bax and inhibited Bcl-2 and Survivin. As a pro-apoptotic protein, Bax can directly regulate apoptosis target protein and promote apoptosis [17, 18]. Bcl-2 is a typical anti-apoptotic protein and Bcl-2 overexpression is common in SCC [19, 20]. Survivin is associated with the immortality of cells, it is tumor-specific and is only expressed in tumors and embryonic tissues [21, 22]. Previous studies have confirmed the existence of abnormal apoptosis in SCC and tumor apoptosis could be affected by many factors [23]. Therefore, we further explored the mechanism regarding the effects of NBPF1 on cell cycle and apoptosis.

As an important factor in cell signaling pathways, Akt regulates a variety of signaling pathways and plays an important role in tumor proliferation, anti-apoptosis and migration [24–26]. Cyclin D1 is one of the most important protein that regulates cell cycle. Moreover, it can bind and activate the unique cyclin-dependent kinase CDK4 during G1 and promote cell cycle progression from G1 to S, therefore promoting cell proliferation [27, 28]. As a tumor suppressor gene, p53 has inhibitory effects on cell proliferation by tissue cycle [29, 30]. Cyclin D1 and p53 are important Akt downstream factors that directly regulate cell cycle [31, 32]. The results of this study showed that after overexpression of NBPF1, the expression of p53 was upregulated, while Cyclin D1 level was downregulated. Overexpression of NBPF1 had no significant effect on Akt protein, however, it could inhibit the phosphorylation of Akt protein. Qin et al. also found that NBPF1 promoted apoptosis of cervical cancer cells via inhibiting PI3K/mTOR pathway [12]. Another study observed that NBPF1 gene had arresting effects on cells in G1 phase [11]. Furthermore, the NBPF1 knockdown promoted the cell growth of A431 and increased the expression of p-Akt, suggesting that NBPF1 could inhibit the activation of Akt-related pathway by inhibiting the phosphorylation of Akt protein, therefore regulating the expression levels of downstream cell cycle-related proteins and apoptosis-related proteins, organizing the cell cycle and promoting apoptosis.

To conclude, NBPF1 was lowly expressed in SCC tissues. Upregulation of NBPF1 could inhibit the activation of Akt-related pathway by inhibiting Akt phosphorylation, therefore promoting apoptosis of A431 cells by regulating cell cycle-related and apoptosis-related proteins and blocking A431 cells in the G1 phase.

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