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NUSAP1 regulates basal cell carcinoma migration, invasion and DNA damage through activation of the Hedgehog signaling pathway

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ABSTRACT

Background: Basal cell carcinoma (BCC) is a prevalent cutaneous cancer with an increasing incidence. Nucleolar and spindle associated protein 1 (NUSAP1) is a cell proliferation-related protein that participates in the development of various cancers. However, its role and mechanism in BCC remain elusive. *Methods:* The expression of NUSAP1 was detected by western blot. Gain- and loss-of-function assays were performed through the transfection of overexpression plasmid of NUSAP1 and si NUSAP1 into TE354.T cells. The role and mechanism of action of NUSAP1 in BCC were explored by cell counting kit-8 (CCK-8), colony formation, transwell, flow cytometry and western blot assays. *Results:* NUSAP1 was highly expressed in TE354.T cells. Overexpression of NUSAP1 enhanced cell viability, colony forming numbers, numbers of migrated and invasive cells and the relative protein expression of RAD51, but reduced the apoptosis rate and the relative protein expression of γH2AX in TE354.T cells. Inverse results were obtained in these indicators after TE354.T cells were downregulated with NUSAP1. Moreover, the relative expression of proteins involved in the Hedgehog signaling pathway was increased by transfection of the overexpression plasmid of NUSAP1 into TE354.T cells, but decreased by the transfection of si NUSAP1 into TE354.T cells. *Conclusion:* Both gain- and loss-of-function results revealed that NUSAP1 promoted proliferation, migration and invasion but attenuated apoptosis and DNA damage in BCC, which was involved in the activation of the Hedgehog signaling pathway.

KEYWORDS

basal cell carcinoma, NUSAP1, migration, invasion, DNA damage, hedgehog

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INTRODUCTION

Basal cell carcinoma (BCC) is a common type of cutaneous cancer with a rising incidence all over the world [1]. It has been shown that BCC accounts for approximately three-quarters of all skin cancers, as well as 80–90% of non-melanoma skin cancer [2–4]. The head and neck are the most vulnerable areas affected by BCC [5]. Multiple factors are involved in the process of BCC, including environmental factors (sunlight exposure, industrial chemical agents and smoking), fair skin, nevi, inflammatory response and genetic factors [1]. BBC generally progresses tardily and rarely metastasizes, but it can result in prominent morbidity through local invasion and tissue destruction [6]. Surgical approach remains the gold standard for BCC therapy, and several topical drugs, such as imiquimod 5% cream and 5-fluorouracil 5% cream have been approved by the US Food and Drug Administration (FDA) [7]. In addition, many novel and emerging topical techniques and agents for the treatment of BCC are summarized in a recent review [7]. On the other hand, advanced BCCs including metastatic BCCs and locally advanced BCCs are rare, but the treatment is still challenging and the prognosis is poor [8]. Therefore, further understanding of the pathogenesis of BCC remains a necessary and urgent topic.

Dysregulated DNA damage repair mechanism is crucial in the development of cancers [9]. In the DNA damage response (DDR) pathway, RAD51, a type of homologous recombination protein exhibits a significant role in the S phase that is associated with fork reboot both following collapse and earlier in fork remodeling and DNA protection [10, 11]. γH2AX generally serves as a marker of DNA damage, during which the phosphorylation of γH2AX indicates the recognition and repair of DNA double-strand breaks (DSBs) [12]. In addition, Hedgehog signaling is a developmentally conserved pathway in various embryonic tissues, which has been demonstrated to be dysregulated in various tumors [13, 14]. In the Hedgehog pathway, the bond of patched receptor PTCH1 and the Hh ligand evokes the release of the smoothened receptor (Smo), which next elicits the release of GliFL from cytoplasmic retention. The released GliFL finally converts into the active form GliA (Gli1 and Gli2), which migrates to the nucleus to induce the activation of the target genes [15].

Nucleolar and spindle associated protein 1 (NUSAP1) is a cell proliferation-related protein with a molecular weight of 55 KDa, which was first discovered in melanoma in 2007 [16]. NUSAP1 is strongly involved in the modulation of chromosome segregation, spindle formation and stability, and cytokinesis [17]. The expression of NUSAP1 peaks in the G2 phase and then declines during cell division [18]. High level of NUSAP1 has been proven to promote progression and resistance in a variety of cancers. For instance, NUSAP1 is highly expressed in gastric cancer, and its silencing inhibits the proliferation, mobility and invasion of gastric cancer [19]. High-level expression of NUSAP1 also strengthens chemotherapeutic resistance in breast cancer [20], glioblastoma [21] and chronic lymphocytic leukemia [22]. Upregulation of NUSAP1 enhances metastasis through the AMPK/PPARγ axis in breast cancer [23]. Moreover, the level of NUSAP1 is closely associated with the prognosis of cancers. It is reported that the downregulation of NUSAP1 after neoadjuvant chemotherapy predicts good survival in patients with breast cancer [24]. Consistently, upregulation of NUSAP1 predicts poor prognosis in hepatocellular carcinoma [25], breast cancer [23, 26], ovarian cancer [27], papillary thyroid carcinoma [28], glioblastoma [29], bladder urothelial carcinoma [30], chronic lymphocytic leukemia [22] and non-small cell lung cancer [31]. However, the role of NUSAP1 in BCC remains uncharted.

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Therefore, the present study was conducted to explore the role of NUSAP1 in BCC migration, invasion and DNA damage through both gain-of-function and loss-of-function assays. Additionally, the related mechanism was also further investigated.

MATERIALS AND METHODS

Cell culture

Human basal cell carcinoma cell line TE354.T (CRL-7762) was obtained from the American Tissue Culture Collection (Manassas, VA, USA). The corresponding control cell line, human immortalized keratinocyte line HaCaT (C6282) [32] was bought from Beyotime (Shanghai, China). Both cells were hatched in DMEM (PM150210, Procell, Wuhan, China) with 10% FBS (164210-50, Procell) and 1% penicillin-streptomycin (PB180120, Procell) at 37 °C with 5% CO_2.

Cell transfection

Small interfering RNA targeting NUSAP1 (si NUSAP1) and the corresponding negative control (si NC) were prepared by GenePharma (Shanghai, China). The sequences of NUSAP1 were inlayed into the pcDNA vector plasmids to overexpress NUSAP1. Then, pcDNA vector plasmids harboring NUSAP1 (NUSAP1), empty pcDNA vector plasmids (NC), si NUSAP1 and si NC were transfected into TE354.T cells with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After transfection for 72 h, cells were yielded for further examination.

Cell counting kit-8 (CCK-8) detection

Following transfection, TE354.T cells were plated into 96-well plates at 40000 cells per well, and then cultured with 5% CO_2 at 37 °C for 24, 48, 72 and 96 h. Subsequently, 10 µl of CCK-8 regents (Dojindo Laboratories, Kumamoto, Japan) were added into every well and further incubated for 2 h. Cell viability was assessed through the optical density (OD) measured by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm.

Colony formation assay

Based on a previous report [33], transfected TE354.T cells with 6×10^5 cells per well were sowed into 6-well plates and incubated for 14 days at 37 °C. The colonies were then immobilized with 4% paraformaldehyde (P1110, Solarbio, Beijing, China) and stained with 0.1% crystal violet (G1063, Solarbio) for half an hour, separately. The clone numbers were captured and counted manually.

Transwell assay

The migration and invasion of TE354.T cells were examined by transwell assay [34]. Transwell plates (353097) were purchased from the Corning Company (New York, NY, USA), and the upper chambers of the transwell plates were pretreated with or without Matrigel matrix (354234, Corning Company). TE354.T cells following transfection were yielded, resuspended in DMEM, and plated into the upper chambers of transwell plates. The lower chambers were filled with

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DMEM with 10% FBS. After being hatched for 24 h at 37 °C with 5% CO_2, cells in the lower chambers were immobilized with 4% paraformaldehyde and stained with 0.1% crystal violet for half an hour. Cells were photographed under an inverted microscope (BX51, Olympus, Tokyo, Japan), and ten random fields were chosen for the assessment of the numbers of migrated or invaded cells.

Flow cytometry

The apoptosis of TE354.T cells was determined by flow cytometry assay based on previous descriptions [35, 36]. Transfected TE354.T cells were seeded into 24-well plates at a density of 2.5×10^5 cells per well and maintained with 5% CO_2 at 37 °C. Then, TE354.T cells were harvested and rinsed with phosphate-buffered saline (PBS) (P1020, Solarbio). Next, cell apoptosis was determined by using the Annexin V-FITC Apoptosis Detection Kit (CA1020, Solarbio) on a FACScan flow cytometer with CellQuest software (BD Biosciences, NJ, USA).

Western blot

Following published protocols [37, 38], TE354.T cells were split with RIPA buffer (R0010, Solarbio) to obtain the total protein, whose concentration was analyzed by the BCA Protein Assay Kit (PC0020, Solarbio). 20 μg protein samples were dissolved and electrically transferred onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). After being blocked with 5% skim milk (D8340, Solarbio) at room temperature for 1 h, the membranes were probed with primary antibodies, including anti-NUSAP1 (1:500, ab137230, Abcam, Cambridge, UK), anti-histone H2AX (γH2AX) (1:1000, ab11175, Abcam), anti-RAD51 (1:5000, ab176458, Abcam), anti-patched1 (PTCH1) (1:1000, ab90438, Abcam), anti-glioma-associated oncogene homolod-1 (GLI1) (1:2000, ab273018, Abcam), anti-hedgehog-interacting protein (HIP1) (1:10000, ab181238, Abcam), anti-histone deacetylase 1 (HDAC1) (1:100, ab19845, Abcam) and anti-GAPDH (1:10000, ab181602, Abcam) overnight at 4 °C. The membranes were then treated with Goat Anti-Rabbit IgG H&L (HRP) (1:20000, Abcam) for 2 h at room temperature and visualized by the ECL Western Blotting Substrate (PE0010, Solarbio). The gray value was determined by QUANTITY ONE software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and the difference was tested by the Student's *t*-test using SPSS 26.0 software (IBM, Armonk, New York, USA). $P < 0.05$ was regarded as significant difference.

RESULTS

Knockdown of NUSAP1 inhibited BCC proliferation

To assess the role of NUSAP1 in BCCs, the expression of NUSAP1 was first studied in human basal cell carcinoma cell line TE354.T. Compared with that in human immortalized keratinocyte line HaCaT, the relative protein expression level of NUSAP1 was observably increased in TE354.T cells (Fig. 1A1-2). Meanwhile, transfection of overexpression plasmid of NUSAP1

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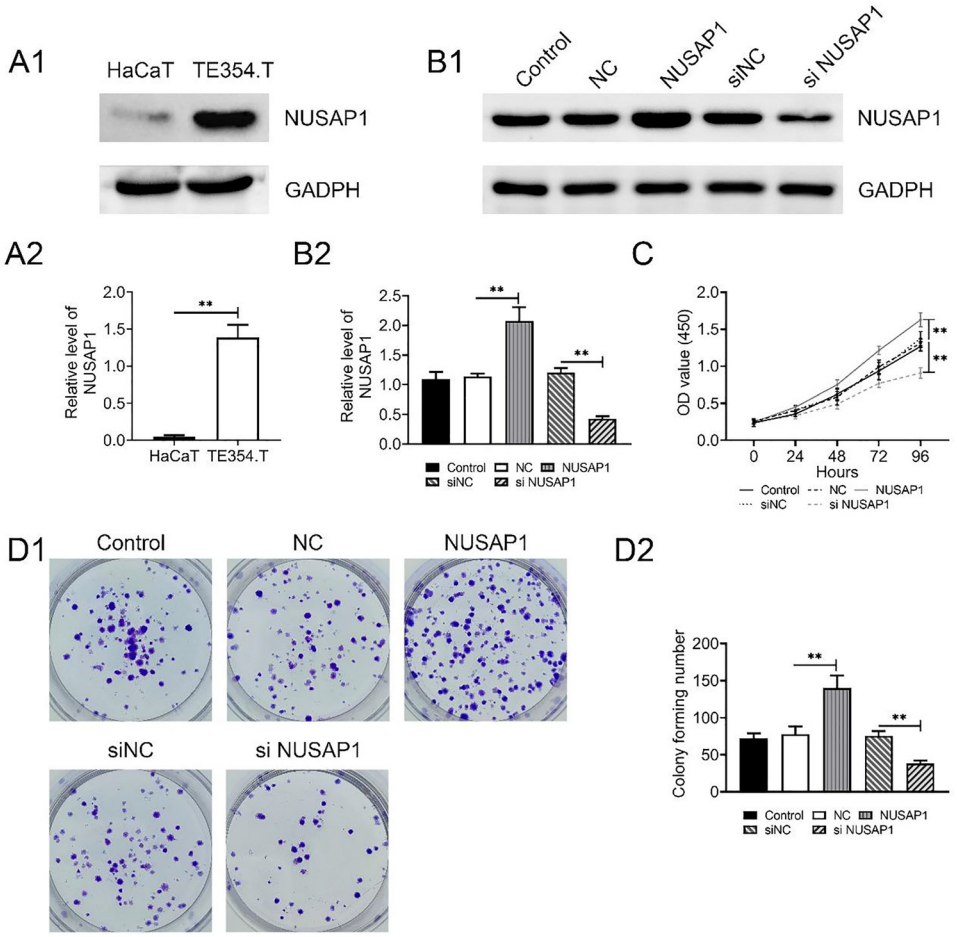

Fig. 1. Downregulation of NUSAP1 repressed BCC proliferation. (A1 and 2) The relative protein expression of NUSAP1 was detected in TE354.T and HaCaT cells. Data were expressed after normalization with GAPDH. (B1 and 2) The relative protein expression of NUSAP1 was examined after TE354.T cells were transfected with overexpression plasmid of NUSAP1, si NUSAP1 and their corresponding NC. Data were expressed after normalization with GAPDH. (C) The cell viability was measured by CCK-8 assays after transfected TE354.T cells were hatched with 5% CO_2 at 37 °C for 24, 48, 72 and 96 h. (D1 and 2) Colonies formed were manually counted after transfected TE354.T cells were incubated for 14 days at 37 °C, fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Data were presented as mean ± SD and the difference was tested by the Student's *t*-test using SPSS 26.0 software. $^{**}P < 0.01$

and si NUSAP1 into TE354.T cells significantly enhanced and reduced the relative protein expression level of NUSAP1, respectively (Fig. 1B1-2). Moreover, overexpression of NUSAP1 prominently elevated cell viability and colony forming number, whereas knockdown of NUSAP1 markedly diminished these two parameters in TE354.T cells (Fig. 1C and D1-2). These results demonstrated that silencing of NUSAP1 suppressed BCC proliferation.

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Interference of NUSAP1 impeded the migration and invasion of BCCs

To investigate the role of NUSAP1 in BCC migration and invasion, transwell assays were conducted in transfected TE354.T cells. As demonstrated in Fig. 2, transfection of overexpression plasmid of NUSAP1 into TE354.T cells prominently increased the migrated and invasive cell numbers, and transfection of si NUSAP1 into TE354.T cells notably decreased the migrated and invasive cell numbers. Thus, downregulation of NUSAP1 restrained the migration and invasion of BCCs.

Downregulation of NUSAP1 induced apoptosis and DNA damage in BCCs

In addition, the role of NUSAP1 in apoptosis and DNA damage was also explored in TE354.T cells. Upregulation of NUSAP1 in TE354.T cells notably reduced the apoptosis rate, whereas interference of NUSAP1 in TE354.T cells observably enhanced the apoptosis rate (Fig. 3A1 and 2). Meanwhile, the relative protein expression of γH2AX was significantly

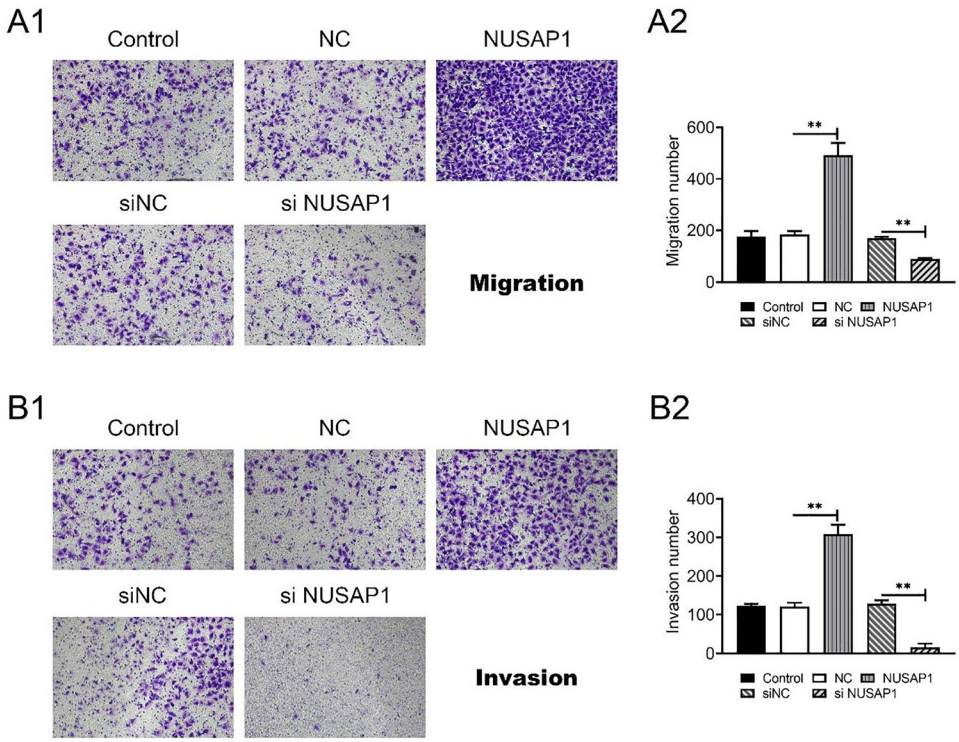

Fig. 2. Silencing of NUSAP1 dampened the migration and invasion of BCCs. (A1 and 2) The numbers of migrated TE354.T cells were determined by transwell assays with Matrigel matrix in the upper chamber. (B1 and 2) The numbers of invasive TE354.T cells were determined by transwell assays without Matrigel matrix in the upper chamber. Data were presented as mean ± SD and the difference was tested by the Student's *t*-test using SPSS 26.0 software. $^{**}P < 0.01$

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decreased with the transfection of overexpression plasmid of NUSAP1 into TE354.T cells, whereas it was markedly elevated by the transfection of si NUSAP1 into TE354.T cells (Fig. 3B1 and 2). Conversely, upregulation of NUSAP1 observably increased the relative protein level of RAD51, and silencing of NUSAP1 prominently diminished the relative protein level of RAD51 (Fig. 3B1 and 2).

NUSAP1 activated the hedgehog signaling pathway in BCCs

Moreover, the underlying mechanism of action of NUSAP1 in TE354.T cells was also addressed through the detection of the expression of proteins involved in the Hedgehog signaling pathway. Results from Fig. 4 showed that transfection of the overexpression plasmid of NUSAP1 into TE354.T cells notably enhanced the relative protein expression of GLI1, PTCH1, HIP1 and HDAC1, and transfection of si NUSAP1 into TE354.T cells observably reduced the relative protein expression of GLI1, PTCH1, HIP1 and HDAC1. Therefore, NUSAP1 activated the Hedgehog signaling pathway in BCCs.

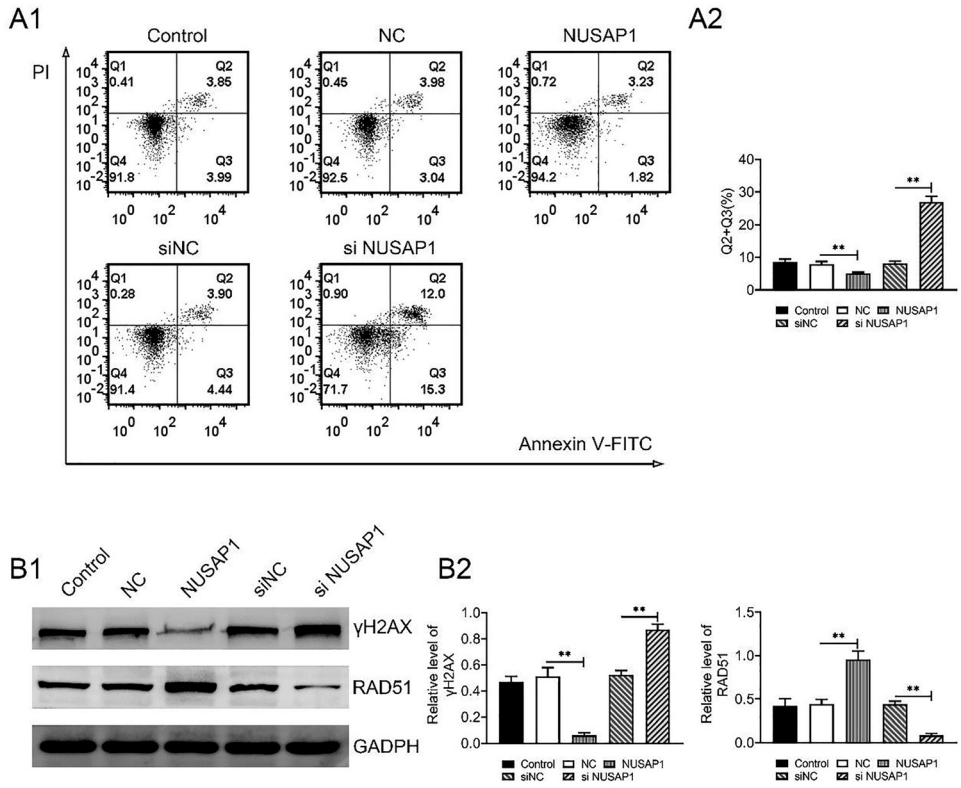

Fig. 3. Knockdown of NUSAP1 enhanced apoptosis and DNA damage in BCCs. (A1 and 2) The apoptosis of TE354.T cells was determined by flow cytometry. (B1 and 2) The relative protein level of γH2AX and RAD51 was examined by western blot. Data were expressed after normalization with GAPDH. Data were presented as mean ± SD and the difference was tested by the Student's *t*-test using SPSS 26.0 software. $^{**}P < 0.01$

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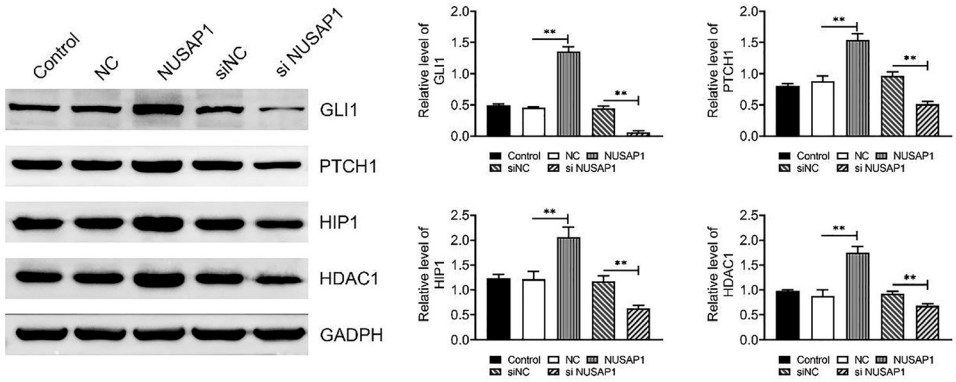

Fig. 4. NUSAP1 activated the Hedgehog signaling pathway in BCCs. The relative protein expression of GLI1, PTCH1, HIP1 and HDAC1 was examined by western blot. Data were expressed after normalization with GAPDH. Data were presented as mean ± SD and the difference was tested by the Student's *t*-test using SPSS 26.0 software. $^{**}P < 0.01$

DISCUSSION

In the current study, gain- and loss-of-function assays were performed through the transfection of the overexpression plasmid of NUSAP1 and si NUSAP1 into TE354.T cells. The results showed that NUSAP1 was highly expressed in TE354.T cells. Inhibition of NUSAP1 attenuated proliferation, migration and invasion, but enhanced apoptosis and DNA damage in TE354.T cells. Mechanically, the relative expression of proteins involved in the Hedgehog signaling pathway was decreased by the transfection of si NUSAP1 into TE354.T cells. Inverse results were obtained in these indexes after NUSAP1 was overexpressed in TE354.T cells. Taken together, both gain- and loss-of-function results demonstrated that NUSAP1 facilitated proliferation, migration and invasion, but suppressed apoptosis and DNA damage in BCC, which was associated with the activation of the Hedgehog signaling pathway.

Mounting evidence has highlighted that NUSAP1 serves as an oncogene with high-level expression in a wide variety of cancers, such as ovarian cancer [27], hepatocellular carcinoma [25], glioblastoma [29], breast cancer [23], nephroblastoma [39], bladder cancer [40] and non-small-cell lung cancer [41]. Consistent with these results, the level of NUSAP1 was also highly expressed in TE354.T cells. Moreover, high expression of NUSAP1 promotes the development of cancers, thus NUSAP1 is an alternative molecular target for the treatment of cancers. For instance, silencing of NUSAP1 suppresses glioblastoma growth and invasion, and enhances apoptosis by inhibiting the expression of TOP2A [29]. Knockdown of NUSAP1 impedes proliferation, mobility and invasion of breast cancer cells, as well as lung metastasis *in vivo* [23]. Interference of NUSAP1 also prevents growth and metastasis by modulating the BTG2/PI3K/Akt axis in non-small-cell lung cancer [41]. Here, our results consistently revealed that NUSAP1 enhanced proliferation, migration and invasion with reduced apoptosis in BCC cells. It is acknowledged that proliferation, apoptosis, migration and invasion are significant hallmarks of cancer [42]. Therefore, these results indicate that NUSAP1 accelerated the progression of BCC, thus interference of NUSAP1 may be a promising strategy for the treatment of BCC.

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DNA damage also plays a crucial role in BCC progression. It has been revealed that DNA damage of the skin can be directly or indirectly elicited upon a superfluous exposure of ultraviolet radiation, which promotes changes in inflammatory response and mutations in tumor suppressor genes and oncogenes, ultimately resulting in the initiation and progression of BCC [43]. In the present study, overexpression of NUSAP1 reduced the relative protein expression of γH2AX with increased relative protein expression of RAD51, whereas NUSAP1 silencing enhanced the relative protein expression of γH2AX with decreased relative protein expression of RAD51. More importantly, it has been proved that NUSAP1 influences the DNA damage response, in which NUSAP1 depletion causes the obstruction of double strand DNA break repair [44]. In addition, the enhanced role of NUSAP1 in chemoresistance is also involved in the DNA damage repair pathway in chronic lymphocytic leukemia [22]. Collectively, these outcomes illustrate that inhibition of NUSAP1 induces DNA damage in BCC.

The Hedgehog signaling pathway is one of the pivotal pathways involved in the tumorigenesis of BCC [45]. Correspondingly, several Hedgehog pathway inhibitors approved by the US FDA, such as sonidegib and vismodegib have been applied in the treatment of BCC in clinical practice [46, 47]. In the current study, the relative protein expression of GLI1, PTCH1, HIP1 and HDAC1 was decreased by the transfection of si NUSAP1 into TE354.T cells, and reverse results were obtained in these indexes after overexpression of NUSAP1 in TE354.T cells. It has been substantiated that NUSAP1 activates the Hedgehog signaling pathway to promote invasiveness in astrocytoma [48]. In line with these findings, our results also demonstrated that NUSAP1 induced the activation of the Hedgehog signaling pathway in BCC.

In conclusion, the present study clarified that NUSAP1 was expressed at high levels in BCC cells. NUSAP1 contributed to proliferation, migration and invasion, but attenuated apoptosis and DNA damage in BCC, which involved the activation of the Hedgehog signaling pathway. Nevertheless, several limitations remain to be addressed in subsequent research. Firstly, the clinical profiles of NUSAP1 in BCC can be analyzed in the future. Secondly, the direct role of the Hedgehog signaling pathway should be further confirmed by the application of pharmacological inhibitors or other effective interference. In addition, the role of NUSAP1 in BCC needs to be investigated *in vivo* to consolidate the results. Briefly, our study recognized that NUSAP1 was an oncogene and might be a therapeutic target of BCC.

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Availability of data and materials: All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Competing interests: The authors state that there are no conflicts of interest to disclose.

Author contributions: Yanjun Zhu designed the experiments. Yan Liu, Liwen Zhang performed the experiments. Shihua Zeng, Wen Xu analyzed and interpreted the results of the experiments. Yanjun Zhu was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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