Involvement of the Wnt/β-Catenin signaling pathway in the heterogeneous nuclear ribonucleoprotein K-driven inhibition of proliferation and migration in head and neck squamous cell carcinoma

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Abstract. The abnormal upregulation of heterogeneous nuclear ribonucleoprotein K (hnRNP K) expression levels were reported to be involved in the progression of various types of cancer. Therefore, it is hypothesized that hnRNP K may serve as a useful diagnostic marker and antitumor target; however, only a few studies to date have investigated the exact role of hnRNP K in head and neck squamous cell carcinoma (HNSCC) and the potential downstream signaling pathway involved. The present study aimed to identify the roles of hnRNP K in the proliferation and migration of HNSCC, and the possible signaling pathways hnRNP K may be associated with in HNSCC. hnRNP K expression levels in clinical HNSCC samples were analyzed using the Oncomine and UALCAN databases, and its association with the survival of patients with HNSCC was analyzed using the tumor-immune system interactions database. Short hairpin RNA targeting hnRNP K was transfected into the CAL-27 cell line to establish HNSCC cells with stable hnRNP K-knockdown. Cell viability was analyzed using a Cell Counting Kit-8 assay and an absolute count assay, and cell proliferation was measured using 5-ethynyl-2’-deoxyuridine incorporation and colony formation assays. Migratory ability of cells was analyzed using wound healing assay and transwell assay. The growth of xenografts derived from hnRNP K-knockdown cells was also evaluated, and bioinformatics analyses were performed using the Gene Ontology and Kyoto Encyclopedia for Genes and Genomes databases to determine the possible downstream signaling pathways of hnRNP K. Furthermore, the status of the Wnt/β-Catenin signaling pathway in hnRNP K-knockdown cells mediated by small interfering RNA was determined using reverse transcription-quantitative PCR and western blotting. The results revealed that the expression levels of hnRNP K were upregulated in HNSCC cell lines and tissues. Moreover, the upregulation of hnRNP K expression levels was associated with poor survival of patients with HNSCC. The knockdown of hnRNP K also decreased HNSCC cell proliferation and migration, and inhibited tumor growth in nude mice. Bioinformatics analyses identified the Wnt/β-Catenin signaling pathway as a possible downstream signaling pathway of hnRNP K. Knockdown of hnRNP K significantly downregulated the expression levels of Wnt/β-Catenin signaling pathway-related proteins; while with knockdown of hnRNP K and overexpression of β-Catenin, the expression levels of Wnt/β-Catenin signaling pathway-related proteins were partially rescued. In conclusion, the present findings indicated that hnRNP K may serve as a candidate diagnostic biomarker and a promising therapeutic target for HNSCC.

Introduction

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a docking molecule that integrates nucleic acid-directed processes with signal transduction pathways (1) and was initially identified as a member of the hnRNP family in 1992 (2). Over the past 20 years, clinical and basic science studies have attempted to determine whether hnRNP K...
serves as an oncogene or tumor suppressor gene; for example, numerous studies have reported the oncopgenic role of hnRNP K in gastric cancer (3), bladder cancer (4), colorectal cancer (5), malignant melanoma (6), hepatocellular carcinoma (7) and renal cell carcinoma (8). In contrast, Gallardo et al (9) directly implicated hnRNP K as a tumor suppressor in acute myeloid leukemias by generating an hnRNP K haploinsufficient mouse model. In addition, Bastidas et al (10) demonstrated that the inactivation of hnRNP K was a potential driver in mycosis fungoides development. However, there are currently conflicting results for the same tumor. For example, previous studies have reported that the hnRNP K protein was upregulated in gastric cell lines and tissue microarrays, where it was associated with the poor survival of patients with gastric cancer (3,11). However, our previous study concluded that hnRNP K served a tumor suppressive role in gastric cancer (12), which contradicts previous studies. These findings are because hnRNP K has demonstrated the capacity to regulate both tumor suppressive and oncogenic pathways, and both the overexpression and knockdown result in cell proliferation and apoptotic defects (13). Therefore, it remains necessary to perform basic scientific studies to determine the function of hnRNP K and its associated downstream signaling pathways.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer, with a worldwide incidence of 600,000 cases reported annually (14). Previous studies, including the tissue protein analysis of clinical samples, have indicated that hnRNP K may be a biomarker for predicting the potential for the malignant transformation of precancerous lesions and the poor prognosis of patients with HNSCC (15,16). However, to the best of our knowledge, few studies have performed phenotypic experiments to verify the genetic role of hnRNP K in HNSCC cell lines. In addition, the underlying molecular mechanisms of hnRNP K in the development and metastasis remain to be determined.

The present study aimed to investigate the roles of hnRNP K in the proliferation and migration of HNSCC and to determine the possible associated signaling pathways.

Materials and methods

Oncomine, UALCAN and TISIDB database analysis. The Oncomine database (17,18) (www.oncomine.org) was used to search for the fold changes in hnRNP K expression levels in different types of tumor using the following screening conditions: i) Gene name, hnRNP K; ii) analysis type, cancer vs. normal; and iii) data type, mRNA. The associations between hnRNP K expression levels and different clinicopathological parameters of HNSCC were analyzed using the UALCAN online database (http://ualcan.path.uab.edu/index.html). In the UALCAN database, hnRNP K and HNSCC were used as the search criteria, and the different clinical pathological parameters, such as sample type, sex, age, ethnicity, tumor grade, individual cancer stages, nodal metastasis status and HPV status, were selected and the corresponding figures were downloaded. In the tumor-immune system interactions database (TISIDB; http://cis.hku.hk/TISIDB), HNRNPK was used as the gene symbol, with the search terms ‘clinical’ and ‘head and neck squamous cell carcinoma’ as the cancer type to generate survival curve.

Cell culture and transfection. CAL-27 (human tongue squamous cell carcinoma) and WI-38 (human embryo lung fibroblast) cell lines were cultured in DMEM, while the FaDu (human pharyngeal squamous cell carcinoma) cell line was cultured in MEM. All cells were purchased from National Infrastructure of Cell Line Resource (http://www.cellresource.cn/), and the cells were supplemented with 10% FBS and 100 U/ml penicillin and streptomycin, and maintained in a humidified atmosphere with 5% CO2 at 37°C. The short hairpin RNA (shRNA/sh) sequence targeting hnRNP K (shhnRNP K; 5’-GGCTGATATTTGAAAACAT-3’) (GV248-hnRNP K) and the negative control (NC) lentivirus expressing green fluorescence protein (shNC; 5’-TTCTCCGAGCTGTCAGCTGTG-3’) (GV248-NC) were provided by Shanghai GeneChem Co., Ltd. The shhnRNP K and shNC (MOI=20) were transfected into CAL-27 cells (5x10^3 cells per well) using polybrene according to the manufacturer’s protocol. The duration of transfection was 12 h at 37°C followed by changing the fresh medium. Transfected cells were used for subsequent experiments after 72 h.

As the frozen shRNA after resuscitation was not sufficient to perform experiments, siRNA was used to verify the results of microarray results in order to save time. Small interfering RNA (siRNA/si) targeting hnRNP K (sihnRNP K; 5’-GAGCUUGCAGUCAAUAUGAGTT-3’) and a non-specific siNC siRNA (5’-UUUCAGAGGUACUGACUUTT-3’) were purchased from Guangzhou Ribobio Co., Ltd. The target sequences in the two groups were identical to those previously described (12). The overexpression plasmid of β-Catenin (GV362-β-Catenin) and the plasmid-NC (empty vector, GV362-NC) were provided by Shanghai GeneChem Co., Ltd. The transfections were performed with Lipofectamine 2000, and cells with sihnRNP K and siNC (30 pmol) were harvested after 48 h of transfection at 37°C for further experimentation.

Immunofluorescence. The cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100. The slides were then incubated with mouse anti-β-Catenin (cat. no. 8480), anti-disheveled (Dvl)2 (cat. no. 2978), anti-c-Myc (cat. no. 5605), anti-matrix metalloproteinase (MMP)7 (cat. no. 3801) and anti-β-actin (cat. no. 3700) primary antibodies were purchased from Cell Signaling Technology, Inc. and anti-mouse IgG, HRP-linked antibody (cat. no. 7074) and anti-rabbit IgG, HRP-linked antibody (cat. no. 7076) were purchased from Abcam, while mouse anti-β-Catenin (cat. no. ab52600) was purchased from Abcam, while anti-β-Catenin (cat. no. 8480), anti-disheveled (Dvl)2 (cat. no. 3224), anti-c-Jun (cat. no. 9165), anti-Met (cat. no. 8198), anti-Cyclin-D1 (cat. no. 2978), anti-c-Myc (cat. no. 5605), anti-matrix metalloproteinase (MMP)7 (cat. no. 3801) and anti-β-actin (cat. no. 3700) primary antibodies were purchased from Cell Signaling Technology, Inc. Moreover, anti-rabbit IgG, HRP-linked antibody (cat. no. 7074) and anti-mouse IgG, HRP-linked antibody (cat. no. 7076) were purchased from Cell Signaling Technology, Inc. To confirm the overexpression of β-catenin gene was expressed and the plasmid transfection was successful, flag expression level was analyzed using anti-flag (cat. no. M185-3L; Medical & Biological Laboratories).

Western blotting. Total protein was extracted from CAL-27, FaDu and WI-38 cells using cold RIPA lysis buffer (Sigma-Aldrich; Merck KGaA). Total protein was quantified using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). The protein samples were boiled with 2X SDS-PAGE protein loading buffer (Beyotime Institute of Biotechnology) for 15 min and then the cell lysates

Antibodies and chemical reagents. FBS, DMEM and minimum essential medium (MEM) were purchased from Gibco; Thermo Fisher Scientific, Inc. The anti-hnRNP K primary antibody (cat. no. ab52600) was purchased from Abcam, while anti-β-Catenin (cat. no. 8480), anti-disheveled (Dvl)2 (cat. no. 3224), anti-c-Jun (cat. no. 9165), anti-Met (cat. no. 8198), anti-Cyclin-D1 (cat. no. 2978), anti-c-Myc (cat. no. 5605), anti-matrix metalloproteinase (MMP)7 (cat. no. 3801) and anti-β-actin (cat. no. 3700) primary antibodies were purchased from Cell Signaling Technology, Inc. Moreover, anti-rabbit IgG, HRP-linked antibody (cat. no. 7074) and anti-mouse IgG, HRP-linked antibody (cat. no. 7076) were purchased from Cell Signaling Technology, Inc. To confirm the overexpression of β-catenin gene was expressed and the plasmid transfection was successful, flag expression level was analyzed using anti-flag (cat. no. M185-3L; Medical & Biological Laboratories).
(20 μg per lane) were separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto nitrocellulose filter membranes (Pall Corporation) and blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 at room temperature for 1 h. The membranes were then incubated with the following primary antibodies overnight at 4°C: Anti-hnRNP K primary antibody (1:10,000, cat. no. 8480; Abcam), anti-β-Catenin (1:1,000, cat. no. 8480; Cell Signaling Technology), anti-Dvl2 (1:1,000, cat. no. 3224; Cell Signaling Technology), anti-c-Jun (1:1,000, cat. no. 9165; Cell Signaling Technology), anti-Met (1:1,000, cat. no. 8198; Cell Signaling Technology), anti-Cyclin-D1 (1:1,000, cat. no. 2978; Cell Signaling Technology), anti-c-Myc (1:1,000, cat. no. 5605; Cell Signaling Technology), anti-MMP7 (1:1,000, cat. no. 3801; Cell Signaling Technology), anti-Flag (1:1,000, M185-3L; Medical & Biological Laboratories) and anti-β-actin (1:1,000, cat. no. 3700; Cell Signaling Technology). Following the primary antibody incubation, the membranes were incubated with anti-rabbit IgG, HRP-linked antibody (1:5,000; cat. no. 7074) or anti-mouse IgG, HRP-linked antibody (1:5,000; cat. no. 7076) which was purchased from Cell Signaling Technology, Inc., for 1 h at room temperature. An enhanced chemiluminescence kit (cat. no. 32106; Thermo Fisher Scientific, Inc.) was used to visualize the membrane. β-actin was used as the loading control. Densitometric analysis was performed using ImageJ software (version 1.8.0; National Institutes of Health).

Patient studies. A total of 20 paired tumor and adjacent normal tissues (17 males and 3 females; median age, 63; age range, 44-84 years) were collected from patients with HNSCC at Beijing Tongren Hospital (Beijing, China) between March 2018 and March 2019. The patients had no history of radiotherapy or chemotherapy prior to surgery. Written, informed consent was obtained from all patients and the use of patient specimens for the experiments was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University (Beijing, China).

Immunohistochemistry. The patient tissue were fixed with 10% formalin at room temperature for 24 h. Briefly, 5-μm sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated in a descending series of ethanol at room temperature. The deparaffinized sections were incubated with 3% H₂O₂ at room temperature for 15 min to inhibit the endogenous peroxidase activity. The sections were then boiled in antigen retrieval solution for 8 min and blocked with 10% goat serum (cat. no. ZLI-9017; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 30 min at 37°C. Subsequently, the tissue sections were incubated with an anti-hnRNP K monoclonal antibody (1:250) overnight at 4°C, followed by incubation with anti-rabbit IgG, HRP-linked secondary antibody (1:5,000; cat. no. 7074) which was purchased from Cell Signaling Technology, Inc., for 1 h at room temperature. Fresh 3,3’-diaminobenzidine (DAB substrate solution:DAB concentrate, 20:1) was added to the sections for a color reaction at room temperature, and then, the samples were counterstained with hematoxylin for 10 sec at room temperature. Finally, the sections were dehydrated with an ascending series of ethanol and cleared with xylene at room temperature, sealed with a coverslip and visualized under a Zeiss Axio Imager Z2 Upright light microscope (magnification, x20; Zeiss AG).

Absolute cell count assays. CAL-27 cells were seeded into 96-well plates at a density of 1,000 cells/well with three replicates per condition. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 24, 48, 72, 96 or 120 h, and the cell number was checked by cell counter every 24 h.

Cell Counting Kit (CCK)-8 assay. CAL-27 cells were seeded into 96-well plates at a density of 800 cells/well with three replicates per condition. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 24, 48, 72, 96 or 120 h and the cell viability was analyzed every 24 h. Briefly, every 24 h, 100 μl serum-free DMEM containing 10 μl CCK-8 reagent (Dojindo Molecular Technologies, Inc.) was added to each well and incubated for 2 h according to the manufacturer's protocol. The absorbance was measured for 450 nm using microplate reader at room temperature. Data were analyzed from ≥3 independent experiments.

Colony formation assay. CAL-27 cells were seeded into 6-well culture plates (Corning Inc.) at a density of 1,000 cells/well, with each condition set up in triplicate wells. The cells were cultured for 10 days at 37°C constant temperature CO₂ incubator to induce colony formation (>50 cells per colony). Following the incubation, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min, and then stained with 1% crystal violet at room temperature for 30 min. The colonies were counted and images were captured using a Zeiss Axio Imager Z2 light microscope.

5-Ethynyl-2′-deoxyuridine (EdU) assay. The EdU incorporation assay was performed with transfected CAL-27 cells using the Cell Light EdU Apollo® 567 In Vitro Imaging kit (Guangzhou RiboBio Co., Ltd.). For each group, 1x10⁶ cells/well were seeded into 24-well plates and incubated overnight at 37°C constant temperature CO₂ incubator. The EdU assay was performed according to the manufacturer's protocol; however, the cell nuclei were stained with DAPI instead of Hoechst 33342 for 30 min at room temperature, and the fixative was used 4% paraformaldehyde for 30 min at room temperature. Five randomly selected fields of view of EdU-positive cells were visualized using Leica DMi8 fluorescence microscope (magnification, x20). Image analysis was performed using ImageJ software (version 1.8.0; National Institutes of Health). Data were analyzed from ≥3 independent experiments.

Wound healing assay. A total of 1x10⁵ CAL-27 cells/well were plated into 6-well plates and cultured with complete medium in an incubator at 37°C overnight until 100% confluence. Subsequently, a linear scratch wound was generated in the cell monolayer using a 200-μl pipette tip. After washing with PBS 3 times, the cells were cultured with serum-free DMEM at 37°C for 24, 48, 72, 96 or 120 h, and the cell number was checked by cell counter every 24 h.
Transwell assay. CAL-27 cells were transfected with shhnRNP K and shNC as described above. A total of 5x10^4 cells/well were resuspended in 200 μl DMEM without FBS and seeded into the upper compartments of Boyden chambers (Falcon; Corning Inc.) The lower chamber was filled with 700 μl DMEM containing 10% FBS. Following 12 h of incubation at 37°C, the non-migratory cells remaining in the upper chamber were removed with cotton swabs and the migratory cells in the lower chamber were washed with PBS three times, fixed with 4% paraformaldehyde at room temperature for 30 min and stained with crystal purple for 15 min at room temperature. The stained cells were visualized in five randomly selected fields using a Zeiss Axio Imager Z2 light microscope (magnification, x100).

Mouse xenograft model. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Sciences, Peking Union Medical College (approval no. GR-16002; Beijing, China). The nude mice were housed in pathogen-free environment with 12-h light/dark cycle, controlled humidity (50%) and temperature (26°C) and had free access to food and water.

Equal numbers of shhnRNP K- and shNC-transfected cells (5x10^6 cells) which were resuspended with PBS were subcutaneously injected into the axilla of the left forelimb of 12 male BALB/c nude mice (six mice/group; age: 6-weeks-old; weight: 21 g; HFK Bioscience, http://www.hfkbio.com/). The tumor volume (mm^3) was determined every 2 days from the 5th day after injection to the study endpoint using the following formula: Tumor volume = (a x b^2)/2, where ‘a’ was the longest tumor diameter and ‘b’ was the shortest tumor diameter. The maximum tumor volume of the study was 603.7 mm^3. All animals were sacrificed 4 weeks later by exposure to CO_2 (25 l/min) at a chamber displacement rate of 30% and subsequent cervical dislocation, and the tumors were excised. Death was verified by complete cessation of breathing, the lack of a heartbeat and dilated pupils.

Microarray analysis and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cultured shhnRNP K and shNC cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Gene expression profiles were analyzed by Shanghai OE Biotechnology Corporation (https://www.oebiotech.com/). The hybridized signals were assessed with Agilent SurePrint G3 Human gene expression v3 microarrays (Agilent Technologies, Inc) and Genomes (KEGG) database (20) using GeneSpring GX software (version 14.9; Agilent Technologies, Inc.) for the downregulated genes. A fold change threshold of ≤-2 and P≤0.05 were considered to indicate a statistically significant difference. The differentially expressed genes were then subjected to functional term enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (20) and signaling pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (20) using GeneSpring GX software (version 14.9; Agilent Technologies, Inc.);

RT-qPCR was performed as previously described (12). The reverse transcription protocol was: 42°C for 60 min and 70°C for 5 min. The protocol of PCR was as follows: 95°C, 2 min; 95°C, 10 sec; 60°C, 30 sec for 40 cycles in total. The primers used for the qPCR are provided in Table I and the expression levels were quantified using the 2^-ΔΔCq method (21).

| Gene                                      | Primer sequence (5’-3’) |
|-------------------------------------------|------------------------|
| Heterogeneous nuclear ribonucleoprotein K | F: AGAAGTTGAGACCGTATC  |
|                                           | R: ATAAGCCATCTGCACTT   |
| β-Catenin                                 | F: GCGCATTATTTAAGCTCTCG|
|                                           | R: AAATACCTCGGGAGGAC   |
| Disheveled 2                              | F: CTCCTATCCTTCCACCTTAAT|
|                                           | R: CATGCTACTGTCCTTCTTTC|
| c-Jun                                     | F: TGGAGACCGGCAGACCTTTCA|
|                                           | R: TTTCCTAAAGGACGCACTCCA|
| Met                                       | F: CGACACGCTAGCTGCTGAGA|
|                                           | R: AGGTATTATCTTCTGCTGAG|
| Cyclin-D1                                 | F: GATCAAGTGTGACCCGACT|
|                                           | R: CTTGGGCTCATTGTTCTGCT|
| c-Myc                                     | F: TACAAACCCCGAGACGAC  |
|                                           | R: CGGGAGCTGCTTCTCCA   |
| Matrix metalloproteinase 7                | F: GTCTCTGAGCCGACGATG  |
|                                           | R: GATGACTCTGAGCCGATGCC|
| GAPDH                                     | F: CATCAAGAAGGGTGGTAAGCAG|
|                                           | R: CGTCAAAGGTGAGGAGTGG  |

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.) and data are presented as the mean ± SD from three independent experiments. Group comparisons between two groups were performed using an unpaired two-tailed Student's t-test. Multiple group comparisons were performed using a one-way ANOVA followed by a Bonferroni's post hoc test. The overall survival analysis for low and high expression levels of hnRNP K was performed using the Kaplan Meier method and a log-rank test was used to determine the P-value. P<0.05 was considered to indicate a statistically significant difference.

Results

Aberrant expression levels of hnRNP K in HNSCC tissues and cells. To obtain an initial insight into hnRNP K expression patterns, data mining using the Oncomine and UALCAN databases was performed and the results are presented in Figs. 1A and S1. Compared with the normal tissues, the mRNA expression levels of hnRNP K were upregulated in brain and CNS cancer, cervical cancer, melanoma, myeloma and head and neck cancer (Fig. 1A), and the expression levels of hnRNP K in primary tumor of HNSCC was more upregulated than normal head and neck tissues. Furthermore, hnRNP K expression levels were positively associated with the grade, human papillomavirus status and lymph node metastasis of patients with HNSCC (Fig. SIE, G and H), while no significant difference were recorded between the expression levels of hnRNP K and the sex, age, ethnicity or tumor stage of the patients with HNSCC (Fig. SIB-D and F). In addition, data mining in the TISIDB revealed that high expression levels...
of hnRNP K were associated with the significantly poorer survival of patients with HNSCC compared with patients with low expression levels (Fig. 1B). Furthermore, the expression levels of hnRNP K were determined in two HNSCC cell lines (CAL-27 and FaDu) and human embryonic lung cells (WI-38) using RT-qPCR and western blotting analysis. The results indicated that hnRNP K expression levels were upregulated in CAL-27 and FaDu cells compared with the WI-38 cells (Fig. 1C and D). Next, 20 pairs of paraffin-embedded cancerous and adjacent normal tissue samples from patients with HNSCC were analyzed using immunohistochemistry. It was revealed that hnRNP K was mainly expressed in the nuclei of tumor tissue compared with the adjacent normal tissue, the expression levels of hnRNP K in the cancer specimens were markedly increased (Fig. 1E). Collectively, these data indicated that hnRNP K may have an important role in the development of HNSCC.

hnRNP K silencing reduces the viability, proliferation and migration of CAL-27 cells. To investigate the function of hnRNP K in HNSCC cells, shRNA was used to establish a stable hnRNP K-knockdown CAL-27 cell line. Both hnRNP K mRNA and protein expression levels in the shhnRNP K-transfected cells were downregulated compared
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with the shNC-transfected cells (Fig. 2A and B). The results of the CCK-8 and absolute cell count assays indicated that the knockdown of hnRNP K significantly inhibited CAL-27 cell viability and proliferation compared with the shNC-transfected cells following 120 h of incubation (Fig. 2C and D). Furthermore, the results of the colony formation assay revealed that the hnRNP K-knockdown CAL-27 cell line formed significantly fewer and smaller clones compared with the clones formed in the shNC group (Fig. 2E and F). The EdU incorporation assay also demonstrated that hnRNP K silencing resulted in a markedly reduced percentage of CAL-27 cells compared with the shNC group (Fig. 2G). Taken together, the present results indicated that hnRNP K may promote the cell viability, proliferation and migration of CAL-27 cells.
hnRNP K knockdown effectively suppresses CAL-27 growth in vivo. To verify the oncogenic role of hnRNP K in CAL-27 cells in vivo, shhnRNP K- or shNC-transfected CAL-27 cells were inoculated into BALB/c nude mice. Tumors derived from the shhnRNP K cells exhibited slower growth compared with the tumors derived from the shNC group (Fig. 3A-C). The differences in the tumor volume and weight between the two groups were significant, which was shown as the tumor weight and volume significantly decreased in the shhnRNP K group compared with the shNC group (Fig. 3A-C), while there were no significant differences recorded in the body weight of the mice between the two groups (Fig. 3D). These results indicated that hnRNP K may promote the tumor formation by CAL-27 in vivo.

hnRNP K inhibits the activation of the Wnt/β-Catenin signaling pathway. To determine the molecular mechanisms underlying the phenotypic changes in hnRNP K-knockdown HNSCC cells, mRNA microarray analysis was performed. GO functional term enrichment analysis of the significantly differentially expressed genes of shhnRNP K knockdown cells compared with the NC was used to identify the role of differentially expressed hnRNP K. The results identified that ‘Cell adhesion’ was the most highly enriched biological process of the downregulated genes, which suggested that hnRNP K may be an important modulator of HNSCC metastasis, while receptor agonist activity was the most highly enriched molecular function and extracellular region was the most highly enriched cellular component (Fig. 4A). Furthermore, the downregulated genes were also relevant to the ‘Response to virus’ and ‘Defense response to virus’, suggesting that hnRNP K may be involved in the immune response (Fig. 4A).

KEGG signaling pathway enrichment analysis of the significantly differentially expressed genes was used to elucidate the pathways and molecular interactions of hnRNP K in HNSCC. The top 30 pathways associated with the downregulated mRNAs are listed in Fig. 4B. The ‘Wnt signaling pathway’ was the top pathway enriched by the downregulated genes, which implied that the Wnt signaling pathway may be a target for hnRNP K (Fig. 4B). Next, CAL-27 cells were transfected with shhnRNP K and siNC, and RT-qPCR (Fig. 4C) and western blotting analysis (Fig. 4D and E) revealed that hnRNP K, β-catenin, Dvl2, c-Jun, Met, Cyclin-D1, c-Myc and MMP7 expression levels were significantly downregulated in the shhnRNP K-transfected cells compared with the siNC-transfected cells. To determine if the β-Catenin pathway was involved in hnRNP K regulating phenotype of HNSCC, β-Catenin was overexpressed with plasmid (Fig. 4F). Following the simultaneous transfection with si-hnRNP K and a β-Catenin overexpression plasmid, the expression levels of β-Catenin, c-Myc and c-Jun were significantly restored compared with the cells transfected with the si-hnRNP K only (Fig. 4G and H).

These results indicated that the Wnt/β-Catenin signaling pathway may be involved in the hnRNP K-driven inhibition of proliferation and migration in HNSCC.

Discussion

The overexpression or amplification of oncogenes, mutations and the de novo promoter methylation of tumor suppressor genes have frequently been detected in HNSCC (22). hnRNP K is a member of the hnRNP family; the hnRNP family comprises 19 hnRNP genes termed hnRNP A1 through to U (23). It is well established that hnRNP K is
overexpressed in various types of cancer, such as hepatocellular carcinoma, bladder, pancreatic and lung cancer (24-27). Wiesmann et al (28) proved that the downregulated expression levels of hnRNP K had the potential to improve the success rate of HNSCC radiotherapy. However, to the best of our knowledge, previous studies on hnRNP K in HNSCC did not perform any molecular investigations directly demonstrating the exact function of hnRNP K and only a few studies
have explored the downstream regulatory mechanisms of hnRNP K (9,10,29). In the present study, functional molecular studies were performed to identify the exact roles of hnRNP K in HNSCC and the downstream mechanisms involved.

The Oncomine and UALCAN databases were used to confirm the upregulation of hnRNP K expression levels in HNSCC, which indicated that hnRNP K may serve as an oncogene in the development of HNSCC. Furthermore, the present study also revealed that hnRNP K expression levels were significantly upregulated in HNSCC tissues and cell lines, which was consistent with the results from the Oncomine and UALCAN databases and previous studies (15,16). Further data mining in the TISIDB database suggested that the expression levels of hnRNP K were negatively associated with the overall survival of patients with HNSCC, which suggested that the hnRNP K mRNA expression levels may serve as a potential biomarker to evaluate the prognosis of patients with HNSCC.

In order to investigate the function of hnRNP K in tumor development, hnRNP K was silenced in HNSCC cells, and the subsequent in vitro and in vivo assays performed indicated that hnRNP K knockdown attenuated the malignant and migration properties of the cells. Similarly, Gao et al (30) revealed that normal NIH3T3 mouse embryonic fibroblasts overexpressing hnRNP K were characterized by enhanced malignant properties both in vitro and in vivo. Thus, the present results further confirmed the oncogenic activity of hnRNP K in promoting cell proliferation and migration in HNSCC.

Although accumulating evidence has indicated that hnRNP K has an important role in oncogenesis and metastasis, the mechanisms remain unclear and controversial (31-33). Therefore, to clarify the potential molecular mechanisms of the regulation of hnRNP K, gene expression microarray analysis was performed on hnRNP K-knockdown cells and the corresponding NCs. KEGG signaling pathway enrichment analysis revealed that the differentially expressed genes between the shhnRNP K and shNC groups were enriched in the ‘Wnt signaling pathway’, which indicated hnRNP K place an important role in HNSCC development through Wnt signaling pathway. The knockdown of hnRNP K in HNSCC cells blocked the Wnt signaling pathway, which was demonstrated through the downregulated expression levels of β-Catenin, Dvl2, c-Jun, Met, Cyclin-D1, c-Myc and MMP7; however, following the simultaneous transfection with shhnRNP K and β-Catenin overexpression plasmid, the expression levels of β-Catenin, c-Myc and c-Jun were almost completely restored compared with the shhnRNP K knockdown cells.

Previous studies have proved that the Wnt/β-Catenin signaling pathway had a critical role in the development and metastasis of HNSCC (34,35). β-Catenin is the central effector of the Wnt signaling pathway, and aberrant β-Catenin expression was identified to contribute to HNSCC therapy resistance and disease relapse (36). In addition, Cyclin-D1 is a target downstream effector activated by β-Catenin in the Wnt signaling pathway, and the overexpression of cyclin D1 was discovered to promote the proliferation and metastatic potential in HNSCC (37-39). Furthermore, when the Wnt/β-Catenin signaling pathway is activated, specific target oncogenes, including c-Jun, MMP7 and c-Myc, were found to also be aberrantly activated (40).

MMPs are a group of enzymes that cleave extracellular matrix components, growth factors and cell-surface receptors (41); they are deeply involved in the process of metastasis and they were reported to be overexpressed in patients with head and neck cancer with metastasis compared with patients without metastasis (42). Previous studies have suggested that MMP7 may promote tumor development and metastasis in HNSCC (42-44). Thus, it was hypothesized that hnRNP K may positively regulate the Wnt signaling pathway during the proliferation and metastasis of HNSCC. To the best of our knowledge, the present study was the first to suggest that hnRNP K may regulate the development and metastasis of HNSCC through the Wnt signaling pathway. Therefore, the suppression of the Wnt/β-Catenin signaling pathway through hnRNP K may represent a promising approach for the treatment of HNSCC.

Although this research has made progress in our understanding, there are some limitations. Firstly, no normal head and neck cell lines were available to use as the control cell line, thus the present study used human embryo lung cells (WI-38). This was considered to be acceptable, as the Human Protein Atlas network (https://www.proteinatlas.org) indicated that the expression levels of hnRNP K are conserved in all cell lines. Secondly, the present study identified that hnRNP K knockdown significantly impaired the cell proliferation and migration of HNSCC cell lines; however, it did not investigate whether hnRNP K overexpression had the opposite effects. Thus, this will be investigated in future studies, if possible.

In summary, the present study observed that the knockdown of hnRNP K markedly attenuated the viability, proliferation and migration of HNSCC cells. In addition, the knockdown of hnRNP K led to the downregulation of the protein expression levels of β-Catenin, Dvl2, c-Jun, Met, Cyclin-D1, c-Myc and MMP7 in HNSCC cells, which confirmed that the Wnt/β-Catenin signaling pathway may be involved in the hnRNP K-driven inhibition of proliferation and migration in HNSCC. These results may provide a novel therapeutic biomarker for the treatment of HNSCC.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
RG and ZH conceived and designed the study, and critically revised the manuscript. HL performed all experiments and drafted the manuscript. XY, ML, WZ, GZ, XZ, LC and WL helped perform experiments and helped analyze some data
of the study. XC participated in initial design of the study and provided the samples in the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Animal experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Sciences, Peking Union Medical College (approval no. GR-16002; Beijing, China). Written, informed consent was obtained from all patients and the use of patient specimens for the experiments were approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University (Beijing, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References
1. Barboro P, Ferrari N and Balbci C: Emerging roles of heterogeneous nuclear ribonucleoprotein K (hnRNP K) in cancer progression. Cancer Lett 352: 152‑159, 2014.
2. Matunis MJ, Michael WM and Dreyfuss G: Characterization and primary structure of the poly(C)-binding heterogeneous nuclear ribonucleoprotein complex K protein. Mol Cell Biol 12: 164‑171, 1992.
3. Yang R, Zeng Y, Xu H, Chen Z, Xiang M, Fu Y, Yin Y, Zhong J, Zeng M, Wang P, et al: Heterogeneous nuclear ribonucleoprotein K is overexpressed and associated with poor prognosis in gastric cancer. Oncol Rep 36: 929‑935, 2016.
4. Chen X, Gu P, Xie R, Han J, Liu H, Wang B, Xie W, Xie W, Zhong G, Chen C, et al: Heterogeneous nuclear ribonucleoprotein K is associated with poor prognosis and regulates proliferation and apoptosis in bladder cancer. J Cell Mol Med 21: 1266‑1279, 2017.
5. Carpenter B, McKay M, Dundas SR, Lawrie LC, Telfer C and Murray GI: Heterogeneous nuclear ribonucleoprotein K is over expressed, aberrantly localised and is associated with poor prognosis in colorectal cancer. Br J Cancer 95: 921‑927, 2006.
6. Wen F, Shen A, Shanans R, Bhattacharya A, Lian F, Hostetter G and Shi J: Higher expression of the heterogeneous nuclear ribonucleoprotein K in melanoma. Ann Surg Oncol 17: 2619‑2627, 2010.
7. Guo Y, Zhao J, Bi J, Wu Q, Wang X and Lai Q: Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a tissue biomarker for detection of early hepatocellular carcinoma in patients with cirrhosis. J Hematol Oncol 5: 37, 2012.
8. Otsuki T, Tanaka T, Morimoto K and Nakatani T: Cytoplasmic accumulation of heterogeneous nuclear ribonucleoprotein K strongly promotes tumor invasion in renal cell carcinoma cells. Jpn J Cancer Res 106: 624‑630, 2015.
9. Gallardo M, Lee HJ, Zhang X, Bueso‑Ramos C, Pageon LR, McArthur M, Multani A, Nazha A, Manshouri T, Parker‑Thornburg J, et al: hnRNP K is a haploinsufficient tumor suppressor that regulates proliferation and differentiation programs in hematologic malignancies. Cancer Cell 28: 486‑496, 2015.
10. Bastidas Torres AN, Cats D, Mei H, Suzhai K, Willemsen R, Vermeer MH and Tenten CP: Genomic analysis reveals recurrent deletion of JAK‑STAT signaling inhibitors HNRNPK and SOCS1 in mycosis fungoides. Genes Chromosomes Cancer 57: 653‑664, 2018.
11. Zhao Y, Jin X, Tian T and Yu DH: Expression of hnRNPK in gastric carcinoma and its relationship with Helicobacter pylori L‑form infection. Zhonghua Zhong Liu Za Zhi 33: 759‑763, 2011.
12. Huang H, Han Y, Yang X, Li M, Zhu R, Hu J, Zhang X, Wei R, Li K and Gao R: HNRNPK inhibits gastric cancer cell proliferation through p53/p21/CCND1 pathway. Oncotarget 8: 103364‑103374, 2017.
13. Gallardo M, Hornbaker MJ, Zhang X, Hu P, Bueso‑Ramos C and Post SM: Aberrant hnRNP K expression: All roads lead to cancer. Cell Cycle 15: 1552‑1557, 2016.
14. Lemal A, Bray F, Center MM, Fertlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69‑90, 2011.
15. Matta A, Tripathi SC, DeSouza LV, Grigull J, Kaur J, Chauhan SS, Srivastava A, Thakar A, Shukla NK, Duggal R, et al: Heterogeneous ribonucleoprotein K is a marker of oral leukoplakia and corilates with poor prognosis of squamous cell carcinoma. Int J Cancer 125: 1398‑1406, 2009.
16. Wu CS, Chang KP, Chen LC, Chen CC, Liang Y, Hseuh C and Chang YS: Heterogeneous ribonucleoprotein K and thymidine phosphorylase are independent prognostic and therapeutic markers for oral squamous cell carcinoma. Oncol OncoL 48: 516‑522, 2012.
17. Estilo CL, O‑characterist P, Talbot S, Socci ND, Carlson DL, Ghossein R, Williams T, Yonekawa Y, Ramanathan Y, Boyle JO, et al: Oral tongue cancer gene expression profiling: Identification of novel potential prognosticators by oligonucleotide microarray analysis. BMC Cancer 9: 11, 2009.
18. Ginos MA, Page GP, Michalowicz BS, Patel KJ, Volker SE, Pambuccian SE, Ondrey FG, Adams GL and Gaffney PM: Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. Cancer Res 64: 55‑63, 2004.
19. Ashburne M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al: The gene ontology consortium: Gene ontology: Tool for the unification of biology. Nat Genet 25: 25‑29, 2000.
20. Kanelmis A, Goto S, Sato Y, Furumichi M and Tanabe M: KEGG for integration and interpretation of large-scale molecular datasets. Nucleic Acids Res 40: D109‑D114, 2012.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression using real‑time quantitative PCR and the 2(‑Delta Delta C(T)) method. Methods 25: 402‑408, 2001.
22. Mao L, Hong WK and Papadimitrakopoulou VA: Focus on head and neck cancer. Cancer Cell 5: 311‑316, 2004.
23. Carpenter B, MacKay C, Alnabuls A, MacKay M, Telfer C, Melvin WT and Murray GI: The roles of heterogeneous nuclear ribonucleoproteins in tumour development and progression. Biochim Biophys Acta 1765: 85‑100, 2006.
24. Shu Y, Hu J and Deng H: miR‑1249‑5p accelerates the malignancy phenotype of hepatocellular carcinoma by directly targeting HNRNPK. Mol Genet Genomic Med 7: e00867, 2019.
25. Aboushousta T, Hammad M, Halal N and El Dahshan S: Impact of Cyclin D1 and heterogeneous nuclear ribonucleoprotein‑K (hnRNP‑K) on urinary bladder carcinogenesis. Asian Pac J Cancer Prev 19: 513‑519, 2018.
26. He D, Huang C, Zhou Q, Liu D, Xiong L, Xiang H, Ma G and Zhang Z: HnRNPK/miR‑223/FBXW7 feedback cascade promotes pancreatic cancer cell growth and invasion. Oncotarget 8: 20165‑20178, 2017.
27. Chen L, Li W, Chen Y, Wang D, Han J and Liu D: Downregulation of hnRNP K by RNAi inhibits growth of human lung cancer cells. Oncol Lett 7: 1073‑1077, 2014.
28. Wiesmann N, Strozynski J, Beck C, Zimmermann N, Mandler S, Gieringer R, Schmittmann I and Brieger J: Knockdown of hnRNP K leads to increased DNA damage after irradiation and reduces survival of tumor cells. Carcinogenesis 38: 321‑328, 2017.
29. Liu L, Luo C, Luo Y, Chen L, Liu Y, Wang Y, Han J, Zhang Y, Wei N, Xie Z, et al: MRPL33 and its splicing regulator hnRNP K are required for mitochondria function and implicated in tumor progression. Oncogene 37: 6096‑6104, 2018.
30. Gao R, Yu Y, Inoue A, Widoed N, Kaul SC and Wadhwa R: Heterogeneous nuclear ribonucleoprotein K (hnRNP‑K) promotes tumor metastasis by induction of genes involved in extracellular matrix, cell movement, and angiogenesis. J Biol Chem 285: 15026‑15036, 2010.
31. Hope NR and Murray GI: The expression profile of RNA‑binding proteins in primary and metastatic cutaneous cancer: Relationship of heterogeneous nuclear ribonucleoproteins with prognosis. Hum Pathol 42: 393‑402, 2011.
32. Du Q, Wang L, Zhu H, Zhang S, Xu L, Zheng W and Liu X: The role of heterogeneous nuclear ribonucleoprotein K in the progression of chronic myeloid leukemia. Med Oncol 27: 673‑679, 2010.
33. Inoue A, Sawata SY, Taira K and Wadhwa R: Loss‑of‑function screening by randomized intracerebral antibodies: Identification of hnRNP K as a potential target for metastasis. Proc Natl Acad Sci USA 104: 8983‑8988, 2007.
34. Yang F, Zeng Q, Yu G, Li S and Wang CY: Wnt/beta-catenin signaling inhibits death receptor-mediated apoptosis and promotes invasive growth of HNSCC. Cell Signal 18: 679-687, 2006.

35. Aminuddin A and Ng PY: Promising Druggable target in head and neck squamous cell carcinoma: Wnt Signaling. Front Pharmacol 7: 244, 2016.

36. Roy S, Kar M, Roy S, Saha A, Padhi S and Banerjee B: Role of beta-catenin in cisplatin resistance, relapse and prognosis of head and neck squamous cell carcinoma. Cell Oncol (Dordr) 41: 185-200, 2018.

37. Sales KU, Giudice FS, Castilho RM, Salles FT, Squarize CH, Abrahao AC and Pinto DS Jr: Cyclin D1-induced proliferation is independent of beta-catenin in head and neck cancer. Oral Dis 20: e42-e48, 2014.

38. Hanken H, Gröbe A, Cachovan G, Smeets R, Simon R, Sauter G, Heiland M and Blessmann M: CCND1 amplification and cyclin D1 immunohistochemical expression in head and neck squamous cell carcinomas. Clin Oral Investig 18: 269-276, 2014.

39. Zhang B, Liu W, Li L, Lu J, Liu M, Sun Y and Jin D: KAI1/CD82 and cyclin D1 as biomarkers of invasion, metastasis and prognosis of laryngeal squamous cell carcinoma. Int J Clin Exp Pathol 6: 1060-1067, 2013.

40. Liang S, Zhang S, Wang P, Yang C, Shang C, Yang J and Wang J: LncRNA, TUG1 regulates the oral squamous cell carcinoma progression possibly via interacting with Wnt/beta-catenin signaling. Gene 608: 49-57, 2017.

41. Gonzalez-Avila G, Sommer B, Mendoza-Posada DA, Ramos C, Garcia-Hernandez AA and Falfan-Valencia R: Matrix metalloproteinases participation in the metastatic process and their diagnostic and therapeutic applications in cancer. Crit Rev Oncol Hematol 137: 57-83, 2019.

42. Rosenthal EL and Matrisian LM: Matrix metalloproteases in head and neck cancer. Head Neck 28: 639-648, 2006.

43. Fingleton B, Vargo-Gogola T, Crawford HC and Matrisian LM: Matrilysin [MMP-7] expression selects for cells with reduced sensitivity to apoptosis. Neoplasia 3: 459-468, 2001.

44. Vento SI, Jouhi L, Mohamed H, Haglund C, Mäkitie AA, Atula T, Hagström J and Mäkinen LK: MMP-7 expression may influence the rate of distant recurrences and disease-specific survival in HPV-positive oropharyngeal squamous cell carcinoma. Virchows Arch 472: 975-981, 2018.

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