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STIL is upregulated in nasopharyngeal carcinoma tissues and promotes nasopharyngeal carcinoma proliferation, migration and invasion

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Nasopharyngeal carcinoma (NPC) is the most common primary malignancy that originates from the nasopharynx. Some regulatory networks involved in nasopharyngeal carcinoma have been reported, but the relevant genes have not been fully identified. We have used mRNA microarray to identify differential expression genes between NPC tissues and adjacent normal tissues. Then high-content shRNA screening was carried out to screen the genes that may control proliferation in nasopharyngeal carcinoma. Cell proliferation was monitored by MTT assays and Celigo image cytometry *in vitro* and by subcutaneous transplantation model *in vivo*. Flow cytometric analysis was carried out to detect the distribution of cell cycle stages and apoptosis. Transwell assay was performed to measure the migratory and invasive capacities of NPC cells. We identified 20 genes that potentially play an important role in the proliferation of nasopharyngeal carcinoma by mRNA microarray and functional analysis. The result of high-content shRNA screening indicated that STIL had the greatest effect on reducing the proliferation rate of NPC cells. The analysis of The Cancer Genome Atlas (TCGA) data showed that STIL was upregulated in several human cancer tissues, and higher STIL expression level was correlated with shorter survival time. STIL knockdown also inhibited NPC cell migration and invasion, promoted G1/S phase transition and apoptosis. Three genes including ITGA2, SMAD2, JAK1, associated with molecular mechanisms of cancer were influenced by downregulating STIL. Our study confirmed STIL as a key regulator that promotes the proliferation of NPC, providing insight into the molecular mechanisms of nasopharyngeal carcinoma and suggesting a novel therapeutic strategy.

Key words: STIL, nasopharyngeal carcinoma, cell proliferation, high-content shRNA screening, mRNA microarray

Nasopharyngeal carcinoma (NPC) is generally acknowledged as being one of the most common primary malignancies that originates from the nasopharynx, with the highest incidence in southeast Asia [1]. Annually, more than 70% of the 87,000 new cases of NPC are diagnosed as locoregionally advanced disease worldwide [2]. Radiotherapy combined with chemotherapy is the standard treatment for locoregionally advanced NPC patients [3, 4]. Currently, locoregional control of NPC has improved substantially and distant metastasis is known to be the main reason for treatment failure [5, 6]. The tumor-node-metastasis (TNM) staging system is the key determinant for treatment decisions. NPC patients with the same TNM stage receive similar treatments, but 30–40% of patients eventually develop distant metastasis after treatment [7]. Hence, the current anatomical-based staging system is not sufficient to predict the distant metastasis. The carcinogenesis of NPC is a complex process that involves the accumulation of genetic and epigenetic alterations [5, 8]. Identifying genes associated with NPC rapid growth and distant metastasis might lead to novel therapeutic strategies or more accurate prognostic prediction.

To screen for abnormally overexpressed genes in NPC, six pairs of matched nasopharyngeal carcinoma and adjacent normal tissues were subjected to a gene expression profile microarray analysis. High-content shRNA screening was carried out to verify the function of the identified candidate genes. We found that the centriolar replication factor STIL, may play a vital role in NPC proliferation.

The human STIL gene was initially identified in a common chromosomal rearrangement in T-cell acute lymphoblastic leukemia and named SCL/TAL1 Interrupting Locus (SIL/STIL) [9, 10]. STIL contains conserved regions and interacts

with several proteins, such as PLK4, CDK1, SASS6 and CPAP [11, 12]. Increasing evidence indicates that upregulation of STIL in differentiated tissues triggers centrosomal amplification and is associated with an increased metastatic potential in multiple cancers [13–15]. Thus, targeting STIL may be a promising anticancer strategy.

In this study, we demonstrate that STIL can promote NPC proliferation *in vitro* and *in vivo* and find some STIL-interaction genes, which were involved in molecular mechanisms of cancer. This result provides insight into the molecular mechanisms of NPC and suggests a novel therapeutic strategy.

Materials and methods

Ethical statement. This study was approved by the Human Research Ethics Committees at the Affiliated Foshan Hospital of Sun Yat-Sen University (Guangdong, China), and was carried out in accordance with the principles embodied in the Declaration of Helsinki. All animal treatments were carried out according to the US Public Health Service Policy on the Humane Care and Use of Laboratory Animals. All surgical procedures were conducted under sodium pentobarbital anesthesia and every effort was made to minimize animal suffering.

Cell culture. CNE-2Z cell lines were purchased from Genechem (Shanghai, China). The cell line was previously authenticated via STR typing (Suppl. file 1). CNE-2Z cells were grown in RPMI1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

Human tissue samples. All clinical samples in this study were obtained from the Affiliated Foshan Hospital of Sun Yat-Sen University. Tumor and paired adjacent normal tissue specimens were collected from 6 patients diagnosed with NPC. None of them had received chemoradiotherapy or surgery. All of the tissue specimens were obtained during endoscopic biopsy of the nasopharynx. The adjacent non-tumor tissues were taken at least 1 cm apart from the nasopharyngeal carcinoma tissue.

RNA microarrays. Total RNA was extracted from the tissue samples and cell line using TRIzol (Invitrogen, USA). RNA quantity and quality were measured by NanoDrop ND-1000 and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology).

High-content screening and cell growth curve analysis. CNE-2Z cells were transfected with a gene knockdown (GENE-KD) or negative control (NC) lentivirus and seeded into 96-well plates. GFP expression was observed using a fluorescence microscope. The cells were collected for further experiments when they reached 80–90% confluency. A total of 2,000 cells per well were analyzed once a day using a Celigo System (Nexcelom). Cells were quantified by measuring the green fluorescence signal in each well. Data were collected for statistical analysis using 5-day cell proliferation curves. Image analysis software was used to count the cells on the scanned image. The number of cells at each time point was compared with the cell count on day 1 to obtain a cell proliferation ratio for each time point for each group, and the fold change in proliferation was used to produce a cell growth curve. The cell proliferation ratio was calculated as follows: fold change (NC vs GENE-KD group) = proliferation ratio on day 5 for the NC group/proliferation ratio on day 5 for the GENE-KD group.

Animals. Female 4-week-old BALB/c nude mice were purchased from LingChang Bioscience (Shanghai, China). All mice were maintained under specific pathogen-free conditions.

Subcutaneous transplantation of CNE-2Z cells in BALB/c nude mice. Female 4-week-old BALB/c nude mice were used in all experiments. A total of 4×10^6 transfected cells were subcutaneously injected into the right armpit of BALB/c nude mice. The weight of each mouse and the tumor diameter were measured every workday from day 8 post-injection. All mice were killed 2 weeks after injection.

Statistical analyses. Results for continuous variables are presented as means ± S.D. unless otherwise stated. Comparisons between two groups were analyzed using the independent sample t-tests, paired t test or Mann-Whitney U tests if appropriate. Two-sided p-value of <0.05 was considered statistically significant. These analyses were performed using Graphpad Prism software (version 5.1) and SPSS (version 20.0).

Results

Identification of differentially expressed mRNAs in tumor tissues compared with adjacent normal tissues. To identify critical gene that contributes to NPC tumorigenesis, NPC tissues and paired adjacent normal tissues from 6 patients were subjected to mRNA microarray analysis. As shown in Figure 1A, the results identified 3,191 upregulated and 2,546 downregulated mRNAs in the NPC tissues. On the basis of a functional analysis and literature review, 20 genes were found to potentially play an important role in the proliferation of cancer (Suppl. file 2).

Identification of STIL as a gene that promotes NPC proliferation. All 20 candidate genes were then silenced in CNE-2Z cells to examine the potential effect on the proliferation of NPC cells *in vitro* (Figure 1B, Suppl. file 3). Knockdown of two candidate genes, STIL and KLHL42, in CNE-2Z cells reduced the cell proliferation rate to >2 fold than negative control (Figures 1B and C). STIL knockdown showed the greatest effect on reducing the proliferation rate and thus we focused on STIL in subsequent studies.

Upregulation of STIL in tumor is associated with poor patient prognosis. Interestingly, we analyzed the TCGA databases and found that STIL expression was also signifi-

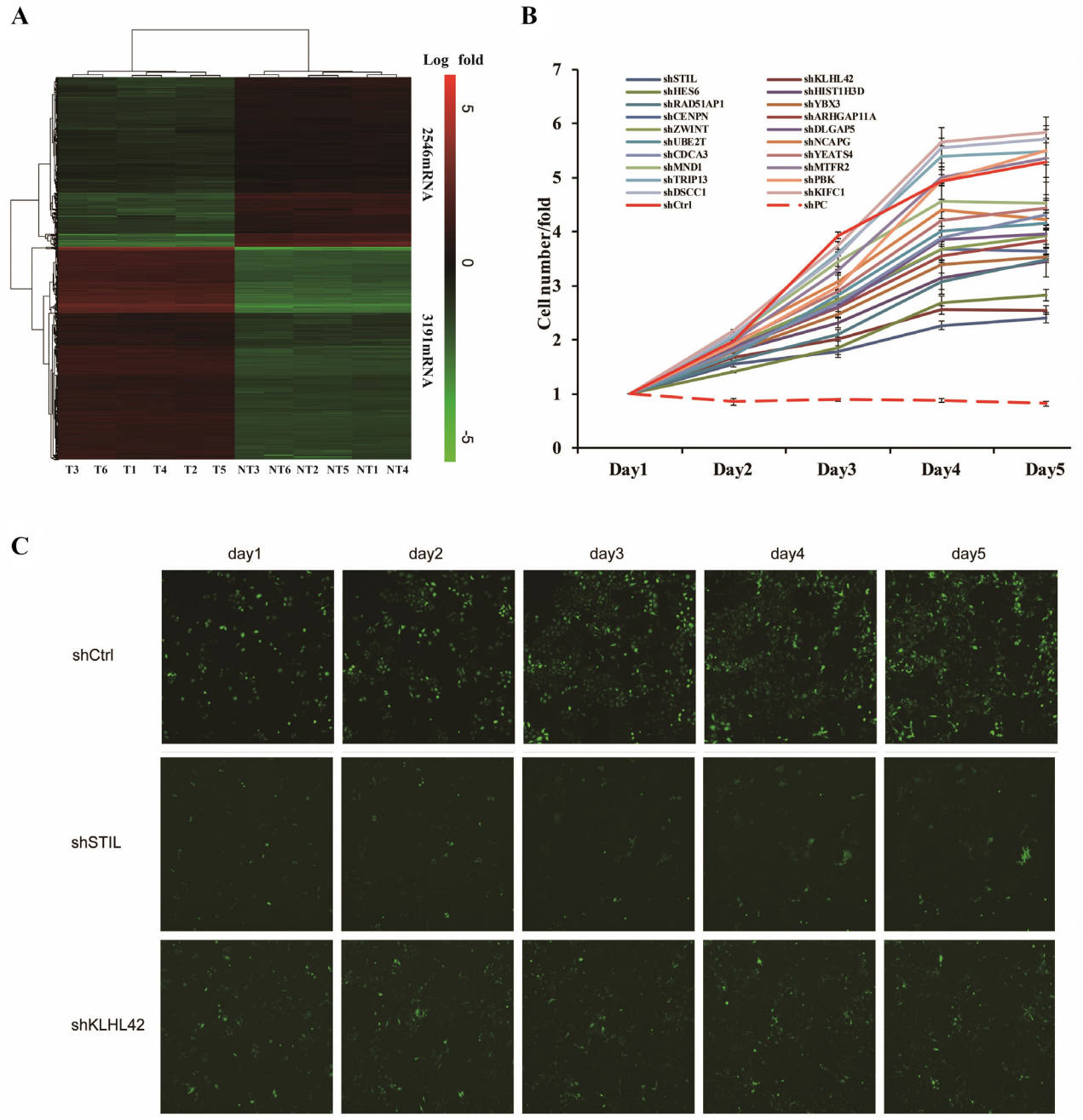

Figure 1. Agilent mRNA microarray and high-content shRNA screening identified STIL as a critical gene in promoting NPC proliferation. A) Heat map showing gene expression profiles. Each row represents a gene and each column represents a sample. Red indicates high expression, whereas green indicates low expression. T, tumor; NT, adjacent normal tissue. B) A total of 20 genes were selected for validation by high-content screening. NC: negative control shRNA, PC: positive control shRNA targeting β-actin. C) Representative fluorescence images of high-content shRNA screening for STIL and KLHL42.

cantly upregulated in glioblastoma multiforme, kidney renal clear cell carcinoma, head and neck squamous cell carcinoma, lung squamous cell carcinoma, liver hepatocellular carcinoma, breast invasive carcinoma and they are consistent with our results [16, 17] (Figures 2A–F). Moreover, higher expression of STIL is correlated with poor prognosis in kidney renal clear cell carcinoma and liver hepatocellular carcinoma [16], further supporting the oncogenic role of STIL in cancer (Figures 2G and H).

Promotion of STIL in NPC cell proliferation, migration and invasion. To reveal the role of STIL in nasopharyngeal carcinoma progression, we designed a STIL shRNA

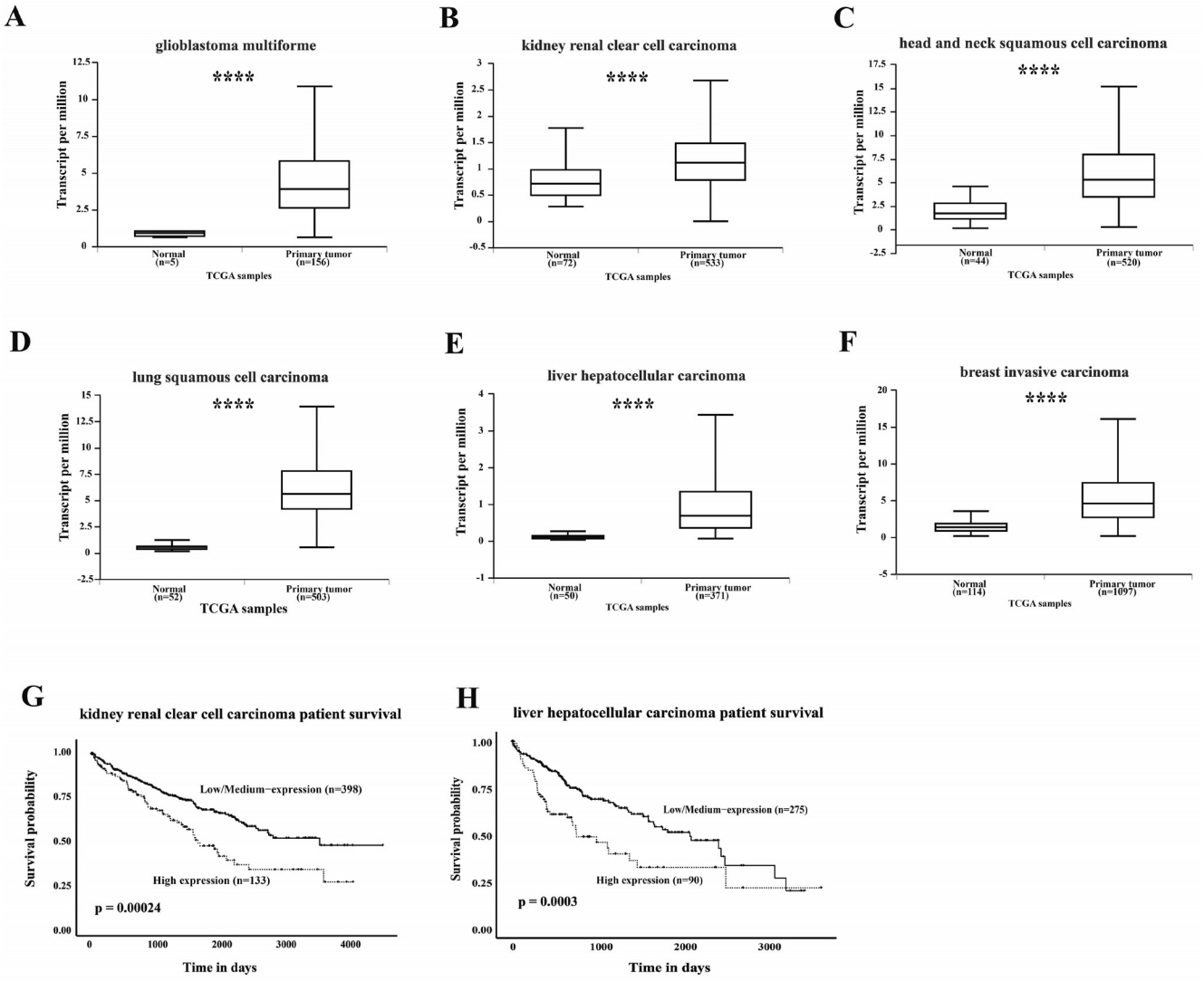

Figure 2. STIL is overexpressed in multiple types of human cancer. A–F) Data from TCGA databases were analyzed in different types of cancers as indicated. The Mann-Whitney U test was used to compare differences between the two groups. ****p<0.0001. G–H) TCGA data showed that STIL overexpression correlates with poor prognosis of kidney renal clear cell carcinoma and liver hepatocellular carcinoma.

with stable silencing efficiency by lentiviral transduction and confirmed downregulation of both STIL protein and mRNA in CNE-2Z cell line (Figures 3A and B). Cell growth curve analysis using a fluorescence imaging system and MTT assay indicated that STIL knockdown suppressed NPC cell proliferation (Figure 3C and D). To further explore whether STIL plays a key role in NPC proliferation *in vivo*, a subcutaneous xenograft model of CNE-2Z cells in BALB/c nude mice was established. The STIL knockdown (STIL-KD) group showed a slower increase in tumor volume and weight compared with negative controls (Figures 3E–G). Furthermore, cell migration assay and cell invasion assay showed that STIL knockdown suppressed the migration and invasion of CNE-2Z cells (Figure 4).

Promotion of G1/S phase transition and apoptosis and influence of the genes involving in cancer by STIL knockdown. We used flow cytometry to explore whether STIL promotes NPC proliferation through regulating the cell cycle and/or apoptosis. Flow cytometry analysis of the cell cycle in STIL-KD and NC CNE-2Z showed that the fraction of cells in S phase increased, while the fraction of cells in G2/M phase decreased compared with NC group. Moreover, the apoptotic ratio increased significantly in STIL-KD group (Figures 5A and C). Independent experiments were performed to validate the results (Figure 5B and D).

To further elucidate the mechanisms underlying STIL promotion of NPC proliferation, mRNA microarray was performed in STIL-KD CNE-2Z cells and empty vector-

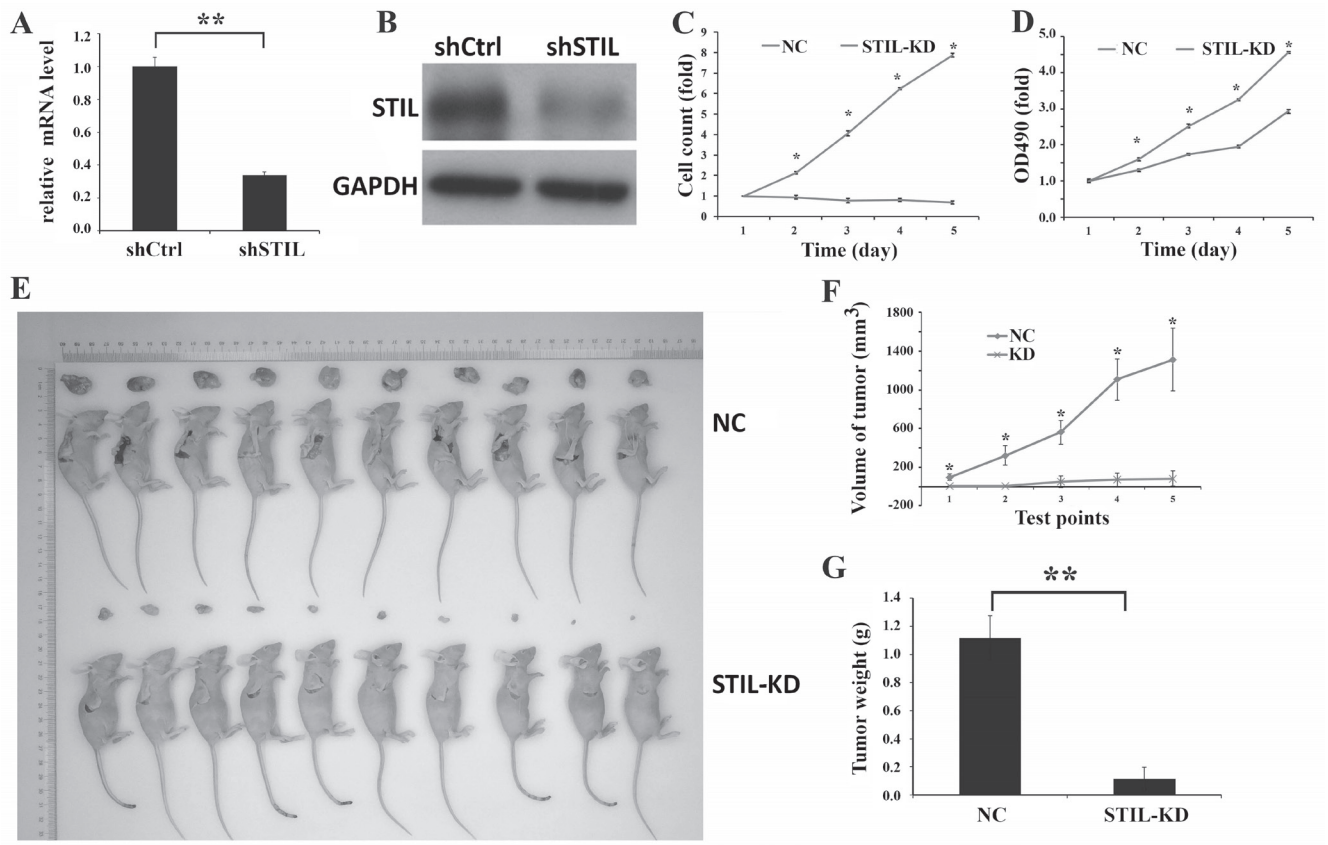

Figure 3. STIL knockdown affects NPC cell proliferation *in vivo* and *in vitro*. A and B) CNE-2Z cells were transfected with control (ctrl) shRNA, STIL1 shRNA. qRT-PCRs (A) and western blot (B) were performed to detect STIL levels. C) Cell growth curve analysis comparing STIL knockdown (STIL-KD) with negative control (NC) NPC cells. n=3, *p<0.05. D) MTT assays comparing proliferation of STIL-KD and NC NPC cells. n=3, *p<0.05. E) Images of the subcutaneous xenografts from the STIL-KD and NC groups. n=10. Tumor volume growth curves (F) and tumor weight (G) for subcutaneous xenografts. *p<0.05. Tumor volume = π/6 × long diameter × the short diameter × the short diameter. *p<0.05, **p< 0.01.

transduced cells. We found 325 upregulated and 457 downregulated genes with significant difference (|Fold Change|>1.5 and FDR<0.05). Network analysis using data obtained from mRNA microarrays predicted some STIL-associated genes (Figures 6A). Western blot and q-PCR analysis showed that ITGA2, ITGAV, SMAD2, JAK1 were downregulated significantly in STIL-KD cells (Figures 6B and C). Most of them (3/4, ITGA2, SMAD2, JAK1) are the key genes involved in molecular mechanisms of cancer, an ingenuity canonical pathway [18].

Discussion

The centrosome is a cytoplasmic organelle built around microtubule-based core components called centrioles. Centrosomes are essential for chromosomal stability, and abnormalities of their number, or structure affect cell division [12]. Many kinds of cancers are associated with centrosome dysfunction [13–15]. STIL gene encodes a regulatory protein necessary for centriole biogenesis and plays the central role in maintaining centrosome integrity in highly proliferating cells [11, 13–15]. Overexpression of STIL, which results in supernumerary centrosomes, could lead to cancer because of chromosomal instability [19]. Although STIL has been found upregulated in several cancers, such as lung cancer, colon carcinoma, ovarian cancers and so on, the role of STIL in regulating the proliferation of nasopharyngeal carcinoma cells has not yet been elucidated.

STIL promoting SHH signaling could be another pathway to account for its association with cancer [11]. Sonic hedgehog (SHH) is a morphogen involved in proliferation and survival of neural stem cells and STIL participates in the control of SHH signaling [20]. STIL can bind to suppressor-of-fused homolog (SUFU) and SUFU is a negative regulator of SHH [21]. In PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla, downregulating STIL would decrease the SHH signaling and cell proliferation [22]. Similar results have been evidenced in other cancer cell lines, such as H1299, Hela, PC3 and LS174T cells [23]. These further suggested that STIL plays a role in cell proliferation through the SHH pathway [24].

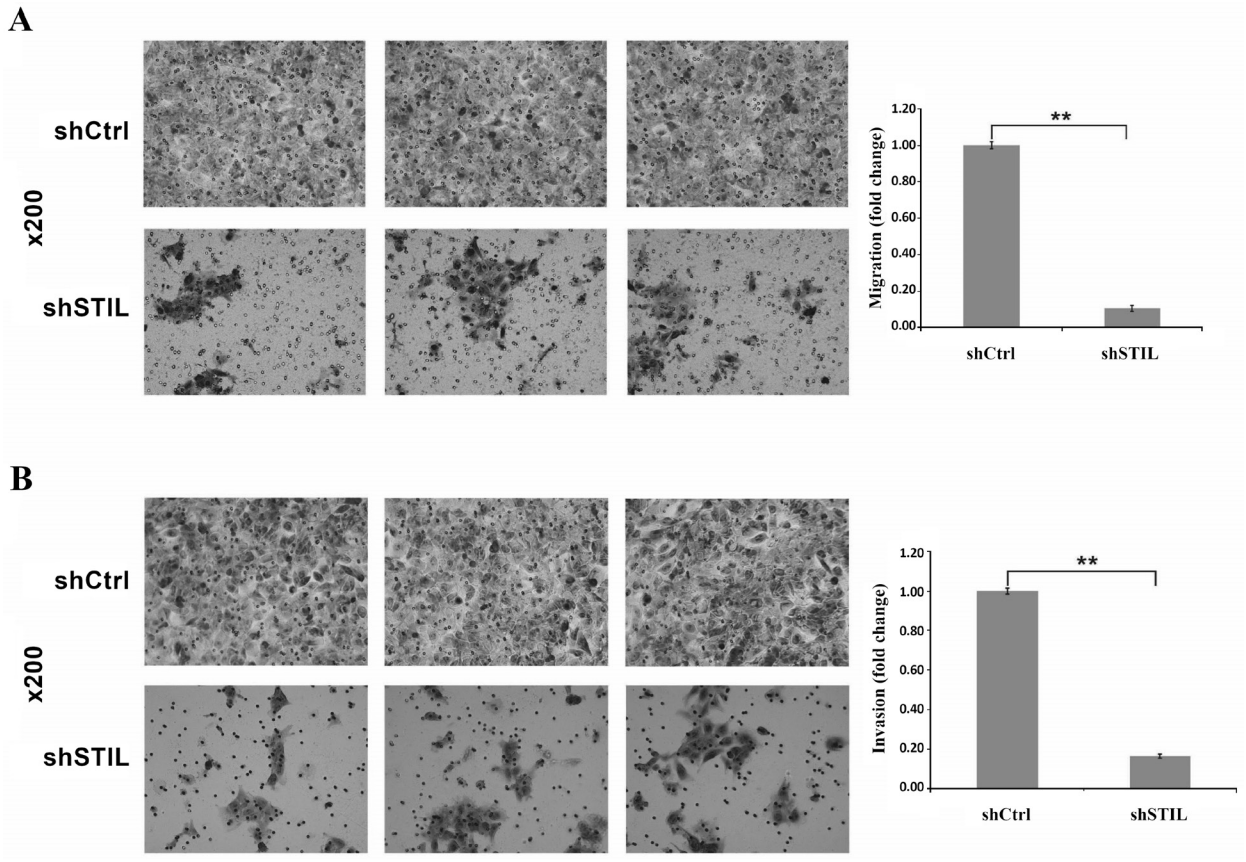

Figure 4. Transwell migration assay (A) and invasion assay (B) showed that STIL knockdown suppressed NPC cell migration and invasion. **p<0.01.

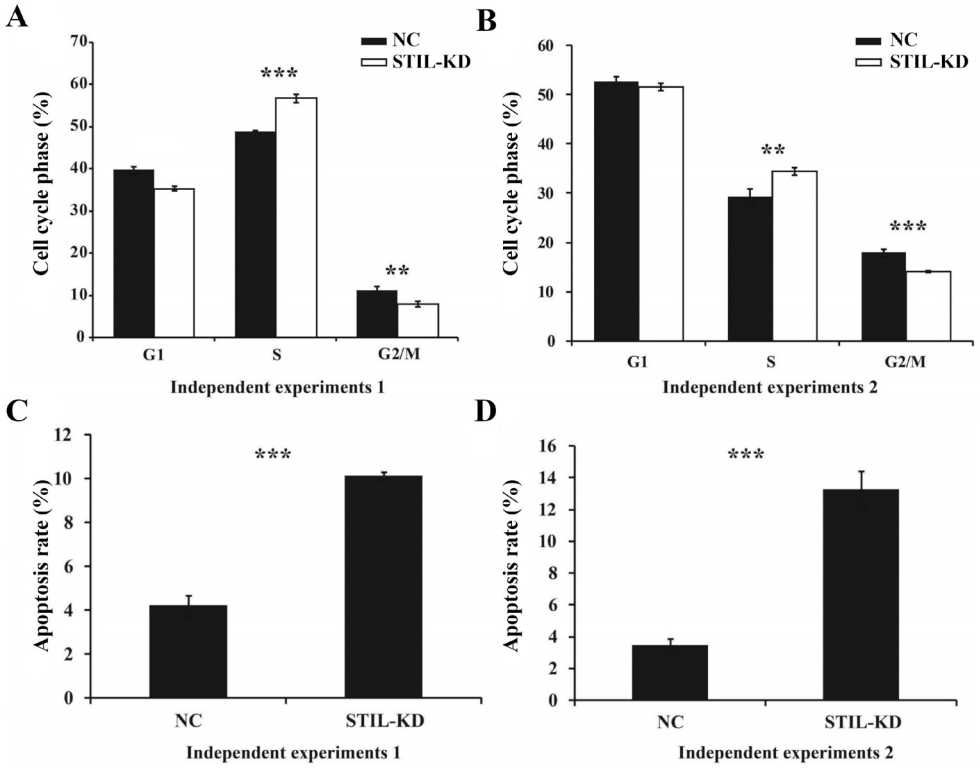

Figure 5. STIL knockdown promotes G1/S phase transition and apoptosis. A and B) Flow cytometry analysis of the cell cycle in STIL knockdown (STIL-KD) and negative control (NC) CNE-2Z cells. C and D) Flow cytometry analysis of apoptosis in STIL-KD and NC CNE-2Z cells. n=3. **p<0.01, ***p<0.001.

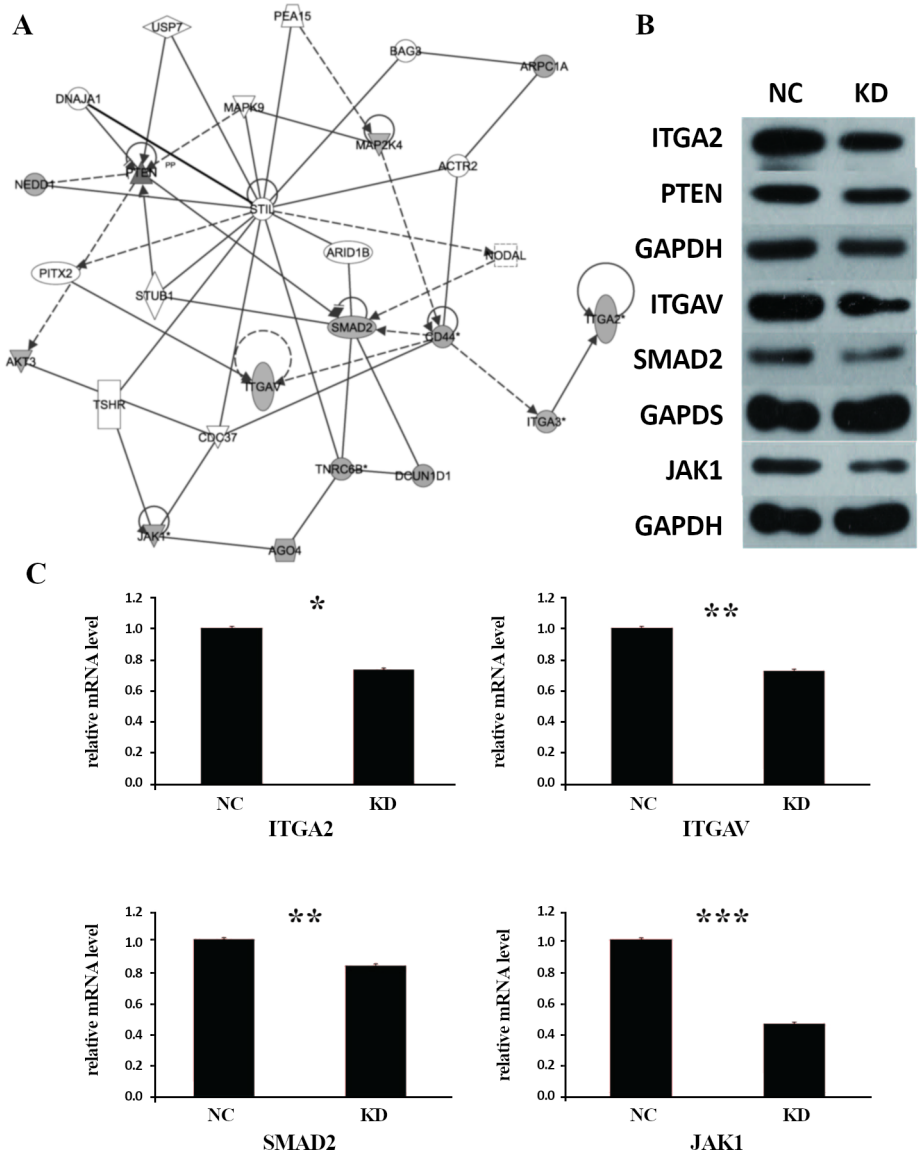

Figure 6. STIL knockdown influence the genes that participate in molecular mechanisms of cancer. A) Network analysis of STIL-associated genes using mRNA microarrays data obtained from STIL knockdown and empty vector-transduced CNE-2Z cells. B–C) Protein and relative mRNA expression levels (in relation to GAPDH) of STIL-associated genes were analyzed by western blotting (B) and qRT-PCRs (C) in STIL knockdown (KD) and negative control (NC) CNE-2Z cells. *p<0.05, **p<0.01, ***p<0.001.

participate in centromeric replication and number regulation. Among them, CPAP/HsSAS-4 is required for centromeric replication; STIL/SIL binds to SAS-6 to participate in the assembly of the original centrosome. Mutations in these proteins can cause centrosome abnormalities in varying degrees. In addition, the stability of STIL expression levels in the cytoplasm is affected by STILT (STIL truncating mutations). More importantly, STILT can also be clustered around and separated from the centrosome like STIL. The two genes play a consistent role in promoting centrosome synthesis, indicating that the true role of STILT is to affect centrosome amplification.

This study reports that STIL promotes the proliferation of NPC cells and STIL knockdown influences the NPC cell cycle and apoptosis. We also found that the expressions of 3 genes (ITGA2, SMAD2 and JAK1), which were associated with molecular mechanisms of cancer, were influenced by downregulating STIL. Based on the results of our research, we suggest that STIL may promote the migration and invasion of nasopharyngeal carcinoma through the mechanistic association with the three abovementioned genes. This study did not investigate the role of STIL in regulating centromere function during the metastasis in nasopharyngeal carcinoma, so it cannot be ruled out whether this metastatic activity responds

to STIL's role in regulating centrosome biogenesis. To confirm further the role of STIL in nasopharyngeal carcinoma, we will next explore the oncogenic mechanism of STIL and the aforementioned three genes, and further discuss whether the role of STIL in regulating centrosome biogenesis will affect the metastasis activity of nasopharyngeal cancer cells.

In conclusion, our study provides evidence that supports STIL as a key regulator that promotes the proliferation of NPC. The result provides insight into the molecular mechanisms of nasopharyngeal carcinoma and suggests a novel therapeutic strategy.

Supplementary information is available in the online version of the paper.

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