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ORIGINAL ARTICLE

Non-Coding RNA Pvt1 Promotes Cancer Stem Cell–Like Traits in Nasopharyngeal Cancer via Inhibiting miR-1207

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Abstract

Nasopharyngeal carcinoma (NPC) is a kind of head-neck malignant tumor. lncRNA-PVT1 can promote the proliferation of carcinoma cells, and induce cells to have stem cell-like potentials. However, the function of PVT1 in NPC cells is not clear. The expressions of lncRNA-PVT1 and the expressions of the stem cell markers in NPC tissues or cell lines were investigated by qRT-PCR or western blot. The cell proliferation, and the ability of NPC cells to form spherical, clonal colonies were investigated by MTT assay, colony formation assay, and tumor-sphere formation assay. Cancer stem cells surface markers were detected by flow cytometry and western blot. PI3K/AKT signal activation in NPC cells was determined by western blot. PVT1 was significantly up-regulated in both NPC tissues and cell lines and associated with poor prognosis. PVT1 knockdown reduced NPC cells viability, clonogenicity, the cell surface CD44+/CD24− stem phenotype, and the expressions of the stem cell markers in NPC cells, including Oct4, c-Myc, SOX2, and ALDH. Furthermore, PVT1 negatively regulates the expression levels of miR-1207 in NPC cells and spheres cells, which is critical for NPC stemness. Knockdown of miR-1207 promoted stem phenotype and the expressions of the stem cell markers in NPC cells. Moreover, phosphor-PI3K (p-PI3K) and phosphor-AKT (p-AKT) were found to be down-regulated after PVT1 siRNAs transfection in NPC cells. And miR-1207 inhibitor transfection reversed the all the effects brought by PVT1 knockdown. Pvt1 promotes cancer stem cell–like properties in NPC cells via inhibiting miR-1207 and activating the PI3K/AKT signal pathway.

Keywords PVT1 · Nasopharyngeal carcinoma · miR-1207 · PI3K/AKT · Tumor stem cell

Introduction

Nasopharyngeal carcinoma (NPC) is a kind of head-neck malignant tumor with its distribution featured by distinct territoriality and ethnicity [1]. Compared with other head-neck tumors, NPC at the early stage is easier to transfer to cervical lymph nodes [1]. Besides, patients have always had an unsatisfactory treatment effect, mostly suffering from death due to recurrence or distant metastasis after treatment [1, 2]. The incidence of NPC is a multi-factor, multi-step process, involving multiple factors and extremely complicated molecular mechanisms, which currently are still unclear [1, 2]. Tumor stem cells (CSCs), a small group existing in tumor cells, have the abilities of tumorigenesis and self-renewal, and their "stemness" is the main cause leading to tumor formation, proliferation, and recurrence and metastasis [3, 4]. CSCs are associated with tumor recurrence and proliferation, which has been well verified [3, 4]. Most solid tumors, such as breast cancer, cerebral cancer, prostate cancer, uterus cancer and colorectal cancer, contain those tumor-initiating cells of a small number [4, 5]. Conventional anticancer therapies can inhibit/kill some heterogeneous tumor cells, leading to tumor volume reduction, but the presence of CSCs may result into tumor recurrence [4, 5].

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With the wide application of RNA-sequencing (RNA-seq) technology, people have gotten a deeper understanding of biological transcriptome [6]. Initially, there was a consensus in the scientific community that only less than 2% of transcripts of mammalian genomescould encode proteins, and over 98% didn't have the ability of protein coding, thus named as noncoding RNA (ncRNA) and viewed as "evolutionary junk" [7]. However, there is growing evidence that those ncRNAs, as an important regulatory factor of gene expression, are participating in the evolution process of a wide range of human diseases, playing an especially prominent role in the development of tumors [8]. LncRNAs are a class of ncRNAs that are more than 200 nt in length and usually transcribed from RNA polymerase II, having the structural features similar to those of mRNAs, such as 5′ cap, poly (A) tail, and splice sites [9]. LncRNA is the necessary regulatory factor of many developmental pathways, including maintaining the pluripotency of stem cells, regulating apoptosis, producing erythrocytes, and differentiating horn cells [7, 10]. In osteosarcoma, the expression of lncRNA HIF2PUT affects the proliferation and metastasis of stem cells and is associated with the expression of HIF-2α, suggesting its potential as a therapeutic target [11]. In breast cancer cells, lncRNA-Hh enhances the stemness of breast cancer cells by activating Twist-induced SHH-GLI1 pathway [12]. LncRNA is becoming an important regulator in stem cell biology studies.

Human PVT1 gene is also known as plasmacytoma variant translocation gene 1, of which the transcript belongs to the intergenic lncRNA, homologous to the transcript of mouse Pvt1 gene [13]. The PVT1 gene is located in the chromosome 8q24 region and on the plus strand of the chromosome, spanning over a genome interval of more than 300 kb. PVT1 often generates translocation in human Burkitt lymphoma and mouse plasmacytoma [14]. For the spatial position, PVT1 is at the end of the oncogene c-myc 3' [13, 14]. These findings indicate that the abnormal expansion of PVT1 is likely to be associated with the development and progression of tumors [13]. Moreover, in consideration of the wide distribution of c-myc RNA, the expression level of PVT1 in normal tissues is relatively low [13, 15, 16]. In addition, Huppi K et al. analyzed the 8q24 gene desert region where chromosome breakpoint is often occurring, and found by bioinformatics analysis and q-PCR technical verification that the PVT1 loci in 8q24 region can transcribe six miRNAs, namely miR-1204, miR-1205, miR-1206, miR-1207-3p, miR-1207-5p and miR-1208 [17]. Therefore, the relationship of PVT1 with tumor genesis and development has attracted widespread attention.

Studies found that the abnormal expression of PVT1 is associated with the genesis and development of several cancers (reviewed in [18]). Currently, the high expression of PVT1 has been found in many tumors, such as lymphoma, pancreatic cancer, prostate cancer, gastric cancer, liver cancer, non-small cell lung cancer, colorectal cancer, ovarian cancer, and breast cancer, suggesting that PVT1 is of great guiding significance in the early diagnosis of cancers, prognostic assessment and targeted therapy [18–26]. Wang et al. [27] found that in fetal and adult mouse livers, Pvt1 as a carcinoembryonic RNA can promote cell proliferation and also induce the stem cell-like potentialsof mouse cells, while PVT1 is also highly expressed in human hepatocellular carcinoma, which indicates a poor prognosis. In vitro and *invivo* functional experiments showed that PVT1 can also promote the proliferation of hepatocellular carcinoma cells, and induce cells to have stem cell-like potentials. However, the role of PVT1 in NPC, especially on the regulation of cancer "stemness", is not yet clear. In this study, we found that the expression of PVT1 in NPC tissues and cell lines were significantly higher than that in the control tissues or cell lines. PVT1 can promote cancer stem cell–like properties in NPC cells via inhibiting miR-1207 and activating the PI3K/AKT signal pathway. Our novel findings provide a potential target for the treatment of NPC patients, and have great theoretical and clinical value to improve the survival rate of patients.

Materials and Methods

Clinical Specimens and Cell Culture

Fifteen NPC samples and corresponding noncancerous nasopharyngeal epithelial tissues were collected from Affiliated Cancer Hospital of Zhengzhou University. Written informed consent was obtained from all patients. All tissue samples were stored at −80 °C until use. Normal nasopharyngeal epithelial cells NP69 was cultured in keratinocyte-SFM (Invitrogen, USA) supplemented with growth factor and bovine pituitary extract. NPC cell lines 5-8F, HNE1, HNE2, CNE1 and CNE2 were provided by the cancer center of Central South University (Changsha, China),were cultured in RPMI-1640 medium (Gibco, USA) or Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C, and 5% CO2. All the experiments were approved by the Ethics Committees of Affiliated Cancer Hospital of Zhengzhou University.

RNA Extraction and qRT-PCR

RNA isolation was performed with RNeasy Plus Micro Kit (QIAGEN, USA) according to the manufacturer's instructions. Then, reverse transcription was conducted to synthesize the cDNA by utilizing the SuperScript® IV First-Strand Synthesis System (Invitrogen, USA). qRT-PCR was performed in Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, USA), using 20 ng template in 25 μl reaction volume with 1 x Power SYBR® Green PCR Master Mix (Invitrogen, USA) and gene specific primer pairs. The GAPDH was used as an internal control. The sequences of PCR primers were listed in Table 1. All data are displayed as the mean ± SD of three independent experiments.

Table 1 The sequences for RT-qPCR primer

gene name	sequence (5′-3′)
Oct4-F	CCCGAAAGAGAAAGCGAACC
Oct4-R	GCAGCCTCAAAATCCTCTCG
c-myc-F	GGACTTGTTGCGGAAACGAC
c-myc-R	CTCAGCCAAGGTTGTGAGGT
Sox2-F	CATGTCCCAGCACTACCAGA
Sox2-R	TTTGAGCGTACCGGGTTTTC
ALDH1-F	GATCCCCGTGGCGTACTATG
ALDH1-R	TGGATCTTGTCAGCCCAACC
PVT1-F	AAAACGGCAGCAGGAAATGT
PVT1-R	GGAGTCATGGGTGTCAGACA
hsa-miR-1204-F	AGCTCGTGGCCTGGTCTC
hsa-miR-1204-RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACATAATG
hsa-miR-1204-R	CGGCCCAGTGTTCAGACTAC
hsa-miR-1207-F	AGTGGCAGGGAGGCTGG
hsa-miR-1207-RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCCCCTC
hsa-miR-1207-R	CGGCCCAGTGTTCAGACTAC
hsa-miR-1205-F	GCGTGTGCAGGGTTTGC
hsa-miR-1205-RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCTCAAA
hsa-miR-1205-R	CGGCCCAGTGTTCAGACTAC
hsa-miR-1206-F	TCGCGCTGTTCATGTAGATGT
hsa-miR-1206-RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACGCTTAA
hsa-miR-1206-R	CGGCCCAGTGTTCAGACTAC
hsa-miR-1208-F	CGCGCTCACTGTTCAGACA
hsa-miR-1208-RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTCCGCC
hsa-miR-1208-R	CGGCCCAGTGTTCAGACTAC
GAPDH-F	CCAGGTGGTCTCCTCTGA
GAPDH-R	GCTGTAGCCAAATCGTTGT
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT

F, forward; R, reverse; RT, reverse transcription

Cell Transfection

Hsa-miRNA-1207 inhibitor/negative control inhibitor and siRNAs against PVT1(si-PVT1) were purchased from GenePharma (Shanghai, China). The sequences for hsa-miRNA-1207 inhibitor were 5′-CCCCUCCCAGCCUCCCUGCCA-3′, The si-RNA sequence for PVT1 were si-PVT1–407, 5′-GCUGAAUGCCUCAUGGAUUTT-3′, si-PVT1–1907, 5′-CCGGCCUCGUGUCUAUUAATT-3′. The cells were plated in 6-well plates for 12 h and then transfected with the si-PVT1 or miR-1207-inhibitor using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

MTT Assay

Cell proliferation of NPC cells was detected using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma, USA). The transfected cells were cultured onto 96-well plates with 100 μL of growth medium. After culturing for different days (0 d, 1 d, 2 d, 3 d, or 4 d), the medium was replaced with fresh culture medium containing 10 μL of 5 mg/mL MTT dye. After 4 hincubation at 37 °C, the MTT solution was replaced with 150 μL of dimethyl sulfoxide (DMSO). The absorbance at 490 nm was then detected with amicroplatereader (BioTek, USA).

Colony Formation Assay

The transfected cells were cultured onto 96-well plates for 24 h. Then, 2×10^2 cells were plated in 6-well plates and cultured for 2 more weeks. The plates were then washed with PBS for twice, and cells were fixed with methanol-acetic acid. At last, the cells were stained with 0.5% cristal violet. The number of colonies was counted under a microscope (Olympus, Japan).

Tumor Sphere Formation Assay

The transfected cells were cultured onto 6-well plates for 48 h, and the cells (1×10^4) were seeded in a ultra-low attachment T25 culture flask (Corning, USA) for 20 days. A DMEM/F12 serum-free medium (Invitrogen, USA) containing 5 μg/ml insulin, 20 ng/ml epidermal growth factor (EGF), 2% B27, and 20 ng/ml basic fibroblast growth factor (bFGF) was applied to culture the spheres. The numbers of tumor spheres were counted under a phase-contrast microscope (40×; Olympus, Japan).

Flow Cytometry Analysis for CSCs Surface Marker

The formed tumor spheres of NPC cells (HNE1 and CNE1) were trypsinized and resuspended (1×10^6 cells/

ml). Then, cells were washed with PBS, and stained with antibodies specific forcell surface markers used to distinguish CSCs from the bulk-tumor population: CD24-PE, and CD44-FITC (BD Bioscience, CA). The cells were incubated on ice for more than 30 min and washed twice with PBS before analysis by FACSCanto II cytometer Flow Cytometry (BD Biosciences, Germany).

Western Blot Analysis

Cells were harvestedand lysed in the RIPA buffer (Sigma-Aldrich, USA). Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, USA). Proteins (30 μg) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with BSA, the membranes were then incubated with primary antibodies against Oct4, c-Myc, SOX2, ALDH1, PI3K, p-PI3K, AKT p-AKT, and GAPDH (Abcam, USA). GAPDH was loaded as an internal reference. Bands were then treated withthegoat anti-rabbit IgG-HRP secondary antibody (1:2000; Abcam, USA). Bands were developed using chemiluminescence substance (Thermo Scientific, USA).

Statistical Analysis

Data were analyzed with Prism 5.0 (GraphPad Software, USA). The overall survival (OS) was estimated with Kaplan–Meier method. All data were expressed as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) with multiple comparisons using Dunnett's test was applied to compare the difference between groups. $P < 0.05$ was considered significantly different.

Results

PVT1 Is Up-Regulated in both NPC Tissues and Cell Lines and Associated with Poor Prognosis

The expressions of lncRNAPVT1 in 15 pairs of NPC samples and corresponding noncancerous nasopharyngeal epithelial tissues (control), and NPC cell lines (5-8F, CNE1, CNE2, HNE1 and HNE2) were investigated by qRT-PCR. As shown in Fig. 1a, the expression of PVT1 in NPC samples was significantly higher than that in the control tissues ($P < 0.001$). Furthermore, the expression of PVT1 was barely detectable in the normal nasopharyngeal epithelial cells NP69, while a much higher expression in NPC cells was observed (Fig. 1b). In order to study the correlation between the expression of PVT1 and the patient overall survival (OS), the Kaplan-Meier test was applied. As shown in Fig. 1c, patients with low PVT1 expression had significantly longer OS ($P = 0.0385$). Consistently, patients with low PVT1 expression had significantly longer disease-free survival (DFS) ($P = 0.0261$; Fig. 1d).

Knockdown of PVT1 Suppresses the Proliferation and Clonogenicity of NPC Cells

In order to further investigate the role of PVT1 in NPC tumorigenesis, two independent siRNAs were used to knock down the PVT1 expression in CNE1 and HNE1 cells(the siRNA targeted sites were described in [28]). The PVT1 expressions were significantly reduced in both CNE1 and HNE1cells when transfected with siRNAs ($P < 0.05$, versus the NC group; Fig. 2a). Next, the MTT assay was performed to identify the effect of PVT1on the cell proliferation. As shown in Fig. 2b, the cell viability of both CNE1 and HNE1cells was significantly decreased after transfection with siRNAs ($P < 0.001$, versus the NC group). Furthermore, in the colony formation assay, much fewer colonies were observed when the PVT1 was silenced in CNE1 and HNE1cells (Fig. 2c). All these indicate that PVT1could serve as an oncogene in NPC.

Knockdown of PVT1 Attenuates Cancer Stem Cell-like Properties in NPC Cells

The ability of NPC cells to form spherical, cancer stem cell-like properties was further investigated. The tumor-sphere formation assay (Fig. 3a, b) showed that PVT1 siRNAs transfection reduced the ability of NPC cells to form spheres when compared with the NC group ($P < 0.05$). Then, the role of PVT1 expression on the induction of cancer stem cell-like properties in NPC was firstly studied with cell surface markers of CSCs CD24 and CD44 by flow cytometry. As shown in Fig. 3c, the percentage of $CD44^{+}/CD24^{-}$ stem phenotype was decreased when the NPC cells were transfected with the siRNAs. Furthermore, the expressions of the stem cell markers in NPC cells, including Oct4, c-Myc, SOX2, and ALDH1, were further studied by qRT-PCR and Western blot. As expected, the mRNA (Fig. 3d) and protein (Fig. 3e) expression levels of the markers were down-regulated after the siRNAs transfection ($P < 0.05$, versus the NC group). All of these results indicate that PVT1can stimulate the stem cell-like properties of NPC cells.

PVT1 Negatively Regulates the Expression of miR-1204 and 1207 in NPC Cells

The locations of six PVT1-derived miRNAs, including miR-1204, −1205, −1206, −1207-5p/3p, and − 1208, and the siRNA targeted sites were indicated in Fig. 4a. The relative

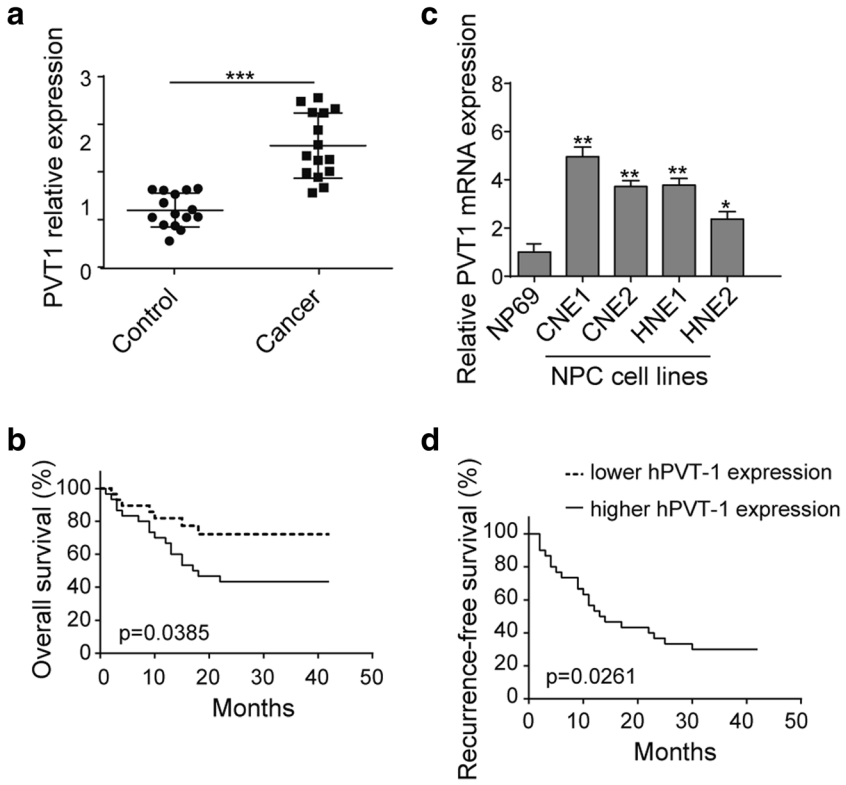

Fig. 1 PVT1 is up-regulated in both NPC tissues and cell lines and associated with poor prognosis. **a** LncRNA PVT1 is overexpressed in NPC samples ($n = 15$) and cell lines (5-8F, CNE1, CNE2, HNE1 and HNE2) detected by qRT-PCR (**a**) The mRNA expression level of PVT1 in NPC samples and corresponding noncancerous nasopharyngeal epithelial tissues (control) detected by qRT-PCR ($n = 15$). **b** The mRNA expression level of PVT1 in NPC cell lines (5-8F, CNE1, CNE2, HNE1, and HNE2) and normal nasopharyngeal epithelial cells NP69 detected by qRT-PCR. **c** Patients with low PVT1 expression had significantly longer OS ($P = 0.0385$; $n = 15$). **d** Patients with low PVT1 expression had significantly longer disease-free survival ($P = 0.0261$; $n = 15$). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$, versus the control groups

expression levels of these five miRNAs in PVT1 siRNAs transfected NPC cells were up-regulated when compared with the NC group (Fig. 4b). More specifically, the expression levels of miR-1204 and miR1207 were significantly up-regulated in CNE1 and HNE1, compared with the NC group($P < 0.05$). While the expression levels of miR -1205, −1206 and − 1208 in si-PVT1 have no difference with NC group. This indicates that knockdown PVT1 might negatively regulates the expression of miR-1204 and 1207 in NPC cells.

miR-1207 Inhibits the NPC Cell Proliferation and Stem Cell-like Properties

MiR-1204 and -1207 expression levels in the spheres formed by PVT1 siRNAs or NC siRNA transfected NPC cells were detected by qRT-PCR. As shown in Fig. 5a, the expression of miR-1207 was significantly up-regulated about 3 times in si-PVT1 group compared with the NC group ($P < 0.05$), while the change on the expression of miR-1204 was not significant. This indicates that miR-1207 might involve in the regulation of stem cell-like properties of NPC cells. Therefore, the miR-1207 was applied for the following studies. In order to investigate the effect of miR-1207 on the induction of cancer stem cell-like properties in NPC, we took the tumor-sphere formation assay. As showed in Fig. 5b, c, the ability of NPC cells to form spheres was enhanced by the transfection of miR-1207 inhibitor, which reversed the effect brought by PVT1 knockdown. Consistently, the cell surface markers of CSCs CD24- and CD44+ were studied by flow cytometry. As shown in Fig. 5d, the percentage of $CD44^{+}/CD24^{-}$ stem phenotype was increased when the NPC cells were transfected with the miR-1207 inhibitor. Also, the decreases brought by PVT1 siRNA transfection can be reversed by the co-transfection of miR-1207 inhibitor. Consistently, Furthermore, the mRNA (Fig. 5e) and protein (Fig. 5f) expression levels of the stem cell markers in NPC cells were significantly up-regulated in the miR-1207 inhibitor only transfection group ($P < 0.05$, versus the NC group), as well as in the miR-1207 inhibitor and PVT1 siRNA co-transfection group ($P < 0.05$, versus the si-PVT group). All these results indicate that miR-1207 may inhibit the NPC cell proliferation and stem cell-like properties.

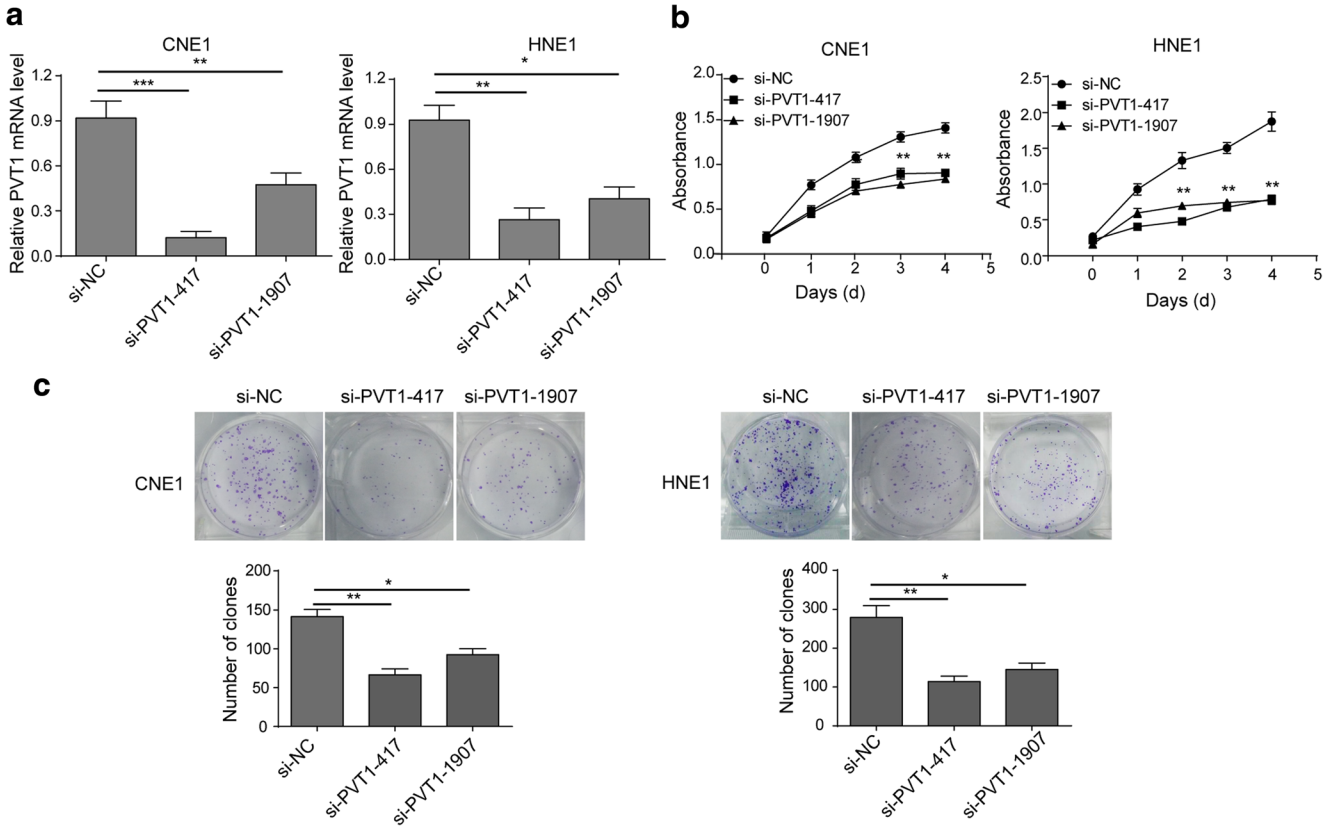

Fig. 2 Knockdown of PVT1 suppresses the proliferation and clonogenicity of NPC cells. a PVT1 expressions were significantly reduced in both CNE1 and HNE1 cells when transfected with PVT1 siRNAs. **b** The cell viability of both CNE1 and HNE1cells was significantly decreased after transfection with PVT1 siRNAs determined by MTT assay. **c** Fewer colonies were observed when the PVT1 was silenced in CNE1 and HNE1 cells. The numbers of colonies were also quantified. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, as indicated in the figure

Knockdown of PVT1 Inhibits PI3K/AKT Signal Pathway

In order to further investigate the underlying mechanism of the effect of PVT1 on the cancer stem cell-like properties in NPC cells, PI3K/AKT signal activation in NPC cells was determined by western blot analysis. As shown in Fig. 6a-d, phosphor-PI3K (p-PI3K) and phosphor-AKT (p-AKT) were found to be down-regulated after PVT1 siRNAs transfection, while this inhibition can be reversed by the co-transfection of miR-1207 inhibitor and PVT1 siRNA. These indicate that Pvt1 can promote cancer stem cell–like properties in NPC cells via inhibiting miR-1207, and further activating the PI3K/AKT signal pathway.

Discussion

Nasopharyngeal carcinoma is different from other head-neck cell carcinomas, for its patients are prone to see lymph node metastasis at the early stage and to suffer from local recurrence and distant metastasis after treatment due to drug resistance, which are the main reasons for patient's death [1, 2, 29]. In recent years, cancer stem cell (CSC) has become one of the hot spots in cancer research, and it is considered as the root of tumor growth, proliferation and recurrence [3, 4]. Previous reports indicated that CSCs,similar to embryonic stem cells (ESCs), have"stemness", i.e., the abilities of self-renewal and self-differentiation [3, 4]. This suggests that there may be common signal transduction pathways and molecular markers between CSCs and ESCs. LncRNAs are the necessary regulatory factors for many developmental pathways, including maintaining the pluripotency of stem cells, regulating apoptosis, generating red blood cells, and differentiating horn cells [7, 10]. Some studies have shown that lncRNAs can regulate the pluripotency of ESCs and induce pluripotent stem cells [30, 31]. It was reported that lincRNA-ROR was up-regulated in induced pluripotent stem cells (iPSCs), and pluripotent transcription factors (OCT4, SOX2 and NANOG) can also directly regulate the expression of ROR [32]. Wang et al. [32] verified the ROR function in ESCs, and found that ROR regulates the self-renewal and differentiation of ESCs. In addition, intracellular transcription factors (SOX2, OCT4, and KLF4) can activate tumor stem cells, while the regulation over OCT4, SOX2 and KLF4 in cells may be involved in the feedback loop of lncRNA, thus regulating the differentiation of

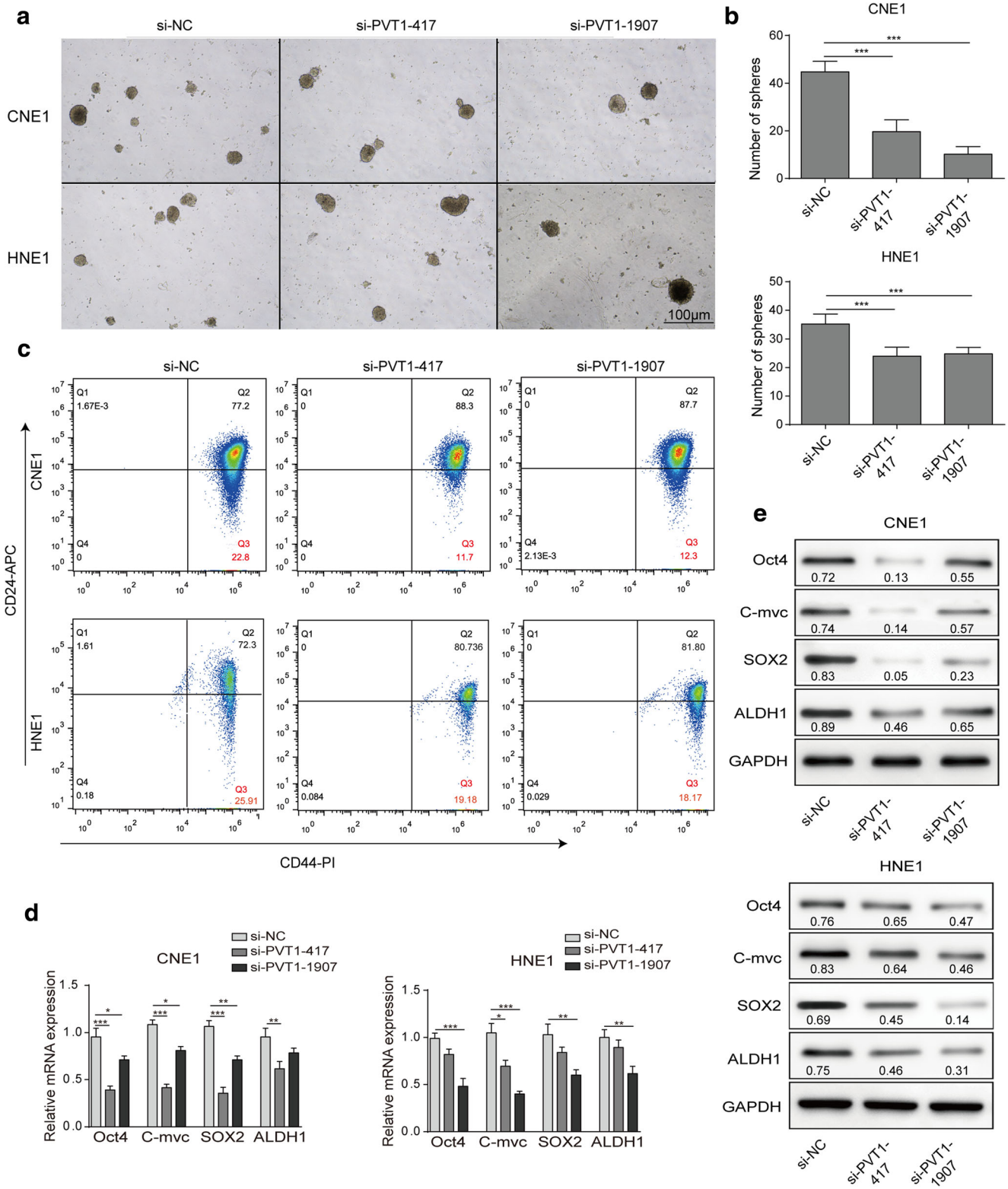

Fig. 3 Knockdown of PVT1 attenuates cancer stem cell-like properties in NPC cells. a PVT1 siRNAs transfection reduced the ability of NPC cells to form spheres when compared with the NC group. **b** The numbers of spheres were quantified. **c** The percentage of $CD44^{+}/CD24^{-}$ stem phenotype was decreased when the NPC cells were transfected with the PVT1 siRNAs detected by flow cytometry. The mRNA (**d**) and protein (**e**) expression levels of the stem cell markers in NPC cells, including Oct4, c-Myc, SOX2, and ALDH1, were down-regulated after the PVT1 siRNAs transfection. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, as indicated in the figure

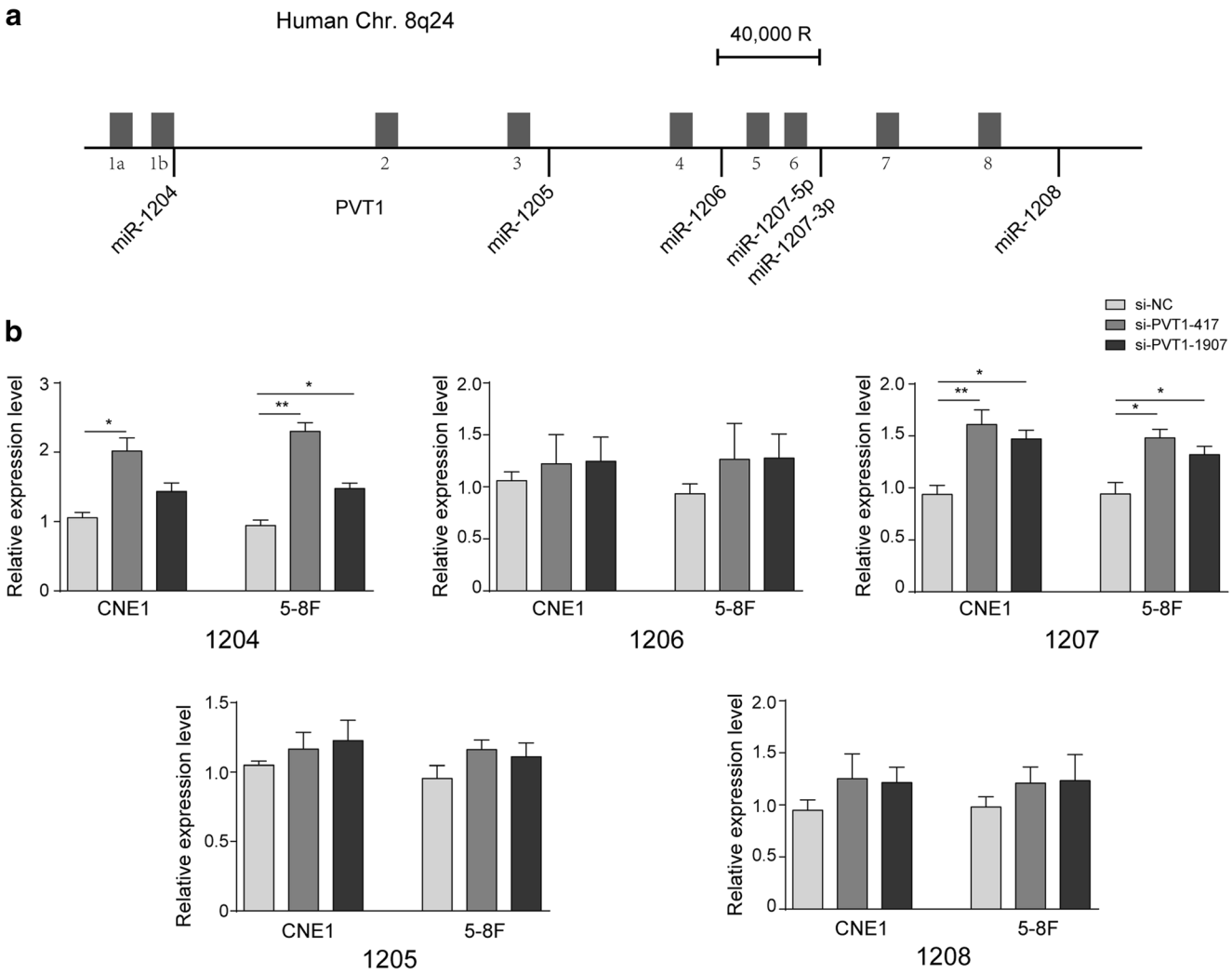

Fig. 4 PVT1 negatively regulates the expression of miR-1204 and 1207 in NPC cells. a Location of the validated PVT1-derived miRNAs. **b** Relative expression of PVT1-derived miRNAs in CNE1 and HNE1 cells when tranfected by PVT1 siRNAs or NC siRNA detected by RT-PCR. *, $P < 0.05$, **, $P < 0.01$, versus the NC siRNA group

tumor stem cells [32]. LncRNA is becoming an important regulator in stem cell biology studies.

The specificity of lncRNA PVT1's structure and location indicates that its abnormal expression is closely related to tumor formation [13, 18]. In recent years, studies have shown that PVT1 presents a high expression in many tumor tissues and cell lines, such as gastric cancer, liver cancer, lung cancer, colon cancer and breast cancer, and it can promote tumor cell proliferation, invasion and metastasis, and increase the resistance of tumor cells to chemotherapy drugs, hence playing a role similar to that played by oncogene [18–26]. In this study, we also found that the expression of PVT1 in NPC tissues and cell lines was significantly higher than that in the control tissues or cell lines ($P < 0.001$). Patients with low PVT1 expression had significantly longer OS ($P = 0.0385$) and disease-free survival (DFS) ($P = 0.0261$). These indicate that PVT1 could serve as an oncogene in NPC. In studying NSCLC, Yang et al. [33] found that PVT1 was highly expressed both in NSCLC tissues and lung cancer cell lines, which was associated with tissue typing and lymph node metastasis, and that after the in-vitro knockout of PVT1 in cancer cells, the proliferation, invasion and metastasis of tumor cells were inhibited. Wang et al. [27] found that PVT1 was highly expressed in hepatocellular carcinoma tissues, which had promoted the proliferation, cell cycle and stemness of hepatocarcinoma cell lines, and the increase of PVT1 was associated with poor prognosis. Consistently, we also found that PVT1 knockdown reduced NPC cells viability ($P < 0.001$), the ability of NPC cells to form spheres ($P < 0.05$), the cell surface CD44+/CD24− stem phenotype. Knockdown of PVT1 attenuates cancer stem cell-like properties in NPC cells, and all these indicate that PVT1 can stimulate the stem cell properties of NPC cells.

Huppi K et al. [17] analyzed the 8q24 gene desert region where chromosome breakpoint is of frequent occurrence, and found that PVT1 locus can transcribe six annotated miRNAs, namely miR-1204, miR-1205, miR-1206, miR-1207-5p,

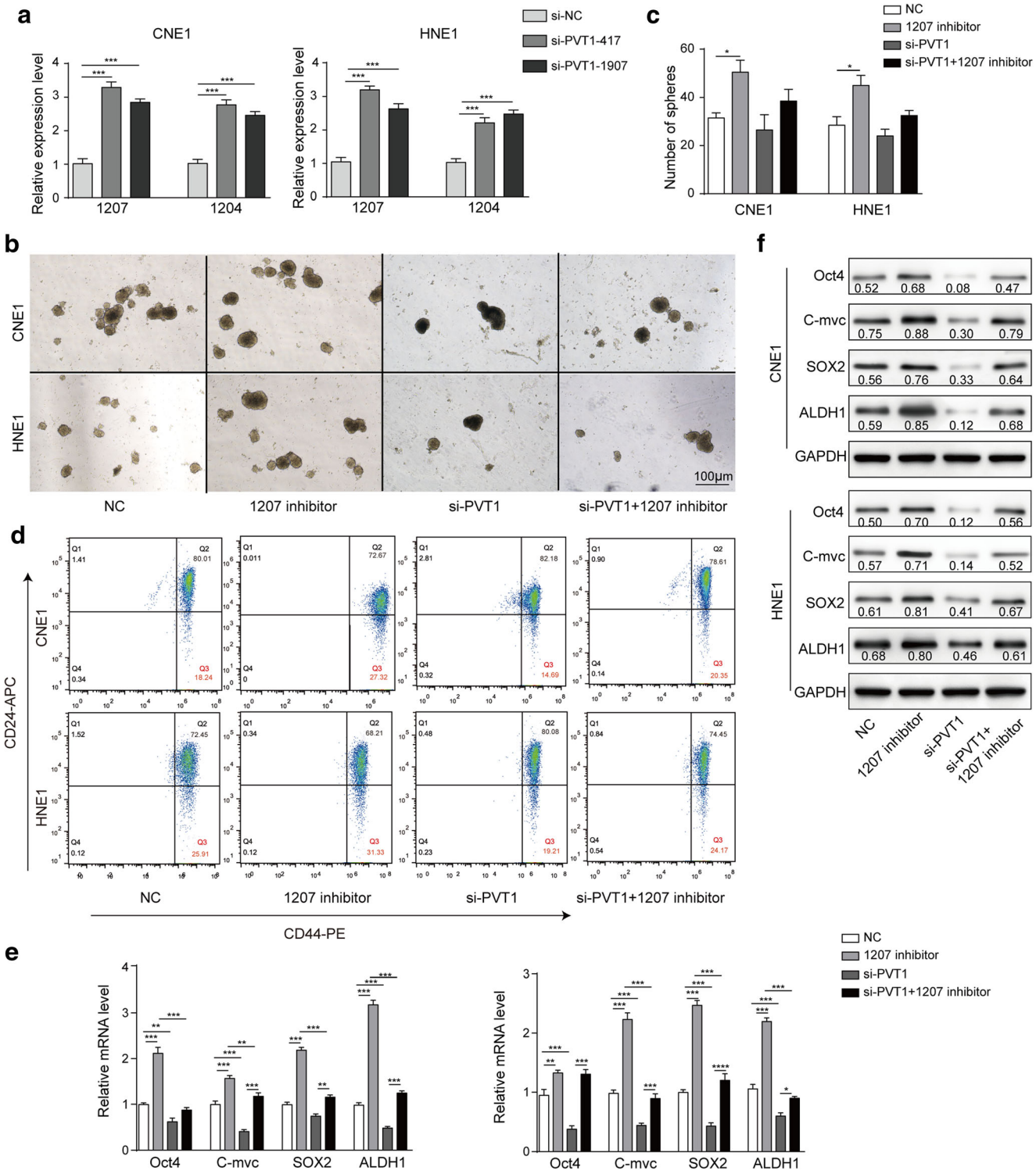

Fig. 5 MiR-1207 inhibits the NPC cell proliferation and stem cell-like properties. a The expression of miR-1207 was significantly up-regulated in si-PVT1 group compared with the NC group, while the change on the expression of miR-1204 was not significant, detected by RT-PCR. **b** MiR-1207 inhibitor transfection enhanced the ability of NPC cells to form spheres, and the numbers of spheres were quantified (**c**). **d** The percentage of $CD44^{+}/CD24^{-}$ stem phenotype was increased when the NPC cells were transfected with the miR-1207 inhibitor detected by flow cytometry. The mRNA (**e**) and protein (**f**) expression levels of the stem cell markers in NPC cells, including Oct4, c-Myc, SOX2, and ALDH1, were up-regulated miR-1207 inhibitor only transfection group, as well as in the miR-1207 inhibitor and PVT1 siRNA co-transfection group. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, as indicated in the figure

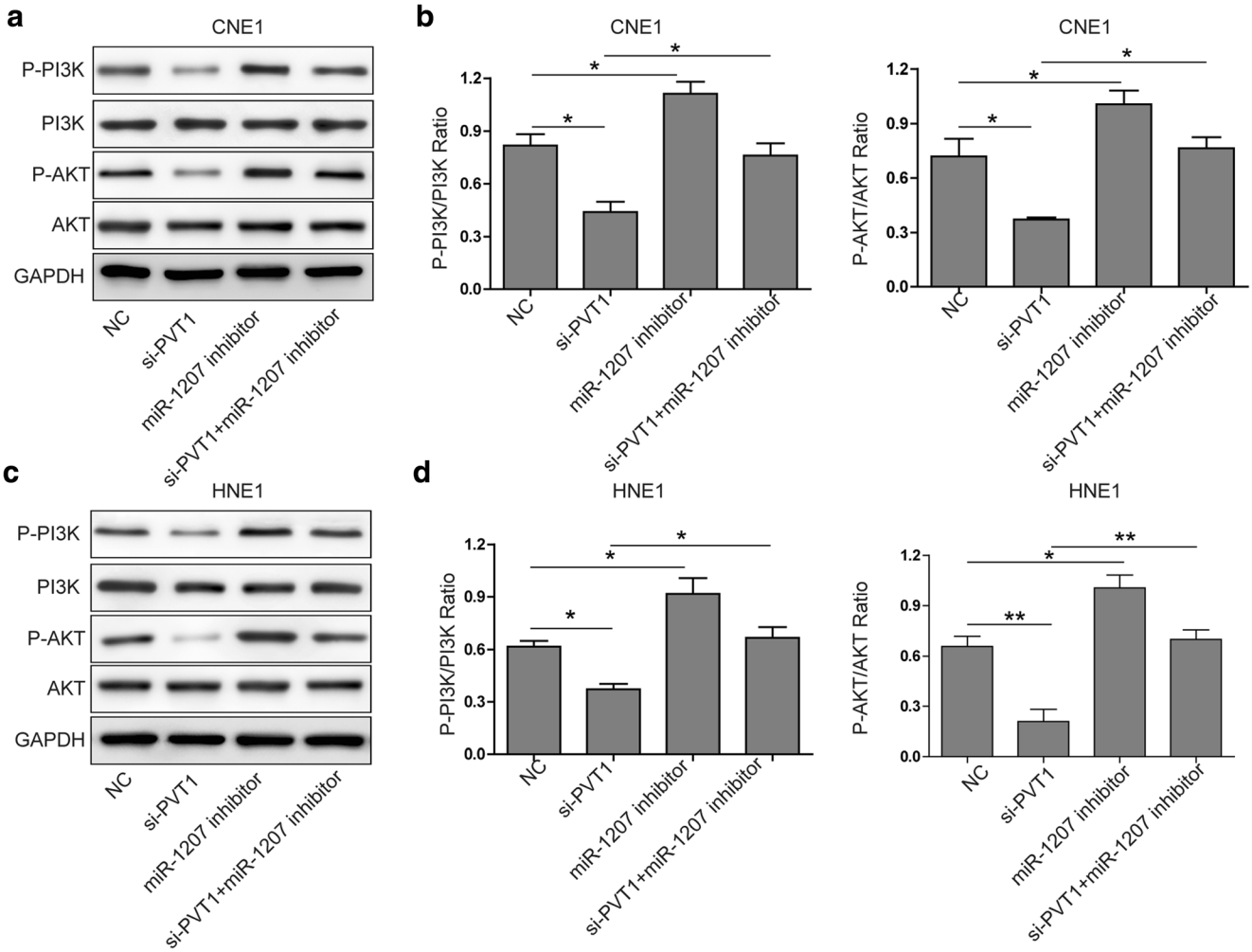

Fig. 6 Knockdown of PVT1 inhibits PI3K/AKT signal pathway. a p-PI3K and p-AKT were down-regulated after PVT1 siRNAs transfection, while this inhibition can be reversed by the co-transfection of miR-1207 inhibitor and PVT1 siRNA in both CNE1 (**a**) and HNE1 (**c**) cells. The ratio of PI3K/p-PI3K and AKT/p-AKT in both CNE1 (**b**) and HNE1 (**d**) cells were quantified, respectively. *, $P < 0.05$, **, $P < 0.01$, as indicated in the figure

miR- 1207-3p and miR-1208. At present, the study of these six miRNAs is still superficial. Many studies reported that the expression of miRNAs can be affected by their host gene expression [34]. And there is evidence that most of intron miRNAs are consistent with their host genes in terms of expression, and are controlled by host gene promoters [34]. In 2012, BarsottiAM et al. [35] reported that the expression of p53-induced endogenous PVT1 up-regulated the expression of miR-1204 to a certain extent, and the abnormal expression of miR-1204 leaded to the p53-dependent apoptosis of some cells. Riquelme et al. [36] demonstrated through q-PCR that the increase in PVT1 copy number in MSTO-211H cells increased the expression of miR-1204 and miR-1208, but not increased the expression of miR-1206 ormiR-1207-3p. In these studies, PVT1 could act as a host gene to regulate the expression of the miRNAs in the intron region of its transposon by sharing the promoter, thus influencing the genesis of the cancer. As described above, the high expression of PVT1 has been found in many tumors, such as pancreatic cancer, gastric cancer and esophageal cancer. However, interestingly, Chen et al. [37] found that the expression of miR-1207-5p in gastric cancer tissues was down-regulated, and the antitumor effect of miR-1207-5p is realized by influencing the expression of hTERT. Yang et al. [33] reported that miR-1207-5p was down-regulated in esophageal cancer cells and it was associated with the typing, pathology and lymph node metastasis of esophageal cancer. In addition, the increase in PVT1 copy number in MSTO-211H cells increased the expression of miR-1204 and miR-1208, but not increased the expression of miR-1206 or miR-1207-3p [36]. In our study, we found that the expression levels of miR-1207 were significantly up-regulated in PVT1 siRNAs transfected NPC cells($P < 0.05$). MiR-1207 inhibitor transfection increased the percentage of CD44+/CD24− stem phenotype, the ability of NPC cells to form spheres, and the expression levels of the stem cell markers in NPC cells. These results indicate that miR-1207

may inhibit the NPC cell proliferation and stem cell-like properties, but the molecular mechanism under this regulation still need to further study.

The transcription factors of S0X2, OCT4 and c-myc are important parts of the cell transcriptional regulation system, and play a key regulatory role in ESCs pluripotency and self-renewal [38, 39]. Therefore, these transcription factors closely related to stem cell phenotype are likely to be associated with CSCs. Recently, it has been reported that these transcription factors are closely related to the "stemness" of CSCs [40]. Leis et al. [41] reported that the increased expression of S0X2 can promote the formation of breast cancer tumor sphere, while inhibiting S0X2 expression can lead to a significant reduction in the number of animal tumors. In addition, OCT4 and c-myc also play an important role in dedifferentiating tumor cells and promoting the formation of CSC phenotype [40]. Acetate deoxygenase (ALDH1) is a kind of cytoplasmic lysate that can oxidize aldehydes into acetic acid [42]. Its increased expression in embryonic and adult stem cells plays an important role in maintaining the "stemness" of stem cells, and meanwhile serves as a means of sorting adult stem cell markers [42]. In this study, we found that the expressions of Oct4, c-Myc, SOX2, and ALDH1 were down-regulated after PVT1 knockdown ($P < 0.05$, versus the NC group). Furthermore, the expression levels of these stem cell markers in NPC cells were significantly up-regulated in the miR-1207 inhibitor only transfection group ($P < 0.05$, versus the NC group), as well as in the miR-1207 inhibitor and PVT1 siRNA co-transfection group ($P < 0.05$, versus the si-PVT group),which further indicates that PVT1 can stimulate the stem cell properties of NPC cells, and miR-1207 may inhibit the NPC cell proliferation and stem cell-like properties.

Studies have shown that PI3K / AKT signaling pathway plays an important role in the genesis, development and metastasis of cancers, and therefore inhibiting the activation of PI3K/AKTsignaling can depress the self-renewal of tumor stem cells and reduce their proportion [43]. Tumor cell signaling pathways are prone to be abnormal, which is especially more common for PI3K/AKT signaling pathway [43]. Research has shown that PI3K / AKT signaling pathway plays a vital role in maintaining the functions of tumor stem cells of glioma, pancreatic cancer, liver cancer, etc. [43–46]. AKT is a key molecule of PI3K / AKT signaling pathway, and its sustained activation is closely related to the incidence and development of tumors [43]. PI3K activates AKT by making it phosphorylated, thus promoting the growth, proliferation, invasion, metastasis, angiogenesis, and apoptosis inhibition of tumor cells [43]. In present study, p-PI3K and p-AKT were found to be down-regulated after PVT1 knockdown, and this inhibition can be reversed by the co-transfection of miR-1207 inhibitor and PVT1 siRNA.

Taken together, our study indicates that Pvt1 can promote cancer stem cell–like properties in NPC cells via inhibiting miR-1207 and activating the PI3K/AKT signal pathway, while the underlying regulatory mechanism still need to be further investigated. Studying the molecular mechanism of nasopharyngeal carcinomarecurrence is of great theoretical and clinical value to improve the survival rate of patients.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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