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SAMHD1 is down regulated in lung cancer by methylation and inhibits tumor cell proliferation

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ABSTRACT

The function of dNTP hydrolase SAMHD1 as a viral restriction factor to inhibit the replication of several viruses in human immune cells was well established. However, its regulation and function in lung cancer have been elusive. Here, we report that SAMHD1 is down regulated both on protein and mRNA levels in lung adenocarcinoma compared to adjacent normal tissue. We also found that SAMHD1 promoter is highly methylated in lung adenocarcinoma, which may inhibit its gene expression. Furthermore, over expression of the SAMHD1 reduces dNTP level and inhibits the proliferation of lung tumor cells. These results reveal the regulation and function of SAMHD1 in lung cancer, which is important for the proliferation of lung tumor cells.

1. Introduction

SAMHD1. (sterile alpha motif and histidine-aspartic domain containing protein 1) is a deoxynucleoside triphosphate phosphohydrolase which cleaves deoxynucleoside triphosphates (dNTP) into deoxynucleosides (dN) and inorganic triphosphates, thus depleting the cellular dNTP pool required for cellular DNA polymerase [1,2]. It can efficiently prevent HIV-1 replication by degrading the dNTP pool, thus, inhibits the early steps of reverse transcription. Blockade at this step prevents the synthesis of full-length double-strand DNA and disrupts later stages of the viral life cycle, including nuclear translocation and integration of proviral DNA [3–5]. This function of SAMHD1 has been recognized predominantly in non-cycling cell types including monocytes [6], macrophages [7], dendritic cells [8,9] and resting CD4+ T cells [10].

The function of SAMHD1 in human immune cells was well established. It can be regulated by a number of factors, including phosphorylation state, the production of type 1 IFN and combination of IL-12 and IL-18. Recently, three studies demonstrate that the antiretroviral activity of SAMHD1 is regulated by phosphorylation in a cell-cycle-dependent manner [11–13]. IFN-α can up regulate SAMHD1 expression in primary monocytes [6] and a combination of IL-12 and IL-18 can lead to an increase in SAMHD1 expression in monocyte-derived macrophage (MDM), resulting in the generation of cells that are more resistant to HIV-1 infection [14]. However, its regulation and function in cancer have been elusive. Lately, one group found that SAMHD1 is mutated recurrently in chronic lymphocytic leukemia and the acquired SAMHD1 mutations are associated with high variant allele frequency and reduced SAMHD1 expression and occur in 11% of relapsed and refractory CLL patients [15], indicating it may play a role in tumorigenesis and development.

In this study, we studied the regulation and function of SAMHD1 in lung cancer and found that SAMHD1 is down regulated both on protein and mRNA levels in lung adenocarcinoma compared to adjacent normal tissue, which may be regulated by methylation on its promoter. Furthermore, over expression of the SAMHD1 reduces dNTP level and inhibits the proliferation of lung tumor cells. These results reveal the regulation and function of SAMHD1 in lung cancer, which is important for the proliferation of lung tumor cells.

2. Materials and methods

2.1. Cell lysis and immunological procedures

Cells were lysed in an NP40 buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4 and 1 mM PMSF. Western blot analysis was performed according to standard methods.

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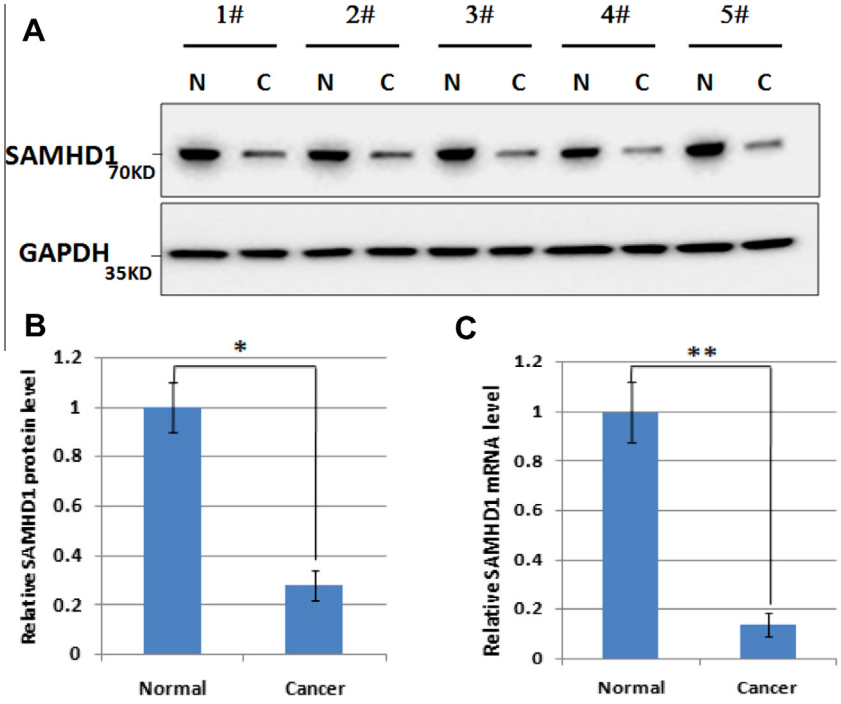

Fig. 1. SAMHD1 expression level is down regulated in lung adenocarcinoma. (A) SAMHD1 is down regulated on protein level in lung adenocarcinoma. Five pairs of lung adenocarcinoma sample and corresponding adjacent normal tissue were subject to Western blot with SAMHD1 antibody. N: adjacent normal tissue; C: lung adenocarcinoma. (B) Relative SAMHD1 protein level was quantified. The protein level of SAMHD1 was quantified by densitometry and normalized to GAPDH. Error bars represent ± SD for quintuplicate experiments. (C) SAMHD1 is down regulated on mRNA level in lung adenocarcinoma. The mRNA level of SAMHD1 in lung adenocarcinoma and corresponding adjacent normal tissue was determined by quantitative RT-PCR analysis. Error bars represent ± SD for triplicate experiments.

Antibodies specific to SAMHD1 (Abcam) and GAPDH (Santa Cruz) were purchased commercially.

2.2. Preparation and analysis of lung adenocarcinoma

Lung adenocarcinoma samples were acquired from Huadong Hospital affiliated to Fudan University. An informed consent was obtained from the patients. The procedures related to this study were approved by Ethic Committee of Huadong Hospital, Fudan University. RNA of lung adenocarcinoma samples and matched surrounding normal tissues was extracted using RNeasy Mini Kit (QIAGEN), reverse transcribed to cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen) following the protocol provided by the manufacturer and SAMHD1 mRNA level was determined by RT-PCR.

2.3. DNA methylation analysis of the SAMHD1 promoter in lung adenocarcinoma

Two methods were employed to confirm the methylation level of the SAMHD1 promoter in lung adenocarcinoma. (1) For methylation-specific PCR (MSP) [16], genomic DNA was extracted from lung adenocarcinoma and corresponding adjacent normal tissue using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Genomic DNA (0.75 μg) was bisulfite-converted using the MethylDetectorTM bisulfite modification kit (Active Motif) according to the manufacturer's guidelines. Next, 1–2 ng of sodium bisulfite-converted genomic DNA was used as a template for PCR analysis using methyl-specific primers. The sequences of the methyl-specific primers (M_fwd, M_rev, U_fwd, and U_rev) were previously described [17]. The PCR conditions for amplifications were 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. A final extension at 72 °C for 10 min was conducted. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. (2) For HpaII digestion of genomic DNA, followed by SAMHD1 promoter-specific PCR, genomic DNA (1 μg) was digested with 10 units of HpaII endonuclease (New England Biolabs) for 3 h at 37 °C. The enzyme was heat-inactivated, and DNA was purified using DNA Clean and Concentrator TM-5. Purified DNA (50 ng) was used as a template for PCR amplification with the primer pair (P1 and P2) [17] that flanks the predicted CpG island containing the HpaII sites. To serve as an input control, a 0.25 kb fragment of the GAPDH gene [18] lacking HpaII sites was PCR amplified using the same template DNA. The PCR conditions and the sequences of the GAPDH gene specific primers have been described previously [19].

2.4. Cell proliferation analysis

5×10^4 A549cells were seeded in triplicate in 6-well plates, and cell numbers were counted every 24 h over a 5-day period.

2.5. Statistical analysis

Data were analyzed using Student's *t* test, and statistical significance was defined as $^{*}P < 0.05$ and $^{**}P < 0.01$.

3. Results and discussion

3.1. SAMHD1 expression level is down regulated in lung adenocarcinoma

SAMHD1 is the first identified mammalian dNTP triphosphohydrolase, its regulation and function in cancer have been unclear. A

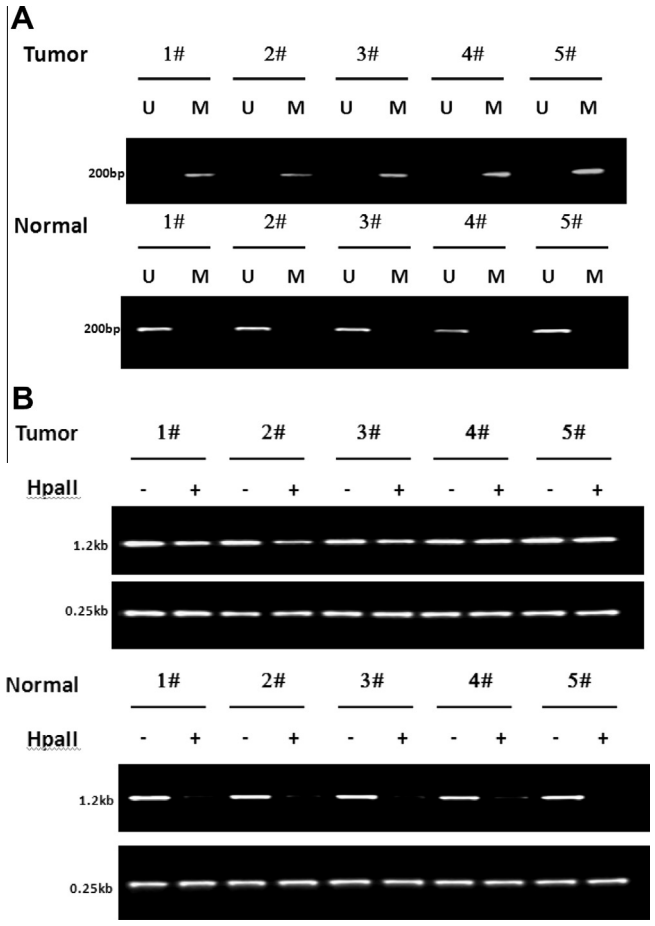

Fig. 2. The promoter of SAMHD1 is methylated in lung adenocarcinoma. (A) The DNA methylation status of the endogenous SAMHD1 promoter in lung adenocarcinoma was analyzed by MSP (upper panel). MSP was also carried out on the corresponding adjacent normal tissue (lower panel), which expresses high level of SAMHD1. A 2% agarose gel stained with ethidium bromide depicting the PCR products from MSP is presented. M: primer pair designed to amplify methylated SAMHD1 promoter sequence; U: primer pair designed to amplify unmethylated SAMHD1 promoter sequence. (B) HpaII-treated (+) and untreated (−) genomic DNAs from lung adenocarcinoma and corresponding adjacent normal tissue were PCR-amplified using the P1/P2 SAMHD1 promoter sequence-specific primer pair (1.2 kb bands). The same template DNA was used in a separate PCR amplification reaction using primers complementary to a sequence within the GAPDH gene that does not contain HpaII restriction sites, which served as an input control (0.25 kb bands). PCR products were resolved on a 1% agarose gel and stained with ethidium bromide, and gel images depicting the resolved PCR products are presented. One representative experiment of three is shown.

recent paper reported that acquired SAMHD1 mutations are associated with reduced SAMHD1 expression and occur in 11% of relapsed and refractory chronic lymphocytic leukemia patients compared to a mutation frequency of 3% in the pretreatment group, suggesting a potential role of SAMHD1 in tumor. So we compared SAMHD1 protein level between five pairs of lung adenocarcinoma sample and corresponding adjacent normal tissue by Western blot and found that SAMHD1 protein level in lung adenocarcinoma was down regulated significantly (Fig. 1A). The reducing of SAMHD1 was quantified by densitometry and normalized to GAPDH, which demonstrated that SAMHD1 protein level in lung adenocarcinoma was reduced to 28.2% of normal tissue (Fig. 1B), confirming that SAMHD1 protein level was down regulated in lung adenocarcinoma.

To find out at which level SAMHD1 is down regulated in lung adenocarcinoma, we checked the mRNA level of SAMHD1 in the same five pairs of lung adenocarcinoma sample and corresponding adjacent normal tissue by q-PCR. Analysis of SAMHD1 mRNA level demonstrated that SAMHD1 is down regulated on mRNA level in lung adenocarcinoma (Fig. 1C).

3.2. The promoter of SAMHD1 is methylated in lung adenocarcinoma

Because our data suggested that SAMHD1 expression is down regulated at the level of transcription and Suresh de Silva et al. found that methylation on the promoter of SAMHD1 can regulate its gene expression in human CD4+ T cells recently [17], we speculated that methylation status of SAMHD1 promoter maybe involved in the down-regulation of SAMHD1 expression in lung adenocarcinoma tissues. To examine whether methylation of the SAMHD1 promoter correlates with transcriptional repression of SAMHD1 expression in lung adenocarcinoma, we analyzed the methylation status of the SAMHD1 promoter in the five pairs of lung adenocarcinoma sample and corresponding adjacent normal tissue using methylation-specific PCR (MSP). PCR amplification of bisulfite-modified genomic DNA using primers designed to discriminate methylated DNA from unmethylated DNA revealed that the SAMHD1 promoter was indeed methylated in lung adenocarcinoma sample (Fig. 2A, upper panel), but not in corresponding adjacent normal tissue (Fig. 2A, lower panel), which has high level of SAMHD1 expression (Fig. 1).

To further confirm our findings obtained using MSP, genomic DNA from the five pairs of lung adenocarcinoma sample and corresponding adjacent normal tissue was individually digested with the methylation-sensitive HpaII endonuclease or left untreated and then subjected to PCR amplification using primers complementary to the *SAMHD1* promoter sequence. Note that the *SAMHD1* promoter contains five HpaII sites surrounding the transcription start site. Methylation of the HpaII sites in the *SAMHD1* promoter would prevent the digestion by HpaII endonuclease, and the intact sequence would serve as a template for PCR amplification using *SAMHD1* promoter-specific primers (P1 and P2) that flank the HpaII sites. To serve as an input control, a 0.25-kb region within the GAPDH gene lacking HpaII sites was PCR-amplified. A single PCR band corresponding to the size of the *SAMHD1* promoter (1.2 kb) was detected in all control genomic DNA samples from lung adenocarcinoma sample and corresponding adjacent normal tissue that were left untreated with HpaII and also in lung adenocarcinoma sample genomic DNA samples that were digested with HpaII (Fig. 2B, upper panel), but not in corresponding adjacent normal tissue genomic DNA samples that were digested with HpaII (Fig. 2B, lower panel). These results confirmed that the *SAMHD1* promoter is methylated in lung adenocarcinoma. The lack of a 1.2-kb PCR product in HpaII-digested corresponding adjacent normal tissue genomic DNAs confirmed that the *SAMHD1* promoter is in an unmethylated state in normal lung tissue, which results in high expression of SAMHD1.

3.3. Inhibition of DNA methylation up regulates SAMHD1 expression level in lung cancer cells

To determine whether methylation indeed down regulates SAMHD1 expression in lung cancer, we treated A549 and H1299 with a DNA methyltransferase inhibitor 5-AzadC [20] and checked mRNA level of SAMHD1. Treatments of H1299 and A549 with 5-AzadC increased *SAMHD1* mRNA expression 7.5-fold and 8.2-fold (Fig. 3A), respectively. Given the significant induction in *SAMHD1* mRNA observed in H1299 and A549 cells upon treatment with 5-AzadC, Western blot was performed to examine whether the increase in *SAMHD1* mRNA resulted in the increased expression of SAMHD1 protein. Consistently, SAMHD1 protein was induced in H1299 and A549 cells compared with untreated control cells (Fig. 3B) by 4.8-fold and 5.3-fold respectively (Fig. 3C). The increase of SAMHD1 protein level is not as high as mRNA level, which sug-

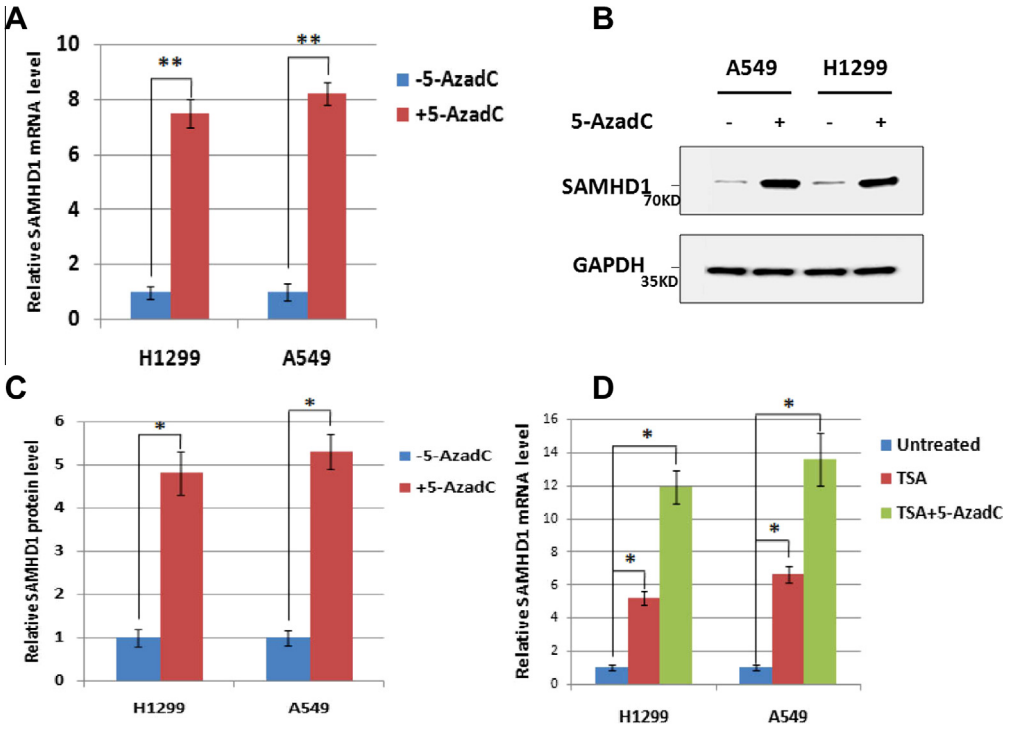

Fig. 3. Inhibition of DNA methylation up regulates SAMHD1 expression level in lung cancer cells. (A) Inhibition of DNA methylation up regulates SAMHD1 mRNA level. Lung adenocarcinoma cell lines were treated with a DNA methyltransferase inhibitor 5-AzadC (10 μM for 72 h). SAMHD1 mRNA level was quantified by real-time PCR analysis. The SAMHD1 mRNA level in cells without inhibitor was set to 1 and the relative mRNA level after 5-AzadC treatment is shown. Error bars represent ± SD for triplicate experiments. (B) Inhibition of DNA methylation up regulates SAMHD1 protein level. The protein level of SAMHD1 in cells without or with 5-AzadC treatment (10 μM for 72 h) was determined by Western blot. GAPDH served as a loading control. The data represent average results of three independent experiments. (C) Relative SAMHD1 protein level after 5-AzadC treatment (10 μM for 72 h) was quantified. The SAMHD1 protein level in cells without inhibitor was set to 1 and the relative protein level after 5-AzadC treatment is shown. The protein level of SAMHD1 was quantified by densitometry and normalized to GAPDH. Error bars represent ± SD for triplicate experiments. (D) Inhibition of histone deacetylation up regulates SAMHD1 mRNA level. Lung adenocarcinoma cell lines were treated with a histone deacetylase inhibitor TSA (1 μM for 18 h). Combination drug treatments were performed by treating cells with 5-AzadC (10 μM) for 72 h, followed by an 18 h treatment with TSA (1 μM). SAMHD1 mRNA level was quantified by real-time PCR analysis. The SAMHD1 mRNA level in cells without treatment was set to 1 and the relative mRNA level after TSA and TSA plus 5-AzadC treatment is shown. Error bars represent ± SD for triplicate experiments.

gests that post-transcriptional and/or post-translational regulations might also contribute to SAMHD1 protein level in lung cancer cells. On the basis of the significant induction of *SAMHD1* gene transcription observed in H1299 and A549 cells by blocking DNA methylation, we speculate that methylation of the *SAMHD1* promoter might be one of the key regulatory mechanisms of *SAMHD1* gene expression in lung cancer. To find out if histone acetylation is involved in the SAMHD1 regulation, we treated H1299 and A549 cells with the histone deacetylase inhibitor TSA or TSA plus 5-AzadC. Result showed that treatments of H1299 and A549 with TSA increased *SAMHD1* mRNA level 5.2-fold and 6.6-fold (Fig. 3D), respectively. Moreover, combination treatments with TSA and 5-AzadC up regulated *SAMHD1* mRNA level cumulatively (Fig. 3D). These results strengthen the notion that SAMDH1 expression is epigenetically regulated in lung cancer cells.

3.4. Over expression of SAMHD1 inhibits the proliferation of lung cancer cells

To find out the benefit of down regulation of SAMHD1 by methylation in lung cancer, we set up an A549 cell line which over expressed SAMHD1 stablely (Fig. 4A). Then we compared the dNTP level and cell proliferation rate of SAMHD1 over expressed cells and control cells and found that over expression of SAMHD1 reduced dNTP level by 38.4% (Fig. 4B). As dNTP is the primary material for DNA synthesis in proliferating cells, we checked the cell proliferation rate of SAMHD1 over expressed cells and control cells. Result demonstrated that a growth disadvantage was conferred to lung tumor cells by the over expression of SAMHD1 (Fig. 4C), which further indicates that the level of SAMHD1 is critical for tumor cell proliferation.

Tumorigenesis is a complicated process induced by the interaction of endogenous and exogenous factors. Unquestionably, a genetic change must have occurred in this process. Nucleotides participate in most biochemical processes as activated precursors for DNA and RNA synthesis and as intermediate products in biosynthesis. The metabolic pathway for synthesis of nucleotides and for recycling bases and nucleosides depletion is subject to alteration as a normal cell goes through the steps which ultimately yield a cancerous cell. Many anticancer and antiviral drugs interfere with the synthesis of DNA and RNA and/or their precursors, notably dNTP and NTP. dNTP pools are limiting for DNA replication, their levels in the cell being only about 1% of the amount necessary for a single round of chromosomal replication [21] and reducing the concentration of dNTP can cause a decrease in the rate of DNA replication in vivo [22]. Thus, the regulation of intracellular dNTP pools is critical for DNA synthesis during DNA replication in organisms, especially in rapid proliferating tumor cells [23]. In the present study, we report that SAMHD1 is down regulated both on protein and mRNA levels in lung adenocarcinoma by methylation on its promoter. Furthermore, over expression of the SAMHD1 reduces dNTP level and inhibits the proliferation of lung tumor cells. These results reveal the regulation and function of SAMHD1 in lung cancer, which is vital for the proliferation of lung tumor cells and provide a potential target for clinical cancer research and treatment. And combined with the previous finding made by Suresh de Silva et al. that promoter methylation also regulates SAMHD1 gene expression in human CD4+ T cells [17], we conclude

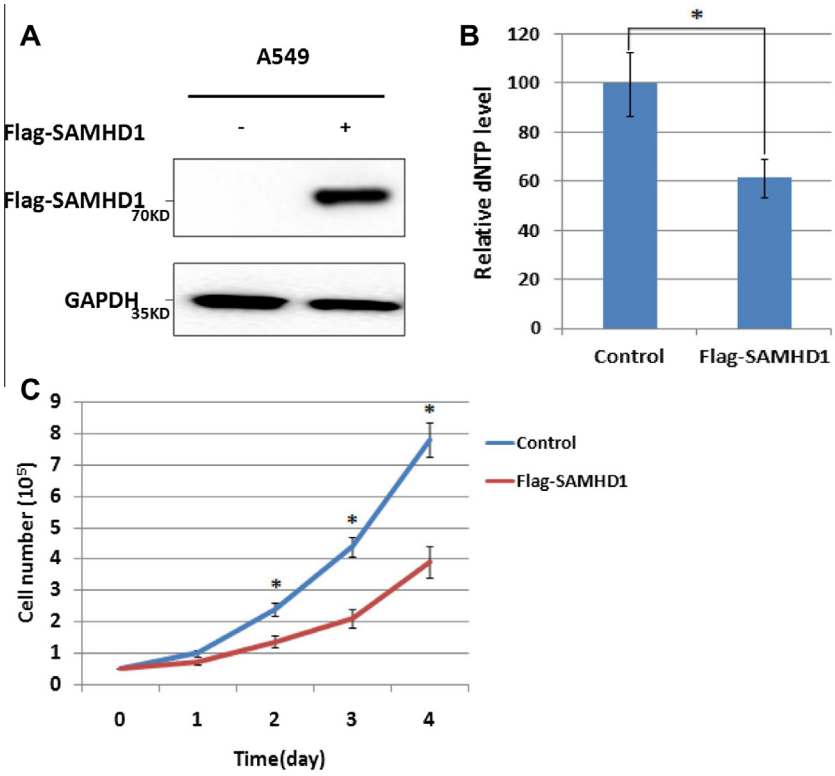

Fig. 4. Over expression of SAMHD1 inhibits the proliferation of lung cancer cells. (A) Expression identification of SAMHD1 in A549 cells. Whole cell lysates from stable cell line expressing SAMHD1 were prepared and analyzed by Western blot. (B) Over expression of SAMHD1 reduces dNTP level of A549 cells. dNTP level of SAMHD1 over expressed A549 cells and control cells was measured following a method previously described [24]. Error bars represent ± SD for triplicate experiments. (C) Over expression of SAMHD1 inhibits the proliferation of lung cancer cells. 5×10^4 SAMHD1 over expressed A549 cells and control cells were seeded in each well. Cell numbers were counted every 24 h. Error bars represent ± SD for triplicate experiments.

that it may be a universal mechanism that methylation on promoter down regulates SAMHD1 in many cells, which needs further validation.

Conflict of interest

The authors declare that they have no conflict of interest.

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