Hyperoside exhibits anticancer activity in non-small cell lung cancer cells with T790M mutations by upregulating FoxO1 via CCAT1

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Abstract. Acquired epidermal growth factor receptor (*EGFR) T790M* mutation is the most common mechanism that accounts for EGFR-TKI (tyrosine kinase inhibitor) resistance of non-small cell lung cancer (NSCLC). High expense and acquired resistance weaken support for the use of osimertinib for T790M-positive NSCLC treatment, and limit the efficacy and application of this drug. Hyperoside, a flavonol glycoside compound, extracted from *Hypericum perforatum,* has been reported to inhibit the growth of a variety of tumors. The present study aimed to investigate the role of hyperoside in treating NSCLC with *T790M* mutations, and to elucidate the underlying molecular mechanisms. Cell viability assays, apoptosis analysis, reverse transcription-quantitative PCR, western blot analysis, animal experiments and immunohistochemistry were performed to examine the anticancer activity of hyperoside. Hyperoside inhibited the proliferation and induced the apoptosis of T790M-positive NSCLC cells. Hyperoside upregulated forkhead box protein O1 (FoxO1) expression and downregulated the level of long non-coding RNA (lncRNA) colon cancer associated transcript 1 (CCAT1) in T790M-positive NSCLC cells. In the *in vivo* study, hyperoside inhibited the growth of T790M-positive NSCLC xenografts. In conclusion, hyperoside inhibited proliferation and induced apoptosis by upregulating FoxO1 via CCAT1 in T790M-positive NSCLC both *in vitro* and *in vivo*, suggesting that hyperoside is a novel candidate for T790M-positive NSCLC treatment.

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Introduction

Epidermal growth factor receptor (EGFR) mutations have been reported to play a vital role in the oncogenesis of non-small cell lung cancer (NSCLC). Exon 19 deletion and exon 21 L858R point mutations are the most common mutations associated with a positive response to first- and second-generation EGFR-tyrosine kinase inhibitors (EGFR-TKIs), and improved progression-free survival, compared with conventional chemotherapy (1). However, although patients show an initial response to EGFR-TKIs, the development of acquired resistance may appear after 9-14 months (2). The mechanisms underlying acquired resistance to first- and second-generation EGFR-TKIs include *EGFR T790M* secondary mutation, *MET* amplification, *IGF1R* activation, *HGF* overexpression, *BRAF V600E* mutation and epithelial-mesenchymal transition (EMT) (3-5). Among them, the acquired *T790M* mutation is the most common mechanism that accounts for more than 50% of resistant cases. Osimertinib, a third-generation EGFR-TKI, was developed to overcome T790M-positive NSCLC that obtained acquired resistance to EGFR-TKIs. Currently, osimertinib is the only drug approved by the Food and Drug Administration for T790M-positive NSCLC treatment, but with high financial cost (6). On the other hand, resistance to osimertinib has been reported (7). Such findings weaken the support for the use of osimertinib for T790M-positive NSCLC treatment and limit the efficacy and application of this drug. Therefore, it is necessary to search for an anti-T790M-positive NSCLC agent that exhibits high efficacy and low economic cost to the patient.

The development of anticancer agents from herbs has emerged as a novel strategy for potential cancer treatment and these agents show desirable efficacy with fewer adverse effects and low cost. These agents have been reported to present specific anti-proliferative, chemo-sensitizing or radio-sensitizing effects in various types of malignancies by targeting multiple signaling pathways (8-18). Hyperoside, a flavonol glycoside compound, extracted from *Hypericum perforatum*, is cultivated worldwide. Hyperoside has been studied extensively due to its anti-inflammatory, anti-oxidative, analgesic and anticancer activities. Hyperoside has been reported to

inhibit the growth of a variety of malignancies, including lung cancer, colorectal cancer, pancreatic cancer, renal cancer, ovarian cancer, prostate cancer and osteosarcoma, without severe side effects and drug resistance (19-25).

Recent studies have demonstrated that hyperoside inhibited lung cancer cell proliferation by inducing cell cycle arrest, autophagy and apoptosis through multiple signaling pathways (26,27). However, to the best of our knowledge, the anticancer effect of hyperoside on NSCLC with *T790M* mutation, and the underlying molecular mechanisms, have not been previously investigated. The present study investigated the anticancer activity of hyperoside in T790M-positive NSCLC cells and a xenograft model, and aimed to elucidate the underlying molecular mechanisms. In addition, the anticancer potential of hyperoside as a novel candidate for T790M-positive NSCLC treatment was investigated, and the associated target signaling pathway was identified.

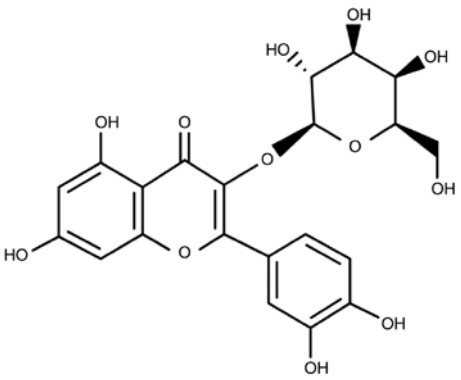

Figure 1. Chemical structural formula of hyperoside ($C_{21}H_{20}O_{12}$).

Materials and methods

Drugs and cell lines. Hyperoside ($C_{21}H_{20}O_{12}$) (Fig. 1) was obtained from Sigma-Aldrich; Merck KGaA (batch no. 00180585). The adenocarcinoma lung cancer cell line PC-9 and the T790M-positive NSCLC cell line NCI-H1975 were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO_2 at 37˚C (seeding density: $4x10^4$ cells/cm^2, subculture every 4-5 days, 1:5 split).

Cell viability assay. Cells were plated into 96-well plates at a density of $5x10^3$ cells per well. After incubation with different concentrations of hyperoside (0, 30, 60, 90, 120 and 150 μM) for 24, 48 and 72 h, MTT reagent (Sigma-Aldrich; Merck KGaA) was added and the cells were incubated for another 4 h. The supernatant was then replaced by dimethyl sulfoxide and the absorbance was detected at 490 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Cell viability curves were generated.

For the clonogenic assay, cells were plated into 6-well plates at a density of $1x10^3$ cells per well. Cells were treated with hyperoside (0, 30, 60, 90, 120 and 150 μM) for 48 h and further cultured in a humidified atmosphere containing 5% CO_2 at 37˚C for 14 days. The colonies were then fixed with paraformaldehyde prior to 0.5% crystal violet staining for 30 min at room temperature. Colonies were counted using an inverted light microscope (magnification, x200; Olympus Corp.).

Apoptosis analysis. Cells were plated into 6-well plates at a density of $1x10^4$ cells per well. After incubation with different concentrations of hyperoside (0, 30, 60, 90, 120 and 150 μM) for 48 h, the cells were trypsinized, washed and collected for apoptosis detection by flow cytometry. Annexin V-FITC and propidium iodide (Sigma-Aldrich; Merck KGaA) were added and the cells were incubated in the dark at 37˚C for 15 min. Cell apoptotic rates were detected using a FACSCalibur flow cytometer.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells and xenograft tumor specimens using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The total RNA was reverse transcribed into complementary DNA using a PrimeScript RT reagent kit (Takara). PCR amplification was performed using a SYBR premix Taq kit (Takara). The primer sequences used were as follows: CCAT1: Forward, 5'-CAT TGGGAAAGGTGCCGAGA-3' and reverse, 5'-ACGCTT AGCCATACAGAGCC-3'; FoxO1: Forward, 5'-AGGATCCGA TGTCACCATGGCCG-3' and reverse, 5'-AAAGGATCCACC ATGGCCG-3'. Amplification conditions for relative expression analysis were as follows: Denaturation at 95˚C for 2 min; 40 cycles of 98˚C for 20 sec, 55˚C for 20 sec, and 68˚C for 30 sec, and finally extension at 72˚C for 4 min. All RT-qPCR reactions were performed using the ABI StepOne™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to β-actin (28).

Western blot analysis. Total protein was extracted from cells with RIPA buffer. The protein concentration was quantified using a BCA Protein Assay kit. Equal amounts of protein were separated by 10% SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked in TBST with 5% non-fat milk at 37˚C for 2 h, followed by incubation with primary antibody (FoxO1; C29H4; rabbit monoclonal antibody; cat. no. 2880; dilution 1:1,000; Cell Signaling Technology, Inc.) at 4˚C overnight. The membranes were washed with TBST and further incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, cat. no. 7074; dilution 1:10,000; Cell Signaling Technology, Inc.) at 37˚C for 1 h. Enhanced chemiluminescence was used to detect protein bands (Image Lab version 5.2; Bio-Rad Laboratories, Inc.). GAPDH was used as the endogenous control.

Cell transfection. Short hairpin RNA (shRNA) that specifically targets lncRNA colon cancer associated transcript 1 (CCAT1) or forkhead box protein O1 (FoxO1) (shCCAT1, shFoxO1) and amplified full-length CCAT1 or FoxO1 cDNA for overexpression (CCAT1, FoxO1) were synthesized by Genechem. The primer sequences used were as follows: shCCAT1-1, CCA TTCCATTCATTTCTCTTTCCTA and shCCAT1-2, CAU ACCAAUUGAACCGAGCCUUGUA; shFoxO1, GCTGCA TGCTACCACCTTACA. The cells in the logarithmic growth phase were collected and then cultured in 6-well plates for transfection. Cell transfection was performed using lentivirus

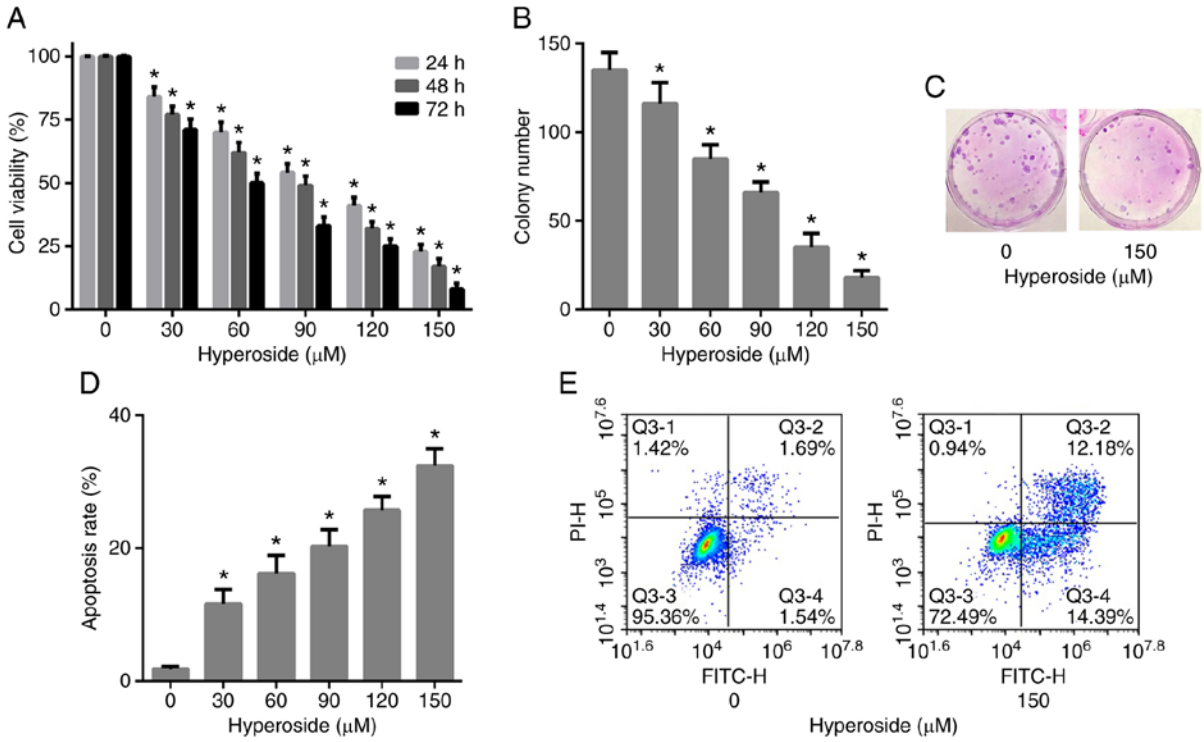

Figure 2. Hyperoside inhibits proliferation and induced apoptosis of T790M-positive non-small cell lung cancer cells. (A) MTT assay revealed that hyperoside (0-150 μM) significantly inhibited the cell viability of H1975 cells in a dose- and time-dependent manner at 24, 48 and 72 h. (B) Hyperoside (0-150 μM) significantly inhibited the clonogenic ability of H1975 cells in a dose-dependent manner at 48 h. (C) Colony formation in the hyperoside (150 μM) group was less than that in the untreated hyperoside (0 μM) group. (D) Hyperoside (0-150 μM) significantly induced the apoptosis of H1975 cells in a dose-dependent manner at 48 h. (E) FACS results showed that early and late apoptosis rates in the hyperoside (150 μM) group were significantly higher than that in the untreated hyperoside (0 μM) group. *P<0.05 vs. the untreated hyperoside (0 μM) group.

according to the manufacturer's protocols. Cells were transfected with lentiviral plasmid and particles (Sino Biological, Inc.), and then harvested for further evaluation at 48 h after transfection. RT-qPCR was performed in order to determine the transfection efficiency.

Animal experiment. Ten nude male mice of 4 weeks of age (weight 20±1 g) were purchased from SLAC Laboratory Animal, Co. H1975 cells, at a density of $1x10^6$, were collected and subcutaneously injected into the flank of mice to form xenograft tumors. The mice were were randomly divided into a hyperoside group and a control group, and injected with hyperoside (25 mg/kg) once daily for 3 weeks, or injected with saline intraperitoneally once daily. The tumor volume was calculated as: Volume (mm^3) = width^2 (mm^2) x length (mm) ÷ 2. Mice were sacrificed by cervical dislocation after 3 weeks, and the tumors were removed and weighed. The animal experimental procedures were approved by the Ethics Committee of Zhejiang Hospital and were in accordance with the National Institutes of Health Guidelines for Animal Care and Use (https://www.nap.edu/read/5140/chapter/1).

Immunohistochemistry. Briefly, tumor specimen sections were cut into 4-μm-thick sections and used for immunohistochemistry staining according to the manufacturer's protocol (Cell Signaling Technology, Inc.). Sections were deparaffinized and rehydrated. Incubation with the primary antibody (FoxO1; C29H4; rabbit monoclonal antibody; cat. no. 2880; dilution 1:1,00; Cell Signaling Technology, Inc.) was performed at 4˚C overnight, and the sections were then incubated with secondary antibody [SignalStain® Boost IHC Detection Reagent (HRP, rabbit) cat. no. 8114; Cell Signaling Technology, Inc.)] for 30 min at room temperature, followed by DAB staining. Positive cells that exhibited brownish-yellow or tan coloring were scored (the staining intensity was scored as 0 (negative-weak), 1 (medium), 2 (strong), or 3 (very strong). The percentage of the staining area was scored as 0 (0-10%), 1 (11-50%), and 2 (51-100%) relative to the total tumor area) and observed under light microscopy (magnification, x200; Olympus Corp.).

Statistical analysis. The data are presented as the mean ± standard deviation. One-way ANOVA followed by SNK-q post hoc test was performed using SPSS software (version 17.0; SPSS Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Hyperoside inhibits the proliferation of T790M-positive NSCLC cells. H1975 cells were exposed to increasing doses of hyperoside (0-150 μM) for 24, 48 and 72 h and the cell viability was assessed by MTT assay. The viability of H1975 cells was significantly inhibited following hyperoside treatment in a dose- and time-dependent manner (Fig. 2A). The IC_{50} values of hyperoside at 24, 48 and 72 h were 104.1, 87.4 and 70.6 μM, respectively. Clonogenic assay was further performed to confirm the anti-proliferative activity of hyperoside, and the results revealed that hyperoside significantly inhibited the

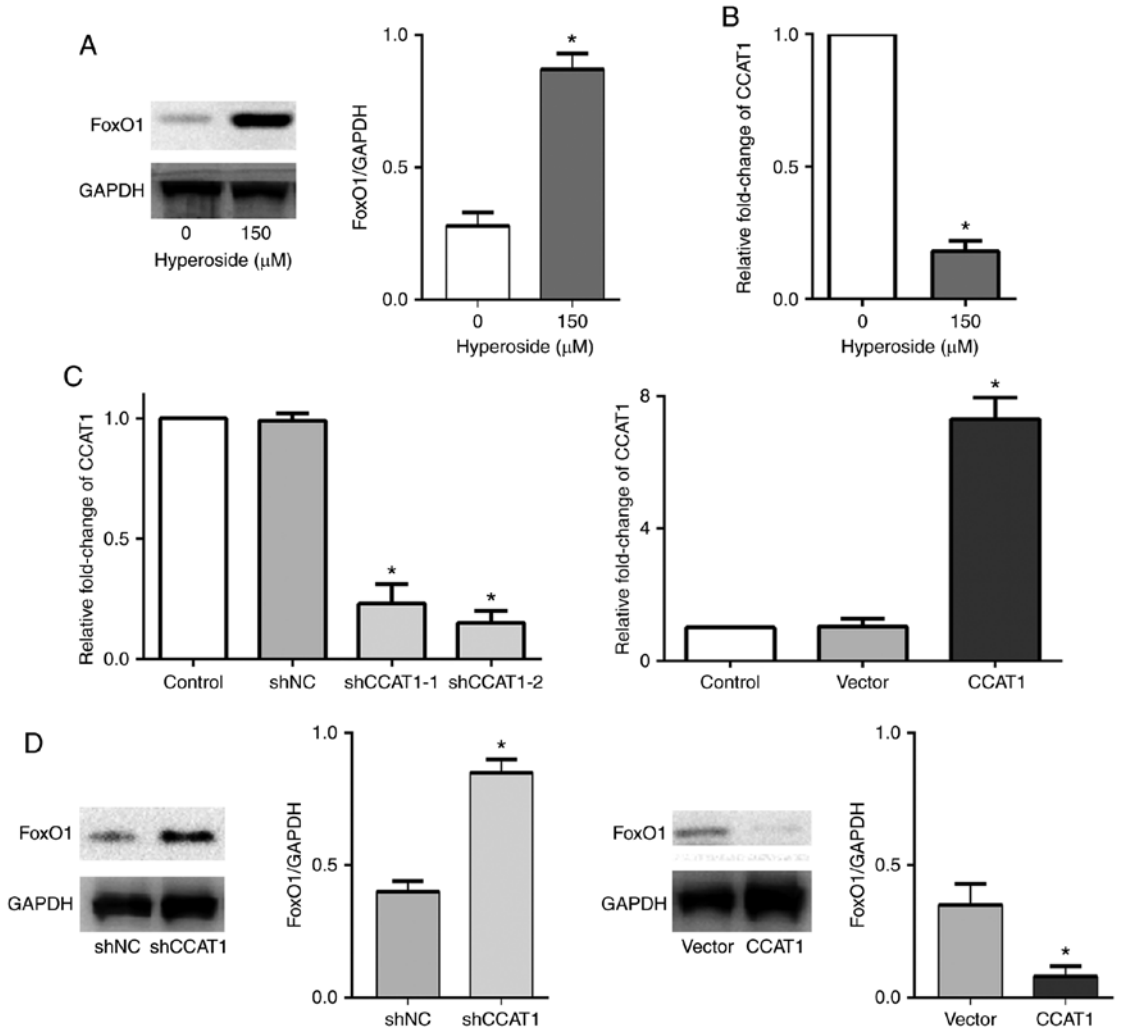

Figure 3. Hyperoside upregulates FoxO1 expression and downregulates the level of CCAT1 in T790M-positive non-small cell lung cancer cells. (A) Western blot analysis revealed that hyperoside (150 μM) upregulated FoxO1 protein expression. *P<0.05 vs. the untreated hyperoside (0 μM) group. (B) RT-qPCR revealed that hyperoside (150 μM) downregulated the level of CCAT1 expression at 48 h. *P<0.05 vs. the untreated hyperoside (0 μM) group. (C) H1975 cells were transfected with CCAT1 shRNA (shCCAT1-1 and shCCAT1-2) for knockdown or amplified full-length CCAT1 cDNA for overexpression and the level of CCAT1 expression was determined by RT-qPCR. *P<0.05 vs. the shNC or Vector group. (D) Western blot analysis revealed that FoxO1 protein expression was upregulated in CCAT1-knockdown (shCCAT1) H1975 cells and downregulated in CCAT1-overexpressing (CCAT1) H1975 cells when compared with the negative control (shNC) or Vector groups. *P<0.05 vs. the shNC or Vector group. FoxO1, forkhead box protein O1; CCAT1, colon cancer associated transcript 1; RT-qPCR, reverse transcription-quantitative PCR.

clonogenic ability of H1975 cells in a dose-dependent manner at 48 h (Fig. 2B and C). These data indicated that hyperoside effectively inhibited the growth of T790M-positive NSCLC.

Hyperoside induces the apoptosis of T790M-positive NSCLC cells. Flow cytometric analysis was performed in order to quantify the cellular apoptosis of H1975 cells induced by hyperoside. H1975 cells were exposed to increasing doses of hyperoside (0-150 μM) for 48 h, stained with Annexin V and PI and subjected to flow cytometry. The results revealed that hyperoside treatment led to a significant increase in the apoptosis rate in a dose-dependent manner when compared with the control group (Fig. 2D and E). These findings indicated that cell proliferation suppression by hyperoside was associated with the induction of apoptosis.

Hyperoside upregulates FoxO1 expression and downregulates the level of CCAT1 in T790M-positive NSCLC cells. In order to investigate the potential mechanisms underlying hyperoside in suppressing proliferation and inducing apoptosis in H1975 cells, western blotting and RT-qPCR assays were performed to examine the forkhead box protein O1 (FoxO1) protein expression levels and the level of long non-coding RNA (lncRNA) colon cancer associated transcript 1 (CCAT1). FoxO1 is a key protein that plays a crucial role in tumor cell apoptosis, and the results of the present study revealed that the expression of FoxO1 was downregulated and CCAT1 was upregulated in the T790M-positive H1975 cells compared with the wild-type PC-9 cells (Fig. S1). However, FoxO1 protein expression was observed to be upregulated following hyperoside (150 μM) treatment (Fig. 3A) in the H1975 cells, demonstrating that hyperoside-induced apoptosis was associated with FoxO1 upregulation. Meanwhile, hyperoside (150 μM) significantly downregulated the level of CCAT1 expression at 48 h (Fig. 3B). The present study further investigated whether CCAT1 regulates FoxO1 expression in T790M-positive H1975 cells. CCAT1

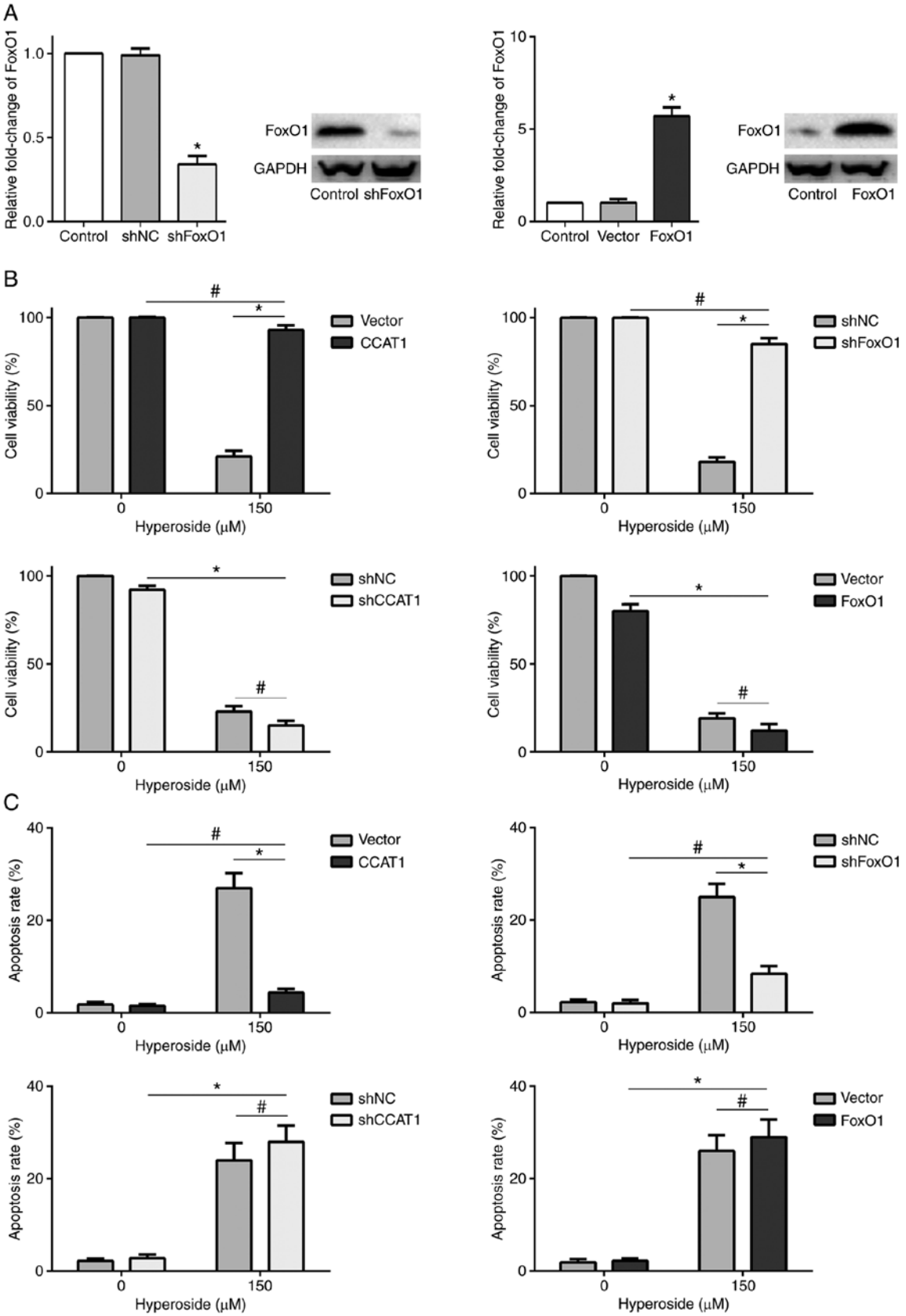

Figure 4. Hyperoside inhibits proliferation and induces apoptosis through upregulation of FoxO1 via CCAT1 in T790M-positive non-small cell lung cancer cells. (A) H1975 cells were transfected with FoxO1 shRNA (shFoxO1) for knockdown or amplified full-length FoxO1 cDNA (FoxO1) for overexpression and the level of FoxO1 expression was determined via reverse transcription-quantitative PCR. Western blot analysis showed that FoxO1 expression was decreased or increased in the FoxO1 knockdown or overexpression H1975 cells. *P<0.05 vs. the shNC or Vector group. (B) MTT assay revealed that cell proliferation did not decrease in the hyperoside-treated CCAT1-overexpressing (CCAT1) or FoxO1-knockdown (shFoxO1) H1975 cells. *P<0.05; #P>0.05, no statistically significant difference. (C) Annexin V-FITC/PI apoptosis assay revealed that the apoptosis rate did not increase in the hyperoside-treated CCAT1-overexpressing (CCAT1) or FoxO1-knockdown (shFoxO1) H1975 cells. *P<0.05; #P>0.05, no statistically significant difference. FoxO1, forkhead box protein O1; CCAT1, colon cancer associated transcript 1.

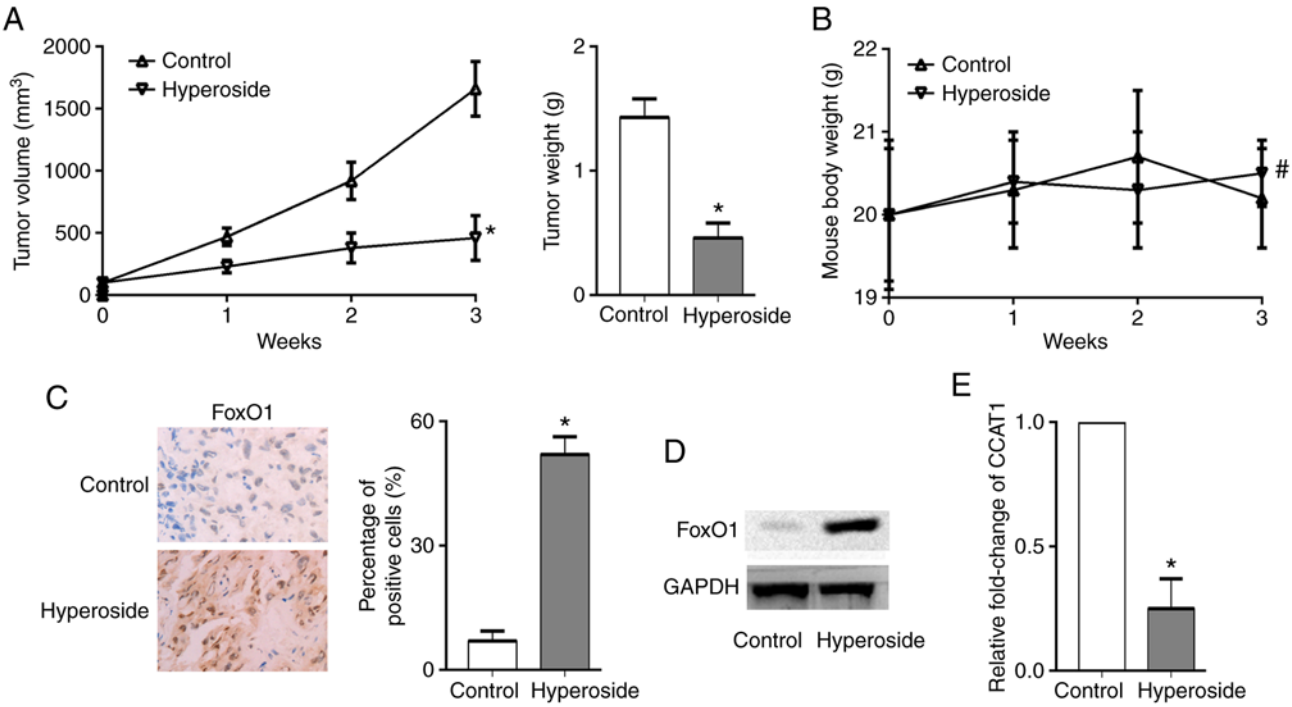

Figure 5. Hyperoside inhibits the growth of T790M-positive non-small cell lung cancer mouse xenografts. (A) Hyperoside inhibited the growth (tumor volume and weight) of H1975 xenograft tumors. (B) Nude mice did not exhibit significant weight loss. (C) Immunohistochemical assay revealed that hyperoside upregulated the FoxO1 expression in the xenograft tumor samples. (D) Western blot analysis revealed that hyperoside upregulated the FoxO1 expression in the xenograft tumor samples. (E) Reverse transcription-quantitative PCR assay revealed that hyperoside downregulated CCAT1 expression in the xenograft tumor sample. *P<0.05 vs. the control group; $^{\#}$P>0.05 (not significant) vs. the control group. FoxO1, forkhead box protein O1; CCAT1, colon cancer associated transcript 1.

knockdown or overexpressing H1975 cells were established and the CCAT1 expression level was determined (Fig. 3C). It was revealed that FoxO1 protein expression was upregulated in the CCAT1-knockdown H1975 cells, while FoxO1 protein expression was downregulated in the CCAT1-overexpressing H1975 cells (Fig. 3D).

Hyperoside inhibits proliferation and induces apoptosis through upregulation of FoxO1 via CCAT1 in T790M-positive NSCLC cells. MTT assay and Annexin V/PI apoptosis analysis were performed in order to investigate the anticancer activity of hyperoside in CCAT1-knockdown or -overexpressing and FoxO1-knockdown or -overexpressing H1975 cells (Figs. 3C and 4A). The results revealed that hyperoside did not decrease the cell proliferation or increase the apoptosis rate in the CCAT1-overexpressing or FoxO1-knockdown H1975 cells (Fig. 4B and C), suggesting that CCAT1-mediated FoxO1 signaling was essential for hyperoside in treating T790M-positive NSCLC.

Hyperoside inhibits the growth of T790M-positive NSCLC xenografts. A xenograft tumor model was established by transplanting H1975 cells into nude mice in order to investigate the anticancer effect of hyperoside *in vivo*. Hyperoside significantly inhibited the growth of H1975 xenograft tumors (Fig. 5A), and the nude mice did not exhibit significant weight loss in the hyperoside group compared with the control group (Fig. 5B). Finally, tumor tissues were removed and prepared for RT-qPCR analysis, immunohistochemistry staining and western blot analysis. The results revealed that FoxO1was highly expressed in the hyperoside group compared with the control group, and the level of CCAT1 was significantly downregulated by hyperoside treatment (Fig. 5C-E).

Discussion

Previous studies have demonstrated that hyperoside exhibits anticancer effects in various types of cancer cell lines by modulating multiple signaling pathways. Hyperoside was found to exert an inhibitory effect on lung cancer growth by inducing apoptosis and cell cycle arrest through phosphorylation of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK), activation of P53 signaling and caspase-3 and -9, and inhibition of NF-κB transcriptional activity (19,29,30). Hyperoside induced both autophagy and apoptosis in non-small cell lung cancer (NSCLC) cells by inhibiting the phosphorylation of Akt, mTOR, p70S6K and 4E-BP1, but increased the phosphorylation of ERK1/2 (27). Hyperoside was found to regulate microRNAs such as miR-21 or miR-27 to inhibit prostate or renal cancer growth and metastasis (23,24). However, to the best of our knowledge, the molecular mechanisms underlying hyperoside in treating T790M-positive NSCLC have not yet been elucidated.

Apoptosis is considered to be an important biological process in cell survival, and resisting apoptosis is one of the main hallmarks of carcinogenesis. Apoptosis is controlled by a variety of apoptotic-associated genes, and current evidence supports the fact that forkhead box protein O1 (FoxO1) is critical for cell survival (31). FoxO1, regarded as a tumor-suppressing factor, can inhibit carcinogenesis, while

FoxO1 disruption may promote carcinogenesis. Activation of FoxO1 was found to trigger cancer cell apoptosis, leading to inhibition of tumor growth (32). The present study demonstrated that hyperoside inhibited proliferation and induced apoptosis in H1975 cells, and the suppression of cell proliferation by hyperoside was associated with the induction of apoptosis. Further investigation revealed that FoxO1 protein expression was upregulated as a result of hyperoside treatment, suggesting that the anticancer activity of hyperoside was associated with FoxO1 upregulation.

An increasing amount of evidence has demonstrated that lncRNAs, >200 nucleotides in length, play an important role in cellular biological processes, including carcinogenesis. lncRNAs, exerting gene transcription regulatory function, have been increasingly studied in cancer diagnosis and therapy. Notably, the aberrant expression of lncRNAs has been demonstrated to contribute to the development of cancer. lncRNA colon cancer associated transcript 1 (CCAT1), located on chromosome 8q24.21, was first observed as highly expressed in colorectal cancer. However, CCAT1 has been reported to be an oncogenic lncRNA and is upregulated in a variety of human cancer types, including lung cancer, gastric cancer, hepatocellular cancer, breast cancer, gallbladder cancer, ovarian cancer and acute myeloid leukemia (33). Particularly in NSCLC, it has been reported that aberrant CCAT1 expression may induce epithelial-to-mesenchymal transition (EMT) by regulating the expression levels of E-cadherin, N-cadherin and vimentin (34). CCAT1 was found to be upregulated in cisplatin-resistant NSCLC and contributed to cisplatin-resistance by downregulation of miR-130a-3p (35). CCAT1 was also found to contribute to docetaxel-resistance in lung adenocarcinoma, and CCAT1 downregulation decreased chemoresistance, promoted apoptosis and reversde the EMT phenotype of docetaxel-resistant cells (36). However, the expression levels of CCAT1 in T790M-positive NSCLC and whether this lncRNA is involved in the anticancer effects of hyperoside remain unclear. In the present study, it was revealed that hyperoside notably downregulated the level of CCAT1 expression. CCAT1 regulated the FoxO1 expression in H1975 cells. Hyperoside could not decrease cell proliferation or increase the apoptosis rate in CCAT1-overexpressing or FoxO1-knockdown H1975 cells, demonstrating that hyperoside inhibited T790M-positive NSCLC tumor growth and promoted apoptosis by upregulating FoxO1 via CCAT1.

In conclusion, the present study demonstrated that hyperoside inhibited proliferation and induced apoptosis by upregulating FoxO1 via CCAT1 in T790M-positive NSCLC cells, providing a theoretical basis for hyperoside in treating T790M-positive NSCLC. Further studies are required in order to apply hyperoside to the clinical setting.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HX conceived and designed the study. ZH and PZ performed the experiments. ZH wrote the paper. HX reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal experimental procedures were approved by the Ethics Committee of Zhejiang Hospital and were in accordance with the National Institutes of Health Guidelines for Animal Care and Use.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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