

Mitochondrial creatine kinase 1 in non-small cell lung cancer progression and hypoxia adaptation

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Research

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Abstract

Background

Hypoxia is a prominent feature of solid cancer. This research aims to expose the role of mitochondrial creatine kinase 1 (CKMT1) in non-small cell lung cancer (NSCLC) progression and hypoxia adaptation.

Methods

The mRNA and protein expression of CKMT1 in NSCLC tissues and cells were detected using GEPIA web, immunohistochemistry, qRT-PCR and western blot. Cells were exposed to a hypoxic chamber with atmosphere containing 5% CO₂, 1% O₂ and residual N₂. The protein levels of HIF-1α and CKMT1 in H1650 and H1299 cells exposed to hypoxia were determined by western blot. Luciferase activity assay and HIF1 specific inhibitor (LW6) assay indicated the related function of HIF-1 and CKMT1. The role of CKMT1 to NSCLC cells biological function on hypoxic condition was measured by CCK8, colony formation, transwell and apoptosis assay.

Results

CKMT1 was highly expressed in NSCLC tissues and cells using GEPIA web, immunohistochemistry, qRT-PCR and western blot. Hypoxia induced the accumulation of HIF-1α and the expression of CKMT1 in H1650 and H1299 cells. The results of luciferase activity assay and HIF1 specific inhibitor (LW6) assay indicated that HIF-1, as a transcription factor of CKMT1, up-regulated the expression of CKMT1 under hypoxic conditions. Further, knockdown of CKMT1 inhibited the cell proliferation and invasion of H1650 and H1299 cells, which could be rescued by hypoxia.

Conclusions

In summary, CKMT1 has the potential as a target for NSCLC hypoxic targeted therapy.

Background

There are more than 1.30 million newly diagnosed cases of lung cancer and more than 1.20 million deaths every year¹. Although the early diagnosis and therapeutic methods of non-small cell lung cancer (NSCLC) patients have been greatly improved, the overall 5-year survival rate has not improved significantly, due to primary or secondary drug resistance², displaying little global variation³.

Hypoxia is a prominent feature of solid cancer, which is mainly due to the rapid proliferation of tumor cells and the abnormal formation of blood vessels⁴. In NSCLC, hypoxia is an important factor in treatment resistance and poor survival⁵. Tumor cells change the expression of specific genes and

activate stress response pathways to facilitate the proliferation and growth of cells, and to confer aggressive phenotype and resistance to treatment under hypoxic conditions⁶. Hypoxia is an attractive therapeutic target, but it has not been successfully developed in most cancers, including NSCLC⁷. Hypoxia inducible factor-1 (HIF-1) is a core transcriptional regulator that responds to hypoxia⁸. We believe that HIF-1-induced glycolysis plays an important role in promoting the malignant phenotype and drug resistance of NSCLC.

Mitochondrial creatine kinase 1 (CKMT1) is responsible for transferring the phosphate group of mitochondrial ATP to the creatine (Cr). Recent studies have shown that CKMT1 plays different roles in multiple tumor types. The expression of CKMT1 is significantly up-regulated in hepatocellular carcinoma⁹. CKMT1 promotes the proliferation and migration of nasopharyngeal carcinoma cells, and affects the sensitivity to radiation¹⁰. However, it is downregulated during the carcinogenesis of oral cancer¹¹ or prostate cancer¹². However, the effect of CKMT1 on the progression of NSCLC has not been studied so far.

In this study, we found that CKMT1 contributed to the hypoxia-induced malignant phenotype of NSCLC cells, and elucidated the transcriptional regulation of HIF-1 on CKMT1.

Materials

Tumor sample collection

NSCLC tissues and matching normal lung tissues were obtained at Shandong Provincial Chest Hospital, after obtaining patient informed consent according to the protocol approved by the Shandong Provincial Chest Hospital institutional review board. All patients were diagnosed with NSCLC at Shandong Provincial Chest Hospital and underwent surgical resection. All tumor tissues were diagnosed by histopathology.

RT-qPCR

Total RNA from NSCLC tissues was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA). The cDNA was synthesized by PrimeScript Reverse Transcription Reagent Kit, and then qPCR was performed using SYBR Premix Ex Taq™. β -actin acted as the endogenous control. The specific primers of *CKMT1* and *β -actin* were as follows, F: 5'-CTTCACCTCACTTTACCTTC-3', R: 5'-TCTTTTACTTCTCTGCGTCT-3' and F: 5'-CGTGACATTAAGGAGAAGCTG-3', R: 5'-CTAGAAGCATTTGCGGTGGAC-3'. The relative levels of CKMT1 mRNA were calculated with $2^{-\Delta\Delta C_t}$ method.

Immunohistochemistry (IHC)

All samples were routinely fixed in formalin and then embedded in paraffin. The paraffin samples were continuously sliced into 5 μ m sections. All tissues were stained using the streptavidin-peroxidase immunohistochemistry. Briefly, sections were deparaffinized in xylol and rehydrated in gradient ethanol.

Subsequently, the sections were immersed in a sodium citrate solution (pH 6.0) and microwaved. To eliminate nonspecific staining, slides were incubated with 5% goat serum for 1 h. Then, the slides were incubated with rabbit polyclonal CKMT1 antibody (anti-CKMT1, 1:200, 15346-1-AP, Proteintech) for 2 h and incubated with a labeled polymer-HRP for 1 h. DAB chromogen solution was used for color reaction, and hematoxylin was used for counterstaining.

The scores for IHC were quantified independently by two trained pathologists at three 200X fields. The score for each sample was multiplied by the staining intensity score and the percentage score of positive cells. Staining intensity for CKMT1 was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Percentage of positive cells was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). A score > 3 points was considered to CKMT1 high expression.

Cell culture and transfection

The human normal pulmonary epithelial cell line (Beas-2B) and four NSCLC cell lines (H1650, H1299, A549 and H524) were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were cultured in RPMI-1640 medium (Sigma Chemical Co, St Louis, MO) supplemented with 10% fetal bovine serum (FBS, Sigma), 10 U/mL penicillin and 10 µg/mL streptomycin in a humidified atmosphere of 5% CO₂/21% O₂ at 37°C. Cells were exposed to a hypoxic chamber (MACS V A500 microaerophilic workstation, Don Whitley Scientific, Bingley, UK) with atmosphere containing 5% CO₂, 1% O₂ and residual N₂ at 37°C to hypoxic conditions.

Specific siRNA sequences targeting CKMT1 (si-CKMT1) were synthesized by Genepharma Co. (Shanghai, China), and were transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The CKMT1-specific siRNA sequences were designed as follows: 5'-ACGGTACCATGGCTGGTCCCTTCTCCCGT-3'.

Western blot

Total protein was lysed using RIPA Buffer (Beyotime, Beijing, China) for 15 minutes on ice. The equal amounts of proteins (20–40 µg/lane) were separated by SDS-PAGE and transferred to PVDF membranes (EMD Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk for 1 h and incubated with the following primary antibodies: anti-CKMT1 antibody (1:1000, 15346-1-AP), anti-HIF-1α antibody (1:1000, 20960-1-AP), anti-E-cadherin antibody (1:2000, 20874-1-AP), anti-N-cadherin antibody (1:2000, 16617-1-AP), anti-Snail1 antibody (1:2000, 13099-1-AP), and anti-Tubulin antibody (1:5000, 11224-1-AP), at 4°C overnight. All antibodies were purchased from Proteintech. Tubulin was used as an endogenous control. Then, membranes were incubated with appropriately HRP-conjugated secondary antibody for 1 h and visualized using the ECL system (GE Healthcare, Little Chalfont, UK).

Luciferase activity assay

The CKMT1 wild type (WT) or mutant type (MUT) promoter sequence was cloned into pGL3.0 vector, and the pGL3.0 recombinant plasmid was transfected into H1650 and H1299 cells using Lipofectamine 2000.

After cultured under normoxic or hypoxic conditions for 48 h, cells were harvested and luciferase activity was detected using a luciferase assay system (Promega, Madison, Wisconsin).

CCK8 assay

Cells transfected or not transfected with si-CKMT1 or si-NC were seeded in 96-well plates. After incubating for a specified time, 10 μ L of CCK8 solution was added to each well and incubated for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader.

Clone formation

The colony formation ability was determined with colony formation assay. Cells transfected or not transfected with si-CKMT1 or si-NC were seeded in 6-well plates, and cultured under normoxic or hypoxic conditions for 24 h. After another 2 weeks of cultivation, visible colonies were formed. The colonies were fixed with methanol and stained with 0.1% crystal violet. Colonies were analyzed by Elispot system (CTL) and pictured.

Transwell

Transwell chamber (pore size of 8 μ m) coated with Matrigel was used to evaluate the cell invasion. Cells transfected or not transfected with si-CKMT1 or si-NC were suspended in FBS-free RPMI-1640 medium and were seeded to the upper chamber at a density of 2×10^3 cells/well, and the lower chamber was filled with RPMI-1640 medium containing 10% FBS. Invasion cells on the lower surface of the chambers were fixed by 4% paraformaldehyde and stained with 0.1% crystal violet. Finally, the cells were counted in five randomly selected fields under an inverted microscope (200x magnification, Nikon TE2000).

Apoptosis

Cells transfected or not transfected with si-CKMT1 or si-NC were cultured in FBS-free RPMI-1640 medium for 24 h, and were continue cultured under normoxic or hypoxic conditions for 24 h. Then, cells were harvested and washed twice with Annexin V binding buffer. Subsequently, cells were double stained with 5 μ L Annexin V and 5 μ L PI, and analyzed by a flow cytometer (C6 Accuri, BD Biosciences).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software. Data are presented as the mean \pm SD. The differences between the groups were calculated using a Student's t-test. $P < 0.05$ was considered statistically significant.

Results

CKMT1 is highly expressed in NSCLC tissues and cells

We first examined the CKMT1 mRNA expression in NSCLC tissues using the GEPIA web, and we found that CKMT1 mRNA levels increased significantly in lung adenocarcinoma (LUAD) (n = 483) and lung

squamous cell carcinoma (LUSC) tissues (n = 483), compared into normal lung tissues respectively ($P < 0.05$, Fig. 1A). The mRNA expression of CKMT1 in NSCLC and normal lung tissues were further surveyed by qRT-PCR using clinically collected samples, and we got consistent results from the GEPIA web that CKMT1 mRNA was highly expressed in NSCLC tissues (n = 32) ($P < 0.05$, Fig. 1B). The protein expression and cellular location of CKMT1 in NSCLC tissues also detected by IHC using clinically collected samples. The percentage of high CKMT1 expression was significantly higher in NSCLC tissues ($P < 0.05$, Fig. 1C and Table 1). Furthermore, the results of statistical analysis indicated that the high level of CKMT1 was significantly correlated with the high pathological grade of NSCLC patients ($P < 0.05$, Table 2). In addition, we observed the mRNA and protein expression of CKMT1 was more prominent in NSCLC cell lines (H1650, H1299, A549 and H524), compared to normal Beas-2B cells ($P < 0.05$, Fig. 1D **and E**). Furthermore, H1650 and H1299 cells were selected for further experiments.

Table 1
CKMT1A expression in NSCLC tissues compared with normal lung tissues

Group	n	CKMT1A expression		P
		Low (n%)	High (n%)	
NSCLC	32	12 (37.5)	20 (62.5)	0.001**
Normal	32	26 (81.2)	6 (18.8)	

Table 2
CKMT1A expression associated with the clinicopathological parameters in NSCLC patients

Clinicopathological parameters	n	CKMT1A Low (n%)	CKMT1A High (n%)	P
Gender				
Male	20	5 (25)	15 (75)	0.131
Female	12	7 (58.3)	5 (41.7)	
Age (years)				
< 60	19	8 (42.1)	11 (57.9)	0.780
≥ 60	13	4 (30.8)	9 (69.2)	
Tumor diameter (cm)				
< 5	15	7 (46.7)	8 (53.3)	0.522
≥ 5	17	5 (29.4)	12 (70.6)	
Pathological grade				
I-II	17	10 (58.8)	7 (41.2)	0.022*
II-III	15	2 (13.3)	13 (86.7)	

CKMT1 is induced to express by hypoxia and HIF-1α is the transcription factor of CKMT1 in NSCLC cells.

We detected the protein levels of HIF-1α and CKMT1 in H1650 and H1299 cells exposed to hypoxia for 6, 12, 24 and 48 h by western blot. Hypoxia induced the increased expression of HIF-1α and CKMT1 (Fig. 2A). In addition, until 24 h of hypoxia, the protein level of HIF-1α gradually increased with the extension of hypoxia time; while the protein level of CKMT1 was the highest at 24 h of hypoxia and subsequently decreased, which may be due to the long period of hypoxia leading to the decline of protein expression ability and cell viability¹³. The binding sequence of transcription factor HIF-1 to the *CKMT1* promoter region was predicted (Fig. 2B). The luciferase report assay showed that hypoxia induced the expression of CKMT1 (Fig. 2C). Further, when the predicted binding site was mutated, the induced expression of CKMT1 was significantly reduced (Fig. 2C). LW6 is a specific inhibitor of HIF, which effectively inhibited the accumulation of HIF-1α by degrading HIF-1α without affecting the level of HIF-1α mRNA under hypoxic conditions. As shown in Fig. 2D, the addition of LW6 effectively suppressed the protein levels of HIF-1α and CKMT1 in the cells. These results indicated that HIF-1α can indeed transcriptionally regulate the expression of CKMT1. Hypoxia upregulated intracellular CKMT1 levels by inducing HIF-1α accumulation.

CKMT1 in hypoxia adaptation and NSCLC progression

To examine the correlation of CKMT1 expression and hypoxia adaptation in vitro, we knocked down the protein expression of CKMT1 in H1650 and H1299 cells with siRNA (si-CKMT1, Fig. 3A and B). Hypoxia promoted the proliferation, colony formation, invasion and EMT of H1650 and H1299 cells (Fig. 3C-D and Fig. 4A-B). In addition, we observed that knockdown of CKMT1 inhibited cell proliferation, colony formation, invasion and EMT of H1650 and H1299 cells, which could be rescued by hypoxia (Fig. 3C-D and Fig. 4A-B). Furthermore, hypoxia significantly inhibited the cellular apoptosis, while knockdown of CKMT1 had no effect on apoptosis, as examined by Annexin V and PI staining (Fig. 4C).

Discussion

The cellular energy supplied by ATP closely matches the demand. Even if the load suddenly increases, the ATP level of the cells will not change in the short term, which is due to the CK phosphagen system. PCr acts as a mobile energy storage reservoir for ATP regeneration. When demand continues, free calcium levels rise and activate mitochondrial dehydrogenase, promoting the synthesis of nascent ATP¹⁴. CKMT1 is a mitochondrial creatine kinase, which plays a vital role in cells with high energy requirements or tumor cells with altered energy metabolism.

In this study, we found that the expression of CKMT1 were up-regulated in NSCLC tissues and cells. Although studies have shown that the expression of CKMT1 is significantly down-regulated in oral squamous cell carcinomas (OSCC)¹¹ or prostate cancer¹² tissues and cell lines. In addition, CKMT1 Y153 phosphorylation is generally upregulated in HER2⁺ breast cancer¹⁵. This may depend on the state of specific tumor cells, such as hypoxia, p53 mutation or methylation. CKMT1 is hardly expressed in PC3 and DU145 cell lines, which are related to the lack or mutation of p53 expression, respectively¹². In addition, the low expression of CKMT1 in OSCC-derived cell lines may be due to frequent methylation of its CpG island region¹¹.

In this study, we also found that hypoxia induced the expression of CKMT1, and induced-expression of CKMT1 was transcriptional regulated by HIF-1 α accumulation. Mammalian cells encode three HIF homologous genes (HIF1-3). HIF is a heterodimer consisted of constitutively expressed β -subunit and O₂-adjusted α -subunit. When oxygen is present, the two proline residues of HIF- α are hydroxylated¹⁶. Prolyl hydroxylated HIF- α undergoes rapid ubiquitination and subsequent proteasome degradation. Under hypoxic conditions, prolyl hydroxylation is inhibited, HIF- α accumulates, forms a heterodimer with HIF- β , enters the nucleus and binds to the hypoxia response element (HRE) to induce transcriptional activation of target genes, such as CAIX, GLUT1 and VEGF^{17,18}. The protein level of CKMT1 increased with the increase of hypoxia time (increase of HIF-1 α expression level) within 24 h of hypoxia. The protein level of CKMT1 was the highest at 24 h of hypoxia and subsequently decreased, which may be due to the long period of hypoxia leading to the decline of protein expression ability and cell viability¹³. This still requires a lot of further research. In addition, the mutation of HIF1 binding site did not completely eliminate the

luciferase activity. We confirmed that there are no other HIF-1 α binding site on the CKMT1 promoter sequence. However, the CKMT1 promoter sequence has other hypoxia-induced transcription factor binding sites, such as p53 and RUNX1^{19,20}. These transcription factors also participate in the transcriptional regulation of CKMT1 under hypoxic conditions. Our results indicated that CKMT1 was also a target gene of HIF1, which was reported for the first time. In addition, the transcriptional activity of HIF is determined by a variety of factors, including the presence of inhibitors and different sensitivities to HIF hydroxylation²¹. LW6 (HIF inhibitor) effectively inhibited the accumulation of HIF-1 α and the protein expression of CKMT1 in NSCLC cells.

In addition, knockdown of CKMT1 inhibited the cell proliferation, colony formation, invasion and EMT of H1650 and H1299 cells. CKMT1 seems to have different effects on the biological function of different types of tumor cells. For example, inhibition of CKMT1 reduces the viability of human EVI1-positive acute myeloid leukemia (AML) cell lines, and promotes cell cycle arrest and apoptosis¹⁹. In addition, CKMT1 overexpression induces apoptosis of OSCC cells, but does not affect cell invasion¹¹. This may be because the behavior of tumor cells is regulated by various factors, or the combined effect of the tumor microenvironment. We found that hypoxia promoted the proliferation and invasion of H1650 and H1299 cells by up-regulating the expression of CKMT1.

Conclusions

Molecular events changes caused by hypoxia can enhance tumor cell metastasis and drug resistance, and help them escape immune surveillance. However, NSCLC hypoxic targeted therapy trials have not translated into patient benefit. This study found that CKMT1 is significantly over-expressed in NSCLC. In addition, HIF-1, as a transcription factor of CKMT1, up-regulated the expression of CKMT1 under hypoxic conditions. Hypoxia affects the biological function of NSCLC cells by transcriptionally regulating the expression of CKMT1.

Abbreviations

non-small cell lung cancer, NSCLC; Hypoxia inducible factor-1, HIF-1; creatine kinase 1, CKMT1; creatine, Cr; Immunohistochemistry, IHC; siRNA sequences targeting CKMT1, si-CKMT1; wild type, WT; mutant type, MUT; lung adenocarcinoma, LUAD; lung squamous cell carcinoma, LUSC; hypoxia response element, HRE; acute myeloid leukemia, AML.

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of Shandong Provincial Chest Hospital.

Consent for publication

All presentations in this article have been consent for publication by the patient. The written informed consent was obtained from all patients.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

- 1) Conception and design, acquisition of data, or analysis and interpretation of data: All authors.
- 2) Drafting the article or revising it critically for important intellectual content: All authors.
- 3) Final approval of the version to be published: All authors.
- 4) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: Yan Lv and Juan Li.

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Figures

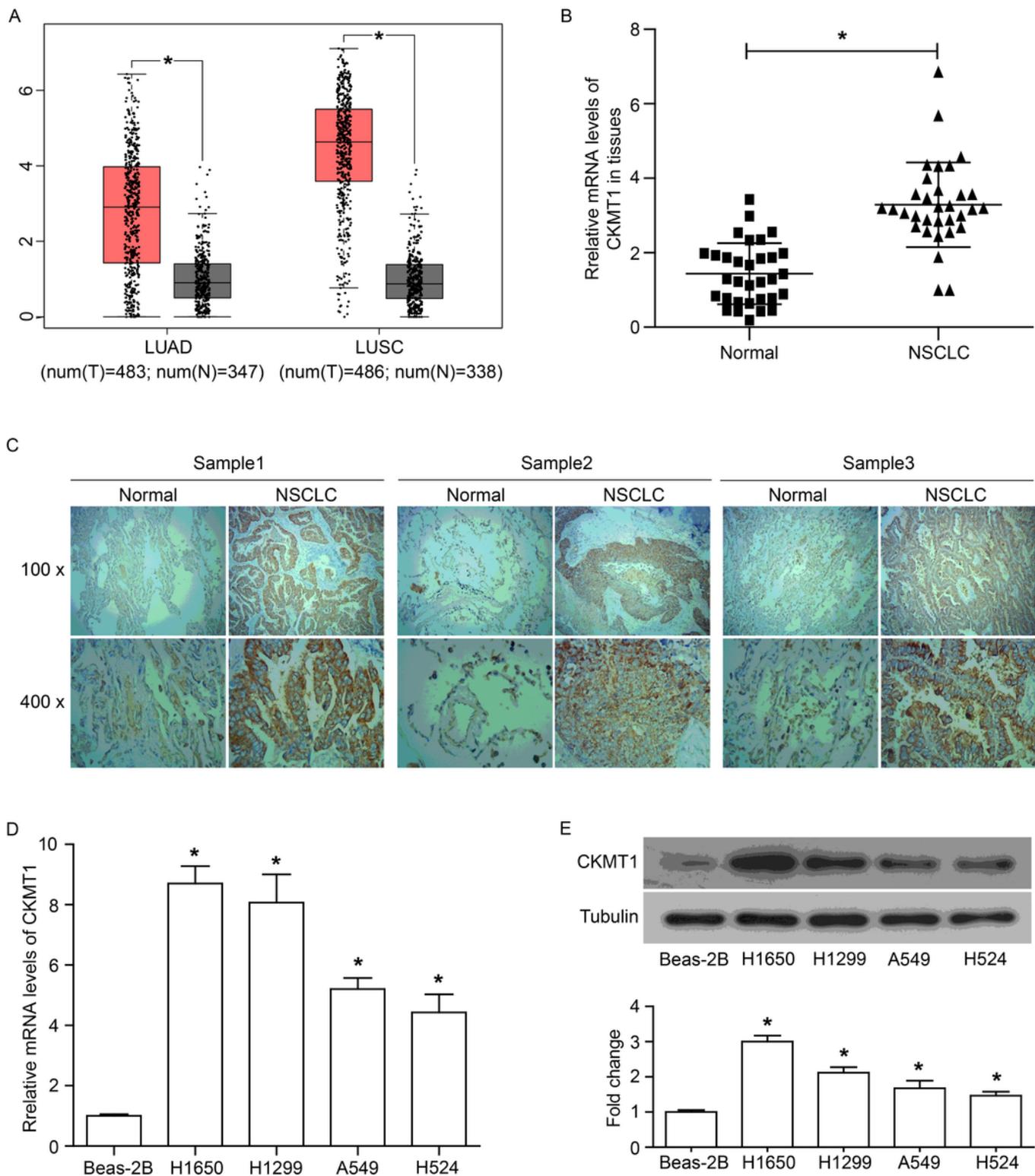


Figure 1

CKMT1 is highly expressed in NSCLC tissues and cells. (A) The boxplot of CKMT1 mRNA expression. Red boxes represent lung adenocarcinoma (LUAD) (n=483) or lung squamous cell carcinoma (LUSC) (n=483) tissues, while gray boxes represent normal bladder tissue, respectively. The data came from the GEPIA database. The mRNA (B) and protein (C) expression of CKMT1 in NSCLC tissues and adjacent normal tissues was examined by qRT-PCR and immunohistochemical (IHC) analysis, respectively. The mRNA (D)

and protein (E) expression of CKMT1 in NSCLC cell lines (H1650, H1299, A549 and H524) and normal Beas-2B cells was detected by western blot. *P<0.05.

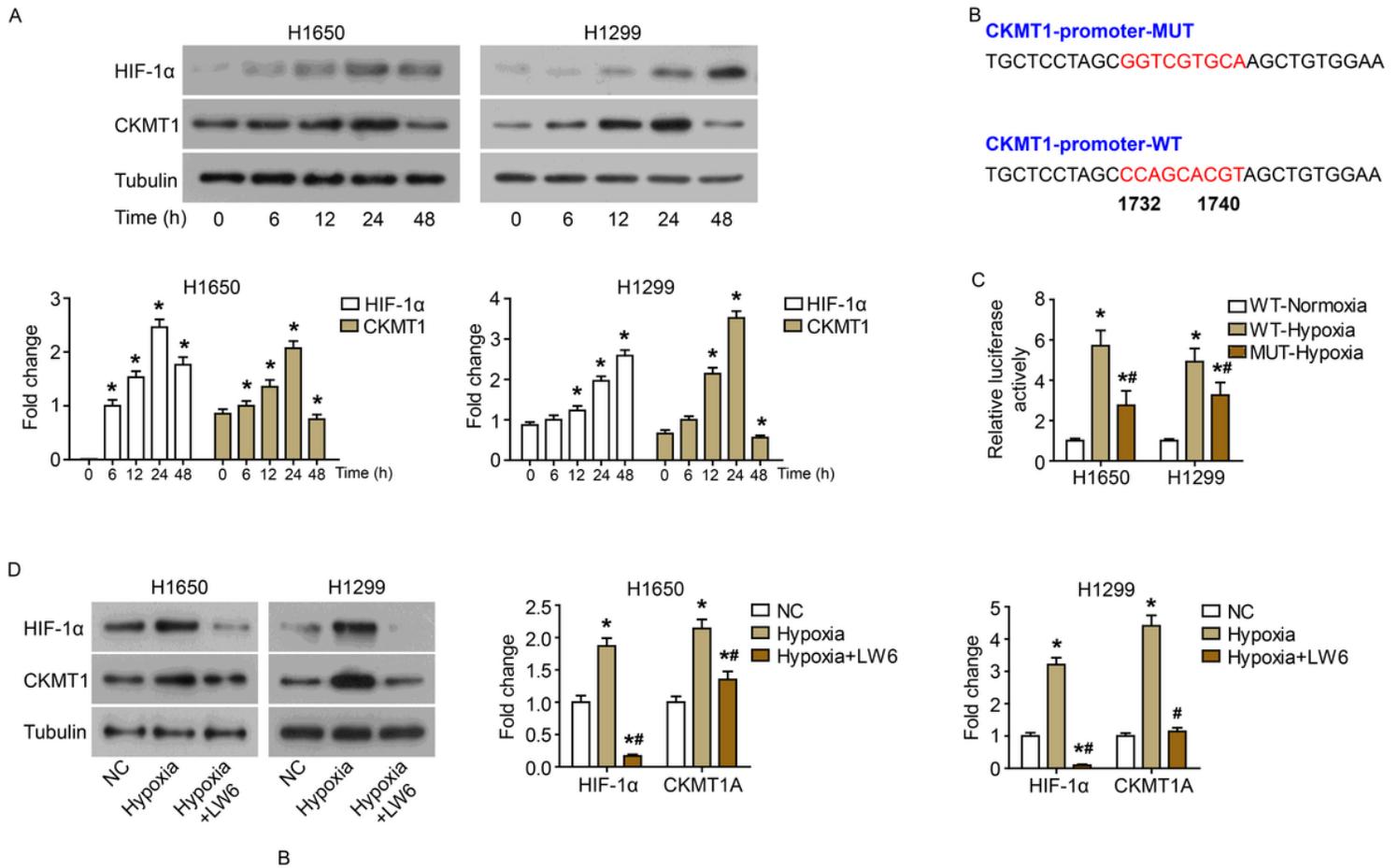


Figure 2

CKMT1 is induced to express by hypoxia and HIF-1 α is the transcription factor of CKMT1 in NSCLC cells. (A) The protein levels of HIF-1 α and CKMT1 in H1650 and H1299 cells exposed to hypoxia for 6, 12, 24 and 48 h were detected by western blot. (B) The wild (CKMT1-promoter-WT) or mutated (CKMT1-promoter-MUT) binding sequence of transcription factor HIF-1 to the CKMT1 promoter region was predicted using the PROMO 3.0 website. (C) The relative luciferase actively in normoxic or hypoxic conditions. (D) The protein levels of HIF-1 α and CKMT1 in H1650 and H1299 cells exposed to hypoxia and LW6 (HIF-1 α inhibitor) treatment were detected by western blot. *P<0.05, compared to NC; #P<0.05, compared to Hypoxia.

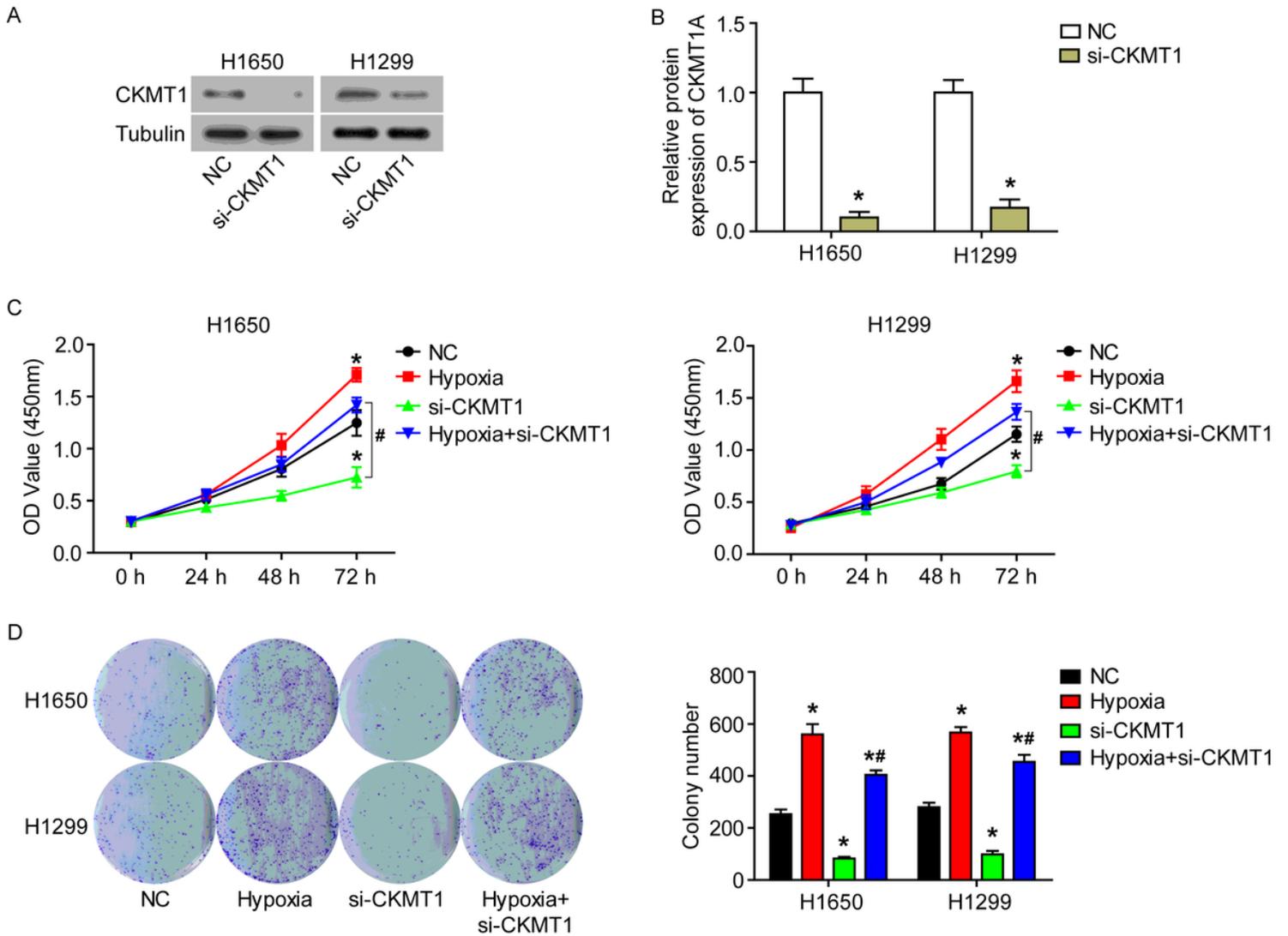


Figure 3

CKMT1 in proliferation and hypoxia adaptation of NSCLC cells. (A and B) After siRNA targeting CKMT1 (si-CKMT1) was transfected into H1650 and H1299 cells, CKMT1 protein expression were detected by western blot. Cell proliferation (C) and colony formation (D) were measured by CCK8 and colony formation assay, respectively. * $P < 0.05$, compared to NC; # $P < 0.05$, compared to si-CKMT1.

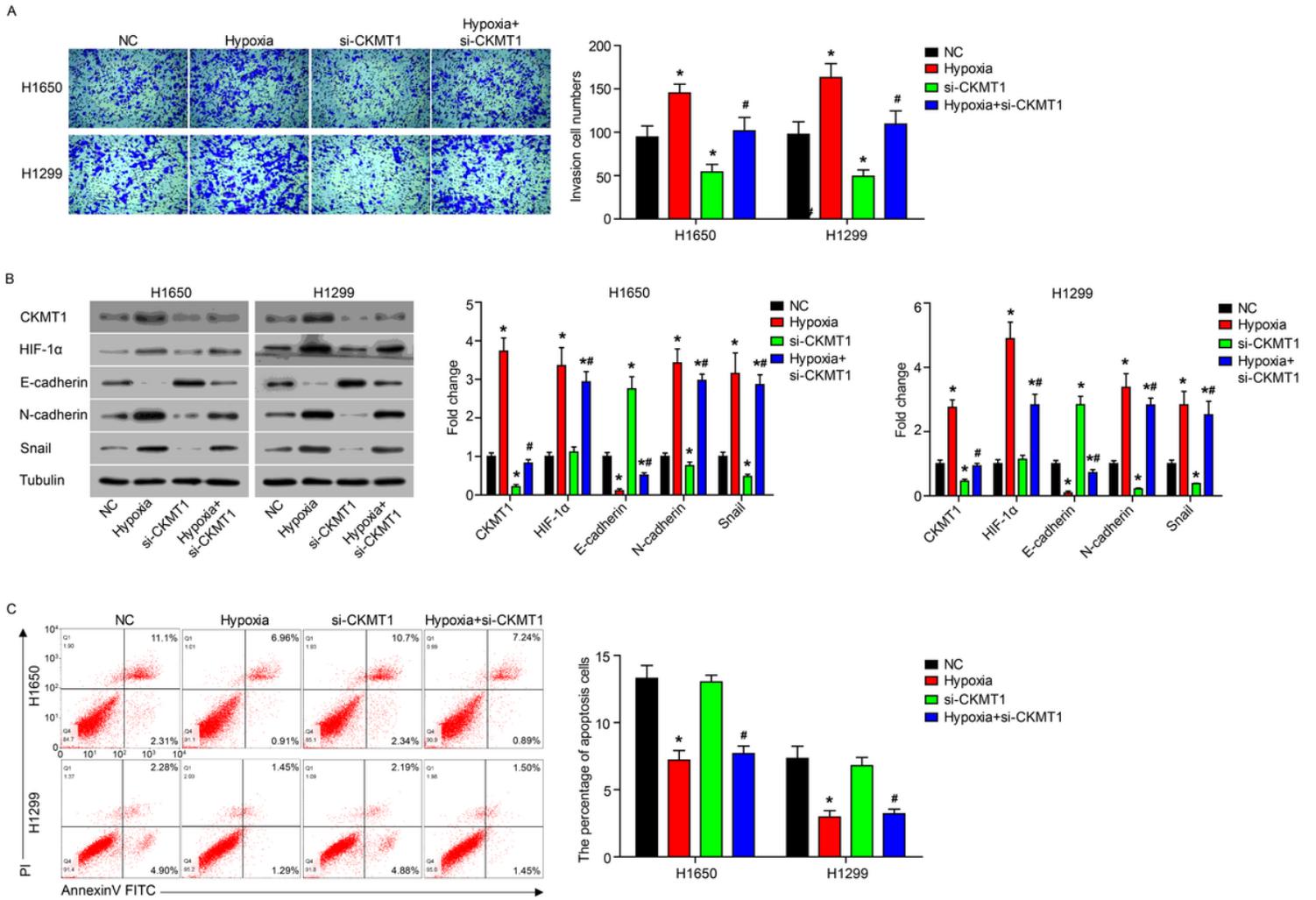


Figure 4

CKMT1 in invasion, apoptosis and hypoxia adaptation of NSCLC cells. (A) The invasion ability was determined by Transwell assay. (B) The expression of EMT-related proteins was detected by western blot. (C) Apoptosis was examined by flow cytometry. * $P < 0.05$, compared to NC; # $P < 0.05$, compared to si-CKMT1.