Insufficient ablation promotes the metastasis of residual non-small cell lung cancer (NSCLC) cells via upregulating carboxypeptidase A4

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ABSTRACT

Background: Thermal ablation is a potentially curative therapy for early-stage non-small cell lung cancer (NSCLC). Early recurrence after thermal ablation necessitates our attention.

Methods: The invasion and migration abilities of NSCLC after sublethal heat stimulus were observed in vitro and in vivo. Sublethal thermal stimulus molecular changes were identified by RNA sequencing. A xenograft model of NSCLC with insufficient ablation was established to explore the epithelial-to-mesenchymal transition (EMT) and metastasis-related phenotypes alteration of residual tumors.

Results: In vitro, the invasion and migration abilities of NSCLC cells were enhanced 72 hours after 44 °C and 46 °C thermal stimulus. Epithelial–mesenchymal transition (EMT) phenotypes were also upregulated under these conditions. RNA sequencing revealed that the expression of carboxypeptidase A4 (CPA4) was significantly upregulated after thermal stimulus. Significant upregulation of CPA4 and EMT phenotypes was also found in the xenograft model of insufficient NSCLC ablation. The EMT process and invasion and migration abilities can be reversed by silencing CPA4.

Conclusions: This study demonstrates that sublethal heat stimulus caused by insufficient ablation can promote EMT and enhance the metastatic capacity of NSCLC. CPA4 plays an important role in these biological processes. Inhibition of CPA4 might be of great significance for improving early-stage NSCLC survival after ablation.

Introduction

Worldwide, lung cancer is a leading cause of cancer incidence and death [1]. The National Lung Screening Trial (NLST) [2] and the Dutch-Belgian Randomized Lung Cancer Screening Trial (NELSON) [3] demonstrated that low-dose computed tomography (CT) screening reduced the mortality of lung cancer by 20%. With the popularity of low-dose CT screening, more early-stage lung cancers were detected early.

Surgery is the recommended treatment for patients with early-stage non-small-cell lung cancer (NSCLC). Referring to National Comprehensive Cancer Network (NCCN) guidelines, for patients with clinical early-stage (Stage I, node-negative) NSCLC who had medical contraindications to surgical resection or who refused surgery, stereotactic body radiotherapy (SBRT) or image-guided thermal ablation was recommended [4]. A large number of studies have shown no significant difference in survival between thermal ablation and SBRT for early-stage NSCLC [5–8]. However, some studies have shown a correlation between tumor size and early recurrence after ablation. Tumors greater than 2 cm in size are especially likely to recur locally after radiofrequency ablation (RFA) [9]. With microwave ablation (MWA), tumors larger than 3 cm in diameter have a better chance of recurrence and result in shorter survival [10].

Thermal ablation is a favorable technique for the treatment of early-stage NSCLC because of its feasibility, effectiveness, and repeatability. Sufficient ablation was the great challenge of this minimally invasive treatment. How to reduce local recurrence and improve survival time are issues of concern. In hepatocellular carcinoma (HCC) [11–13] and breast cancer [14], local recurrence after ablation shows a more malignant phenotype with increased tumor growth and distant metastasis. However, thus far, no study has revealed whether residual tumors exhibit biological behavior changes after insufficient ablation of NSCLC.

In our study, sublethal stimulus induced by insufficient ablation was simulated in vitro to explore the biological behavior changes and molecular changes of NSCLC cells. We...
established a xenograft model of insufficient ablation to verify the molecular changes and mechanisms in vivo.

Materials and methods

Patients and tissue samples

NSCLC samples and matched adjacent tissues from 78 untreated patients were collected from the Characteristic Medical Center of the PLA Rocket Force from February 2012 to December 2017 with the approval of the ethics committee of this institution and written informed consent of all the participants. All protocols were in accordance with the Helsinki Declaration. The clinical characteristics of the recruited patients are presented in Table 1.

Among these 78 patients, 44 (56.4%) patients were male, and 34 (43.6%) were female, with ages ranging from 52 to 91 (mean 74.4 ± 7.0). Squamous cell carcinoma was pathologically diagnosed in 19 patients (24.4%), and non-squamous cell carcinoma was pathologically diagnosed in 59 patients (75.6%). Based on the TNM staging system, 34 patients presented with stage I and stage II disease, followed by 44 with stage III and stage IV disease. The overall survival period was defined as the time from initial diagnosis to the date of death.

Cell culture

A549 and H1975 cells were obtained from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Two cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS, HyClone, Logan, UT).

In vitro heat treatment

Heat treatment was simulated in vitro according to a previously reported procedure [15–18]. Adherent monolayers of A549 and H1975 cells were grown to 70% confluence, and 5 × 10⁶ cells were suspended in 1 ml DMEM, collected in 1.5 ml Boilproof Microtubes (MCT-150-B, Oxygen, Corning, NY), and immediately exposed to heat stimulus using a digital dry bath incubator (CHB-100, KeHuai Instrument, Jiangsu, China) at temperature settings of 37°C, 42°C, 44°C, 46°C, 48°C, and 50°C for 10 min. Cells were then seeded into 60 mm dishes in 10 ml of DMEM with 10% FBS and incubated at 37°C in a humidified atmosphere of 5% CO₂. After 12 h, the cells were trypsinized and centrifuged at 800 rpm for re-inoculation in new dishes to remove debris and dead cells.

Trypan blue exclusion assay

Twenty-four hours after heat treatment, re-adherent cells were harvested by trypsinization. Then, the cells were stained with a 0.4% trypan blue solution (DingGuo Biotech, Shanghai, China) and counted using a cell counting plate.

Cell proliferation assay

Cell proliferation assays were analyzed by a CCK-8 kit (AR1160, Boster, Pleasanton, CA). After 24 h of heat treatment, cells were trypsinized and counted, with 2000 cells seeded per well in 96-well plates. The incubated cells were treated with 10 μl of CCK-8 reagent. The absorbance was recorded 24 h, 48 h, 72 h, and 96 h after thermal stimulus. The absorbance was measured at 450 nm using a Molecular Devices SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

Colony formation assay

Twenty-four hours after heat treatment, re-adherent cells were harvested by trypsinization. The cells were then plated in 6-well plates (500 cells/well) and cultured for 2 weeks. The resulting colonies were stained with crystal violet (DingGuo Biotech, Shanghai, China). Only colonies containing more than 50 cells were regarded as positive colonies and counted.

Apoptosis assay

Apoptosis assays were performed using an apoptosis kit (MK1028, Boster, Pleasanton, CA). A total of 1 × 10⁵ cells/ml were trypsinized and collected 24 h after heat treatment using FACS buffer, washed and stained with 5 μl Annexin V-FITC and 5 μl PI. Each sample was analyzed with flow cytometry (BD FACSScalibur flow cytometer). Data were analyzed by FlowJo software (version 10, SPSS Inc., Chicago, IL).

| Gender | High | Low | χ² | p Value |
|--------|------|-----|-----|---------|
| Male   | 21   | 33  | 2.54 | 0.11    |
| Female | 14   | 10  |     |         |

| Age (year) | High | Low | χ² | p Value |
|------------|------|-----|-----|---------|
| <70        | 25   | 22  | 3.71 | 0.054   |
| ≥70        | 10   | 22  |     |         |

| Smoking | High | Low | χ² | p Value |
|---------|------|-----|-----|---------|
| Yes     | 24   | 27  | 0.29 | 0.59    |
| Non-smoker | 11  | 16  |     |         |

| Histology | High | Low | χ² | p Value |
|-----------|------|-----|-----|---------|
| Squamous  | 7    | 12  | 0.65 | 0.42    |
| Non-squamous | 28  | 31  |     |         |

| Differentiation | High | Low | χ² | p Value |
|-----------------|------|-----|-----|---------|
| Well and moderate | 10  | 30  | 13.1 | <0.01*  |
| Poorly          | 25   | 13  |     |         |

| Tumor size | High | Low | χ² | p Value |
|------------|------|-----|-----|---------|
| ≤5.0 cm    | 17   | 28  | 2.16 | 0.14    |
| >5.0 cm    | 18   | 15  |     |         |

| Regional lymph node involvement | High | Low | χ² | p Value |
|---------------------------------|------|-----|-----|---------|
| N0                              | 13   | 28  | 6.06 | 0.014*  |
| N+                             | 22   | 15  |     |         |
| TNM staging                     |      |     |     |         |
| I + II                         | 10   | 24  | 5.82 | 0.02**  |
| III + IV                       | 25   | 19  |     |         |

*p < 0.05 was considered statistically significant.
**Cell-cycle analysis**

Twenty-four hours after heat treatment, re-adherent cells were harvested by trypsinization. A total of 1 x 10^6 cells were collected and fixed with 70% cold ethanol. After fixation, PI staining solution with RNase A (Abcam Biosciences, Cambridge, UK) was added. After 30 min of incubation, stained samples were run on flow cytometry, and data were analyzed using modfit software LT4.1 (SPSS Inc., Chicago, IL).

**In vivo metastasis assay**

The heat-treated cells above were cultured for 24 h, and an intravenous metastasis model was established. At 24 h after heat stimulus, 5 x 10^6 NSCLC cells were injected into the tail vein of each mouse. Twelve weeks after vein injection, the mice were euthanized and examined. Livers and lungs were harvested, and the numbers of gross metastatic foci were counted. For IHC, tissues embedded in paraffin were sectioned into 5-μm slides. The primary antibodies used in our study are listed below: E-cadherin (CST#3195, 1:200), N-cadherin (CST#13116, 1:200), Vimentin (Abcam#ab8978, 1:200), MMP-2 (CST#40994, 1:200), MMP-9 (CST#13667, 1:200), and CPA4 (Genetex#GTX81517, 1:100). The quantification of IHC was performed by McCarty’s H-score system [19], which incorporates both the intensity of the specific staining and the percentage. The proportion score represented the estimated fraction of positively stained tumor cells (0 = none; 1 = less than 25%; 2 = 25–75%; 3 = greater than 75%). The intensity score represented the average staining intensity of positive tumor cells (0 = none; 1 = weak; 2 = intermediate; 3 = strong). The two scores were multiplied to generate the immunoreactivity score (IS) for each case. Five fields were randomly selected to add up to the total score. CPA4 expression was defined as either high expression (score ≥ 15) or low expression (score < 15). The results were reviewed by pathologists who were blinded to the research purpose.

**Cell migration and invasion assays**

Cell migration and invasion were assessed by transwell assays (Corning Incorporated Costar, Corning, NY). Briefly, in the migration assay, 5 x 10^5 cells suspended in 100 μl serum-free DMEM were seeded into the upper chamber of each well of 24-well plates containing 8.0-μm pore size membranes. FBS (500 μl) was added to the lower chamber of each well. After 24 h, cells that had reached the underside of the membrane were stained with crystal violet, and then 5 randomly selected areas (100× magnification) per well were counted. For the invasion assay, the upper compartment was precoated with 100 μl of Matrigel (0.8 mg/ml, BD Biosciences, Franklin Lakes, NJ), and cells were stained 48 h after cells were seeded onto the membranes.

**Quantitative real-time PCR (RT-PCR)**

Total mRNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA), and cDNA was synthesized using an All-in-One First-Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD). RT-PCR was performed with BlazeTaq SYBR Green qPCR MIX 2.0 Kit (GeneCopoeia, Rockville, MD). The primer sequences used to determine the expression of the target genes were as follows:

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| Snail forward | 5’-GACCCCAATCGGAAGCTCATAA-3’ | 5’-AGGGCTGCGGGAAGCTAAAC-3’ |
| Snail reverse | 5’-GGCAGATGGTCTCCAGAGG-3’ | 5’-CTTCTTCTGGTCTGGTGG-3’ |
| Twist1 forward | 5’-GGACAGTCGAGMCCAGGG-3’ | 5’-GAATGCAAATTTGATGCCGTTG-3’ |
| Twist1 reverse | 5’-GGACAGTGATTCCCAGACGG-3’ | 5’-AGGGCTGCTGGAAGGTAAAC-3’ |
| CPA4 forward | 5’-CGTGTAGTGATCTACATATGGTTG-3’ | 5’-GAATGCAAATTTGATGCCGTTG-3’ |
| CPA4 reverse | 5’-GGATCCCTATGCTGCGAGCA-3’ | 5’-GGATCCCTATGCTGCGAGCA-3’ |
| β-Actin forward | 5’-GGATCCCTATGCTGCGAGCA-3’ | 5’-GGATCCCTATGCTGCGAGCA-3’ |
| β-Actin reverse | 5’-GCCCATGGATAGCACAGC-3’ | 5’-GCCCATGGATAGCACAGC-3’ |

The PCR consisted of 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 1 min at 55°C and primer extension for 1 min at 72°C. The comparative cycle threshold (Ct) method was used to quantitatively gene expression. β-actin was the internal control.

**Immunohistochemistry (IHC) and immunofluorescence staining (IF)**

For IF, the cells were cultured on confocal dishes and fixed, permeabilized with 0.5% Triton X-100 (Thermo Fisher Scientific, Waltham, MA), and incubated with Vimentin (Abcam#ab8978, 1:200) and CPA4 (Genetex#GTX81517, 1:100) overnight at 4°C, followed by incubation with secondary antibodies conjugated with Alexa Fluor 488 (Abbkine#A23220, 1:500) and 594 (Abbkine#A23420, 1:500). Cells were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Images were captured by confocal microscopy (LSM980, Zeiss, Jena, Germany).

**Western blot analysis**

Total cellular proteins were extracted using RIPA lysis buffer (89900, Thermo Fisher Scientific, Waltham, MA) containing protease inhibitor cocktail (78430, Thermo Fisher Scientific, Waltham, MA). Protein concentration was measured according to the instructions of the BCA kit (Thermo Scientific, Waltham, MA). The primary antibodies used in our study are listed below: E-cadherin (CST#3195, 1:1000), N-cadherin (CST#13116, 1:1000), Vimentin (Abcam#ab8978, 1:200), MMP-2 (CST#40994, 1:1000), MMP-9 (CST#13667, 1:1000), CPA4 (Genetex#GTX81517, 1:500), and GAPDH (Am1020b, YTHX Biotechnology, 1:2000). The signals were detected using the Western Blotting Detection System (Bio-Rad, Hercules, CA).

**RNA-sequencing (RNA-seq) and data analysis**

Twenty-four hours after heat treatment, re-adherent cells were harvested by trypsinization. RNA was isolated by an RNA Fast2000 Extraction kit (220011, Fastagen, Oakland, CA). Transcriptome RNA-Seq was performed using Illumina high-
throughput RNA sequencing [20]. In brief, the DNA libraries were sequenced according to the Illumina TruSeq v3 protocol on an Illumina HiSeq2500 sequencer (Illumina, San Diego, CA).

Gene expression was quantified using FeatureCounts software (version 1.4.6) based on the ENSEMBL gene annotation for GRCH38. RNA-Seq data were normalized by trimmed mean of M-values (TMM) using EdgeR’s normalization factor, followed by quantile normalization, and presented by log2-fold change (log2 FC) scales.

Genes with significant down- or upregulation (fold change ≥ 1.5, p < 0.0001) under the indicated conditions were analyzed by the web-based functional analysis tool Ingenuity Pathway Analysis (IPA) to visualize and annotate their biological functions and pathways.

Gene set enrichment analysis (GSEA) was performed using GSEA software (http://software.broadinstitute.org/gsea/).

**RNA interference**

Small interfering RNA (siRNA) targeting human CPA4 was purchased from GenePharma (Shanghai, China).

The siRNA sequences were as follows:

| Si-CAP4-1   | 5′-GACGAGAUCAGCAAAUUGATT-3′ |
| Si-CAP4-2   | 5′-GGCGGAUGUAUGUACUGAATT-3′ |
| Si-NC       | 5′-UUCUCCGAACGUGACGUUGTT-3′ |

siRNA transfections were performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA; Thermo Fisher Scientific, Waltham, MA), and the transfection medium was Opti-MEM (Gibco, Carlsbad, CA; Thermo Fisher Scientific, Waltham, MA). The final concentration of siRNA was 50 nM, the medium was replenished after 24 h, and the cells were harvested 48 h post-transfection. The efficiency of transfection was verified with qRT-PCR and Western blot.

**Xenograft tumor model and insufficient ablation of NSCLC**

A xenograft tumor model was employed as described [21]. Briefly, 1 × 10⁶ A549 cells were injected subcutaneously into the flanks of 3- to 4-week-old BALB/c nude mice (18–20 g), which were purchased from Yiming Bio Co., Ltd. (Beijing, China). The protocol was approved by the ethical committee on Animal Assays of the Animal Care Committee of Characteristic Medical Center of the PLA Rocket Force. Tumor volume was calculated according to the following formula: volume (mm³) = (largest diameter × shortest diameter²)/2. When the tumor volume reached 1000 mm³, the ablation was performed.

A total of 10 mice were randomly divided into two groups: the ablation group and the control group. There were five mice in each group. The ablation device was a microwave ablation device (MTC-100, Fuzhong Medical Hi-Tech Co., Ltd. Nanjing, China). Referring to several previous studies [11,16], insufficient ablation was performed with a lower energy protocol, in which the power was 5 W and the duration was 30 s, as shown in Figure 10(A). This ensured the presence of residual tumors. The control group was sham-operated by inserting a needle electrode into the tumor without performing ablation. All mice were sacrificed on the 10th day. Tumors were placed in a 4% paraformaldehyde solution. Tumor sections were assessed by H&E staining and IHC.

**Statistical analysis**

Statistical analysis was performed by SPSS 23.0 software (SPSS, Chicago, IL) and Prism GraphPad 7.0 (GraphPad Software, La Jolla, CA). All data shown are the results of at least three independent experiments and are expressed as the mean ± standard error of the mean (SEM). The differences between groups were compared using Student’s t test. Pearson’s χ² test was used to analyze differences between CPA4 expression and NSCLC clinicopathological parameters. Correlations of continuous variables were analyzed by the Pearson correlation test. Survival curves were constructed by the Kaplan–Meier method, and a log-rank test was used for comparison. All tests were two-tailed, and p < 0.05 was considered statistically significant.

**Results**

**Sublethal thermal stimulus at 46 °C and 48 °C inhibited the proliferation and increased the apoptosis of NSCLC cells in vitro, and 44 °C and 46 °C stimulus enhanced the invasion and migration ability of NSCLC cells in vitro**

We determined the survival rate after 24 h of heat treatment. The survival rate after heat treatment decreased as the temperature increased. As shown in Figure 1, 24 h after heat treatment at 42 °C, the survival rates of A549 and H1975 cells were 90.0 ± 3.5% and 92.3 ± 4.0%, respectively, while those at 44 °C were 85.3 ± 1.7% and 90.4 ± 3.2%, and the survival rates were 75.5 ± 3.5% and 78.0 ± 4.2%, respectively. After heat treatment at 48 °C, the survival rates of the two cell lines were 50.2 ± 5.7% and 48.5 ± 5.0%, respectively. At 50 °C, the cell survival rates were less than 20% and the cells became round...
and non-adherent. The cell survival rates were significantly reduced by thermal stimulus above or equal to 46 °C. Referring to other studies and trypan blue exclusion assays, 42-48 °C was the sublethal treatment temperature in vitro.

The proliferation of A549 and H1975 cells was inhibited by heat treatment at 46 °C and 48 °C, while the proliferation abilities of A549 and H1975 cells showed no significant change after treatment at 42 °C and 44 °C (Figure 2(A,B)). Consistent with the results of the proliferation assay, 46 °C and 48 °C resulted in cell cycle arrest in the G0/G1 phase of NSCLC cells (Figure 2(C)).

Compared to the control group, the apoptosis of cells was also significantly increased at 46 °C and 48 °C, by 6.2 ± 1.2% (p < 0.05) and 8.8 ± 0.7% (p < 0.05) in A549 cells and 8.8 ± 1.3% (p < 0.05) and 19.0 ± 2.0% (p < 0.05) in H1975 cells, respectively. In the 42 °C and 44 °C groups, there was no significant increase in apoptosis compared to the control group (Figure 2(D)).

Invasion and migration abilities were detected by transwell assay after 72 h of incubation after thermal stimulus. In both A549 and H1975 cells, the invasion and migration abilities were enhanced at 44 °C and 46 °C. Of these, the number of invaded and migrated cells was elevated more in the 44 °C group (Figure 3(A)).

Taken together, the sublethal temperatures of 46 °C and 48 °C can inhibit the proliferation of NSCLC cells. At 44 °C and 46 °C, cell invasion and migration were enhanced, and this enhancement was most obvious at 72 h. About 44 °C is the trigger temperature to stimulate cell invasion and migration, and this temperature does not affect cell proliferation.

**Sublethal thermal stimulus at 44 °C and 46 °C increased the metastatic capacity of A549 cells in tail vein tumor models**

To assess metastatic capacity in vivo, A549 cells treated at different temperatures were injected into the tail veins of nude mice. As shown in Figure 3(B,C), in the 44 °C and 46 °C groups, the numbers of metastases on the lung surface and liver were significantly greater than those in the control group. Of these, the number of metastases was greater in the 44 °C group.

Increases in the invasion and migration abilities of NSCLC cells at 44 °C and 46 °C were confirmed in vivo.
Thermal stimulus at 44 °C and 46 °C led to epithelial-mesenchymal transition (EMT) in NSCLC in vitro

Snai1 and Twist1 are well-known transcription factors that determine EMT. The expression levels of Snai1 and Twist1 were detected by RT-PCR. In A549 cells, the mRNA expression of Snai1 and Twist1 peaked 72 h after 44 °C heat treatment. The same result was also obtained in H1975 (Figure 4(A)). EMT markers were detected by Western blot after incubation for 72 h after thermal stimulus, showing that E-cadherin expression decreased and N-cadherin and Vimentin expression increased in the 44 °C and 46 °C groups compared with the control group, indicating the occurrence of the EMT process (Figure 4(B)). Immunofluorescence also indicated that vimentin fluorescence was significantly increased in the 44 °C and 46 °C groups (Figure 4(C,D)). Between them, the changes in EMT markers were more significant in the 44 °C group.

Furthermore, we observed changes in EMT markers over time under thermal stimulus at 44 °C. At 72 h, NSCLC cells
expressed the lowest amount of E-cadherin and the highest amounts of N-cadherin and Vimentin (Figure 4(E)). This result suggested that EMT was most obvious 72 h after thermal stimulus.

The above results suggested that 44°C thermal stimulus for 72 h most significantly promoted EMT characteristics in NSCLC.

Differentially expressed genes (DEGs) after 44°C heat treatment in A549 cells

About 44°C was the trigger temperature for inducing malignant transition of NSCLC in vitro as determined through the above studies. The mechanism might be that thermal stimulus initiated the EMT process in NSCLC. To explore which molecule plays the key role in this process, RNA sequencing was performed after 24 h of thermal stimulus at 44°C.

Seventy-four upregulated and 20 downregulated differentially expressed genes were identified by RNA sequencing (Figure 5(A)). The top 13 upregulated genes and top 5 downregulated genes are shown in the heatmap by order of fold changes. The expression level of CPA4 mRNA in the thermal stimulus group was significantly higher than that in the control group (fold change = 2.17, p < 0.001) (Figure 5(B)).

The top 20 most significantly enriched pathways were selected to produce the Kyoto Encyclopedia of Genes and Genomes (KEGG) scatter plot. The rich factor was the ratio of the number of DEGs to the number of genes annotated in a given pathway (Figure 5(C)).

GSEA demonstrated a significant enrichment of gene signature-associated cell adhesion molecules (normalized

Figure 4. Sublethal thermal stimulus promoted NSCLC cell EMT in vitro. (A) EMT transcription factors (Snai1 and Twist1) expression of NSCLC cells detected by RT-PCR at 24 h, 48 h, and 72 h after heat treatment. (B) After 72 h of NSCLC cells were heat-treated, the protein levels of EMT markers were detected by western blot. (C) Immunofluorescence analysis of Vimentin expression in A549 and H1975 cells after 72 h of different thermal temperatures treatment. Scale bars, 10 μm. (D) The relative fluorescence intensity statistical graph of Vimentin. (E) After heat treatment at 44°C, EMT markers expression of NSCLC cells was detected at different time by western blot. Presented data were representative one from at least three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.
enrichment score, NES = 1.61, p < 0.01) and EMT (normalized enrichment score, NES = 2.73, p < 0.01) (Figure 5(D,E)).

RNA sequencing revealed that the mRNA expression of EMT-related molecules was upregulated after 44°C treatment, and the expression level of CPA4 was significantly upregulated. We hypothesized that CPA4 played an important role in the malignant transition and EMT of NSCLC after thermal stimulus.

Cpa4 was highly expressed in NSCLC tissues and associated with patient prognosis

To determine CPA4 expression in NSCLC, IHC was performed in 78 primary human NSCLC tissues and 40 paracarcinoma tissues. As presented in Figure 6(A,B), CPA4 was highly expressed in NSCLC specimens and showed stronger staining, whereas CPA4 staining was weaker in paracarcinoma tissues (p = 0.029).

We further analyzed the association between CPA4 expression and clinicopathological parameters. As presented in Table 1, high expression of CPA4 was observed more often in patients with poorly differentiated (65.7% versus 25.0%, p < 0.01) positive lymph node status (59.4% versus 31.7%, p = 0.014) and in advanced-stage patients (56.8% versus 29.4%, p = 0.02).

Moreover, the Kaplan–Meier survival curve showed that high CPA4 expression was correlated with shorter overall survival (p = 0.017) (Figure 6(C)). From the Cancer Genome Atlas (TCGA) datasets, the expression of CPA4 was positively correlated with the EMT markers N-cadherin (R = 0.30, p < 0.01) and Vimentin (R = 0.16, p < 0.01) (Figure 6(D)).

The expression of CPA4 can be induced by sublethal thermal stimulus

In both A549 and H1975 cells, after heat treatment at 44°C, the mRNA of CPA4 was upregulated gradually and peaked at 48 h. It decreased slightly at 72 h (Figure 7(A)).
expression of CPA4 was consistent with the mRNA expression trend, and the expression level peaked at 48 h (Figure 7(B)). We confirmed this conclusion by IF assay. The expression of CPA4 in NSCLC cells was significantly upregulated 48 h after 44 °C thermal stimulus (Figure 7(C,D)).

Silencing CPA4 reversed the malignant biological behavior and EMT of NSCLC caused by sublethal thermal stimulus

The interference efficiency of the siRNA was detected by RT-PCR, Western blot, and IF to confirm the feasibility of the siRNA knockdown. In A549 cells, compared with si-NC cells, the relative mRNA expression levels of the si-CPA4-1 group and si-CPA4-2 groups were 67.0 ± 17.2% and 33.3 ± 10.0%, respectively (p < 0.05). In H1975 cells, compared with si-NC cells, the relative mRNA expression levels of the si-CPA4-1 group and si-CPA4-2 groups were 76.0 ± 12.2% and 45.5 ± 14.3%, respectively (p < 0.05) (Figure 8(A)). Western blotting and IF also confirmed a significant decrease in protein levels after silencing CPA4 (Figure 8(B–D)).

CPA4 knockdown by small interfering RNA reduced the invasion and migration abilities promoted by sublethal thermal stimulus (Figure 9(A)). Consistent with the cell migration and invasion assays, si-CPA4 not only downregulated the background levels of MMP protein but also reversed the upregulation of MMP protein expression caused by thermal stimulus (Figure 9(B)). Western blot and IF showed that the expression of EMT markers was downregulated after silencing CPA4 (Figure 9(C–E)).

Silencing CPA4 can increase apoptosis after thermal stimulus in A549 and H1975 cells

After CPA4 knockdown, A549 and H1975 cells were more sensitive to heat damage and showed increased apoptosis rates. After 44 °C heat treatment, in A549 cells, the apoptosis rates were 6.0 ± 0.5% in the si-CPA4-1 group and 7.5 ± 0.3% in the si-CPA4-2 group, while the apoptosis rate was 4.2 ± 0.3% in the negative control group (p < 0.05). In H1975 cells, the apoptosis rates were 9.2 ± 0.9% in the si-CPA4-1 group and 14.8 ± 1.2% in the si-CPA4-2 group, while the apoptosis rate was 6.7 ± 0.7% in the negative control group (p < 0.05) (Figure 9(F)).

Insufficient ablation increased metastatic potential and induced EMT in NSCLC in vivo

After 10 days of insufficient thermal ablation, the mice were sacrificed, and the tumors were dissected. The tumor volume of the insufficient ablation group was significantly smaller than that of the sham ablation group (246.0 ± 81.2 mm³ versus 1206.0 ± 32.2 mm³, p < 0.01) (Figure 10(A)).

At the sufficient ablation zone, the cells appeared to be necrotic. Compared with the control group, cells in the insufficient ablation zone showed spindle changes, which might result in the EMT process (Figure 10(B)). Compared with the sham ablation group, the expression of E-cadherin decreased significantly in residual tumors, while N-cadherin and vimentin were upregulated (Figure 10(C)). This indicates that EMT occurred in the sublethal ablation zone. The expression of MMP-2 and MMP-9 was also detected and showed significant upregulation in the residual
tumor tissues (Figure 10(D)). The expression of CPA4 was also significantly higher in residual tumors than in the control group (Figure 10(E)).

Discussion

Some recent studies reported local recurrence rates of 30–40% for early-stage NSCLC after ablation [22–25]. Tumor size is widely recognized as an important factor related to the efficacy of lung tumor ablation [26,27], and tumor morphology is also an important risk factor for recurrence [28]. Sufficient inactivation of some early-stage NSCLC was difficult to achieve due to these risk factors. We hypothesize that residual tumor cells from insufficient ablation are the reason for the high relapse rate and shorter 5-year survival rate for early-stage NSCLC.

In HCC, several studies have found that insufficient ablation enhances the invasion and migration abilities of residual tumors. Some have found that sublethal thermal stimulus induced by insufficient ablation can induce EMT in residual HCC cells and breast cancer cells [15,16,18,29]. Other studies

Figure 7. Relationship between CPA4 expression and sublethal thermal stimulus. (A) The RNA expression level of CPA4 at different time after thermal stimulus at 44 °C was detected by RT-PCR. (B) The protein expression level of CPA4 at different time after thermal stimulus at 44 °C was detected by western blot. (C) IF analysis of CPA4 expression in A549 and H1975 cells after 48 h of different thermal temperatures treatment. Scale bars, 10 µm. (D) The relative fluorescence intensity statistical graph of CPA4 in A549 and H1975 cells after 48 h of different thermal temperatures treatment. All groups were compared with the 0 h group. Experiments were independently repeated three times. * p < 0.05, ** p < 0.01, *** p < 0.001.
have also found that sublethal thermal stimulus lead to the differentiation of viable HCC cells into cancer stem cells [11,30–32].

However, no study is currently available that addresses the biological behavior changes and mechanisms of NSCLC after insufficient ablation.

In vitro, 44 °C and 46 °C could enhanced invasion and migration abilities and induced EMT of NSCLC. Between the two temperatures, the induction ability was more obvious at 44 °C. Interestingly, CCK-8 assay and clone formation assay showed that 46 °C inhibit cell proliferation significantly, however, 44 °C did not inhibit proliferation. We believed that 44 °C is the temperature with the most malignant characteristic. In HCC, the malignant behavior of HCC cells could be enhanced at 47 °C [17,29,31–35]. It has been proved that temperatures which induced an increase in malignant behavior were difference in different tumors.

EMT is a process with particular phenotypic changes, through which epithelial cells lose their cell-cell adhesions and cell polarity, increase the expression of mesenchymal cell markers and migratory capacity, resist apoptosis, and produce extracellular matrix components [36,37]. Recent cancer studies have revealed that EMT, which is essential for the transformation of early-stage tumors into aggressive malignancies, is an early event in tumor metastasis [38]. In our study, EMT occurred in residual tumors after insufficient ablation. We inferred that EMT occurred after triggering temperature stimulation of NSCLC cells, leading to enhanced invasion and migration abilities. Then, the tumors recurred and metastasized.

To determine which biological process is regulated, RNA-seq was conducted. By RNA-seq, we detected that the expression of CPA4 was significantly increased after 44 °C treatment. By insufficient ablation in a xenograft model, we found that the expression of CPA4 in residual tumors was upregulated. In vitro, the expression of CPA4 was also upregulated by sublethal thermal stimulus, which was verified by PCR and Western blot.

CPA4 is a member of the metallocarboxypeptidase (MCP) family. CPA4 functions in neuropeptide processing and regulation in the extracellular environment, which are closely related to cancer progression [39]. CPA4 is highly expressed in a variety of solid cancers and is closely related to poor prognosis [40–46]. We found that CPA4 expression in NSCLC was significantly higher than that in para-cancerous tissues, and patients with high expression of CPA4 had shorter survival. Patient samples collected in our study showed that tumor tissue with high CPA4 expression exhibited poor differentiation, positive lymph node status and advanced stage, which predicted worse prognosis. Our findings were consistent with those of another study [40].

In TCGA datasets, there was a positive correlation between CPA4 and EMT marker expression. The expression of CPA4 was also positively correlated with the expression of N-cadherin and Vimentin and negatively correlated with the expression of E-cadherin in the xenograft tumor model of insufficient ablation. In pancreatic cancer, a study showed...
Figure 9. CPA4 altered biological behavior and EMT process of NSCLC. (A) Representative images and quantification of cell invasion and migration results. Silencing CPA4 reversed the enhanced invasion and migration abilities of NSCLC cells caused by sublethal thermal stimulus. Scale bars, 50 μm. (B) Silencing CPA4 reversed the up-regulated expression of MMP-2, MMP-9 caused by sublethal thermal stimulus in NSCLC cells. (C) Silencing CPA4 decreased the expression of the phenotypes associated with EMT in NSCLC cells. It also blocks the EMT process activated by sublethal thermal stimulus. (D) Representative images of Vimentin expression detected by IF assay. Silencing CPA4 reversed the expression of Vimentin caused by thermal stimulus using IF assay. Scale bars, 10 μm. (E) The relative fluorescence intensity statistical graph of vimentin in A549 and H1975 cells after different thermal temperatures treatment and silencing CPA4. (F) Cellular apoptosis was assayed by Annexin V/PI staining and detected by flow cytometry. The apoptosis rates of A549 and H1975 were not significantly increased after CPA4 knockdown. A549 and H1975 with silenced CPA4 stimulated at 44 °C, the apoptosis rates increased significantly. Presented data were representative one from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.
that CPA4 overexpression promoted EMT [47]. To explore whether CPA4 can initiate EMT in NSCLC, we knocked down CPA4 with siRNA and found that silencing CPA4 could reverse EMT induced by sublethal heat treatment. Overall, we confirmed that upregulated CPA4 can induce EMT. To the best of our knowledge, we were the first to demonstrate that CPA4 overexpression can induce EMT in NSCLC. Moreover, the invasion and migration abilities of NSCLC cells were also decreased after silencing CPA4. The enhancement of invasion and migration caused by sublethal heat treatment can also be reversed. This indicated that CPA4 initiated EMT and malignant transition.

In addition, silencing CPA4 can also increase the apoptosis of NSCLC cells caused by sublethal heat treatment, so CPA4 may also have a protective effect against heat damage.

Our findings show that sublethal thermal stimulus induced by insufficient ablation can lead to upregulation of CPA4 expression in NSCLC cells, initiation of EMT, and an increase in malignant characteristics, which were specifically manifested as enhanced invasion and migration abilities and enhanced adaptability to heat damage.

The process and mechanism by which insufficient ablation leads to malignant transition through CPA4 has great significance in the ablation of early-stage NSCLC. First, complete ablation is the key to reducing the recurrence of early-stage NSCLC. Tumors less than 2 cm in diameter and relatively regular in shape might be more appropriate for thermal ablation. Second, CPA4 is a secreted protein, and studies have shown that serum CPA4 levels could be used for the early detection of NSCLC [48], breast cancer [41], pancreatic cancer [45], and liver metastasis in colorectal carcinoma [44]. We can monitor recurrence after ablation by the level of CPA4 in the serum. Third, the carboxypeptidase inhibitor sabellastarte magnifica (SmCI) has been reported to suppress the metallocarboxypeptidase activity of CPA4 by forming a complex with CPA4 [49]. SmCI could be used in adjuvant therapy and to prevent tumor recurrence after NSCLC ablation with high recurrence factors. In addition, one study...
indicated the synergistic effect of the chemotherapy combine with hyperthermia. This treatment pattern might be more effective than hyperthermia or chemotherapy alone [29].

Our data suggest CPA4 as a potential and promising predictor and therapeutic target for preventing recurrence after thermal ablation.

**Disclosure statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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