

Original Research

Dynamic evolution of chaperone-mediated autophagy is associated with tumor microenvironment remodeling and prognostic stratification in lung adenocarcinoma: insights from single-cell transcriptomics, ensemble machine learning, and experimental validation

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ABSTRACT

Background: Lung adenocarcinoma (LUAD) shows prognostic heterogeneity, and tumor-node-metastasis (TNM) staging is limited for individualized management. Chaperone-mediated autophagy (CMA) maintains proteostasis, but its role during adenocarcinoma in situ (AIS)-minimally invasive adenocarcinoma (MIA)-invasive adenocarcinoma (IAC) progression remains unclear.

Methods: Single-cell RNA sequencing (scRNA-seq) data from GSE189357 and bulk transcriptomes from The Cancer Genome Atlas (TCGA)-LUAD and Gene Expression Omnibus (GEO) cohorts were integrated. CMA activity, cell-cell communication, weighted gene co-expression network analysis (WGCNA), tumor-normal differential expression, machine-learning survival modeling, tumor microenvironment (TME) features, drug sensitivity, and EPC1 function were analyzed.

Results: CMA-high tumor epithelial cells increased from AIS (58.1%) to MIA (65.7%) but declined in IAC (44.4%; $p < 0.001$). CMA-low cells preferentially received fibroblast-derived extracellular matrix cues. A CMA-negatively correlated module identified 69 core genes. Random survival forest (RSF) performed best among 117 machine-learning combinations (mean concordance index > 0.873). High-risk patients had worse survival across cohorts, and the risk score was independently associated with overall survival (hazard ratio = 16.013, 95% confidence interval: 9.579–26.768, $p < 0.001$). High-risk tumors showed proliferative activation and M0 macrophage enrichment, whereas low-risk tumors showed stronger immune-related signaling. EPC1 overexpression suppressed malignant phenotypes in A549 cells.

Conclusion: CMA dynamics are associated with stromal and immune remodeling during LUAD progression. A CMA-based model provides robust prognostic stratification and may offer a basis for future TME-guided studies.

Introduction

Lung cancer remains the leading cause of cancer-related mortality worldwide. According to the most recent global cancer statistics, approximately 2.5 million new cases and 1.8 million deaths were attributed to lung cancer in 2022 [1]. Lung adenocarcinoma (LUAD) accounts for nearly half of all non-small cell lung cancers (NSCLC), representing roughly 40%–50% [2]. Although targeted therapies, typified by epidermal growth factor receptor (EGFR) and anaplastic

lymphoma kinase (ALK) inhibitors, as well as immune checkpoint inhibitors (ICIs), have substantially prolonged survival in subsets of patients, the clinical outcomes of LUAD remain profoundly heterogeneous [3–5]. The 5-year survival rate for patients with localized early-stage LUAD can exceed 60%, whereas it declines sharply to below 10% in those with distant metastases [6]. The traditional tumor–node–metastasis (TNM) staging system has shown clear limitations in capturing the highly dynamic molecular evolution of tumors and in guiding individualized interventions [7]. Therefore, there is an urgent need to move

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beyond phenotype-based stratification and to develop prognostic biomarker models with high predictive accuracy and translational potential, grounded in the molecular mechanisms that drive tumor microenvironment (TME) evolution [8,9].

Chaperone-mediated autophagy (CMA) is a highly selective lysosomal degradation pathway that preserves intracellular proteostasis through the specific recognition of substrate proteins harboring KFERQ-like motifs [10,11]. In tumorigenesis and cancer progression, CMA plays a context-dependent dual role [12]. On one hand, in normal or pre-malignant cells, CMA may exert tumor-suppressive effects by targeting oncogenic proteins for degradation, such as mutant p53 and MYC [13,14]. On the other hand, in established malignancies, cancer cells often aberrantly upregulate CMA to degrade pro-apoptotic factors or metabolic rate-limiting enzymes, such as hexokinase 2 (HK2), thereby conferring survival advantages under hypoxia, nutrient deprivation, or chemotherapy-induced stress [15,16]. These stress-adaptive functions may be particularly relevant during the transition from AIS/MIA to IAC, in which tumor cells progressively encounter proteostasis imbalance, hypoxic pressure, extracellular matrix remodeling, and altered stromal and immune interactions. Consistently, CMA has been linked to hypoxia-inducible factor-1 α regulation, tumor-associated extracellular matrix remodeling, and immune/stromal regulation in cancer-related contexts [17–19]. However, during the canonical multistep progression of LUAD from adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA) to invasive adenocarcinoma (IAC), substantial knowledge gaps remain regarding the spatial distribution of CMA activity, the dynamic evolution of its target network, and how dysregulated CMA within the microenvironment drives remodeling of intercellular communication [20].

Recent single-cell and spatial transcriptomic studies have provided important resources for understanding the stepwise evolution of LUAD from preinvasive lesions to invasive carcinoma. In particular, GSE189357 was generated to characterize the spatiotemporal transcriptional landscape of LUAD across AIS, MIA, and IAC, thereby offering a valuable opportunity to examine stage-associated cellular remodeling at single-cell resolution [21]. In parallel, TCGA-LUAD and independent GEO cohorts such as GSE31210 and GSE50081 have been widely used for bulk transcriptomic analysis, early-stage LUAD prognostic assessment, and cross-cohort validation [22,23]. However, these resources have not been fully integrated to investigate how CMA activity changes during the AIS-MIA-IAC sequence, how CMA-related tumor epithelial states interact with stromal and immune components, and whether CMA-associated molecular features can be translated into a robust prognostic model.

In the present study, we integrated single-cell transcriptomic data, bulk transcriptomic cohorts, ensemble machine-learning algorithms, tumor microenvironment analysis, drug sensitivity prediction, and in vitro functional validation to investigate the role of CMA in LUAD progression. We first characterized CMA activity across the AIS-MIA-IAC continuum and analyzed CMA-associated intercellular communication patterns at single-cell resolution. We then identified CMA-related core genes and developed a prognostic risk model with external validation in independent cohorts. Finally, we evaluated immune features, predicted therapeutic sensitivity, and experimentally assessed the functional relevance of EPC1, the top-ranked model gene. Collectively, this study provides a CMA-centered framework for understanding proteostasis-related microenvironmental remodeling and improving prognostic stratification in LUAD.

Methods

Data acquisition and preprocessing

scRNA-seq data were obtained from the Gene Expression Omnibus (GEO) dataset GSE189357, which comprised tissue samples from 9 LUAD patients, including 3 cases of AIS, 3 cases of MIA, and 3 cases of

IAC. Bulk RNA-seq data and corresponding clinicopathological information (including survival time, survival status, and TNM stage) were downloaded from TCGA (TCGA-LUAD cohort), GTEx, and GEO (GSE31210 and GSE50081). Specifically, transcriptomic analyses included 600 tumor samples and 59 adjacent normal samples from TCGA-LUAD. In addition, 578 normal lung tissue samples from GTEx were incorporated to expand the healthy control set. For merged datasets involving multiple sources, batch effects were corrected using the ComBat algorithm implemented in the *sva* R package to mitigate systematic biases across sequencing platforms and cohorts.

scRNA-seq preprocessing, dimensionality reduction, and cell annotation

Seurat (v4.0) was used for quality control and downstream analyses of the GSE189357 scRNA-seq data [24]. Cells were retained if they contained 100–7500 detected genes, <15% mitochondrial gene expression, and 1000–15,000 total RNA counts.

After quality control, data were normalized using LogNormalize. A total of 2000 HVGs were identified with FindVariableFeatures, followed by scaling with ScaleData. PCA was performed for dimensionality reduction, and the top 20 principal components (Dims = 1:20) were selected for visualization using UMAP and t-SNE. Unsupervised clustering was conducted using FindNeighbors and FindClusters (resolution = 0.2). Based on canonical marker genes, clusters were manually annotated into 11 major cell types, for example, B cells expressing CD79A/CD19, endothelial cells expressing CLDN5/VWF, and tumor epithelial cells expressing NKX2-1/NAPSA.

Single-cell CMA activity scoring and inference of cell–cell communication networks

To quantify CMA-related activity at the transcriptomic level, CMA-related genes were derived from the previously cited study by Khawaja et al. [25], in which CMA-associated genes were categorized according to effector functions, positive regulation, and negative regulation. Based on this classification, CMA-related genes were grouped into CMA effector genes, positive regulators, and negative regulators. For each cell, CMA score was calculated using log-normalized expression values according to the following formula:

$$\text{CMA_score} = [2 \times \text{sum}(\text{Effector genes}) + \text{sum}(\text{Positive regulators}) - \text{sum}(\text{Negative regulators})]$$

/ total number of genes included in the signature.

Effector genes were assigned a higher weight because they represent the core machinery directly involved in CMA substrate recognition and lysosomal translocation, whereas positive and negative regulators were used to reflect upstream regulatory effects. Because this score was derived from transcriptomic expression profiles, it was interpreted as a CMA-related transcriptomic proxy rather than a direct measurement of CMA flux.

Tumor epithelial cells were then extracted and dichotomized into CMA-high and CMA-low groups using the median CMA score as the cutoff. The median cutoff was selected to generate balanced subgroups for downstream comparisons and to reduce bias caused by arbitrary or outcome-driven threshold selection. Cell–cell communication patterns between CMA-high/CMA-low tumor cells and other cellular subsets in the microenvironment were inferred using CellChat, with a focus on the strength and directionality of ligand–receptor signaling axes.

Differential expression analysis and weighted gene co-expression network analysis (WGCNA)

Differential expression analysis was performed between CMA-high and CMA-low tumor epithelial subpopulations using FindAllMarkers

based on scRNA-seq data. The resulting single-cell DEGs were merged with established CMA signature genes to generate the input gene set for WGCNA. At the bulk transcriptome level, co-expression networks were constructed using the WGCNA R package based on the expression matrix of the merged gene set in TCGA-LUAD [26]. After examining sample clustering dendrograms to remove potential outliers, the optimal soft-thresholding power was determined using pickSoftThreshold to approximate a scale-free topology, and co-expression modules were identified using a dynamic tree-cutting algorithm. Module eigengenes were correlated with CMA score using Pearson correlation, and the module showing the strongest negative association with CMA score was selected as the core CMA-related module.

To ensure disease relevance and specificity, bulk DEGs were identified between TCGA-LUAD tumor samples and normal lung tissues, where normal controls were defined as the integrated set of TCGA adjacent normal samples and GTEx normal lung samples. Differential expression analysis was performed using limma, and p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) method. Genes with FDR-adjusted $p < 0.05$ and $|\log_2FC| > 0.25$ were considered differentially expressed [27]. This relatively permissive fold-change threshold was used to retain genes with moderate expression changes for subsequent integrative screening, rather than as the sole criterion for final candidate gene selection. The GEO cohorts were not included in WGCNA module construction or module-trait correlation analysis. Finally, overlapping genes between the bulk DEG set and the WGCNA core CMA-related module were retained as the high-confidence CMA core gene set.

Construction of a CMA prognostic risk model using ensemble machine-learning algorithms

To identify prognostically informative features and build a robust model, the CMA core genes were used to develop and validate models in TCGA (training set) and two independent GEO cohorts (GSE31210 and GSE50081). In total, 117 machine-learning algorithm combinations were evaluated, including random survival forest (RSF), Lasso, Ridge, Elastic Net, CoxBoost, GBM, superPC, plsRcox, survival-SVM, and stepwise Cox regression. Models were trained using 10-fold cross-validation, and the mean concordance index (C-index) across the three cohorts was used as the primary performance criterion. The best-performing RSF model ($ntree = 1000$, $splitrule = "logrank"$) was selected to compute the Risk Score for each sample, and patients were stratified into high-risk and low-risk groups using the median Risk Score as the cutoff [28]. All feature selection steps, including WGCNA module identification, bulk differential expression filtering, and CMA core gene selection, were completed before external validation. The GSE31210 and GSE50081 cohorts were used exclusively for independent validation and were not involved in candidate gene selection, model training, or parameter tuning.

Association with clinical characteristics and nomogram development

In this study, the primary clinical outcome was overall survival (OS), defined as the time from diagnosis to death from any cause or last follow-up. Patients who were alive at the last follow-up were treated as censored. Univariable and multivariable Cox proportional hazards regression models were used to assess whether the Risk Score was an independent prognostic factor after adjustment for clinicopathological variables such as age, sex, and TNM stage. A nomogram was constructed using rms by incorporating variables that remained statistically significant in the multivariable Cox model to estimate 1-, 3-, and 5-year overall survival probabilities. Calibration curves were used to evaluate agreement between predicted and observed outcomes. DCA was conducted to quantify clinical net benefit, and time-dependent C-index curves were plotted to characterize how predictive accuracy varied over time.

Functional enrichment and comprehensive TME profiling

To characterize pathway-level biological features associated with risk stratification, differential expression profiles between the high-risk and low-risk groups were analyzed using clusterProfiler for GSEA and the GSVA R package for GSVA. ssGSEA was used to estimate immune-related pathway activity and infiltration scores for 28 immune cell subsets [29]. In addition, CIBERSORT was applied to quantify the relative proportions of 22 tumor-infiltrating immune cell types, and Spearman correlation analyses were performed to assess associations between immune infiltration levels and the Risk Score as well as core model genes.

Drug sensitivity prediction

To explore the potential of the Risk Score in guiding individualized therapy, drug sensitivity was predicted using pRRophetic based on in vitro pharmacogenomic data from GDSC. Half-maximal inhibitory concentration (IC50) values were estimated for multiple chemotherapeutic agents and targeted kinase inhibitors across cohorts. Differences in predicted drug sensitivity between high-risk and low-risk groups were compared using the Wilcoxon rank-sum test [30].

Cell culture and in vitro functional validation

To experimentally assess the functional relevance of EPC1, the top-ranked gene in the CMA-related prognostic model, in vitro gain-of-function assays were performed after EPC1 overexpression, followed by evaluation of proliferation, migration, invasion, and colony formation.

Cell transfection and Western blotting: A549 cells were transfected with a Flag-tagged EPC1 overexpression plasmid. Total protein was extracted 48 h post-transfection, EPC1 expression was detected using an anti-Flag antibody, and GAPDH was used as the loading control [31].

Cell proliferation assay (CCK-8): Transfected A549 cells were seeded into 96-well plates. At 0 h, 24 h, 48 h, and 72 h, CCK-8 reagent was added and absorbance at 450 nm was measured using a microplate reader to generate proliferation curves [32].

Wound-healing assay: When cells reached 100% confluence, a linear scratch was created using a 200 μ L pipette tip. After 24 h of serum-free incubation, images were acquired at 0 h and 24 h under a microscope, and migratory capacity was quantified by relative wound width. All experiments were independently repeated three times [33].

Transwell migration and invasion assays: Transwell chambers without or with Matrigel coating were used to evaluate cell migration and invasion, respectively. Cells suspended in serum-free medium were seeded into the upper chambers, while medium containing 10% FBS was added to the lower chambers. After incubation, cells on the lower membrane surface were fixed, stained, and photographed for quantification [34].

Colony formation assay: Transfected cells were seeded into 6-well plates at a low density and cultured for 1 week. The formed colonies were fixed with methanol, stained with crystal violet, and the relative number of colonies was counted and calculated [35].

Statistical analysis

All statistical analyses and visualizations were performed in R (version 4.3.0 or later). Continuous variables were compared using the Wilcoxon rank-sum test or Student's *t*-test, as appropriate. Comparisons among multiple groups were conducted using the Kruskal–Wallis test. Survival differences were evaluated using the Kaplan–Meier method with the log-rank test. Time-dependent receiver operating characteristic (ROC) curves were used to assess discriminative performance [36]. Unless otherwise specified, all tests were two-sided, and $p < 0.05$ was considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

All analytical processes are illustrated in the flowchart (Fig. 1).

Single-cell transcriptomic analysis reveals a heterogeneous distribution of CMA activity in the LUAD microenvironment

To comprehensively characterize the cellular composition of the LUAD tumor microenvironment and the spatial distribution of CMA activity, we performed scRNA-seq analysis on 102,425 high-quality cells derived from 9 LUAD specimens in GSE189357, including 3 AIS, 3 MIA, and 3 IAC samples.

Using UMAP-based clustering and established cell-type marker signatures, all cells were annotated into 11 major populations (A): T/NK cells, epithelial cells, macrophage cells, B cells, mast cells, plasma cells, endothelial cells, fibroblasts, tumor epithelial cells, proliferating cells, and myeloid cells. Cell-type assignments were validated by canonical marker expression (Fig. 2B). Specifically, B cells highly expressed CD79A, CD79B, and CD19. Endothelial cells specifically expressed CLDN5, PECAM1, and VWF. Epithelial cells expressed EPCAM, KRT18, and KRT19. LUAD tumor cells expressed NKX2-1, NAPS1, and KRT7. Fibroblasts highly expressed COL1A1, DCN, and COL1A2. Macrophage cells expressed MRC1, CD163, and C1QA. Mast cells expressed CPA3, MS4A2, and TPSAB1. Myeloid cells expressed S100A8, S100A9, and VCAN. Plasma cells expressed JCHAIN, IGKC, and IGHG1. Proliferating cells highly expressed MKI67, TOP2A, and UBE2C. T/NK cells expressed CD3D, CD3E, and NKX2-1.

We next compared cellular composition across pathological stages (AIS, MIA, and IAC) (Fig. 2C). As disease progressed from AIS to MIA and IAC, the proportion of tumor epithelial cells showed a biphasic pattern, initially decreasing and then increasing, whereas the proportions of T/NK cells and B cells increased and mast cells gradually declined, indicating progressive immune microenvironment remodeling. Quantitative comparisons across histologic subtypes further confirmed significant shifts in multiple cell populations during the AIS–MIA–IAC continuum (Fig. 2D).

Subsequently, we calculated CMA scores for each cell (CMA score). Visualization of CMA score on UMAP demonstrated pronounced heterogeneity of CMA activity across cell types (Fig. 2E). Notably, CMA score exhibited distinct distribution patterns among AIS, MIA, and IAC. Across most cell types, CMA score in the IAC group was significantly lower than that in AIS and MIA (Fig. 2F), suggesting that decreased CMA activity may be associated with LUAD progression. This observation provided a rationale for subsequent construction of a CMA-related prognostic risk model.

CMA stratification of tumor epithelial cells reveals progression-associated remodeling of intercellular communication

A total of 9915 tumor epithelial cells were extracted for subpopulation analysis (Fig. 3A). Using the median CMA score as the cutoff, cells were stratified into a CMA-high group ($n = 4957$) and a CMA-low group ($n = 4958$). UMAP visualization of the two subgroups is shown in Fig. 3B, and no highly specific spatial segregation was observed.

The proportions of the two subpopulations across histologic subtypes displayed a non-linear dynamic pattern (Fig. 3C). The fraction of CMA-high cells increased from 58.1% in AIS to 65.7% in MIA, followed by a sharp decline to 44.4% in IAC (trend $p < 0.001$). The transient enrichment of CMA-high cells in MIA may reflect a compensatory response to proteostasis stress during early microinvasion. In contrast, the predominance of CMA-low cells in IAC suggests that attenuation of CMA function may confer a selective advantage during invasive growth.

CellChat analysis revealed distinct communication programs between the two subpopulations. In the global interaction network (Fig. 3D), CMA-high cells exhibited markedly stronger communication with macrophages, fibroblasts, and endothelial cells than CMA-low cells,

indicating a greater capacity to sculpt the microenvironment. Outgoing signaling analysis (Fig. 3E) showed that CMA-high cells preferentially activated VEGF (VEGFA–VEGFR1/R2), THBS1 (THBS1–SDC4/CD47), and MDK (MDK–SDC1/LRP1) signaling axes to communicate with endothelial cells and fibroblasts. CMA-high cells also maintained antigen-presentation interactions with T/NK cells via HLA molecules (e.g., HLA-DRA–CD4). These pathways were substantially weakened in CMA-low cells, consistent with reduced immune visibility and diminished pro-angiogenic signaling. Incoming signaling analysis (Fig. 3F) further demonstrated that CMA-low cells received stronger extracellular matrix cues from fibroblasts, including collagen family ligands (COL1A1/A2, COL4A1/A2, COL6A1–A3) as well as FN1 and LAMB1/LAMA family signals, suggesting that CMA-low tumor cells are embedded within a denser extracellular matrix network. This enrichment is unlikely to be purely passive and more plausibly reflects enhanced integrin-mediated matrix adhesion that provides pro-survival and pro-migratory signals, thereby promoting an invasive phenotype.

WGCNA combined with differential expression analysis identifies a core CMA gene set

To systematically identify gene modules most tightly associated with CMA activity at the bulk transcriptome level, we first derived DEGs between CMA-high and CMA-low groups at the single-cell level. These DEGs, together with CMA signature genes, were then used to perform WGCNA in the TCGA-LUAD cohort. The sample clustering dendrogram and CMA trait heatmap (Fig. 4A) showed no apparent outliers. CMA scores exhibited a continuous distribution across samples, supporting the suitability for co-expression network construction. The gene dendrogram with dynamic tree cutting identified five co-expression modules (turquoise, yellow, blue, brown, and the unassigned grey module) (Fig. 4B).

Module-trait correlation analysis (Fig. 4C) demonstrated that the blue module showed the strongest negative correlation with CMA score ($r = -0.8$, $p = 1e-132$), far exceeding the absolute correlations of other modules (turquoise: $r = 0.24$. yellow: $r = 0.17$. brown: $r = 0.1$), thereby defining it as the core CMA-associated module. This strong inverse association indicates that blue-module genes are upregulated in tumors with low CMA activity, suggesting that these genes may either contribute to CMA suppression or represent alternative pathways activated to compensate for CMA deficiency. Consistently, module membership (MM) and gene significance (GS) within the blue module were highly correlated ($\text{cor} = 0.92$, $p = 1.8e-135$) (Fig. 4D), indicating that genes with stronger module affiliation also exhibit stronger associations with CMA score, supporting the biological robustness of the blue module as a CMA-related functional program.

To enhance the validity of the candidate genes, we further performed bulk differential expression analysis between TCGA tumor samples and normal controls defined by the integrated TCGA and GTEx lung tissues (Fig. 4E). The distribution of the top upregulated and downregulated genes is shown in Fig. 4F. To obtain a high-confidence gene set supported by both co-expression and differential expression evidence, we intersected 4283 DEGs with the 330 genes in the blue module (Fig. 4G), yielding 69 core CMA genes. These genes satisfied two independent criteria: membership in the module most strongly and negatively associated with CMA activity, and significant differential expression between tumor and normal tissues. This integrative strategy provides higher specificity than any single approach and establishes a reliable candidate pool for subsequent prognostic modeling and mechanistic investigations.

Construction of a CMA-related prognostic risk model by integrating multiple machine-learning algorithms

To maximize model robustness and generalizability, we systematically evaluated the predictive performance of 117 machine-learning

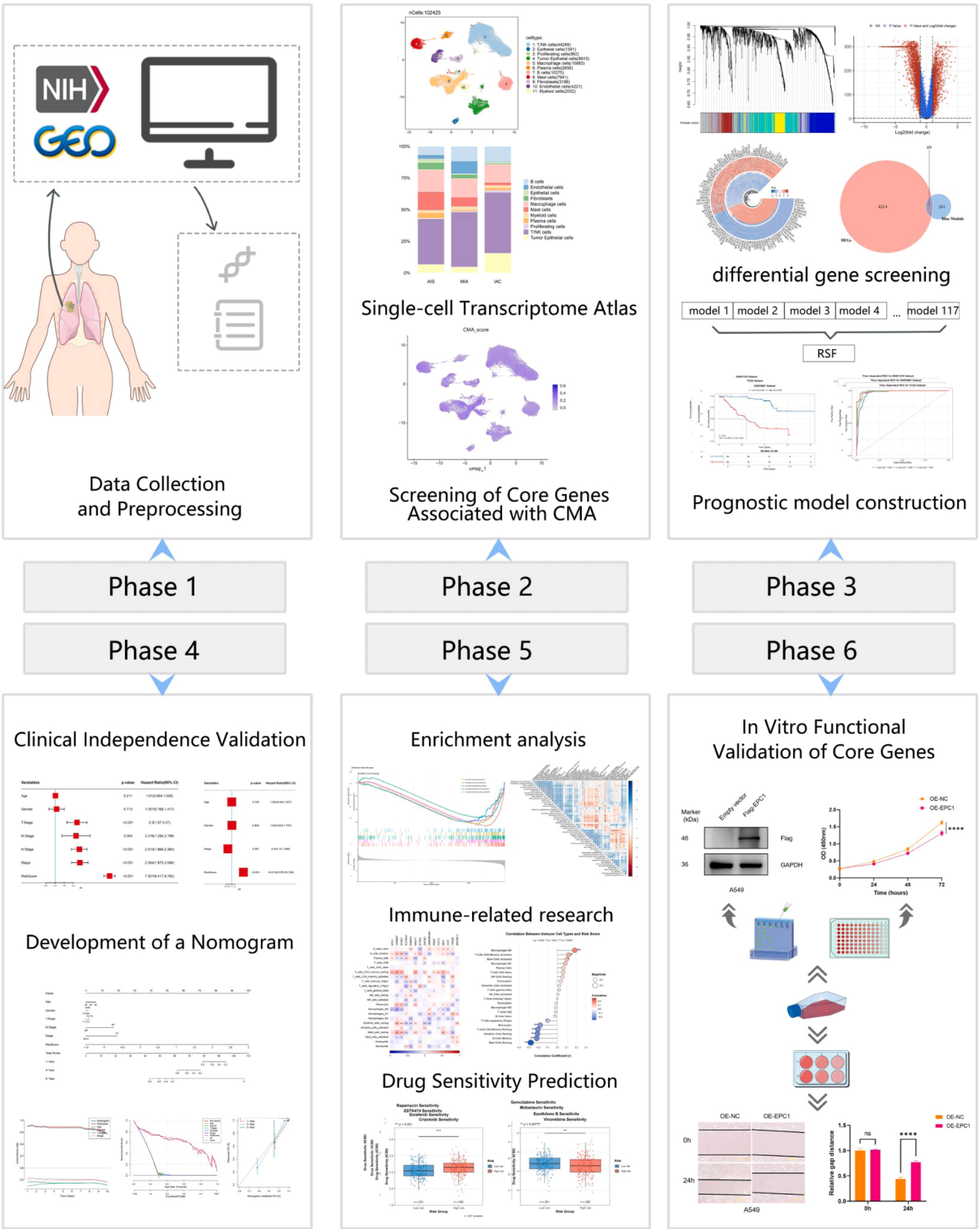


Fig. 1. Study flowchart.

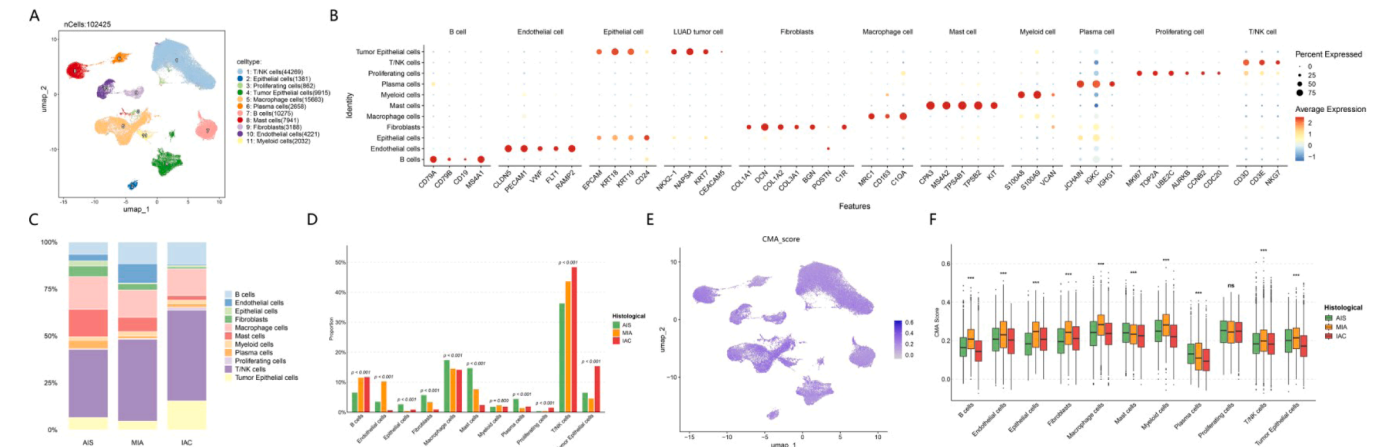


Fig. 2. Single-cell transcriptomic analysis reveals the cellular landscape of the LUAD microenvironment and heterogeneous CMA activity. (A) UMAP plot showing clustering of 102,425 single cells annotated into 11 cell types. Cell types and cell numbers are indicated in the legend (nCells: 102,425). (B) Bubble plot showing marker gene expression across cell types. Bubble size represents the percentage of cells expressing the gene, and color intensity represents the average expression level. (C) Stacked bar plot showing the proportional distribution of cell types across AIS, MIA, and IAC. (D) Bar plot comparing the proportions of each cell type among AIS, MIA, and IAC. (E) UMAP plot showing the spatial distribution of CMA score across all cells. Color intensity from light to dark indicates low to high CMA score. (F) Box plots comparing CMA score among AIS, MIA, and IAC within each cell type (** $p < 0.001$. ns, not significant).

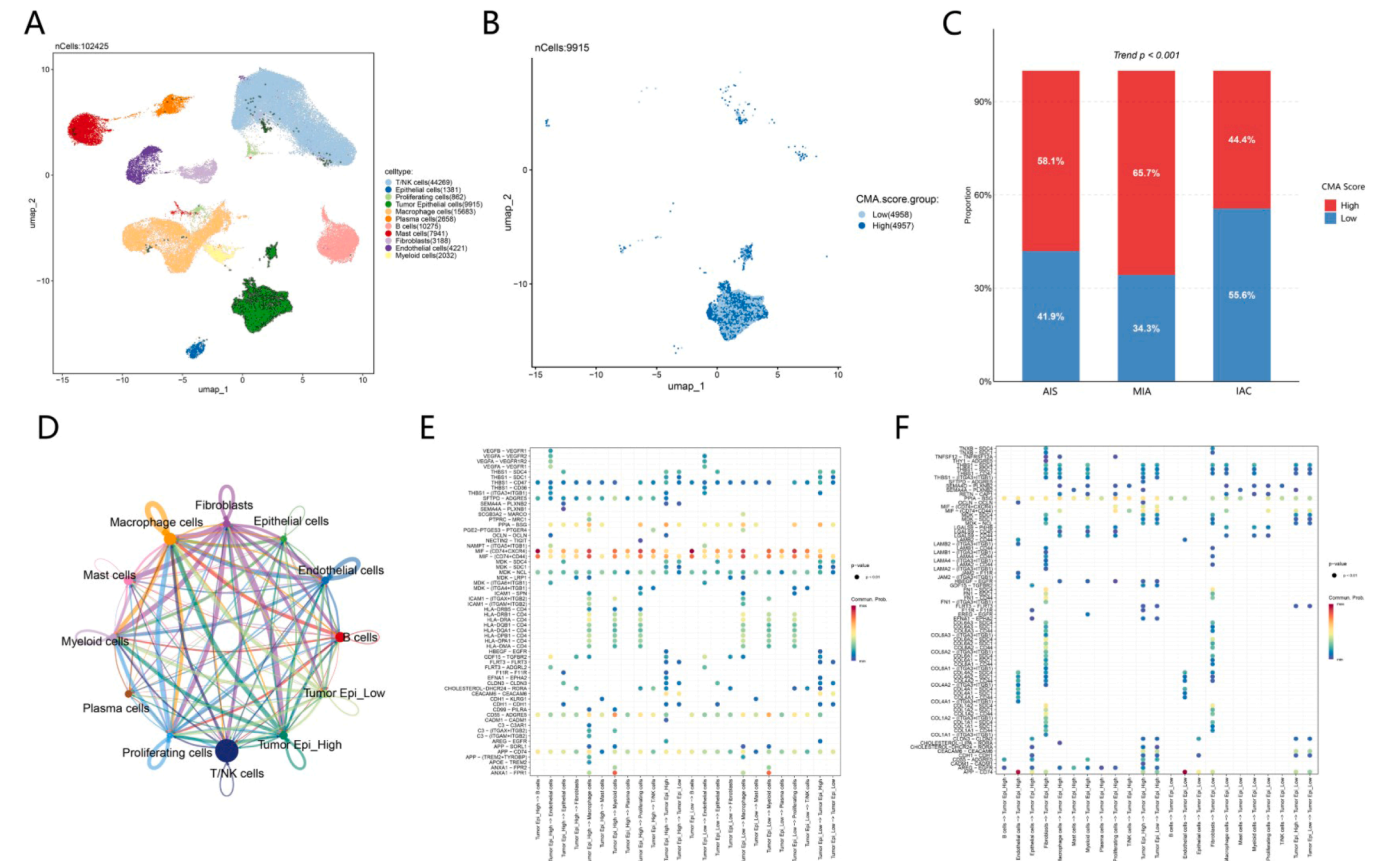


Fig. 3. CMA stratification and cell-cell communication analysis of tumor epithelial cells. (A) UMAP plot showing cell-type distribution of all 102,425 cells. (B) UMAP plot of 9915 tumor epithelial cells stratified into CMA-high (dark blue) and CMA-low (light blue) groups using the median CMA score. (C) Stacked bar plot showing the proportions of CMA-high and CMA-low cells across AIS, MIA, and IAC (trend $p < 0.001$). (D) CellChat chord diagram showing the global communication network between CMA-high/CMA-low cells and microenvironmental cell types. Line thickness indicates interaction strength. (E) Bubble plot showing ligand-receptor communication probabilities from CMA-high or CMA-low cells to other cell types. Bubble size denotes p-value significance, and color from blue to red indicates low to high communication probability. (F) Bubble plot showing ligand-receptor signals received by CMA-high or CMA-low cells from other cell types. Legend definitions are the same as in (E).

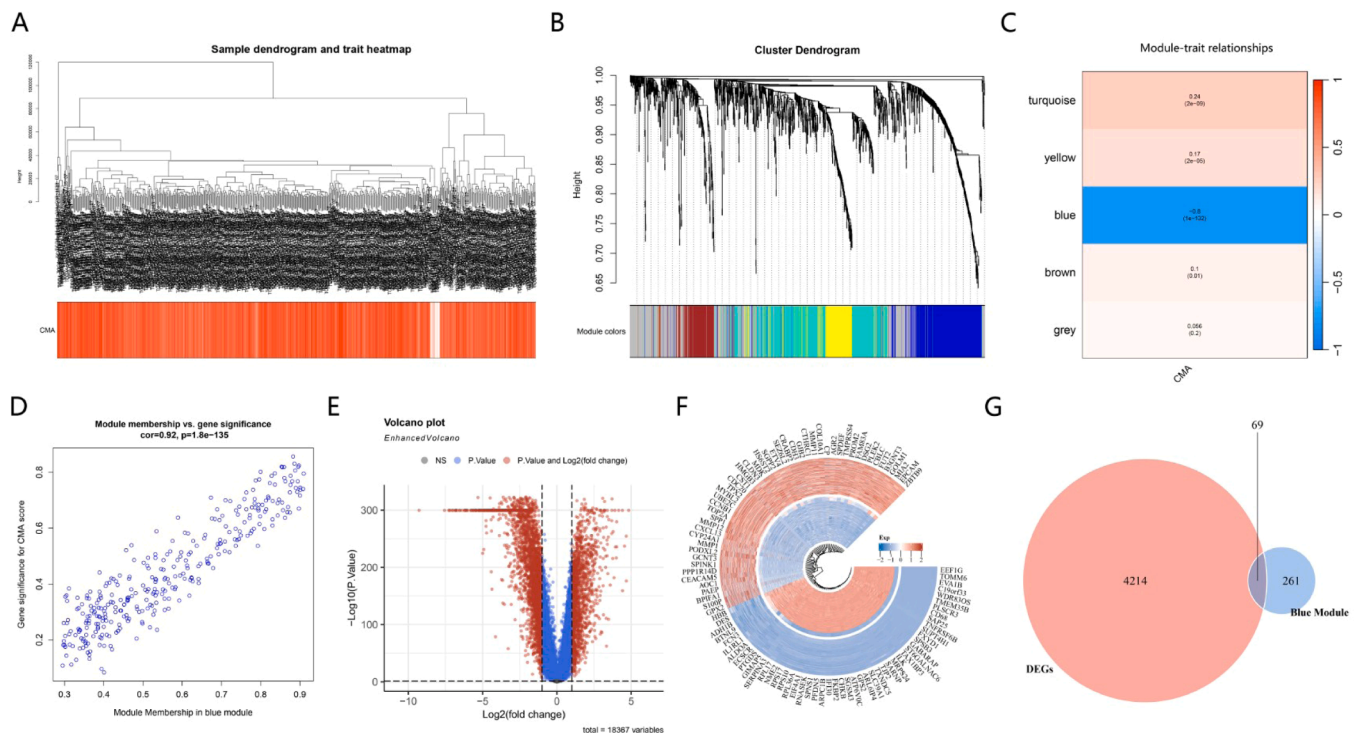


Fig. 4. Identification of a core CMA gene set by integrating WGCNA and differential expression analysis. (A) Sample clustering dendrogram of TCGA-LUAD and the CMA_score trait heatmap. Darker red indicates higher CMA_score. (B) Gene clustering dendrogram and module color assignment. The color bar at the bottom indicates co-expression modules. (C) Module-trait correlation heatmap. Values denote Pearson correlation coefficients, with p-values in parentheses. Colors from blue to red represent negative to positive correlations. (D) Scatter plot of module membership versus gene significance for CMA score within the blue module. (E) Volcano plot of differential expression between tumor and normal tissues. (F) Circos plot showing chromosomal locations and expression changes of DEGs. Outer to inner rings indicate chromosome positions, gene labels, and $\log_2FC$ values, respectively. (G) Venn diagram showing the overlap between DEGs and blue-module genes.

algorithm combinations based on the 69 CMA core genes in the TCGA training set and two independent validation cohorts (GSE31210 and GSE50081) (Fig. 5A). Using the mean C-index across the three cohorts as the integrated metric, RSF and RSF-based hybrid models consistently ranked among the top performers, with mean C-index values exceeding 0.82, markedly outperforming conventional approaches such as Lasso-Cox, Elastic Net, and support vector machine-based models. These results indicate that nonparametric, tree-ensemble models are better suited than linear regression frameworks for capturing nonlinear interactions and higher-order feature relationships within the CMA gene set. Accordingly, the RSF model with the best overall ranking was selected as the final prognostic scoring tool.

Variable importance ranking within the RSF model (Fig. 5B) showed that EPC1 had the highest importance score (0.0143). As a core subunit of the NuA4 histone acetyltransferase complex, EPC1 ranking first suggests that epigenetic regulation may serve as a key intermediary mechanism linking CMA activity to clinical outcomes.

Using the RSF-derived risk score, patients were stratified into high-risk and low-risk groups according to the median cutoff. Kaplan-Meier analyses consistently demonstrated significantly worse overall survival (OS) in the high-risk group across all three cohorts: TCGA (Fig. 5C, HR = 58.73, 95% CI: 26.97–127.90, $p < 0.001$), GSE31210 (Fig. 5D, HR = 21.11, 95% CI: 5.06–88.07, $p < 0.001$), and GSE50081 (Fig. 5E, HR = 6.74, 95% CI: 3.44–13.23, $p < 0.001$).

Time-dependent ROC analysis further quantified discriminative performance at distinct time points. In TCGA (Fig. 5F), the 1-year, 3-year, and 5-year AUCs were 0.966, 0.984, and 0.987, respectively, indicating exceptionally high predictive accuracy without attenuation over time. In GSE31210 (Fig. 5G), the 1-year, 3-year, and 5-year AUCs were 0.825, 0.834, and 0.834, supporting stable mid- to long-term predictive capacity. In GSE50081 (Fig. 5H), the 1-year AUC was 0.853, increasing to 0.921 at 3 years and 0.885 at 5 years. Across the

three cohorts, all time-point AUCs exceeded 0.82. Notably, the 5-year AUC in TCGA approached 1.0, suggesting that the RSF model not only provides strong short-term risk stratification but also maintains excellent long-term prognostic discrimination, underscoring its translational potential.

Association of the CMA risk score with clinicopathological features and subgroup survival analyses

To evaluate the clinical relevance of the CMA-related risk score and its prognostic value beyond conventional clinical parameters, we systematically examined associations between the risk score and major clinicopathological features, and further validated its stratification capacity in key subgroups.

Comparisons of clinicopathological characteristics between risk groups (Supplementary Figure S1A) showed significant differences in vital status ($p < 0.001$), T stage ($p = 4e-04$), N stage ($p < 0.001$), and overall clinical stage ($p < 0.001$), whereas M stage ($p = 0.0857$) and sex ($p = 0.2276$) were not statistically significant. The high-risk group exhibited a markedly higher proportion of death events and was enriched for advanced disease (T3–4, N1–3, and stage III–IV), indicating that the CMA risk score is closely associated with invasive progression.

We next quantified differences in risk score distributions across T stages (Supplementary Figure S1B–C) and clinical stages (Supplementary Figure S1D–E). The risk score increased progressively with higher T stage. After collapsing T stage into T1–2 versus T3–4 (Supplementary Figure S1F), the T3–4 group had a significantly higher risk score than the T1–2 group ($p < 0.0001$), supporting the ability of the risk score to discriminate local tumor burden. With respect to clinical stage (Supplementary Figure S1D), the risk score increased from stage I to stage III ($p < 0.01$), with no significant difference between stage III and stage IV. After regrouping into stage I–II versus stage III–IV (Supplementary

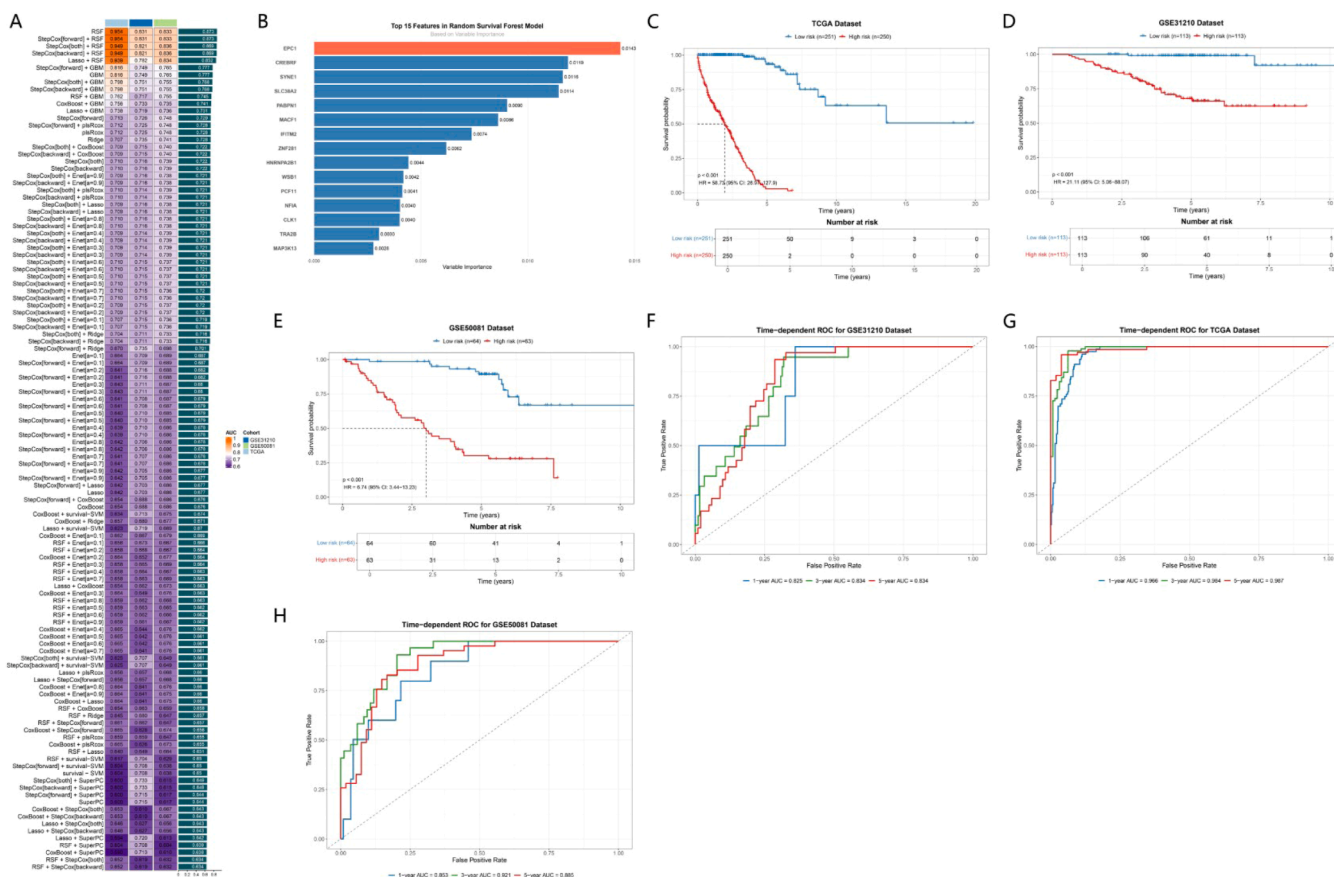


Fig. 5. Construction of a CMA-related prognostic risk model by integrating multiple machine-learning algorithms. (A) Heatmap of C-index values for 117 machine-learning algorithm combinations in the TCGA training set and the GSE31210 and GSE50081 validation cohorts. Colors from blue to red indicate low to high C-index. (B) Variable importance ranking of the top 15 feature genes in the RSF model. The x-axis indicates variable importance. (C–E) Kaplan–Meier OS curves comparing the high-risk group (red) and low-risk group (blue/black) in TCGA (C), GSE31210 (D), and GSE50081 (E). Numbers at risk are shown below. (F–H) Time-dependent ROC curves for TCGA (F), GSE31210 (G), and GSE50081 (H). Red, green, and blue denote 1-year, 3-year, and 5-year AUCs, respectively.

Figure S1E), the stage III–IV group exhibited significantly higher risk scores ($p < 0.0001$). A stacked bar plot (Supplementary Figure S1F) further illustrated stage composition differences between groups. Low-risk patients were predominantly stage I, whereas the high-risk group contained a substantially higher proportion of stage II–IV cases, corroborating the stage-discriminatory capacity of the risk score.

These association analyses suggest that the CMA risk score is not a purely molecular biomarker independent of clinical staging but is strongly coupled with TNM stage. This finding has two implications. First, it indicates that the CMA-related gene set captures biologically meaningful information relevant to invasive progression. Second, it raises a critical question: whether the model provides additional prognostic stratification within the same stage.

To address this question, Kaplan–Meier analyses were performed in age- and stage-stratified subgroups. In age-stratified analyses, both the ≤ 60 -year subgroup (Supplementary Figure S1G. low risk $n = 89$, high risk $n = 78$. HR = 30.44, $p < 0.001$) and the > 60 -year subgroup (Supplementary Figure S1H. low risk $n = 162$, high risk $n = 172$. HR = 125.64, 95% CI: 30.38–519.62, $p < 0.001$) showed consistently shorter survival in the high-risk group. In stage-stratified analyses, the stage I–II subgroup (Supplementary Figure S1I. low risk $n = 218$, high risk $n = 171$. HR = 160.08, 95% CI: 38.57–664.35, $p < 0.001$) demonstrated particularly strong prognostic separation. The stage III–IV subgroup (Supplementary Figure S1J. low risk $n = 27$, high risk $n = 77$. $p < 0.001$) also showed significant survival differences, with a clearly better prognosis in the low-risk group. Together, these findings indicate that even after clinical stage is determined, the CMA risk score provides additional

and clinically meaningful prognostic information within stages, with utility for refined risk stratification among early-stage (stage I–II) patients.

Independent prognostic analysis and nomogram construction

To determine whether the CMA risk score is an independent prognostic factor beyond conventional clinicopathological parameters, we performed univariable and multivariable Cox regression analyses. In univariable analysis (Supplementary Figure S2A), T stage (HR = 2.30, 95% CI: 1.57–3.37, $p < 0.001$), M stage (HR = 2.216, 95% CI: 1.294–3.796, $p = 0.004$), N stage (HR = 2.513, 95% CI: 1.866–3.384, $p < 0.001$), overall clinical stage (HR = 2.564, 95% CI: 1.875–3.506, $p < 0.001$), and the CMA risk score (HR = 7.927, 95% CI: 6.417–9.792, $p < 0.001$) were all significant predictors of OS. Notably, the risk score exhibited a substantially larger HR than any clinical variable, whereas age ($p = 0.211$) and sex ($p = 0.713$) were not significant. In multivariable analysis (Supplementary Figure S2B), only the CMA risk score remained independently significant (HR = 16.013, 95% CI: 9.579–26.768, $p < 0.001$), and its HR increased after adjustment. Although clinical stage was highly significant in univariable analysis ($p < 0.001$), it lost independent significance in the multivariable model ($p = 0.067$), indicating that the risk score largely encompasses and extends beyond the prognostic information provided by conventional staging, while capturing additional molecular signals not reflected by stage.

Based on these results, a nomogram was constructed by integrating the CMA risk score with clinical variables (age, sex, T stage, M stage, and

overall clinical stage) to predict 1-year, 3-year, and 5-year OS probabilities (Supplementary Figure S2E). In this model, the CMA risk score contributed the widest point range (approximately -1.5 to 3.0), dominating the total score and highlighting its central prognostic contribution. Calibration curves (Supplementary Figure S2C) demonstrated close agreement between predicted and observed survival probabilities at each time point, indicating good calibration with minimal systematic bias. Decision curve analysis (Supplementary Figure S2D) showed that across most clinically relevant threshold probabilities, both the nomogram and the risk score achieved higher net benefit than any single clinical variable, as well as the extreme strategies of treating all or treating none. Time-dependent C-index trajectories (Supplementary Figure S2F) further confirmed that the C-index values of the nomogram and the risk score remained above 0.90 throughout the 1- to 10-year follow-up period, whereas C-index values for conventional clinical variables fluctuated around 0.50, approximating random performance. Collectively, these results demonstrate that the CMA risk score is a strong independent prognostic factor. Incorporation into a nomogram enables precise individualized survival prediction with clear translational potential.

Differential pathway activity between high- and low-risk groups

To further delineate the functional context captured by the CMA risk score, we performed pathway enrichment analyses based on Hallmark gene sets. GSEA results (Supplementary Figure S3A–B) showed that pathways significantly enriched in the high-risk group included E2F_TARGETS, G2M_CHECKPOINT, MTORC1_SIGNALING, MYC_TARGETS_V1, and MYC_TARGETS_V2, which represent core regulatory programs of proliferation and metabolic reprogramming, suggesting a stronger proliferative drive in tumors from high-risk patients. In contrast, the low-risk group was enriched for immune-related pathways, including ALLOGRAFT_REJECTION, IL6_JAK_STAT3_SIGNALING, INFLAMMATORY_RESPONSE, INTERFERON_ALPHA_RESPONSE, and INTERFERON_GAMMA_RESPONSE, indicating a more active immune surveillance and inflammatory response within the microenvironment.

GSVA differential analysis (Supplementary Figure S3C) corroborated these findings at a global level. Proliferation-related pathways (e.g., G2M_CHECKPOINT, E2F_TARGETS, and MYC_TARGETS) were significantly upregulated in the high-risk group, whereas immune and inflammatory pathways (e.g., INTERFERON_ALPHA/GAMMA_RESPONSE and IL6_JAK_STAT3_SIGNALING), metabolic pathways (e.g., HEME_METABOLISM, BILE_ACID_METABOLISM, and FATTY_ACID_METABOLISM), and the P53_PATHWAY were more active in the low-risk group. A pathway correlation heatmap (Supplementary Figure S3D) revealed clear separation into two functional modules. Proliferation-associated pathways (E2F_TARGETS, MYC_TARGETS, G2M_CHECKPOINT, DNA_REPAIR, and related programs) were highly positively correlated, forming a tightly coordinated network. Immune-inflammatory pathways (INTERFERON_RESPONSE, INFLAMMATORY_RESPONSE, COMPLEMENT, ALLOGRAFT_REJECTION, and related programs) formed a second positively correlated module. The two modules showed an overall negative correlation trend, reflecting antagonism between proliferative drive and immune surveillance.

Univariable Cox regression of pathway activity (Supplementary Figure S3E) further evaluated the association between key pathways and survival. High activity of proliferation-related pathways such as MYC_TARGETS_V1, MTORC1_SIGNALING, MITOTIC_SPINDLE, and G2M_CHECKPOINT was significantly associated with poor prognosis. In contrast, increased activity of pathways such as HEME_METABOLISM and IL6_JAK_STAT3_SIGNALING was associated with more favorable survival outcomes, consistent with their enrichment patterns in the low-risk group. Collectively, the high-risk phenotype defined by the CMA risk score is characterized by excessive activation of proliferative programs and suppression of the immune microenvironment, whereas the low-risk phenotype exhibits active immune responses and preserved

metabolic homeostasis, revealing a clear functional divergence underlying risk stratification.

Immune microenvironment profiling

To systematically characterize the association between the CMA risk score and the immune microenvironment, we performed integrative evaluations across three dimensions: immune pathway activity, immune cell infiltration abundance, and correlations between model genes and immune cell infiltration. The ssGSEA heatmap of immune-related pathways (Supplementary Figure S4A) showed that the low-risk group exhibited higher enrichment scores across nearly all innate and adaptive immune pathways, including T-cell receptor signaling, B-cell receptor signaling, natural killer cell-mediated cytotoxicity, antigen processing and presentation, Toll-like receptor signaling, NOD-like receptor signaling, RIG-I-like receptor signaling, chemokine signaling, leukocyte transendothelial migration, and Fc receptor-mediated phagocytosis. All between-group differences were statistically significant, indicating a globally activated immune surveillance network in the low-risk microenvironment.

CIBERSORT-based immune deconvolution (Supplementary Figure S4B) further delineated the infiltration landscape. The low-risk group showed significantly higher proportions of activated memory CD4+ T cells ($p = 0.001$), resting dendritic cells ($p < 0.001$), and resting mast cells ($p < 0.001$), consistent with more active antitumor immune responses. In contrast, the high-risk group was characterized by increased infiltration of M0 macrophages ($p < 0.001$), indicating establishment of an immunosuppressive and pro-tumor inflammatory milieu. The correlation matrix between model genes and immune cell infiltration (Supplementary Figure S4C) revealed gene-specific patterns associated with immune remodeling. Consistently, correlation analysis (Supplementary Figure S4D) showed that the risk score was most strongly positively correlated with M0 macrophages and negatively correlated with resting mast cells, monocytes, and resting memory CD4+ T cells.

Comparison of immune cell scores derived from multi-algorithm integration (Supplementary Figure S4E) validated the core findings at a broader cellular granularity. Scores for multiple antitumor effector populations, including activated B cells, activated CD8+ T cells, activated dendritic cells, central memory CD4+/CD8+ T cells, effector memory CD4+/CD8+ T cells, natural killer cells, and type 1 T helper cells, were significantly higher in the low-risk group, whereas memory B cells and type 2 T helper cells had higher scores in the high-risk group, further supporting the dominance of an immunosuppressive microenvironment in the high-risk phenotype.

Drug sensitivity analysis

To explore the potential value of the CMA risk score in guiding individualized treatment in LUAD, we estimated IC50 of candidate drugs using pRRophetic based on the GDSC database, and compared predicted drug sensitivities between high- and low-risk groups (Supplementary Figure S5). The results indicated pronounced divergence in drug response patterns across risk strata, which could be broadly categorized into two groups.

Category 1. Drugs predicted to be more effective in the high-risk group. High-risk patients exhibited lower predicted IC50 values, indicating higher sensitivity, to multiple conventional chemotherapeutic agents and multi-target kinase inhibitors. Specifically, the nucleoside analog gemcitabine ($p = 0.036$; Supplementary Figure S5A), the vinca alkaloid microtubule inhibitor vinorelbine ($p = 0.008$; Supplementary Figure S5B), the microtubule-stabilizing agent epothilone B ($p < 0.001$; Supplementary Figure S5C), and the multi-target FLT3/PKC inhibitor midostaurin ($p = 0.004$; Supplementary Figure S5D) all showed significantly lower IC50 values in the high-risk group than in the low-risk group. These findings suggest that high-risk LUAD patients may derive

greater benefit from cytotoxic chemotherapy and specific multi-target kinase inhibitors. Notably, epothilone B exhibited the most pronounced between-group difference ($p < 0.001$). Sharing a microtubule-stabilizing mechanism with taxanes, it may have potential translational relevance.

Category 2. Drugs predicted to be more effective in the low-risk group. In contrast, low-risk patients showed greater predicted sensitivity to inhibitors targeting the PI3K/AKT/mTOR signaling pathway and to selected targeted agents. The mTOR inhibitor rapamycin ($p < 0.001$; Supplementary Figure S5E) showed the most significant between-group difference among all tested drugs, with substantially lower IC50 values in the low-risk group. The PI3K inhibitor ZSTK474 ($p < 0.001$; Supplementary Figure S5F) demonstrated a consistent pattern, and the two agents provide mechanistic complementarity within the PI3K/AKT/mTOR axis. In addition, the multi-target kinase inhibitor sorafenib, which is widely used as a first-line therapy for renal cancer ($p = 0.011$; Supplementary Figure S5G), and the ALK/MET inhibitor crizotinib ($p < 0.001$; Supplementary Figure S5H) also exhibited lower predicted IC50 values in the low-risk group.

In vitro functional validation of EPC1

To experimentally validate the biological plausibility of the CMA prognostic model, we selected EPC1, the core gene with the largest model weight, for in vitro functional assays. EPC1 is a key subunit of the NuA4/TIP60 histone acetyltransferase complex and is involved in chromatin remodeling and transcriptional regulation. However, its biological role in cancer remains incompletely defined.

Validation of overexpression efficiency. A Flag-tagged EPC1 overexpression plasmid was constructed and transfected into A549 cells. Western blotting demonstrated a distinct Flag-fusion protein band at approximately 46 kDa in the Flag-EPC1 group, whereas no corresponding band was detected in the empty vector control. GAPDH expression was comparable between groups, confirming successful establishment of the EPC1 overexpression system (Fig. 6A).

EPC1 overexpression suppresses cell proliferation. CCK-8 assays showed that the OE-NC and OE-EPC1 groups exhibited comparable proliferative capacity at early time points (0 h and 24 h). Differences became apparent over time. By 48 h, the OE-EPC1 group showed a reduced proliferation rate, and by 72 h, the OD value at 450 nm was significantly lower in OE-EPC1 than in OE-NC (**** $p < 0.0001$), indicating that EPC1 overexpression markedly inhibited tumor cell proliferation (Fig. 6B).

EPC1 overexpression suppresses cell migration. Wound-healing assays further assessed the effect of EPC1 on migratory capacity. Baseline wound widths at 0 h were comparable between groups (ns). After 24 h, the between-group difference was highly significant (**** $p < 0.0001$), demonstrating that EPC1 overexpression substantially impaired tumor cell migration (Fig. 6C).

EPC1 overexpression suppresses cell migration and invasion. Transwell assays were performed to further evaluate cell motility and invasiveness. Consistent with the wound-healing results, the relative migration of A549 cells was significantly reduced in the OE-EPC1 group compared to the OE-NC group (** $p < 0.001$). Furthermore, Matrigel invasion assays revealed that EPC1 overexpression significantly impaired the invasive capacity of the cells (** $p < 0.01$) (Fig. 6D).

EPC1 overexpression impairs colony formation. To assess the long-term proliferative potential, colony formation assays were conducted. The results indicated that the relative number of colonies formed by the OE-EPC1 group was significantly lower than that of the OE-NC group (** $p < 0.01$), reinforcing the persistent inhibitory effect of EPC1 on tumor cell growth (Fig. 6E).

In summary, in vitro functional assays support a tumor-suppressive role of EPC1, as its overexpression significantly attenuated two key malignant phenotypes: proliferation and migration. These results support the functional relevance of EPC1 within the CMA-related prognostic model, namely, higher EPC1 expression was associated with lower risk scores and improved outcomes. However, these phenotypic assays do not establish EPC1 as a direct regulator of CMA activity.

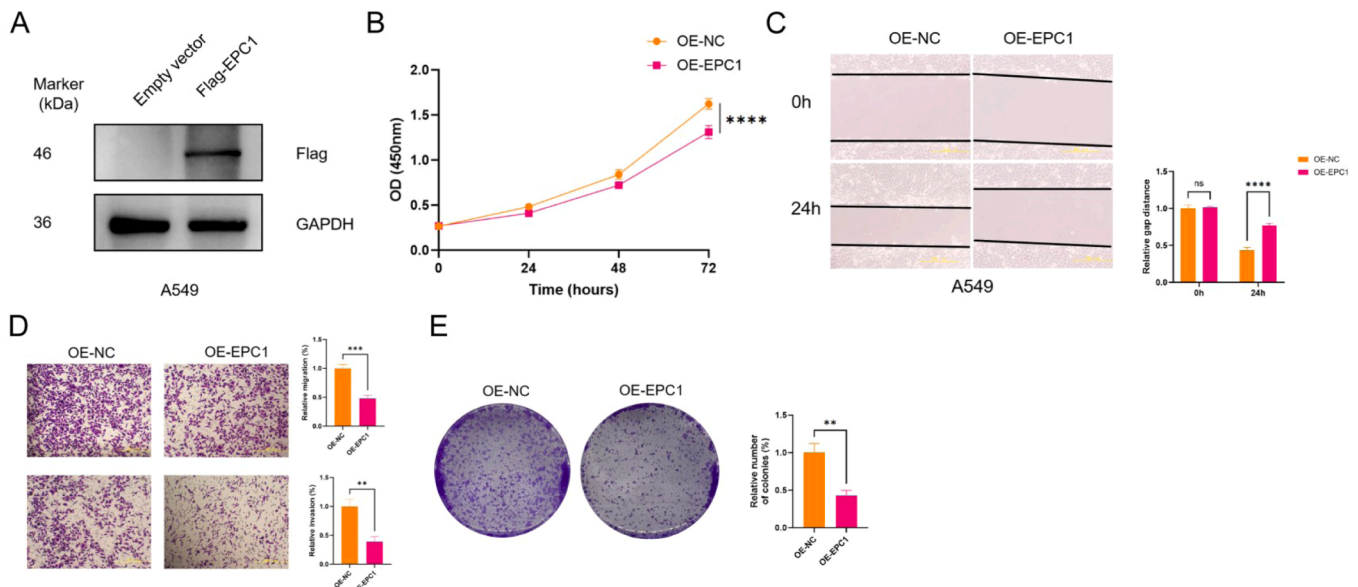


Fig. 6. In vitro validation showing that EPC1 overexpression inhibits tumor cell proliferation and migration. EPC1, the highest-weighted gene in the CMA prognostic model, was selected for functional validation. (A) Western blot analysis of Flag-EPC1 overexpression efficiency in A549 cells, with GAPDH as the loading control. (B) CCK-8 assay showing proliferation curves (OD 450 nm) in OE-NC and OE-EPC1 groups at 0, 24, 48, and 72 h. (C) Wound-healing assay assessing the impact of EPC1 overexpression on migration. Representative microscopy images at 0 h and 24 h are shown on the left, with quantification of relative wound width on the right. (D) Transwell assays evaluating the migration (top) and invasion (bottom) abilities of A549 cells in the OE-NC and OE-EPC1 groups. Representative images are shown on the left, and relative percentages are quantified on the right. (E) Colony formation assay evaluating the long-term proliferative capacity of A549 cells. Representative images of cell colonies are shown on the left, with relative colony numbers quantified on the right. Experiments were independently repeated three times, and data are presented as mean $\pm$ SD. **** $p < 0.0001$. ns, not significant.

Discussion

This study provides evidence that spatiotemporal dysregulation of CMA is associated with malignant progression and microenvironmental remodeling in LUAD. Prior work has largely emphasized the metabolic support role of CMA in advanced tumors [37,38]. Our findings extend this view and highlight CMA as a potentially relevant factor during the transition from early microinvasive lesions to invasive disease. We show that attenuation of CMA activity is associated with altered tumor–stroma communication, immune evasion-related features, and invasive phenotypes. In addition, the CMA-based prognostic model developed here, which was cross-validated using multiple machine-learning algorithms, translated proteostasis-associated signals into a prognostic stratification tool with stable cross-cohort performance. These results may help bridge the conceptual gap between intracellular degradation networks and the macroscopic immune microenvironment, and may provide a CMA-centered framework for future precision risk stratification beyond conventional phenotype-based assessment [39–41]. Several prognostic signatures have been developed for LUAD based on hypoxia, immune infiltration, autophagy, ferroptosis, and metabolic reprogramming [42–44]. These models have provided useful tools for risk stratification from distinct biological perspectives. Compared with these signatures, the present CMA-related model focuses on a specific proteostasis-regulatory process and integrates single-cell tumor epithelial states, CMA-associated intercellular communication, and bulk transcriptomic prognostic modeling. Therefore, our model should be viewed as complementary to existing LUAD prognostic signatures rather than as a replacement for them. Its incremental value lies in linking CMA-associated intracellular degradation programs with tumor microenvironment remodeling and patient survival.

At single-cell resolution, we delineated a nonlinear trajectory of CMA activity across the AIS–MIA–IAC continuum. The transient increase in the proportion of CMA-high cells in MIA may reflect a compensatory stress response to proteostatic burden or hypoxic pressure during early microinvasion. However, at the more aggressive IAC stage, CMA-low tumor cells became predominant. Cell–cell communication analyses further elucidated microenvironmental mechanisms underlying this CMA stratification [45]. CMA-high cells tended to sustain active pro-angiogenic and antigen-presentation interactions through VEGF, THBS1, and MDK signaling axes [46–48]. In contrast, CMA-low cells underwent substantial receptor-level reprogramming and preferentially received extracellular matrix (ECM) cues from fibroblasts, including COL1/4/6 and FN1. This rewiring is unlikely to be stochastic. As demonstrated by mechanistic studies, ECM stiffness and integrin-associated mechanotransduction (including YAP/TAZ signaling) can modulate autophagic flux and link tissue mechanics to autophagy regulation [49,50]. Accordingly, the decline in CMA activity at the IAC stage may form a positive feedback loop with the development of a dense stromal barrier. Tumor cells may actively enhance integrin-mediated ECM adhesion to acquire pro-survival and pro-migratory signals, thereby promoting invasive growth [51].

Aberrant intrinsic CMA function in tumors further triggers transcriptome-wide signaling cascades and facilitates immune escape. Pathways enriched in the high-risk group, including E2F_TARGETS, G2M_CHECKPOINT, and MYC_TARGETS, indicate a strongly proliferative state. Mechanistically, CMA recognizes substrate proteins via KFERQ-like motifs and mediates their lysosomal degradation [52]. As established by the pioneering work of Gomes et al. and Vakifahmetoglu-Norberg et al., oncogenic proteins such as MYC and mutant p53 can be selectively targeted by CMA for degradation [13,14]. We therefore hypothesize that disruption of the CMA core network in high-risk tumors impairs the clearance of these key oncogenic drivers, resulting in toxic accumulation of MYC and related factors and ultimately driving profound cell-cycle deregulation. From an immunological perspective, the high-risk group was characterized by marked enrichment of M0 macrophages. Crotzer et al. have shown that CMA

plays an indispensable role in endogenous antigen processing and major histocompatibility complex class I (MHC-I) antigen presentation [53]. Based on these observations, we speculate that CMA deficiency not only compromises HLA-mediated presentation of tumor-specific antigens but may also reshape the microenvironment through aberrant metabolic signals that constrain macrophages in an undifferentiated M0 state, thereby facilitating immune evasion. In contrast, the low-risk group retained active interferon (IFN- α/γ) responses and IL6/JAK/STAT3-associated inflammatory programs, supporting more effective antitumor immune surveillance.

In exploring key regulatory determinants within the CMA network, EPC1 emerged as the highest-weighted feature gene in the prognostic model. Our *in vitro* assays clearly demonstrated that EPC1 overexpression significantly inhibited proliferation and migration of A549 LUAD cells. As a key scaffold subunit of the NuA4/TIP60 histone acetyltransferase complex, EPC1 is primarily involved in chromatin remodeling and epigenetic regulation of transcription [54]. For example, studies by Lin et al. have demonstrated that TIP60, a central catalytic subunit of this complex, can directly activate the autophagy-initiating kinase ULK1 through acetylation [55]. In addition, work led by Sykes et al. reported that TIP60 precisely regulates transcription of tumor suppressor genes such as p53 [56]. Collectively, these findings suggest that EPC1 may function as a critical “epigenetics–proteostasis” hub. In high-risk LUAD, loss of EPC1 expression may not only release transcriptional constraints on malignant proliferative programs but may also epigenetically silence specific CMA core components, thereby accelerating malignant progression through coordinated dysregulation at both the transcriptional and protein-degradation levels.

This CMA-informed risk stratification also may generate pharmacological hypotheses for future individualized therapeutic studies. High-risk patients were predicted to be more sensitive to gemcitabine, vinorelbine, and the microtubule-stabilizing agent epothilone B, consistent with heightened cell-cycle activity (G2M checkpoint activation) and mitotic spindle dysregulation, which may confer vulnerability to DNA synthesis inhibition and microtubule-targeting cytotoxic agents. Conversely, low-risk patients were predicted to be more sensitive to the mTOR inhibitor rapamycin and the PI3K inhibitor ZSTK474. As reported by Arias et al., mTOR is a central negative regulator of macroautophagy and engages in complex metabolic crosstalk and compensatory feedback with CMA [38]. The apparent dependency of the low-risk subgroup on the PI3K/AKT/mTOR axis provides a theoretical basis for pharmacologically rebalancing metabolism–autophagy networks through targeted therapy.

Several limitations should be acknowledged. First, the scRNA-seq analysis was based on only 9 LUAD samples, with three samples each for AIS, MIA, and IAC. Therefore, the inferred stage-associated cellular dynamics should be interpreted as exploratory and require validation in larger single-cell or spatial transcriptomic cohorts. Second, although the RSF model demonstrated strong robustness in TCGA and independent GEO validation cohorts, its clinical utility, as derived from retrospective datasets, remains preliminary and requires confirmation in prospective, multicenter cohorts with larger sample sizes before clinical application. Third, EPC1 was experimentally validated as a model-associated functional gene, but not as a confirmed upstream regulator of CMA. The present functional experiments were primarily limited to phenotypic validation of proliferation and migration, and the direct molecular interaction network between EPC1 and CMA core components or downstream targets remains unresolved. Further mechanistic studies using chromatin immunoprecipitation sequencing, co-immunoprecipitation, or related approaches, as well as *in vivo* validation, are still required. Finally, the pRRophetic-based drug sensitivity results should be regarded as hypothesis-generating, and further validation using patient-derived organoids or clinical studies is required. Overall, this study offers an epigenetic perspective on proteostasis imbalance in LUAD, and the quantitative prognostic model developed

here holds considerable promise for future clinical translation.

Conclusion

In conclusion, we mapped CMA dynamics during LUAD progression at single-cell resolution and showed that CMA dysregulation is linked to microenvironment remodeling and immune reprogramming. We further developed and validated a robust CMA-based prognostic model across multiple cohorts, and identified EPC1 as a key protective gene with tumor-suppressive effects *in vitro*. These findings suggest that CMA may represent a potentially relevant axis for refined risk stratification in LUAD, while further validation is required before clinical translation.

Data availability statement

The data that support the findings of this study were derived from publicly available resources. Single-cell RNA-seq data were obtained from the Gene Expression Omnibus (GEO) database under accession number GSE189357. Bulk transcriptomic and clinical data were obtained from The Cancer Genome Atlas (TCGA-LUAD) at <https://portal.gdc.cancer.gov/projects/TCGA-LUAD>. External validation datasets were obtained from GEO under accession numbers GSE31210 and GSE50081. Normal lung tissue data were obtained from the Genotype-Tissue Expression (GTEx) project at <https://gtexportal.org/>. Drug sensitivity data were obtained from the Genomics of Drug Sensitivity in Cancer (GDSC) database at <https://www.cancerrxgene.org/>.

Ethical statement

This study was based on publicly available, de-identified transcriptomic and clinical datasets obtained from GEO, TCGA, and GTEx. The original studies had obtained the required ethical approvals and informed consent from participants where applicable. Because no new human tissue samples, identifiable personal information, or animal experiments were involved in the present study, additional ethical approval and informed consent were not required. The *in vitro* experiments were performed using the commercially available A549 lung adenocarcinoma cell line.

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CRediT authorship contribution statement

Kaihong Han: Writing – original draft, Data curation, Conceptualization. **Wenyu Han:** Writing – original draft, Methodology, Investigation, Formal analysis. **Wenyao Xie:** Writing – review & editing, Supervision, Software. **Zibo Zhang:** Writing – review & editing. **Yaqing Han:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2026.102919](https://doi.org/10.1016/j.tranon.2026.102919).

Supplementary Figure S1. Association of the CMA risk score with clinicopathological features and subgroup survival analyses. (A) Doughnut charts comparing clinicopathological feature distributions between the high- and low-risk groups. Chi-square test p-values are

shown below each feature. (B) Violin plots showing risk score distributions across T1–T4 stages. (C) Comparison of risk scores between combined T1–2 and T3–4 groups. (D) Risk score distributions across clinical stages I–IV. (E) Comparison of risk scores between combined stages I–II and III–IV groups. (F) Stacked bar plot showing the distribution of clinical stages in the high- and low-risk groups. (G, H) Kaplan–Meier survival curves in age subgroups: ≤ 60 years (G) and > 60 years (H). (I, J) Kaplan–Meier survival curves in clinical stage subgroups: stages I–II (I) and stages III–IV (J). Red and blue denote high- and low-risk groups, respectively. Numbers at risk are shown below the survival curves. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

Supplementary Figure S2. Independent prognostic analysis and nomogram construction. (A) Forest plot of univariable Cox regression analysis showing hazard ratios, 95% confidence intervals, and p-values for clinicopathological variables and the CMA risk score. (B) Forest plot of multivariable Cox regression analysis showing independent prognostic factors. (C) Calibration curves comparing predicted and observed overall survival probabilities at 1, 3, and 5 years. (D) Decision curve analysis comparing the standardized net benefit of the nomogram, CMA risk score, and clinicopathological variables across threshold probabilities. (E) Nomogram integrating clinical variables and the CMA risk score to predict 1-, 3-, and 5-year overall survival. (F) Time-dependent C-index curves showing dynamic discriminative performance over 1–10 years of follow-up.

Supplementary Figure S3. Hallmark pathway activity differences between high- and low-risk groups. (A) Gene set enrichment analysis plots showing Hallmark pathways enriched in the high-risk group. (B) Gene set enrichment analysis plots showing Hallmark pathways enriched in the low-risk group. (C) Bar plot of differential Hallmark pathway activity based on gene set variation analysis scores. Red denotes pathways upregulated in the high-risk group, and blue denotes pathways upregulated in the low-risk group. (D) Pearson correlation heatmap of Hallmark pathway activities showing the relationship between proliferative and immune-related functional modules. (E) Forest plot of univariable Cox regression analysis for key Hallmark pathway activities, showing associations with overall survival.

Supplementary Figure S4. Immune microenvironment characteristics in high- and low-risk groups. (A) Heatmap of single-sample gene set enrichment analysis scores for immune-related pathways across patients ordered by risk group. Color intensity indicates pathway activity. (B) Violin plots comparing the relative proportions of 22 immune cell types estimated by CIBERSORT between the high- and low-risk groups. (C) Spearman correlation heatmap between model gene expression and the proportions of 22 immune cell types. Red and blue indicate positive and negative correlations, respectively. (D) Bubble plot showing correlations between the CMA risk score and immune cell types. Bubble size reflects correlation strength, and color indicates correlation direction. (E) Box plots comparing scores of 28 immune cell subsets between the high- and low-risk groups. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Supplementary Figure S5. Comparison of predicted drug sensitivity between high- and low-risk groups. Predicted half-maximal inhibitory concentration values were estimated using the GDSC database and the pRRophetic algorithm and compared between the high- and low-risk groups. Lower predicted IC50 values indicate higher predicted drug sensitivity. (A) Gemcitabine. (B) Vinorelbine. (C) Etoposide. (D) Midostaurin. (E) Rapamycin. (F) ZSTK474. (G) Sorafenib. (H) Crizotinib. Group comparisons were performed using the Wilcoxon rank-sum test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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