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Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Deregulation of Casein Kinase-2 in non-small-cell lung cancer: A subunit-specific analysis

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Abstract

Casein kinase-2 (CK2) is a constitutively active kinase that supports neoplastic properties. Although there is an extensive body of preclinical research on CK2, translational and clinical information remains limited and sparse. In this study, we interrogated clinical multiomics databases to examine CK2 deregulation across various cancers. Specifically, we analyzed the mutational frequency, copy-number alterations (CNA), and mRNA expression of CK2 catalytic (*CSNK2A1* and *CSNK2A2)* and regulatory (*CSNK2B)* subunits across two major cancer genomic repositories (The Cancer Genome Atlas/International Cancer Genome Consortium). These genomic and transcriptomic analyses were further focused on lung cancer and complemented by *in situ* assessments of CK2 protein subunits and enzymatic activity in biopsies from non-small-cell lung cancer (NSCLC). Our findings indicate that gene mutations and CNA do not account for the elevated mRNA levels and enzymatic activity of CK2 subunits in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). Particularly, the upregulation of *CSNK2A1* and *CSNK2B* mRNA was associated with a worse prognosis and showed a direct correlation with the infiltration of myeloid-derived suppressor cells in LUAD. At the protein levels, all CK2 subunits and enzymatic activity were markedly elevated in LUAD (n = 103) and LUSC (n = 31) biopsies; however, only *CSNK2A1* expression correlated positively with tumor size and disease stage. Finally, CSNK2A1 appeared to be more tumor-specific than CSNK2A2, suggesting that targeted therapies against this catalytic subunit or its homotetramer may offer a more favorable therapeutic window in NSCLC.

Keywords: Casein Kinase-2; Casein kinase-2 regulation; Lung cancer; Translational study; Oncology target

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1. Introduction

Casein kinase-2 (CK2) is a constitutively active Ser/Thr-protein kinase frequently deregulated in cancer, with broad tumor-supporting roles classified as "non-oncogene addiction".[1] The CK2 holoenzyme comprises two catalytic subunits (CSNK2A1 and CSNK2A2) and two regulatory subunits (CSNK2B), which assemble as either homotetramers ($CSNK2A1_2$/$CSNK2B_2$ or $CSNK2A2_2$/$CSNK2B_2$) or heterotetramers (CSNK2A1/CSNK2A2/$CSNK2B_2$).[2] In addition, CK2's supramolecular structures, spatiotemporal subunit associations, and functional specialization introduce further regulatory intricacies in both health and disease.[3] Notably, CK2 participates in multiple tumor-related signaling cascades, promoting aberrant cell proliferation, survival, and metastasis.[4] However, CK2's unique biochemical features, vast range of substrates, and multifaceted impact on signaling cascades have sparked debates about its viability as an oncology target.[5,6] These discussions include inquiries about CK2's essentiality in cancer versus non-neoplastic tissues, as well as concerns regarding the specificity of current CK2 inhibitors.[7,8]

A myriad of natural and synthetic compounds with diverse CK2 inhibition mechanisms has been described.[9] Recent compounds exhibit ATP-competitive or dual inhibition mechanisms, targeting the ATP-binding site and an allosteric pocket unique to CK2.[7,10] However, only two distinct CK2 inhibitors have reached clinical application: the synthetic peptide CIGB-300 and the small molecule CX-4945.[11,12] Despite extensive pre-clinical research, translational and clinical data on CK2 remain limited and sparse, and its role as a validated oncology target is yet to be established.[13]

In this study, we interrogated multiomics cancer databases to investigate CK2 gene mutations and deregulated expression in clinical samples. We performed meta-analyses to explore potential associations between CK2 subunit expression and overall survival (OS) of non-small-cell lung cancer (NSCLC) patients. Our analysis centers on individual CK2 subunits, assuming subunit-specific functions across cancers, with a particular focus on two major NSCLC types: lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). CK2 protein subunit levels were measured by performing immunohistochemistry (IHC) in LUAD and LUSC patient samples and correlated with selected clinicopathological features. Finally, by measuring two canonical CK2 substrates as indicators of *in situ* CK2 enzymatic activity, we demonstrated that CK2 upregulation in LUAD and LUSC corresponds with increased CK2 enzymatic activity in these NSCLC subtypes.

2. Materials and methods

2.1. *CSNK2* gene mutational burden in cancer cohorts

The cBioPortal for Cancer Genomics (https://www.cbioportal.org/) was used to retrieve data on the mutational burden – specifically, the total frequency and types of alterations – of the *CSNK2A1*, *CSNK2A2*, and *CSNK2B* subunits. This data were sourced from the Pan-Cancer Analysis of Whole Genomes Consortium of the International Cancer Genome Consortium and The Cancer Genome Atlas (TCGA) (Nature 2020).[14,15] Samples with mutations and copy-number alterations (CNA) data were selected across 38 tumor types, comprising 2683 samples from 2565 patients.

For a more detailed analysis within NSCLC cohorts, cBioPortal was further queried for genetic alterations specific to *CSNK2A1*, *CSNK2A2*, and *CSNK2B* subunits in LUAD and LUSC. The select genomic profiles included mutations, putative CNA from the Genome Identification of Significant Targets in Cancer, and mRNA expression *z*-scores relative to normal samples (log RNA Seq V2 RSEM). For LUAD, the TCGA PanCancer Atlas cohort (n = 566 samples) was interrogated, while for LUSC, the TCGA PanCancer Atlas cohort (n = 487 samples) was used.

Driver feature prediction, based on the mutational frequency, distribution, and functional impact of individual mutations, was performed through the IntOgene platform, which interrogates 66 cancer types across 221 studies (n = 28,076 samples; https://www.intogen.org/search).[16] Cancer driver annotations were obtained from OncoKB™MSK's Precision Oncology Knowledge Base (OncoKB) (https://www.oncokb.org/).[17]

2.2. *CSNK2* gene expression in cancer

Expression levels of *CSNK2* genes across pan-cancer studies were interrogated using data from the TCGA available through the University of Alabama at Birmingham database (UALCAN) (https://ualcan.path.uab.edu).[18] Tumor and non-tumor samples are represented in red and blue, respectively. Box-and-whisker plots display gene expression levels, including maximum, upper quartile, median, lower quartile, and minimum values. Statistical comparisons were conducted using the Student's *t*-test with unequal variances. The following TCGA tumor subtypes were included: Bladder urothelial carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma, cholangiocarcinoma, colon adenocarcinoma, esophageal carcinoma, glioblastoma multiforme, head-and-neck squamous cell carcinoma, kidney chromophobe, kidney renal clear cell carcinoma, kidney renal papillary cell

carcinoma, liver hepatocellular carcinoma, LUAD, LUSC, pancreatic adenocarcinoma, prostate adenocarcinoma, pheochromocytoma and paraganglioma, rectal adenocarcinoma, sarcoma, skin cutaneous melanoma, thyroid carcinoma, thymoma, stomach adenocarcinoma, and uterine corpus endometrial carcinoma.

2.3. Survival meta-analysis of *CSNK2* genes in NSCLC

A survival meta-analysis for each *CSNK2* gene was interrogated using the LUAD or LUSC subtype explorer database (https://lce.biohpc.swmed.edu/lungcancer/index.php#about).[19] Output values were displayed based on all curated studies available.

2.4. Metastasis potential of *CSNK2* genes in NSCLC

Differential expression analysis of *CSNK2* genes in tumor, normal, and metastatic tissues was carried out using the TNMplot database (https://tnmplot.com/analysis/).[20] The statistical method was set automatically to the Kruskal–Wallis one-way non-parametric test.

2.5. Correlation of *CSNK2* genes with immune infiltration

The gene module from the TIMER2.0 web server (http://timer.cistrome.org)[21] was used to select genes of interest (*CSNK2* subunits) and visualize the correlation between their expression levels and the immune and stromal cell populations infiltrating tumor samples from TCGA.[22] Immune infiltrate abundances were estimated using multiple immune deconvolution methods, including Tumor Immune Dysfunction and Exclusion (TIDE), Microenvironment Cell Populations-counter (MCPCounter), Estimating the Proportion of Immune and Cancer cells (EPIC), and xCell. The generated heatmap shows purity-adjusted Spearman's rho correlations across various cancer types.

2.6. Immunohistochemical analysis of LUAD and LUSC specimens

A total of 134 pairs of wax blocks were prepared from primary tumor specimens (LUAD = 103; LUSC = 31) and corresponding para-neoplastic tissue (>5 cm from the tumor) collected from patients initially diagnosed at the First Affiliated Hospital of South China University (Hunan, China) since December 2021. This study was approved by the Medical Ethics Committee of Nanhua University Affiliated First Hospital, Issuance, Review Batch Number: 2021LL0115001, Meeting Date: January 15, 2021. Reviewed materials included the Research Plan Version YJFA20210114 and the Informed Consent Form Version ZQTY20210114. Clinical and pathological records provided data on patient age, sex, histological grade, tumor size, invasion depth (T stage), and lymph node metastasis. At the time of specimen collection, patients had not undergone radiotherapy and displayed no other systemic primary tumors besides NSCLC. Post-operative hematoxylin and eosin sections confirmed diagnoses of LUAD or LUSC.

IHC analysis was performed using primary antibodies against CSNK2A1 (Ab76040, Abcam, USA), CSNK2A2 (#PA5-109601, Invitrogen, USA), CSNK2B (Ab#76025, Abcam, USA), AKT (CST#4691, Cell Signaling Technology, USA), AKTs129 (#BS-5188R, Bioss, USA), NPM1 (ab52644, Abcam, USA), NPM1s125 (ab109546, Abcam, USA), and Ki67 (RMA-0542 WWW, Fuzhou Maishin Biotechnology, China), following the immunohistochemical SP method with the Fuzhou Maishin Ultrasensitive SP (Mouse/Rabbit) IHC Kit (Fuzhou Maishin Biotechnology, China). A combined immunoreactivity score (CIS) was calculated based on signal intensity (scored from "0" for non-stained to "3" for strong brown staining) and the frequency of positive cells (from <5% = 0 points to >75% = 4 points) in tumor samples. Each section analyzed was selected randomly from the upper, lower, left, right, and central regions of a magnified field (×400) to ensure representative interpretation. The combined scores (A×B) were classified as follows:

- Score 1 – 3: – (negative)
- Score 4 – 5: + (weak positive)
- Score 6 – 7: ++ (positive)
- Score ≥8: +++ (strong positive)

2.7. Statistical analysis

Statistical analysis of IHC experiments was performed using the Statistical Package for the Social Sciences 27.0. Chi-squared tests and Fisher's exact tests were used to analyze correlations. A two-sided $P < 0.05$ was considered statistically significant for all calculations.

3. Results

3.1. Mutational burden of *CSNK2* subunits across cancer cohorts

Deregulation of CK2 in cancer is regarded as an example of non-oncogene addition. To explore genetic alterations in *CSNK2* genes that may contribute to aberrant kinase activity, we analyzed available cancer genomics databases. Figure 1A illustrates that the genetic alteration rates for *CSNK2A1, CSNK2A2*, and *CSNK2B* in a pan-cancer cohort were 7%, 1.8%, and 6%, respectively. Most alterations were CNA, while missense, splice site, and truncating mutations were below 0.3%. Notably, these uncommon mutations did not exhibit oncogenic potential based on their frequency, distribution, and functional impact, as determined by

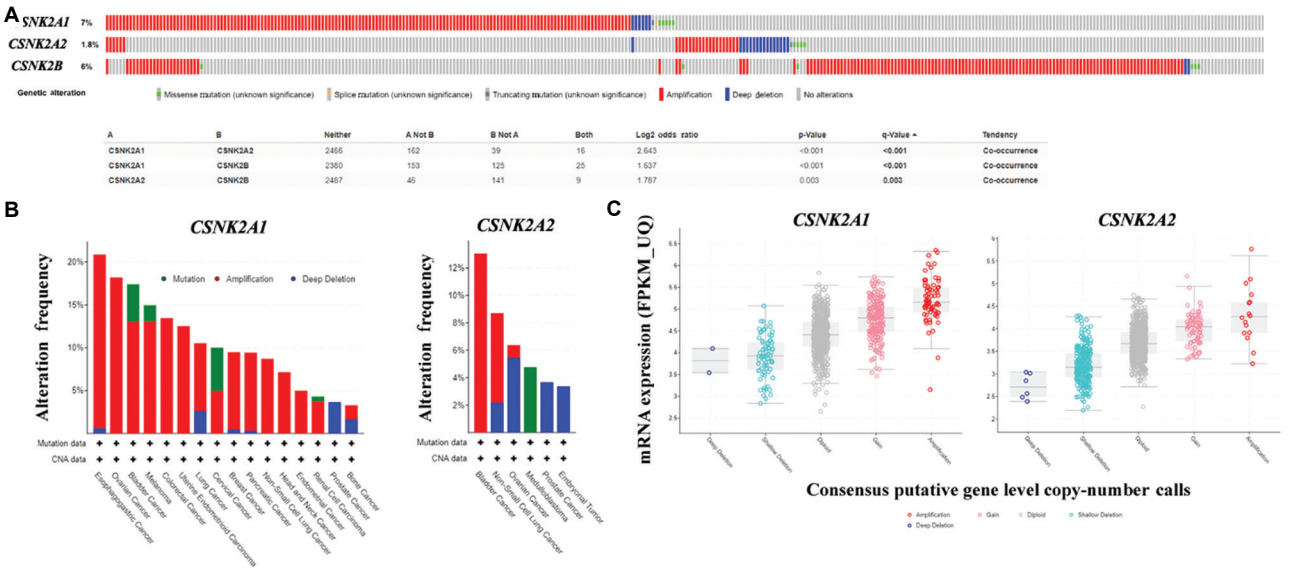


Figure 1. Oncoprint analysis of genetic alterations in pan-cancer cohorts (International Cancer Genome Consortium/The Cancer Genome Atlas, Nature 2020) for catalytic (CSNK2A1/CSNK2A2) and regulatory subunits (CSNK2B). (A) Oncoprint and co-occurrence analysis, including only samples with mutation and CNA information (92% of the cohort, n = 2,683 samples). (B) Cumulative alterations in selected *CSNK2* subunits, displaying only tumors with an alteration frequency of 3% or higher for the target gene. (C) Correlation between CNA (consensus putative gene-level copy-number call) and gene expression (mRNA expression, FPKM_UQ) across the database. In this database, "lung cancer" (n = 38) and "NSCLC" (n = 46) encompass data from LUAD-US (n = 35 plus 3 additional unknown samples) and the LUSC-US study (n = 46), respectively. All annotations and analyses were obtained from cBioPortal, as described in the Materials and Methods section.
Abbreviation: CNA: Copy-number alterations.

IntOgene and annotations from the Precision OncoKB. Overall, genetic alterations in *CSNK2A1*, *CSNK2A2*, and *CSNK2B* frequently co-occurred, both when analyzing CNAs and mutations together (Figure 1A) and when analyzing CNAs alone (data not shown). For *CSNK2A2*, copy-number amplifications and deletions were identified in five tumor types, while *CSNK2A1* and *CSNK2B* amplifications predominated across 14 out of 16 tumor locations, using a 3% threshold for *CSNK2* gene alterations (Figures 1B and S1B).

In addition, cumulative gene alteration frequencies on *CSNK2* subunits exceeded 10% in 15 different tumors (Figure S1A). The highest alteration rates were reported in melanoma and bladder cancer (>30%), with LUAD (denoted as "lung cancer" in Figure 1) and LUSC (denoted as "NSCLC" in Figure 1) being the fifth and sixth most altered cancer types concerning CSNK2 subunits (18.4%), respectively (Figure S1A). In the LUSC cohort, all *CSNK2A1* alterations were amplifications (4/46 cases [9%]), while *CSNK2A2* and CSNK2B exhibited amplifications (3/46 cases [7%] and 2/46 cases [4%], respectively) or deletions (1/46 cases [2%] and 2/46 cases [4%], respectively) (Figures 1B and S1B). In this pan-cancer cohort, CNAs were consistent with mRNA expression levels for the three CSNK2 subunits (Figures 1C and S1C). Furthermore, CSNK2A1 mRNA expression showed a positive correlation with CSNK2A2 mRNA expression (Spearman: 0.24; P = 2.10e-14), while an inverse correlation was found between CSNK2A1 and CSNK2B mRNA expression (Spearman: −0.15; P = 2.91e-6). No correlation was found between CSNK2A2 and CSNK2B mRNA expression in this data cohort (Figure S1D-F).

3.2. Genomic and transcriptomic alterations of *CSNK2* subunits in major types of NSCLC

Given the limited NSCLC data in the pan-cancer cohort,[23] we interrogated more comprehensive datasets for LUAD (n = 566) and LUSC (n = 487). We prioritized samples with complete mutations, CAN, and RNA sequencing data (LUAD = 503; LUSC = 466) (Figure 2A). In cases where full datasets were unavailable, we included samples with mutation and CNA data (LUAD = 507; LUSC = 469) to ensure consistency with the analysis in Figure 1 (Figure 2B and C).

CSNK2A1 exhibited very few mutations in LUAD and LUSC samples (six mutations), and *CSNK2A2* mutations were found only in LUAD (two mutations). No mutations were detected in the regulatory subunit gene *CSNK2B* across the 976 NSCLC samples tested (Figure 2B and C). Overall, these results confirm that mutations in *CSNK2* subunits *CSNK2A1*, *CSNK2A2*, and *CSNK2B* are rare, with a cumulative mutation

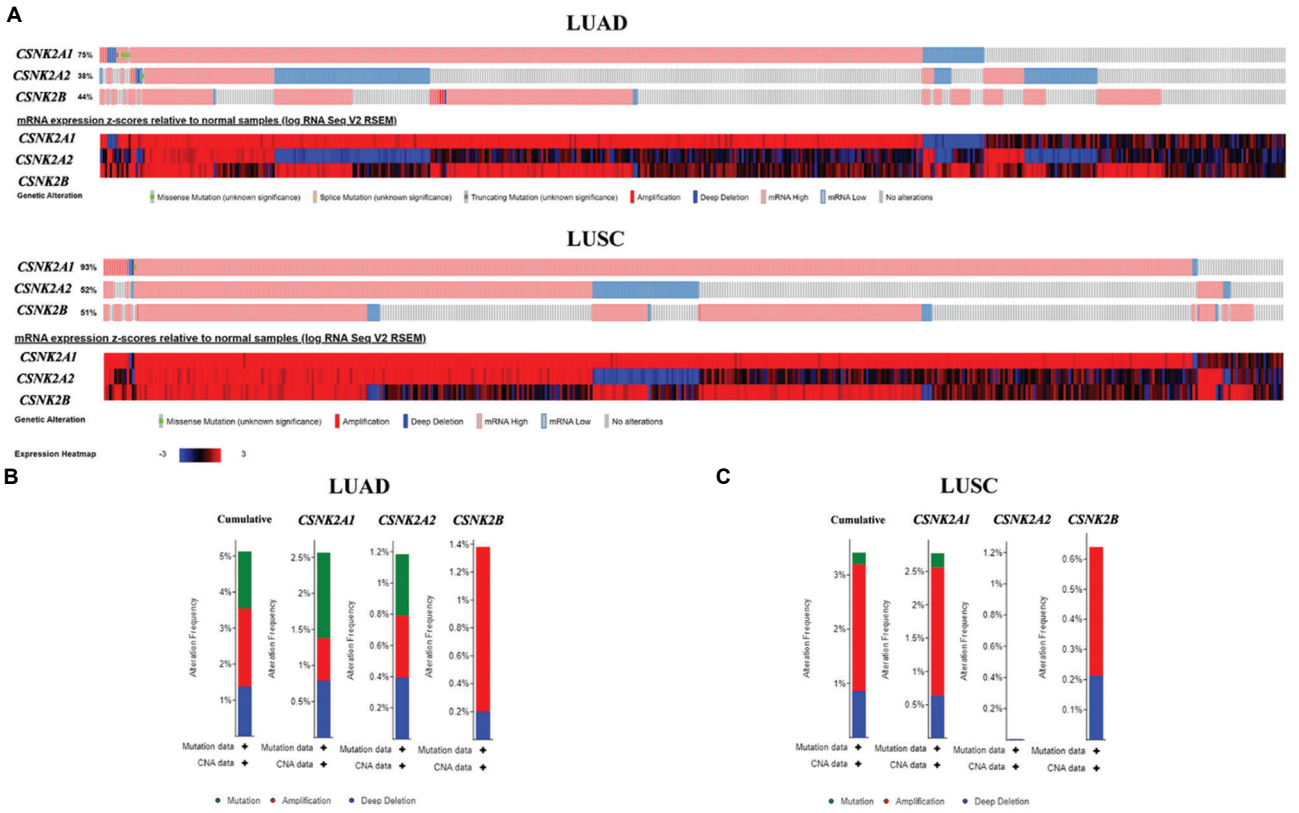


Figure 2. Oncoprint analysis of genetic alterations contained in LUAD and LUSC cohorts. (A) Only samples with mutations, CNA, and mRNA expression data were included (LUAD = 503; LUSC = 466); (B) LUAD samples with mutations and CNA data only (n = 507); (C) LUSC samples with mutations and CNA data only (n = 469). All annotations and analyses were obtained from cBioPortal, as described in the materials and methods section.
Abbreviations: LUSC: Lung squamous cell carcinoma; LUAD: Lung adenocarcinoma; CAN: Copy-number alterations.

frequency of <1.5% across the cohort. In addition, CNA events were uncommon, with amplifications and deletions found in only 3 – 4% of samples (Figure 2B and C). As in the pan-cancer cohort, these CNAs corresponded roughly with target gene expression (Figure S2).

Despite the limited impact of mutations and CNAs on individual *CSNK2* subunits, mRNA overexpression was prevalent across analyzed LUAD and LUSC cohorts (Figure 2A). Particularly, *CSNK2A1* mRNA deregulation was found in over 90% of LUSC samples and 70% of LUAD samples. Overall, catalytic subunit deregulation was more prominent in LUSC than LUAD. While *CSNK2A1* consistently showed overexpression, *CSNK2A2* mRNA levels varied, with both elevated and reduced expression relative to normal-matched tissues. For *CSNK2B*, overexpression was much more common than downregulation, with over 40% of LUAD and LUSC samples displaying higher mRNA levels compared to normal-matched tissues (Figure 2A).

As in the pan-cancer cohorts, *CSNK2A1* mRNA expression showed a weak positive correlation with *CSNK2A2* mRNA expression in both LUAD (Spearman: 0.14; P = 1.214e-3) and LUSC (Spearman: 0.24; P = 1.39e-7). However, no significant correlations were found between the catalytic subunits (*CSNK2A1*/*CSNK2A2*) and regulatory subunit *CSNK2B* at the mRNA expression level in these NSCLC subtypes (data not shown).

To investigate potential regulatory mechanisms, we analyzed methylation data for putative correlations between *CSNK2A1*-TSS200, *CSNK2A2*-TSS1500/1rstExon, and *CSNK2B*-TSS1500/1stExon/5'UTR methylation and mRNA expression levels of *CSNK2* subunits. n LUAD, we found weak negative correlations for *CSNK2A2*-TSS1500 (Spearman: −0.15; P = 8.658e-4) and *CSNK2B*-TSS1500/1stExon/5'UTR (Spearman: −0.14; P = 1.528e-3) (Figure S3A). Similarly, methylation of *CSNK2A2* and *CSNK2B* on promoter regions, but not CSNK2A1, correlated with mRNA expression in LUSC samples, with significant signals observed for *CSNK2A2*-TSS1500 (Spearman: −0.09; P = 0.0443), *CSNK2A2*-1rstExon (Spearman: −0.10; P = 0.0368), and

CSNK2B-TSS1500/1stExon/5'UTR (Spearman: −0.26; P = 9.54e-9) (Figure S3B).

3.3. Prognostic impact of *CSNK2A1* and *CSNK2B* overexpression in LUAD

We investigated whether the overexpression of *CSNK2* subunits correlates with reduced OS in clinical cohorts of NSCLC. Initially, we confirmed that *CSNK2* mRNA levels were consistently overexpressed across various tumor types, including NSCLC, except for *CSNK2A2* in LUAD (Figure S4). Particularly, *CSNK2A1* upregulation was substantially higher than that of *CSNK2A2* in LUAD (fold change: 1.40 vs. 0.95) and LUSC (fold change: 2.13 vs. 1.23) (Figure S4A and B), while *CSNK2B* displayed comparable expression levels in both LUAD and LUSC (LUAD: 1.35 vs. LUSC:1.40) (Figure S4C). In alignment with *CSNK2A1* and *CSNK2B* overexpression, a meta-analysis of mRNA expression versus OS indicated that elevated of both subunits correlated with a poorer prognosis in LUAD but not LUSC patients (*CSNK2A1*: LUAD hazard ratio [HR] = 1.17, P < 0.01; LUSC HR = 1.07, P = 0.72; *CSNK2B*: LUAD HR = 1.11, P < 0.01; LUSC HR = 0.93, P = 0.16) (Figure 3). Conversely, *CSNK2A2* overexpression in LUAD was associated with improved OS (HR = 0.92, P = 0.01) (Figure S5).

It is worth noting that, although data on metastatic samples are limited and not stratified by NSCLC subtypes, preliminary findings suggest that *CSNK2A1* (P = 1.28e-5) and *CSNK2B* (P = 1.33e-6), but not *CSNK2A2* (P = 0.52), could be linked to lung metastasis (Figure S6).

3.4. Correlation of *CSNK2* subunit expression with tumor infiltration by specific cell populations

The oncogenic potential of CK2 has been linked to both tumor-intrinsic and tumor-extrinsic factors.[24] Using various metrics to quantify the infiltration of immune and stromal cells in tumors, we found that elevated *CSNK2A1* mRNA expression levels were correlated with increased infiltration of cancer-associated fibroblasts (CAF) and myeloid-derived suppressor cells (MDSC) across multiple tumor subtypes (Figure 4A).

Tumor infiltration by CAFs showed a positive correlation with *CSNK2A1* mRNA overexpression according to three out of four metrics in both LUAD (CAF_TIDE, Rho = 0.185) and LUSC (CAF_TIDE, Rho = 0.164). MDSC infiltration showed a stronger correlation with *CSNK2A1* in LUAD (MDSC_TIDE, Rho = 0.283) compared to LUSC (MDSC_TIDE, Rho = 0.212) (Figure 4B and C). In contrast, weaker correlations were observed for *CSNK2A2* in both LUAD and LUSC (Figure S7A-C). In addition, *CSNK2B* expression levels correlated with MDSC infiltration in both NSCLC subtypes, with a relatively high correlation in LUAD (Rho = 0.43) (Figure S7D-F). Overall, the strongest and most significant correlations were observed between *CSNK2A1* and MDSC infiltration (Rho = 0.283, P = 1.49e-10) and between *CSNK2B* and MDSC infiltration (Rho = 0.43, P = 1.20e-23) in LUAD specimens.

3.5. Heightened CK-2 protein subunit levels and enzymatic activity in LUAD and lung squamous carcinoma specimens

To assess CK2 protein subunit levels and enzymatic activity in NSCLC, we carried out IHC analysis on primary LUAD and LUSC specimens from treatment-naive patients. CK2 subunit staining frequency and intensity varied across examined tissues but were consistently higher than in tumor-matched para-neoplastic tissues (Figures 5 and S8). CK2 subunits localized to both the nuclear and cytosolic compartments, while the Ki67 proliferation marker predominantly appeared in the nuclear region (Figure 5A).

To quantify protein levels of CSNK2A1, CSNK2A2, and CSNK2B in NSCLC tumors and corresponding para-neoplastic tissues, we applied a CIS incorporating frequency and signal intensity (Table 1). CIS results for Ki67 corroborated the aberrant growth of these tumors.

The CIS analysis demonstrated that all CK2 protein subunits were significantly elevated in NSCLC tumors compared to para-neoplastic tissues (Table 1). To further assess tumor specificity, we calculated CIS ratios (R) by dividing tumor (T) score by para-neoplastic (PN) score, as shown in Equation I:

$$R = T/PN \quad (1)$$

The ratio served as an indicator for tumor-specific marker association. The CSNK2A1 subunit exhibited nearly two-fold higher tumor specificity (R = 3.9) than CSNK2B (R = 2.1) and CSNK2A2 (R = 1.7) (Figure 6A). This specificity was further reinforced by analyzing samples with positive-to-strong positive (++/+++) staining, where CSNK2A1 showed a significantly higher tumor specificity (R = 18.3) than CSNK2A2 and CSNK2B (R = 3.1 – 3.2) (Figure 6A).

Subsequently, we correlated the CIS values for each CK2 subunit with selected pathophysiological features in NSCLC patients (Tables S1 and S2). Protein levels of the CSNK2A1 subunit showed a positive correlation with smoking habit, tumor histology, tumor size, and clinical stage (Table S1). In contrast, elevated levels of the CSNK2A2 subunit correlated only with tumor histology (Table S2). Notably, the catalytic CK2 subunits, CSNK2A1 and CSNK2A2, were more highly expressed in LUSC than LUAD samples (Figure 6B; Tables S1 and S2).

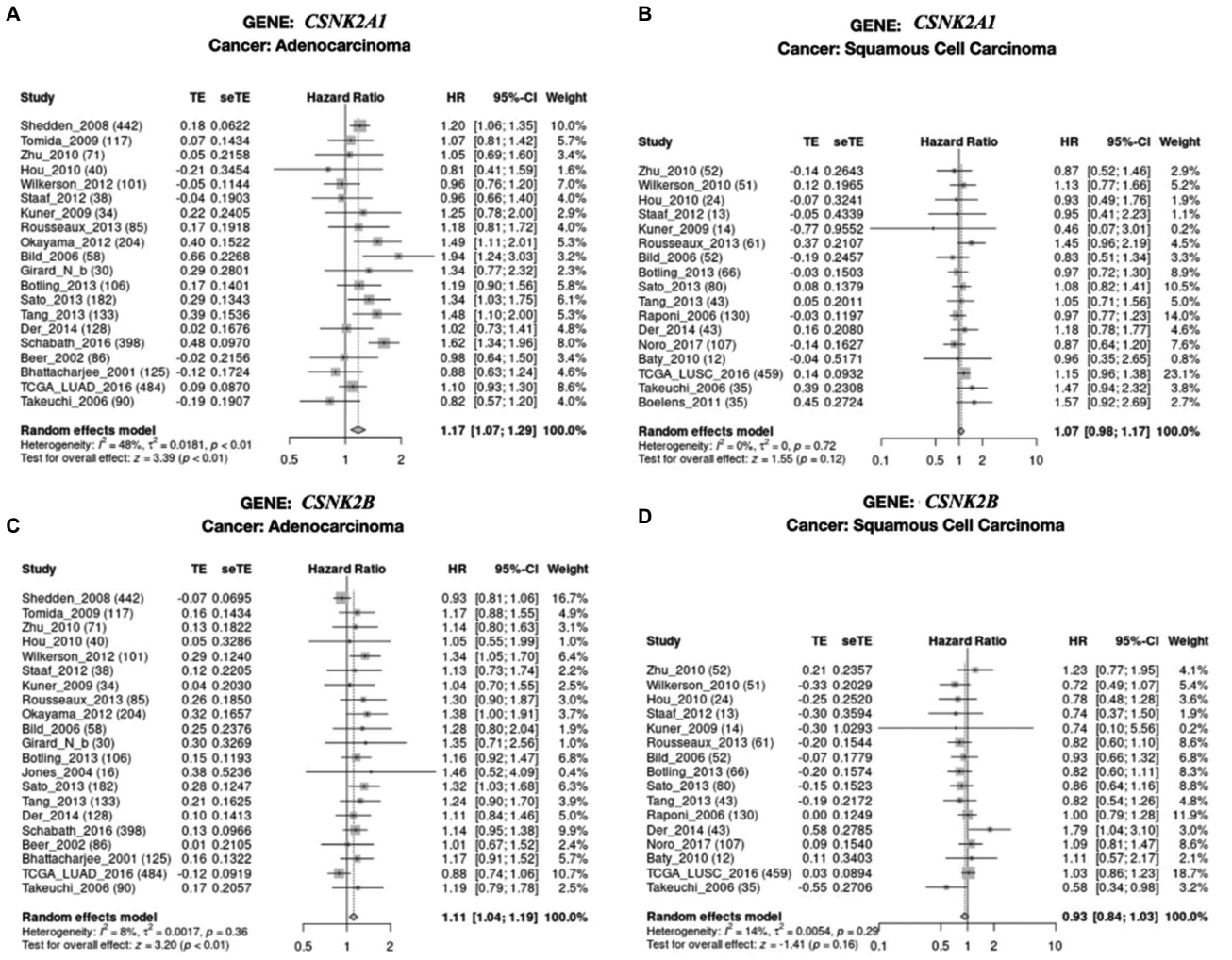


Figure 3. Meta-analysis of *CSNK2* mRNA expression versus overall survival, encompassing 20 independent LUAD and LUSC tumor data cohorts. Hazard ratios for each *CSNK2* gene were independently assessed across studies. Summary statistics and the global score are displayed at the bottom left and right corners of the charts, respectively. Hazard profiles for *CSNK2A1* and *CSNK2B* are shown for (A and C) LUAD and (B and D) LUSC.
Abbreviations: LUSC: Lung squamous cell carcinoma; LUAD: Lung adenocarcinoma.

To quantify AKTs129 phosphorylation as an indicator for CK2 enzymatic activity *in situ*, we performed IHC analysis using a phosphosite-specific antibody on a subset of LUAD (n = 76) and LUSC (n = 36) samples from the same patient cohort (Tables S3 and S4). Both total protein levels (tAKT) and phosphorylated AKTs129 protein (pAKT) were significantly elevated in LUAD and LUSC samples compared to para-neoplastic tissues, with the highest levels observed in LUSC (Figure 6C). Notably, pAKT levels did not correlate with CK2 subunit levels in either LUAD or LUSC specimens (Figure 6D). Finally, CSNK2A1 and CSNK2A2 showed strong correlations with each other in both LUAD (R = 0.504) and LUSC (R = 0.661) samples, whereas CSNK2B positively correlated only with CSNK2A1 in LUAD (R = 0.398) (Figure 6D; Table S5).

Further IHC analysis was conducted on a subset of LUAD (n = 66) and LUSC (n = 16) samples to quantify NPM1s125 phosphorylation as a second *in situ* indicator of CK2 enzymatic activity (Figure S9). Both total NPM1 protein and phosphorylated NPM1s125 protein (pNPM1) were elevated in tumor samples compared to para-neoplastic tissues (Figure S9A and B). Almost all tumor specimens (95%) showed positive-to-strong positive pNPM1 staining, with an R>2 over para-neoplastic tissues (para-LUAD = 45%; para-LUSC = 44%) (Figure S9B). Interestingly, pNPM1 phosphorylation levels correlated with CSNK2A2, but not with CSNK2A1 in LUAD; in contrast, in LUSC, pNPM1 levels correlated with both catalytic subunits, CSNK2A1 and CSNK2A2 (Figure S9C; Table S6). Finally, CK2 catalytic subunits showed strong

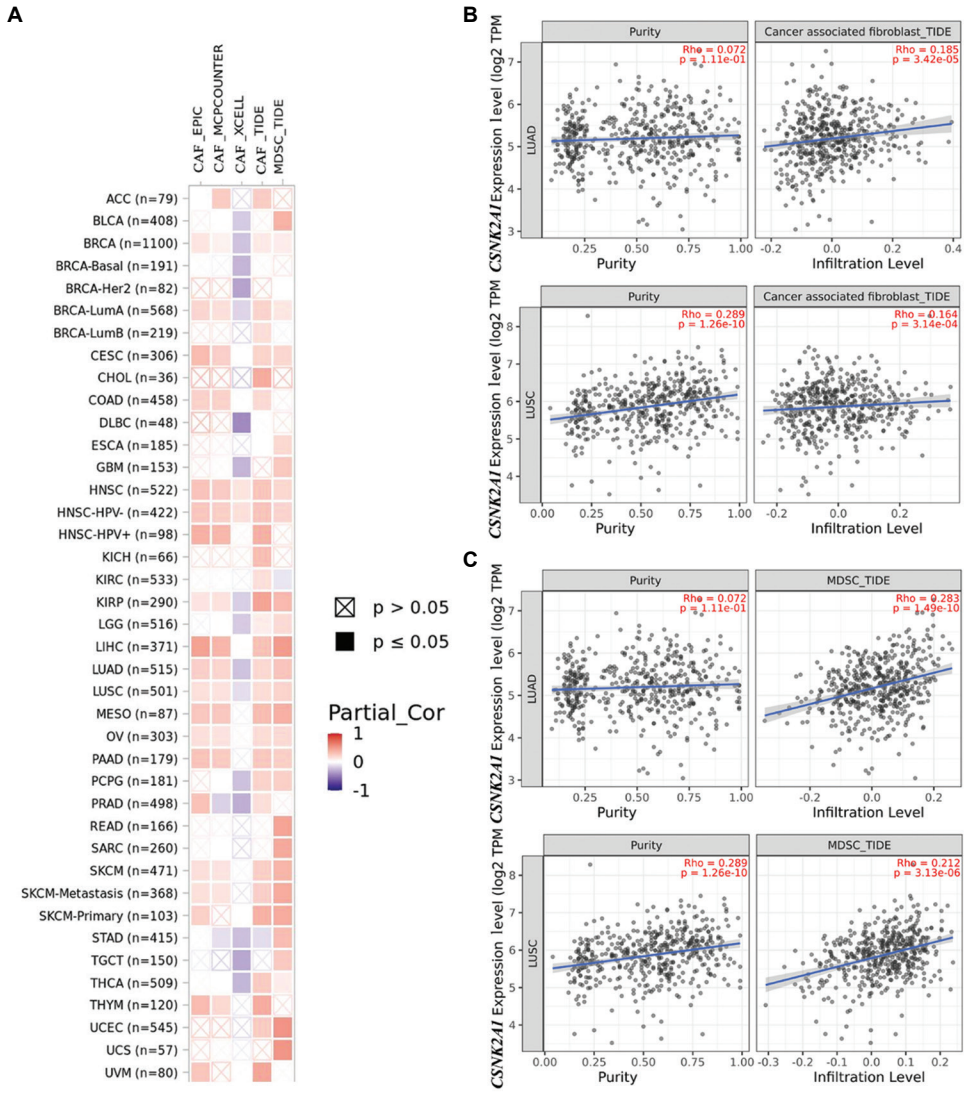


Figure 4. Correlation of CSNK2A1 gene expression levels with infiltrating cell populations in the tumor microenvironment, as analyzed using the TIMER2.0 database. (A) Correlations were assessed using four metrics for cancer-associated fibroblasts (CAFs) (CAF_EPIC, CAF_MCPCOUNTER, CAF_XCELL, CAF_TIDE) and one for myeloid-derived suppressor cells (MDCS) (MDSC_TIDE) across TCGA tumor types; "n" denotes the numbers of clinical samples within each cohort; (B) Detailed correlation analysis of CAF infiltration (CAF_TIDE metric) with CSNK2A1 expression level (Log2- TPM) in LUAD and LUSC; (C) Detailed analysis of MDSC infiltration (MDSC_TIDE metric) with CSNK2A1 expression level (Log2-TPM) in LUAD and LUSC. Each analysis was normalized for sample purity, with significant correlations indicated by color-filled boxes in the heatmap, where color intensity reflects correlation strength. Abbreviations: CAF: Cancer-associated fibroblasts; TPM: transcripts per million; LUAD: lung adenocarcinoma; LUSC: lung squamous carcinoma.

correlations with each other, particularly in LUSC samples (R = 0.683), whereas CSNK2B positively correlated only with CSNK2A1 in LUAD (R = 0.372) (Table S6).

4. Discussion

The deregulation of CK2 and its potential role in neoplastic processes have been revisited in recent studies.[24] CK2 enzyme biochemistry, aberrant expression, phosphorylation cascades, and the enzyme's tumor-supportive activities – both intrinsic and extrinsic – have been extensively investigated in preclinical models.[13,25] In parallel, several CK2 inhibitors have been developed and tested for antineoplastic effects in both *in vitro* and *in vivo*.[9] Despite this wealth of information and the availability of therapeutic compounds targeting CK2, only two CK2 inhibitors have advanced to clinical trials.[11,12]

Notably, translational and clinical data on CK2 remain relatively limited and sparse.[13] In a pioneering study, Ortega *et al.*[26] analyzed *CSNK2* transcript expression in seven tumor types, including lung cancers, using the Oncomine database. *CSNK2A1*, *CSNK2A2*, and *CSNKB* mRNA transcripts were overexpressed in 14/14,

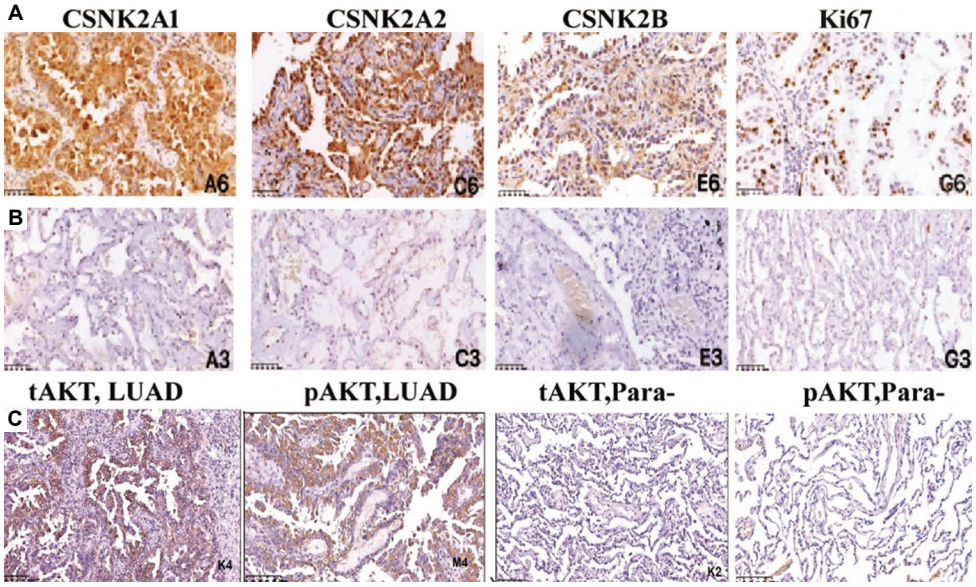


Figure 5. Immunohistochemical detection of CK2 subunits and CK2 substrate phosphorylation (AKT) in LUAD specimens and para-neoplastic tissues. Representative staining results for each CK2 subunit are shown: (A) in tumor and (B) in para-neoplastic tissues as a brown color; (C) Total AKT and phosphorylated AKTs129 (pAKT) protein levels were measured in tumor (LUAD) and para-neoplastic (Para-) tissues as an indicator of CK2 enzymatic activity in situ. Images were captured at 200× magnification. Scale bars: 100 μm.
Abbreviations: LUAD: Lung adenocarcinoma; CK2: Casein kinase-2.

Table 1. Immunohistochemical detection and quantification using a combined immunoreactivity score for independent CK2 subunits in primary tumor specimens of NSCLC and para-neoplastic tissues

Groups	Sample size	Score				Positive rate	z-score	*P*-value
		–	+	++	+++			
CK2A1								
NSCLC	134	30	50	27	27	77.6	−9.864	0.000
Para-neoplastic	134	107	24	2	1	20.1		0.000
CK2A2								
NSCLC	134	16	55	41	21	88.1	−7.248	0.000
Para-neoplastic	134	65	49	16	4	51.5		0.000
CK2B								
NSCLC	134	13	48	35	38	90.3	−8.538	0.000
Para-neoplastic	134	76	35	18	5	43.3		0.000
Ki-67								
NSCLC	134	58	32	20	24	56.7	−9.722	0.000
Para-neoplastic	134	131	3	0	0	2.2		0.000

Notes: Score 1 – 3: – (negative); Score 4 – 5: + (weak positive); Score 6 – 7: ++ (positive); Score ≥8: +++ (strong positive); Two-sided $P<0.05$ was considered statistically significant for all calculations.
Abbreviation: NSCLC: Non-small-cell lung cancer.

2/2, and 3/3 significant unique analyses, respectively, involving lung cancer specimens. In addition, Kaplan–Meier analysis revealed a significant correlation between *CSNK2B* overexpression and reduced OS, while *CSNK2A2* overexpression was unexpectedly associated with improved OS in LUAD.[26] A follow-up analysis analyzing 16 additional tumor types indicated cancer-type-specific up- or downregulation of *CSNK2* transcripts, as well as direct and inverse correlations between *CSNK2* transcripts and OS.[27] Overall, the prevailing notion is that CK2's aberrant expression levels or activity are instrumental to malignant transformation and promotion; however, the

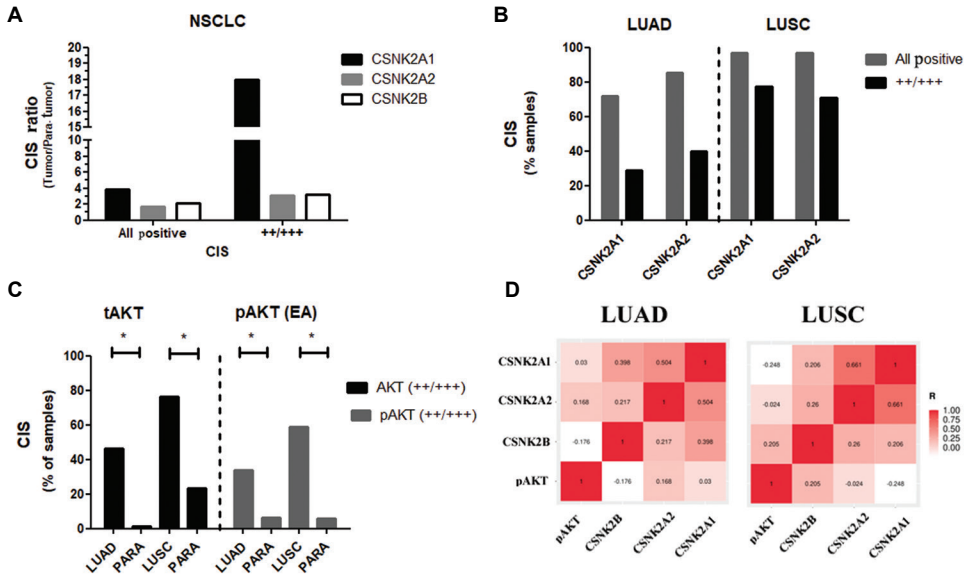


Figure 6. Quantification of IHC results in LUAD and LUSC samples using the combined IHC score. (A) Ratio (R) of tumor (T)/para-neoplastic (PN) positive staining (R = T/PN) as an indicator for tumor-specific association. The analysis included all positive (+/++/+++) or positive-to-strong positive intensity samples (++/+++); (B) Percentage of all positive or positive-to-strong positive staining in LUAD or LUSC samples; (C) IHC results for total AKT protein and phosphorylated AKTs129 protein (pAKT) in LUAD and LUSC samples; (D) Visualization of correlations between CSNK2A1/CSNK2A2/CSNKB protein subunit levels and phosphorylated AKTs129. Statistical analysis is detailed in Tables S3 and S4.
Abbreviations: IHC: Immunohistochemistry; LUAD: Lung adenocarcinoma; LUSC: Lung squamous carcinoma.

efficacy of CK2 inhibition as an anticancer strategy may be context-dependent.[13]

Despite the availability of clinical multiomics databases, there is no comprehensive analysis encompassing CK2 mutational burden, gene/protein expression, and, importantly, CK2 enzymatic activity deregulation in cancer. CK2 subunit-specific contributions to certain tumor phenotypes highlight the necessity for analyzing each subunit's regulatory patterns and functional implications individually.[28-30] Such analyses may provide fresh insights for the CK2 research community, which continues to debate whether CK2 is an actionable and safe target for cancer intervention.[6,8]

In this study, we used cBioPortal to analyze a pan-cancer dataset and two NSCLC cohorts from the TCGA PanCancer Atlas. Our results indicate that mutational events, such as single base insertions, substitutions, deletions, and/or can, are rare within *CSNK2* subunit genes and, when present, do not appear to confer oncogenic potential. These findings align with CK2's roles in the concept of "non-oncogene addiction".[1]

The *CSNK2* gene promoters display characteristics typical of housekeeping genes, including a CpG island near exon 1, which can be methylated.[31] Therefore, we investigated promoter/1stExon/5'UTR methylation and its correlations with *CSNK2* gene expression in LUSC and LUAD cohorts. Promoter/1stExon methylation showed a weak (<−0.30) negative correlation with *CSNK2A2* and *CSNK2B* mRNA levels. Interestingly, *CSNK2A1* mRNA overexpression was nearly twice as common as that of *CSNK2A2* and *CSNK2B*, with higher median expression values, particularly in LUSC samples. However, differential methylation alone does not fully explain the extensive deregulation of *CSNK2* mRNA observed in NSCLC, suggesting that more complex transcriptional and post-transcriptional regulatory mechanisms are involved.[3]

A meta-analysis of 20 LUAD and 17 LUSC clinical studies corroborated that *CSNK2A1* and *CSNK2B* mRNA overexpression correlates with poorer prognosis in LUAD, whereas *CSNK2A2* overexpression is associated with better outcomes. This trend for *CSNK2B* and *CSNK2A2* was previously observed in a microarray-based study.[26] Overall, we found that *CSNK2A1* and *CSNK2B* mRNA levels were particularly elevated in primary LUAD and LUSC samples, as well as in a limited set of metastatic NSCLC clinical samples. In addition, we identified direct correlations between *CSNK2* subunit mRNA levels and tumor infiltration by MDSC/CAF. In LUAD, the strongest correlations were observed between *CSNK2A1* and MDSC infiltration, as well as between *CSNK2B* and MDSC infiltration. These cell populations, known for their array of tumor-supportive functions, rely on secreted molecules and mediators, including CK2 substrates, for their

accumulation within the tumor microenvironment.[32-36] In summary, based on mRNA expression data, we consistently observed *CSNK2A1* and *CSNK2B* overexpression, associations with survival and metastasis, and stronger correlations with tumor-infiltrating MDSC, particularly in LUAD samples. Although internal correlations among *CSNK2* subunits may pose some analysis bias, we did not detect significant internal correlations between *CSNK2A1* and *CSNK2B*. Furthermore, correlations between the catalytic subunits were weaker in LUAD (S = 0.14) than in LUSC (S = 0.24) across analyzed cohorts.

On the other hand, protein information concerning CK2 subunits and enzymatic activity in human lung specimens is limited. Strum *et al.*[13] reviewed four studies measuring overall CK2 levels or activity in LUAD and/or LUSC samples.[37-40] In one of these studies, Liu *et al.*[39] analyzed *CSNK2A2* subunit expression in 160 NSCLC patients, finding it upregulated in both LUAD and LUSC and marginally associated with reduced OS. In our studies, we observed elevated protein levels for all three CK2 subunits in LUAD and LUSC specimens. More importantly, we demonstrate that this upregulation correlates with increased enzymatic activity *in situ*, as indicated by CK2 substrates predominantly localized in the cytoplasm (AKT) or nucleus (NPM1) of the cell. Notably, the catalytic subunits CSNK2A1 and CSNK2A2, and their purported enzymatic activity (as suggested by pAKT levels), were higher in LUSC than in LUAD specimens. We consider the canonical AKTs129 (pAKT) phosphorylation site a more reliable surrogate marker for CK2 *in situ* activity in tumors compared to NPM1s125 (pNPM1).[13] The differing extents of AKT and NPM1 phosphorylation could be explained by variations in phosphorylation turnover for each substrate and phosphorylation site within CK2 signaling, potentially faster for AKT.[41] Finally, *CSNK2A1* and *CSNK2A2* expression correlated strongly and positively with NPM1 (but not AKT), underscoring the complex interplay among kinase-phosphatase activities, CK2 substrates, and subcellular compartments.[41]

Importantly, CSNK2A1 (but not CSNK2A2) protein overexpression correlates with tumor size, stage, and proliferation score in the NSCLC tumors analyzed. This subunit-specific association is evident despite positive internal correlations between both catalytic subunits at the protein (R≈0.5) and mRNA levels (R≈0.2) across independent cohorts. Notably, at the protein level, context-dependent regulatory crosstalk occurs among CK2 subunits, reflecting overlapping activities and, in some instances, isoforms-specific functions.[3] Therefore, our findings suggest that CSNK2A1 may play a distinctive role in NSCLC. Supporting this notion, our IHC studies indicate that CSNK2A1 protein is more tumor-specific than CSNK2A2. Interestingly, a preliminary *in vitro* assessment of dependency scores from knocking out (CRISPR-Chronos) individual *CSNK2* genes in 98 NSCLC cell lines suggests that *CSNK2A1*-KO is approximately 10-fold more "essential" than *CSNK2A2*-KO in these lung cancer models (https://depmap.org/portal/).[42]

5. Conclusion

Our data suggest that transcriptional regulatory mechanisms, rather than mutational and CNA events, drive the increased mRNA expression of CK2 subunits in NSCLC samples. Correspondingly, CK2 subunits and enzymatic activity are significantly upregulated in NSCLC, particularly in LUSC. Correlative studies at both mRNA and protein levels indicate that CSNK2A1, and potentially its homotetramer ($CSNK2A1_2/CSNK2B_2$), may exert specific tumor-supportive functions in LUAD. These compelling yet correlative findings highlight the potential need for isoform-specific CK2-targeted therapies and may further elucidate CK2's role in NSCLC on the development of tailored drugs against specific CSNK2 isoforms or homotetramers.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: George V. Pérez, Yasser Perera
Investigation: All authors
Methodology: George V. Pérez, Qiang Zhao, Zhiwei Zhang, Yasser Perera
Writing – original draft: Yasser Perera
Writing – review & editing: George V. Pérez

Ethics approval and consent to participate

Studies involving human specimens were conducted in accordance with ethical principles from the Helsinki Declaration (2013), the Regulations on Biomedical Ethics Review Involving Humans (2016), the Regulations on

Clinical Research Ethics Review in Traditional Chinese Medicine, and the International Ethical Guidelines for Biomedical Research Involving Human Subjects. This project, titled "Research on Novel Peptide Drugs Targeting CK2-Mediated Phosphorylation for the Treatment of Non-Small-Cell Lung Cancer (NSCLC)" was reviewed and approved by the Nanhua University Affiliated First Hospital Medical Ethics Review Committee. Review information is as follows: Review Batch Number: 2021LL0115001; Meeting Date: January 15, 2021. Reviewed Materials: Research Plan Version: YJFA20210114; Informed Consent Form Version: ZQTY20210114. Signature by Medical Ethics Committee of Nanhua University Affiliated First Hospital Issuance Date: January 15, 2021.

Consent for publication

Written informed consent forms were obtained for the participation and subsequent publication of human data masking or concealing any identifying information of the patients.

Availability of data

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author/s.

Further disclosure

Part of the analysis/data has been uploaded to or deposited as preliminary findings in a preprint server BioRxiv doi: https://doi.org/10.1101/2023.08.04.551954.

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