ALYREF associated with immune infiltration is a prognostic biomarker in hepatocellular carcinoma

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Introduction

Primary liver cancer is very malignant, and its mortality rate has already risen to third place in 2020. The most common pathological type is hepatocellular carcinoma (HCC), which accounts for 75–85% of cases. [1,2]. Due to the insignificant symptoms in the early stage, many patients were unable to undergo hepatectomy at the time of diagnosis [2]. Immunotherapy has emerged as a promising alternative therapy for advanced HCC in recent years, which has brought opportunities for the patient [3]. However, immunotherapy is not suitable for all HCC patients. Prognostic immune markers can be used to screen patients for this treatment [4]. With the increase of combined immunotherapy regimens, more effective prognostic immune markers and immunotherapy targets are needed to be further explored [5].

ALYREF, also termed as THOC4, is an adaptor for mRNA export and is involved in nuclear export of mRNA, 3′ end processing, and regulation of mRNA and genome stability ([6,7]). ALYREF has been found to be upregulated in a number of malignancies and is connected with poor prognosis, including bladder cancer, glioblastoma, and head and neck squamous cell carcinoma. [8–12]. Xue et al. [13] have pointed out that ALYREF might be a prognostic factor for HCC. Nevertheless, the specific biological function of ALYREF in HCC and its relationship to the immune system have not been reported.

It was found in this study, ALYREF is associated with the prognosis of
Table 1
Correlation between ALYREF expression level and clinicopathological characteristics in 120 hepatectomy specimens.

| Characteristics | n | ALYREF Low expression (n=64) | ALYREF High expression (n=56) | \( \chi^2 \) | \( p \) Value |
|-----------------|---|-----------------------------|-------------------------------|----------|---------------|
| Age (year)      |   |                             |                               |          |               |
| ≤60             | 83| 43                         | 40                             | 0.252    | 0.616         |
| >60             | 37| 21                         | 16                             |          |               |
| Gender          |   |                             |                               |          |               |
| Female          | 20| 11                         | 9                              | 0.027    | 0.870         |
| Male            | 100| 53                        | 47                             |          |               |
| Cirrhosis       |   |                             |                               |          |               |
| No              | 27| 15                         | 12                             | 0.069    | 0.793         |
| Yes             | 93| 49                         | 44                             |          |               |
| HBsAg           |   |                             |                               |          |               |
| Negative        | 22| 12                         | 10                             | 0.016    | 0.900         |
| Positive        | 98| 52                         | 46                             |          |               |
| Relapse         |   |                             |                               |          |               |
| No              | 37| 22                         | 15                             | 0.807    | 0.369         |
| Yes             | 83| 42                         | 41                             |          |               |
| BCLC Stage      |   |                             |                               |          |               |
| Stage 0+Stage A | 73| 43                         | 30                             | 4.464    | 0.107         |
| Stage B         | 17| 10                         | 7                              |          |               |
| Stage C         | 30| 11                         | 19                             |          |               |
| Venous infiltration|    |                             |                               |          |               |
| No              | 90| 53                         | 37                             | 4.464    | 0.035         |
| Yes             | 30| 11                         | 19                             |          |               |
| Tumor size (cm) |   |                             |                               |          |               |
| <2              | 16| 13                         | 3                              | 6.805    | 0.033         |
| 2–5             | 46| 20                         | 26                             |          |               |
| >5              | 58| 31                         | 27                             |          |               |
| AFP (ng/ml)     |   |                             |                               |          |               |
| <200            | 72| 44                         | 28                             | 4.375    | 0.036         |
| ≥200            | 48| 20                         | 27                             |          |               |
| AJCC Stage      |   |                             |                               |          |               |
| I               | 72| 45                         | 27                             | 6.275    | 0.043         |
| II              | 11| 5                          | 6                              |          |               |
| III             | 37| 14                         | 23                             |          |               |
| Histologic Grade|   |                             |                               |          |               |
| G1+G2           | 81| 50                         | 31                             | 7.057    | 0.008         |
| G3+G4           | 39| 14                         | 25                             |          |               |

HCC with The Cancer Genome Atlas (TCGA) analysis. The validations of this study include: [1] immunohistochemistry of HCC sections was used to investigate the link between ALYREF expression and patient prognosis; [2] HCC cell lines was adopted to study the biological characteristics of ALYREF with gene transfection and knockout methods; [3] the relationship between ALYREF expression and immunomodulator genes was systematically assessed through online databases; [4] the prognostic immune model was constructed based on ALYREF-related immune genes, which has passed internal validation and can be used to assess the prognosis of HCC patients.

Methods and materials

Public data acquisition and analysis

The liver cancer dataset (LIHC) of the TCGA (https://portal.gdc.cancer.gov/) was used to obtain patient clinical information and mRNA expression profiles (RNA-Seq, FPKM format) containing 374 HCC and 50 paraneoplastic tissue samples. RNA-Seq was analyzed using the R package ‘limma’ to examine the difference in ALYREF expression between HCC and normal liver tissues. The R package “ggpubr” for analyzing the correlation between ALYREF and clinical parameters, and survival curves were constructed by Kaplan-Meier analysis.

Table 2
Sequences of siRNAs.

| Name                      | Sequence (5’-3’)              |
|---------------------------|-------------------------------|
| Negative control          | sense: UUCUCGGAGCUUCAGGUTT, antisense: AGCUUCGAGCUGGAAATT |
| ALYREF siRNA1 (si#1)      | sense: CCAUGAAGCUUCAGCUGUTT, antisense: ACGAAGGAAAGGCUUCGTT |
| ALYREF siRNA2 (si#2)      | sense: GAAUUGGAAGCUUCAGGATT, antisense: UUCUCAGGGGUCUAAUUUTT |

Gene set enrichment analysis (GSEA)

The median expression of ALYREF in LIHC RNA-seq was used as a node to split patients into high and low expression groups. The GSEA software (version 4.1.0) reference molecular signature database (MSigDB) was used to analyze the pathways that ALYREF-related genes may be involved in regulation.

Immunohistochemistry

The data and paraffin specimens from 120 patients with HCC who underwent radical hepatectomy in the First Affiliated Hospital of Anhui Medical University during 2011–2015 were harvested. Survival information of patients was obtained through telephone follow-up. The clinical parameter was present in Table 1. Before the hepatectomy, none of the patients had had any other treatment. SP method was used for the immunohistochemical staining: primary antibody (anti-ALYREF, 1:200, abcam, UK) was added after tissue section dewaxing, hydration, antigen repair, and blocking nonspecific binding sites. The primary and secondary antibodies were incubated for 1 h at 37 ºC and 20 min at room temperature, respectively. Diaminobenzidine (DAB) was used for color development, and the slides were mounted after counterstaining with hematoxylin. There were four categories to record the percentage of positive cells: 1 (0% to 15%), 2 (15% to 50%), 3 (50% to 75%), and 4 (75% to 100%), respectively. According to the staining intensity, they were divided into three categories: 1 (mild), 2 (moderate), and 3 (severe), respectively. The two scores are multiplied to get the final score: low expression group [1–4] and high expression group [6–12]. The objects are classified by pathologists who had no access to clinical data.

Cell culture and transfection

Cell Bank of Chinese Academy of Sciences provided HCC cell lines (Huh7, Hep3B, HepG2, and HCCLM3) as well as hepatocyte cell line HL-7702. DMEM medium with 10% fetal bovine serum was used to cultivate all cells in a cell incubator (5% CO2, 37 ºC). Purchased small interfering RNAs (siRNA; GenePharma, China) were transfected into HCC cell lines Huh7 and Hep3B using lipofectamine 3000 (invitrogen, USA) according to the instructions. After 48 h of cell transfection, the transfection efficiency was checked by real-time fluorescence quantitative PCR (RT-qPCR) and Western Blotting. The siRNA sequences are listed in Table 2.

RNA extraction and RT-qPCR

A reverse transcription kit (TOYOBO, Japan) was used to convert total cellular RNA extracted with TRIzol (Invitrogen, USA) into cDNA. Each sample was analyzed in triplicate by RT-qPCR using SYBR green Master mix (Accurate Biology, China) on an agilent Mx3000p instrument. The gene GAPDH was employed as an internal reference with the primer sequences: forward 5’-CCACTCTCCACCTTTG-3’; reverse 5’-CACGACCTGTTGCTGTC-3’. ALYREF primer sequences: forward 5’-GAACTCTGGTGTCCTGAATC-3’; reverse 5’-CAGCTCTGTTGCCAAC-3’.
Cell proliferation assay

Cell Counting Kit-8 (CCK-8, biosharp, China) assays: cells were transfected for 24 h and inoculated into 96-well plates (3 × 10^3 cells/well) with 100 l complete medium per well. CCK-8 (10 l/well) was added at 0, 22, 46, 70 and 94 h after inoculation, respectively. The absorbance at 450 nm was measured using a microplate reader (BioTek, USA) after 2 h of incubation in the incubator. The experiment was carried out three times, each time with four replicate wells per sample.

Colony formation assay: the cells were transfected for 24 h, inoculated into 6-well plates (1000 cells/well) and cultured for 12 days, washed in PBS, fixed for 30 min with 4% paraformaldehyde, then stained with 0.1% crystal violet for 20 min. The number of cell colonies was calculated by taking pictures after washing.

Scratch healing assay

Cells were inoculated into 6-well plates and transfected when 80% confluency was reached. After the cells formed a monolayer, scratches were made with a 200 l sterile pipette tip, wash out excess cells with PBS, and continue the culture with serum-free DMEM. Photographs were taken under a microscope (Olympus 1×51, Japan) after 24 h, and the percentage of wound healing area was subsequently calculated.

Transwell Invasion assay

Chambers with a membrane pore size of 8 m were covered with Matrigel (Corning, USA) and placed in 24-well plates. The cells were washed in PBS and resuspended in DMEM after being transfected for 24 h. Add 600 l of DMEM containing 20% FBS to the 24-well plate where the chamber is placed, then inoculate 200 l of cell suspension into the chamber (1 × 10^5 cells/well). After culturing for 48 h, the cells were fixed with 4% paraformaldehyde for 30 min, dried and stained with 0.1% crystal violet for 15 min, and the cells inside the chamber were wiped off. After washing off the excess dye, the cells were photographed and counted with a microscope (Leica DM6B, Germany).

Apoptosis assay

Apoptosis was detected with the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA). Cells transfected for 48 h were collected and made into single cell suspensions. After adding Annexin V-FITC, incubate at room temperature for 15 min in the dark. PI was added 5 min before the test, and the flow cytometer CytoFlex (Beckman Coulter, USA) was used for detection.

Western blotting

Total cellular protein was extracted with RIPA lysate containing 1%...
protease inhibitor and quantified by BCA method. SDS-PAGE electrophoresis was used to transfer an equivalent amount of protein samples to PVDF membranes. After incubation with primary antibody (anti-ALYREF: abcam; anti-GAPDH: BBI life sciences) at 4 °C for overnight, incubate in secondary antibody for one hour at room temperature. Enhanced Chemiluminescence Kit (ECL) was used for the imaging with a chemiluminescence imaging system.

**Immune cell infiltration**

The effect of ALYREF on the abundance of infiltrating immune cell subsets in HCC, including macrophages, dendritic cells, neutrophils, CD4⁺ T cells, B cells and CD8⁺ T cells was evaluated by TIMER (https://cistrome.shinyapps.io/timer/) [14]. Since tumor purity can interfere with immune gene expression analysis, the results were corrected for it [15].

**Immunomodulators and related genes**

TISIDB (http://cis.hku.hk/TISIDB/) is a website where tumors and immune systems interact. Tumor-infiltrating lymphocytes (TILs) and immunomodulators associated with ALYREF were queried by TISIDB. These immunomodulatory genes were imported into cBioPortal (www.cbiportal.org) to further acquire the top 50 co-expressed genes. Related genes were submitted to GO annotation and KEGG enrichment analysis.

**Survival analysis**

The LIHC dataset was divided randomly and equally into a training set and a testing set. In the training set, univariate and multivariate Cox regression analysis was performed on ALYREF-related immune genes to construct prognostic markers. The risk score was calculated based on prognosis-related immune genes: Risk score = β1 x 1 + β2 x 2+...+ βixi. The expression of each prognosis-related gene in the tissue is represented by xi, and βi represents the risk factor of the prognostic gene.
calculated by Cox analysis. The relationship between risk score and overall survival was investigated via Kaplan-Meier survival analysis. The risk scores were evaluated via a time-dependent receiver operating characteristic (ROC) curve. A stepwise Cox analysis was used to identify independent prognostic factors. Internal validation is conducted with the testing set.

Table 3
Univariate and multivariate survival analysis of HCC patients in the LIHC database.

| Parameter | Univariate analysis | Multivariate analysis |
|-----------|---------------------|-----------------------|
|           | HR 95%CI P          | HR 95%CI P            |
| Age       | 0.993 0.615-1.601   | 1.137 0.684-1.889     |
| Gender    | 0.780 0.487-1.249   | 0.927 0.560-1.534     |
| Grade     | 1.017 0.746-1.387   | 1.010 0.726-1.404     |
| Stage     | 1.865 1.456-2.388   | 0.913 0.341-2.447     |
| T         | 1.804 1.434-2.270   | 1.889 0.774-4.610     |
| M         | 3.850 1.207-12.281  | 1.281 0.337-4.870     |
| N         | 2.022 0.494-8.276   | 2.176 0.381-12.411    |
| ALYREF    | 1.021 1.011-1.031   | 1.020 1.010-1.031     |

Constructing a nomogram

Nomograms are often applied to evaluate the prognosis of cancer patients because they can be personalized to assess the probability of a clinical event (e.g., recurrence, death), and the effect is mostly better than the TNM staging system [16]. Risk scores and patient clinical parameters were analyzed, and the ‘rms’ package of the R program was used to create nomograms. The deviation between predicted and actual probability is visualized by a calibration curve.

Statistical analysis

The statistics and graphing of the results were implemented by SPSS (version 22.0), GraphPad Prism (version 8), R software (version 4.0.4) and the aforementioned network tools. The R packages ‘survival’ and ‘survival ROC’ were used to plot the Kaplan-Meier survival curve and the time-dependent ROC curve, respectively. Spearman correlation analysis was used to determine relevant immune genes. Statistical significance was defined as a P value < 0.05.

Result

Upregulation of ALYREF in HCC and with poor prognosis

Bioinformatics analysis of the LIHC dataset showed that ALYREF expression was upregulated in HCC compared with paracancerous tissues (Fig. 1A,B). And the expression of ALYREF was related to tumor pathological grade, AJCC stage and T stage, but not to age, gender, M
The mRNA and protein expression of ALYREF in normal hepatocyte line HL-7702 and HCC cell lines (HCCLM3, Huh7, HepG2, and Hep3B) were detected by RT-qPCR and Western Blot (Fig. 4A,B). Based on the expression levels of ALYREF in HCC cell lines, Hep3B and Huh7 cells with higher expression levels were selected and transfected with specific siRNA to knock down ALYREF for further study. RT-qPCR and Western Blot revealed that si#1 and si#2 could effectively inhibit ALYREF expression compared with si#NC (Fig. 4C,D).

CCK-8, colony formation, scratch healing, and transwell invasion assays were used to study the effects of ALYREF on the proliferation, migration, and invasion of Hep3B and Huh7 cells. CCK-8 assay showed that ALYREF-si#1 and ALYREF-si#2 reduced the proliferation rate of cells (Fig. 5A,B). Moreover, cells formed fewer colonies after ALYREF knockdown, further suggesting that ALYREF affects cell proliferation. (Fig. 5C,D). At 24 h after scratching, cells with knockdown of ALYREF had slower scratch healing and weaker migration capacity than si#NC, according to scratch healing assay data. (Fig. 5E,F). Invasiveness assay indicated that the number of cell invasion was dramatically reduced after knocking down ALYREF compared to si#NC (Fig. 5G,H). In addition, the detection of the effect of ALYREF on apoptosis by flow cytometry showed that knockdown of ALYREF promoted apoptosis. (Fig. 5I,J).

**Correlation between ALYREF and biological behavior of HCC cell lines**

The mRNA and protein expression of ALYREF in normal hepatocyte line HL-7702 and HCC cell lines (HCCLM3, Huh7, HepG2, and Hep3B) were detected by RT-qPCR and Western Blot (Fig. 4A,B). Based on the expression levels of ALYREF in HCC cell lines, Hep3B and Huh7 cells with higher expression levels were selected and transfected with specific siRNA to knock down ALYREF for further study. RT-qPCR and Western Blot revealed that si#1 and si#2 could effectively inhibit ALYREF expression compared with si#NC (Fig. 4C,D).

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**ALYREF and immune correlates**

Since the abundance and activity of TILs can affect the survival time of patients with various tumors [17,18], we evaluated the correlation of ALYREF with TILs by TIMER and TISIDB. In TIMER, ALYREF was found to be positively linked with the level of immune cell subset infiltration. (Fig. 6A) and correlated with the abundance of multiple TILs in TISIDB (Fig. 6B). And in the TISIDB data portal, we found that ALYREF was associated with some immune modulator genes (Fig. 6B). These results suggest that alterations in ALYREF expression can affect the immune-phenotype of HCC and influence patient prognosis.

**Immune prognostic models and predictive value**

To explore whether ALYREF-related immune genes can be translated into prognostic indicators for HCC patients, we selected 20 immunostimulators and 9 immunoinhibitors associated with ALYREF and uploaded them to cBioPortal to get the top 50 genes that are closely connected. GO and KEGG results indicated that these genes were implicated in multiple immune functions and immune regulatory pathways (Fig. 6C,D). Stepwise regression analysis was applied on these genes in the LIHC training set, and 4 immune genes affecting prognosis were screened out, thus constituting a prognosis model for HCC (Fig. 7A,B) and to calculate the risk score. Overall survival in the high-risk group was shorter than in the low-risk group, according to the Kaplan Meier survival curve. (Fig. 7C). The great dependability was revealed by the time-dependent ROC curve.
Fig. 5. Effect of ALYREF on proliferation, migration, invasion and apoptosis of HCC cells. (A,B) Proliferative capacity of Hep3B and Huh7 cells after transfection. (C,D) Colony-forming ability of Hep3B and Huh7 cells after transfection. (E,F) Migration ability of Hep3B and Huh7 cells after transfection. (G,H) Invasive ability of Hep3B and Huh7 cells after transfection. (I,J) Apoptosis rates of Hep3B and Huh7 cells after transfection. *P < 0.05, **P < 0.01, ***P < 0.001.
function of a variety of tumors. The overexpression of critical role in the disease levels correlated with multiple clinical parameters. These data indicate In this regard, we first analyzed the TCGA data and obtained similar Nevertheless, the mechanism of action of inhibitors has led to a milestone victory in immunotherapy for HCC [20, advanced HCC patients, and the emergence of immune checkpoint cannot undergo hepatectomy since the limited biomarkers and in HCC is still unclear. Therefore, TILs can be used to identify tumor immunotherapy targets and plays a significant biological function in the malignant progression of HCC.

Discussion

Although early surgery offers a better prognosis, many patients cannot undergo hepatectomy because of the limited biomarkers and advanced stage of the disease [19,20]. Immunotherapy brings hope for the advanced HCC patients, and the emergence of immune checkpoint inhibitors has led to a milestone victory in immunotherapy for HCC [20, 21]. Therefore, more immune-related prognostic biomarkers are needed to be found to provide targets for immunotherapy of HCC or to assess immune efficacy.

Some studies have found that expression of ALYREF is related to the prognosis of HCC patients through public database analysis [13]. Nevertheless, the mechanism of action of ALYREF in HCC is still unclear. In this regard, we first analyzed the TCGA data and obtained similar findings. Subsequently, we demonstrated by immunohistochemical experiments that upregulation of ALYREF expression in HCC was associated with poorer prognosis of patients. In addition, ALYREF expression levels correlated with multiple clinical parameters. These data indicate that ALYREF is a potentially prognostic marker for HCC and it plays a critical role in the disease’s progression.

ALYREF dysregulation can mediate alterations in the biological function of a variety of tumors. The overexpression of ALYREF upregulates PKM2, promoting proliferation and aerobic glycolysis in bladder cancer cells [8]; ALYREF promotes regional lymph node metastasis in oral squamous cell carcinoma by regulating cell migration and invasion [11]. We employed targeted siRNA to knock down ALYREF to learn more about its possible biological role in HCC, and discovered that cell proliferation, migration, and invasiveness were all reduced, while apoptosis was increased. This is consistent with the results obtained by immunohistochemical assays in this study, which showed that ALYREF expression levels were correlated with HCC tumor size and vascular invasion. In addition, GSEA enrichment analysis revealed that the high expression of ALYREF may be involved in the regulation of cell cycle signaling pathways. Oncogenes induce DNA replication stress that drives sustained cell proliferation is regarded as an essential signature of cancer, and cell cycle dysregulation often leads to abnormal cell proliferation [22,23]. These together suggest that ALYREF is closely related to HCC cell proliferation and plays a significant biological function in the malignant progression of HCC.

Immune tolerance and immune escape caused by multiple complex mechanisms can promote the formation and progression of HCC [24]. As an immune organ, the liver is rich in immune cells and immune mechanisms play a significant role in the progression of HCC [25]. TILs are of important value in anti-tumor, and the quantity and status of TILs can affect the survival time of patients with a variety of tumors [17]. Therefore, TILs can be used to identify tumor immunotherapy targets and assess prognosis [26–29]. We found that ALYREF correlated with the abundance of multiple TILs. To investigate the possible mechanisms by which the abundance of TILs is associated with ALYREF, we performed gene co-expression analysis. ALYREF was found to be co-expressed with many immunomodulator genes. GO annotation and KEGG enrichment analysis of ALYREF-related immune genes indicated that these genes have important roles in multiple immune functions and immune-related pathways. These findings imply that ALYREF expression can influence the immunophenotype of HCC. ALYREF may participate in the immune regulation of HCC by affecting tumor-infiltrating
Fig. 7. Develop prognostic gene signatures based on 79 ALYREF-related immune genes. (A) Univariate Cox regression analysis. (B) Multivariate Cox regression analysis. (C) Survival analysis of the LIHC training set risk score. (D) Time-dependent ROC curves for the prognostic model of the LIHC training set. (E) Survival analysis of the LIHC testing set risk scores. (F) Time-dependent ROC curves for the prognostic model of the LIHC testing set.
immune cells in the tumor microenvironment, which in turn affects tumor progression.

Several studies have shown that immune-related genes can be utilized as markers to evaluate the prognosis and responsiveness to immunotherapy in tumor patients [30–32]. Therefore, we developed a prognostic model consisting of four immune-related genes (KDR, ACAP1, TNFRSF4 and CD276) based on ALYREF-related immune genes. KDR has been shown to predict the prognosis of HCC patients and is a target for HCC treatment [33, 34]. Xie et al [35] have stated that TNFRSF4 is strongly associated with the immune microenvironment of HCC and contributes to poor prognosis. CD276 is a potential target for HCC immunotherapy and can be utilized as a predictor of survival in HCC patients [36–38]. It indicated that the prognostic model we constructed provided potential therapeutic targets and prognostic assessment indicators for immunotherapy of HCC.

Conclusions

Our study preliminarily showed that ALYREF can serve as a prognostic marker for HCC and has an important biological function in the progression of HCC. The resulting immune prognostic markers allow for the assessment of patient survival and provide targets for immunotherapy. These provide further insights into the role of ALYREF in HCC progression and immunotherapy. In conclusion, ALYREF plays a crucial role in HCC immunity, affects patient prognosis, and may be a viable immunotherapy target.
Fig. 9. Risk scores were incorporated to construct a prognostic nomogram. (A) Nomogram predicting 1-, 3-, and 5-year survival probabilities of HCC patients. (B) Calibration curves of nomogram-predicted 1-, 3-, and 5-year survival of HCC patients.

CRediT authorship contribution statement

Zhen-Zhen Wang: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft. Tao Meng: Software, Validation, Formal analysis. Ming-Ya Yang: Software, Validation, Formal analysis. Wei Wang: Investigation, Resources. Yan Zhang: Investigation, Resources. Yu Liu: Investigation, Resources. An-Qi Han: Investigation, Resources. Jin Wu: Investigation, Resources. Hui-xiao Wang: Supervision. Bo Qian: Supervision. Li-Xin Zhu: Conceptualization, 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.

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Data Availability Statement

The public datasets used in this study can be found here: The Cancer Genome Atlas (https://portal.gdc.cancer.gov/).

Ethics approval and consent to participate

Approved by the Medical Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Consent for publication

Not applicable

Supplementary materials

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