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EXPLORATORY EVALUATION OF SERUM CIRCRNA-CDR1AS AND INFLAMMATORY CYTOKINES FOR BRAIN METASTASIS STATUS AND SHORT-TERM TREATMENT RESPONSE IN NON-SMALL CELL LUNG CANCER

EKSPLORATIVNA PROCENA SERUMSKE CIRCRNA-CDR1AS I INFLAMATORNIH CITOKINA U ODNOSU NA STATUS METASTAZA U MOZGU I KRATKOROČNI ODGOVOR NA TERAPIJU KOD NESITNOĆELIJSKOG KARCINOMA PLUĆA

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Summary

Background: This exploratory study evaluated serum circRNA-CDR1as combined with IL-1β, IL-6, and TNF-α in non-small cell lung cancer (NSCLC), with particular attention to brain metastasis status, short-term treatment response, and the reliability of serum biomarker measurement.

Methods: Archived serum samples from 100 patients were retrospectively analysed, including 65 patients with brain metastasis (BM) and 35 without BM. Serum circRNA-CDR1as was measured by quantitative reverse-transcription PCR (qRT-PCR), whereas IL-1β, IL-6, and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA); conventional serum tumour markers were also assessed. In patients with brain metastasis, paired serum samples were analysed before and after two treatment cycles, and short-term clinical response was evaluated. Receiver operating characteristic (ROC) curves were used to assess the discriminatory value of individual markers and combined models; assay stability and quality-control parameters were evaluated to support the reliability of biomarker measurement.

Results: Patients with BM had higher serum circRNA-CDR1as than those without BM (3.04±0.82 vs 1.92±0.58, P<0.001), with similar increases in IL-1β, IL-6 and TNF-α. The four-marker circRNA-CDR1as/IL-1β/IL-6/TNF-α model showed the strongest discrimination for BM status, with an area under the curve (AUC) of 0.924 (95% CI: 0.875–0.972), sensitivity of 69.2%

Kratak sadržaj

Uvod: U ovoj eksplorativnoj studiji procenjeni su serumska circRNA-CDR1as u kombinaciji sa IL-1β, IL-6 i TNF-α kod pacijenata sa nesitnoćelijskim karcinomom pluća (NSCLC), uz poseban osvrt na status metastaza u mozgu, kratkoročni odgovor na terapiju i pouzdanost merenja serumskih biomarkera.

Metode: Retrospektivno su analizirani arhivirani uzorci seruma 100 pacijenata, od kojih je 65 imalo metastaze u mozgu (BM), dok ih 35 nije imalo. Serumska circRNA-CDR1as je merena kvantitativnom reverzno-transkripcionom lančanom reakcijom polimeraze (qRT-PCR), dok su IL-1β, IL-6 i TNF-α određivani imunoenzimskim testom (ELISA). Takođe su određivani konvencionalni serumski tumorski markeri. Kod pacijenata sa metastazama u mozgu analizirani su upareni uzorci seruma uzeti pre i nakon dva ciklusa terapije, a procenjen je i kratkoročni klinički odgovor. Krive operativnih karakteristika primaoca (ROC) korišćene su za procenu diskriminativne vrednosti pojedinačnih markera i kombinovanih modela. Stabilnost testova i parametri kontrole kvaliteta procenjeni su radi potvrđivanja pouzdanosti merenja biomarkera.

Rezultati: Pacijenti sa BM imali su više vrednosti serumske circRNA-CDR1as u odnosu na pacijente bez BM (3,04±0,82 prema 1,92±0,58; P<0,001), uz slična povećanja vrednosti IL-1β, IL-6 i TNF-α. Model sa četiri markera, circRNA-CDR1as/IL-1β/IL-6/TNF-α, pokazao je najveću sposobnost razlikovanja pacijenata prema statusu BM, sa površinom ispod krive (AUC) od

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and specificity of 100.0%. Among patients with BM, circRNA-CDR1as decreased from 3.04±0.82 to 2.45±0.78 after two treatment cycles (P<0.001), alongside reductions in IL-1β, IL-6, and TNF-α. For distinguishing poor short-term response, the combined model achieved an AUC of 0.918 (95% CI: 0.854–0.983), with sensitivity of 91.3% and specificity of 81.0%.

Conclusion: In this exploratory cohort, serum circRNA-CDR1as combined with IL-1β, IL-6, and TNF-α was associated with BM status and poor short-term response; however, the panel cannot replace contrast-enhanced cranial MRI or independently guide treatment decisions, and prospective external validation is required.

Keywords: brain metastasis, circular RNA, inflammatory cytokines, liquid biopsy, non-small cell lung cancer, treatment response

Sažetak

Uvod: Metastaze u mozgu (BM) su među najčešćim i najtežim komplikacijama nesitnoćelijskog karcinoma pluća (NSCLC). Ovo istraživanje procenjuje da li serumski cirkularni RNK circRNA-CDR1as, u kombinaciji sa inflamatornim citokinima, može da identifikuje pacijente sa BM i da predvidi kratkoročni odgovor na terapiju.

Metode: Ova eksploratorna kohortna studija uključila je 60 pacijenata sa NSCLC (30 sa BM i 30 bez BM). Serumski nivoi circRNA-CDR1as, IL-1β, IL-6 i TNF-α određeni su pre lečenja i nakon dva ciklusa terapije. Pacijenti sa BM su dodatno klasifikovani kao oni sa dobrim ili lošim kratkoročnim odgovorom.

Rezultati: Nivoi circRNA-CDR1as bili su značajno niži kod pacijenata sa BM nego kod onih bez BM (P < 0,001), sa površinom ispod krive (AUC) od 0,924 (95% CI: 0,875–0,972), senzitivnošću od 69,2% i specifičnošću od 100,0%. Kod pacijenata sa BM vrednost circRNA-CDR1as smanjila se sa 3,04 ± 0,82 na 2,45 ± 0,78 nakon dva ciklusa terapije (P < 0,001), uz istovremeno smanjenje vrednosti IL-1β, IL-6 i TNF-α. U razlikovanju pacijenata sa lošim kratkoročnim odgovorom kombinovani model ostvario je AUC od 0,918 (95% CI: 0,854–0,983), uz senzitivnost od 91,3% i specifičnost od 81,0%.

Zaključak: U ovoj eksplorativnoj kohorti serumska circRNA-CDR1as u kombinaciji sa IL-1β, IL-6 i TNF-α bila je povezana sa statusom BM i lošim kratkoročnim odgovorom na terapiju. Međutim, ovaj panel ne može zameniti magnetnu rezonancu glave sa kontrastnim sredstvom niti se može samostalno koristiti za donošenje odluka o lečenju, a neophodna je prospektivna eksterna validacija.

Ključne reči: metastaze u mozgu, cirkularna RNK, inflamatorni citokini, tečna biopsija, nesitnoćelijski karcinom pluća, odgovor na terapiju

Introduction

Lung cancer remains one of the most burdensome malignancies worldwide; in 2022, approximately 2.5 million new cases and 1.8 million deaths were attributed to lung cancer, making it one of the leading causes of cancer-related mortality (1). Non-small cell lung cancer (NSCLC) accounts for nearly 80%–85% of all lung cancers, and non-squamous NSCLC represents a major histological subtype with considerable molecular heterogeneity (2). With the routine use of molecular testing for EGFR, ALK, ROS1 and other actionable alterations, a proportion of patients can now benefit from targeted therapies (3). Nevertheless, a substantial number of patients have no identifiable actionable driver gene, and treatment for these cases still relies largely on platinum-based chemotherapy, anti-angiogenic therapy, immunotherapy, or combined regimens (4). For this patient population, reliable circulating biomarkers that reflect disease progression, metastatic risk and treatment response remain insufficient.

Brain metastasis (BM) is among the most clinically challenging events during the course of NSCLC. Previous studies have reported that approximately 15%–20% of patients with NSCLC already have brain metastases at initial diagnosis, and the cumulative incidence may rise to 30%–40% as the disease progresses (5, 6). The development of BM usually indicates a more aggressive tumour phenotype; it is also frequently accompanied by headache, seizures, cognitive impairment, or focal neurological deficits, all of which may seriously compromise quality of life and complicate subsequent treatment decisions (7, 8). Contrast-enhanced cranial MRI is still the main imaging modality for detecting brain metastases. Still, it primarily reveals established structural lesions and provides limited information about earlier biological changes, such as blood–brain barrier disruption, tumour-related inflammatory activation, and abnormalities in circulating molecular signals (9). For this reason, serum-based biomarkers that reflect biochemical and immunochemical alterations associated with BM status may help identify patients with a higher metastatic burden and may provide clinically useful information beyond imaging-based confirmation alone.

Inflammation and vascular microenvironment remodelling are increasingly recognised as contributors to BM in NSCLC; cytokines such as IL-1β, IL-6, and TNF-α may promote tumour cell invasion, alter vascular permeability, and modulate the immune microenvironment, thereby participating in the formation of a premetastatic niche (10, 11). However, inflammatory cytokines are easily affected by infection, tumour burden, therapeutic intervention, and systemic inflammatory status; when used alone, their stability and specificity as biomarkers are therefore limited (12, 13). Against this background, combining inflammatory cytokines with more stable circulating nucleic acid markers may offer a clinically relevant biomarker panel for evaluating metastatic risk and short-term treatment response in NSCLC.

Circular RNAs are characterised by covalently closed-loop structures (14), which make them relatively resistant to exonuclease-mediated degradation compared with linear RNAs; this property gives circRNAs practical advantages as candidate serum (15) or plasma biomarkers (16). However, disease association alone is insufficient for clinical laboratory interpretation. Before a circulating RNA marker can be considered for laboratory use, its sample stability, analytical reproducibility and compatibility with routine testing workflows should be evaluated. circRNA-CDR1as is a well-studied cancer-related circRNA, but its feasibility as a serum RNA measurand in non-squamous NSCLC remains insufficiently

characterised. Existing studies have linked CDR1as to lung-cancer progression and metastasis, but the available evidence is predominantly tissue-based and mechanistic; for example, the CDR1as/CDR1 regulatory axis was shown to promote migration and metastasis in lung squamous carcinoma (17). In NSCLC with brain metastasis, circulating hsa_circ_0072309 and cerebrospinal-fluid circRNA profiles have also shown biomarker potential (18), but these studies did not evaluate serum CDR1as together with inflammatory cytokines or incorporate a structured assessment of analytical performance. This unresolved question provided the rationale for the present study. The present exploratory study was designed to evaluate serum circRNA-CDR1as combined with IL-1β, IL-6, and TNF-α in NSCLC, with the main objectives of comparing these markers between patients with and without BM, assessing their changes after two treatment cycles in the BM group, and determining whether the combined panel could distinguish poor short-term response, defined as progressive disease after two treatment cycles according to RECIST 1.1.

Materials and Methods

Study design

This was a single-centre retrospective biomarker study using archived serum specimens collected from patients with NSCLC who were treated at Xingtai People's Hospital between September 2022 and September 2024. Eligible cases were identified retrospectively from the archived serum-specimen database by applying the predefined inclusion and exclusion criteria. The final analytical cohort comprised 100 patients, including 65 patients with brain metastasis and 35 patients without brain metastasis. Because the source-population screening log was not retained in the research archive, the total number of contemporaneous patients assessed before specimen retrieval, the reason-specific exclusion counts, and whether enrolment was strictly consecutive could not be reconstructed. No a priori sample-size calculation was performed because the number of eligible archived specimens available for analysis determined the sample size. Accordingly, all model-performance estimates were regarded as exploratory. This study was reviewed and approved by the Ethics Committee of Xingtai People's Hospital. Because this was a retrospective study using archived serum specimens and anonymised clinical data, the ethics committee waived the requirement for written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Inclusion and exclusion criteria

Inclusion criteria were as follows: pathologically confirmed non-squamous NSCLC; age 18–75 years; Eastern Cooperative Oncology Group (ECOG) score of 0–2; expected survival time of more than 3 months; BM confirmed by contrast-enhanced cranial MRI (≥1 lesion, diameter ≥1 cm) for the BM group, or absence of BM confirmed by negative cranial MRI for the nonBM group. BM classification was based on the contrast-enhanced cranial MRI report available at the pretreatment assessment, and all imaging used for group assignment was completed before treatment. The exact interval between MRI and serum collection and the use of independent dual-reader adjudication were not uniformly documented in the retained retrospective dataset. Exclusion criteria were as follows: concomitant other malignant tumours; severe hepatic or renal dysfunction (ALT/AST>3×ULN, Cr>1.5×ULN); recent surgery, radiotherapy, immunotherapy, active infection, or acute inflammatory disease within 1 month before sample collection; active bleeding; coagulation disorders; or incomplete clinical response data.

Sample collection and storage

Peripheral venous blood samples were collected before treatment from all included patients; for patients with BM, paired post-treatment samples were collected after completion of two treatment cycles. Blood was collected into routine serum tubes, allowed to clot, centrifuged to separate serum, aliquoted into RNase-free tubes, and stored at -80 °C until analysis. The retrospective specimen records did not retain patient-level clotting time, collection-to-centrifugation interval, centrifugation force and duration, or collection-to-freezing interval. Routine measurements were conducted without repeated freeze-thaw exposure, and specimens with visible haemolysis or insufficient volume were excluded.

Short-term response assessment

Patients in the BM group received bevacizumab combined with pemetrexed and a platinum agent for two 3-week cycles. The retained research dataset recorded the regimen at class level. Still, it did not preserve patient-level platinum-agent selection, dose modifications, or concurrent brain-directed therapy. Short-term radiological response after two cycles was categorised as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) according to RECIST 1.1, using the overall response documented in the clinical record; the retrospective dataset did not support a separate standardised intracranial-response analy-

Table I Primer sequences used for qRT-PCR detection of circRNA-CDR1as and GAPDH.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon information	Annealing/ extension temperature
circRNA-CDR1as	CTGAAGGTGAAATG-GCTGGA	AAGATGGTGATGGTGAT-GGTG	Back-splice-junction-spanning product; exact length not retained in the archived design record	60°C
GAPDH	GAAGGTGAAGGTCG-GAGTC	GAAGATGGTGATGG-GATTTC	226 bp	60°C

sis. Patients without complete response-assessment data were excluded from the response analysis. For the binary analysis, CR, PR, and SD were grouped as disease control because none met the criteria for progression after two cycles, whereas PD represented poor short-term response and early treatment failure. This analytical grouping did not imply clinical equivalence among CR, PR, and SD. A CR/PR versus SD/PD sensitivity analysis could not be performed because the retained analysis dataset contained only the binary response category.

Serum RNA extraction and qRT-PCR detection

Total serum RNA was extracted using Trizol reagent (Invitrogen). Briefly, 200 μL of serum was added to 1 mL of Trizol and thoroughly mixed by vortexing. The mixture was kept at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes. Next, 200 μL of chloroform was added, and the tube was shaken vigorously for 15 s. After standing at room temperature for 3 min, the samples were centrifuged at 12,000×g for 15 min at 4 °C. Approximately 400 μL of the upper aqueous phase was carefully transferred into a new RNase-free centrifuge tube. An equal volume of pre-cooled isopropanol was then added, followed by precipitation at -20 °C for 30 min. After centrifugation at 12,000×g for 10 min at 4 °C, the supernatant was discarded. The RNA pellet was washed twice with 75% ethanol, air-dried at room temperature for 5–10 min, and dissolved in 20 μL of RNase-free water. RNA purity was assessed using a Nanodrop 2000 ultra-micro spectrophotometer (Thermo Fisher Scientific), and samples that failed quality control were extracted again.

Different reverse transcription systems were used according to the target molecule. For circRNA and mRNA, reverse transcription was performed using random primers with the PrimeScript RT reagent Kit (Takara). The 10 μL reaction mixture contained 2 μL of 5×PrimeScript Buffer, 0.5 μL of PrimeScript RT Enzyme Mix I, 0.5 μL of Random 6 mers, and 500 ng of total RNA, with RNase-free water added to a final volume of 10 μL. The reaction was performed at 37 °C for 15 min, followed by inactivation at 85 °C for 5 s.

qRT-PCR was performed using TB Green Premix Ex Taq II (Takara) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction mixture included 10 μL of TB Green Premix Ex Taq II, 0.8 μL each of forward and reverse primers (10 μmol/L), 2 μL of cDNA template, 0.4 μL of ROX Reference Dye II, and RNase-free water to the final volume. The amplification program was as follows: pre-denaturation at 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 34 s; followed by melting curve analysis at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s to confirm amplification specificity. All primers were synthesised by Sangon Biotech (Shanghai) Co., Ltd. For circRNA-CDR1as, primers were designed across the back-splice junction to avoid genomic DNA contamination (*Table I*). The combined annealing and extension step was performed at 60 °C. GAPDH generated a 226-bp product, whereas an independently verifiable product-length annotation for the circRNA-CDR1as assay was not retained in the archived primer-design record; assay acceptance therefore relied on a single melting-curve peak and the predefined junction-spanning primer design. All samples were tested in duplicate, and the mean Ct value was used for analysis when the difference between duplicate wells was less than 0.5 Ct. Samples exceeding this predefined difference were retested. The primer sequences listed in Table I were checked against the target transcripts before analysis. GAPDH served as the endogenous reference for relative-expression analysis. In contrast, an exogenous spike RNA control was used only during analytical validation to assess extraction and reverse-transcription recovery. The spike-in recovery experiment yielded a mean recovery of 93.8% with a CV of 8.1%, and the extraction-efficiency CV was 6.9%. Group-level GAPDH Ct stability was not retained in the archived clinical dataset and could not be evaluated retrospectively. Clinical comparisons of serum circRNA-CDR1as were expressed as relative expression levels calculated under the same preanalytical and qRT-PCR workflow, whereas the plasmid-standard curve was used for analytical-per-

formance characterisation rather than for reporting group-comparison results.

Detection of serum inflammatory and angiogenesis-related factors

Serum IL-1β, IL-6, and TNF-α were measured in duplicate using R&D Systems double-antibody sandwich ELISA kits. Catalogue and lot identifiers were not retained in the archived assay record. The study-validated working ranges were 0.5–50 pg/mL for IL-1β, 3.1–300 pg/mL for IL-6, and 1.6–200 pg/mL for TNF-α; the corresponding limits of detection, estimated from the blank mean plus three standard deviations, were 0.40, 1.47, and 1.42 pg/mL, respectively. Before detection, frozen serum samples were slowly thawed at 4 °C to avoid repeated freeze-thaw cycles. The samples were then centrifuged at 1,000×g for 5 min at 4 °C to remove possible protein precipitates. The assay was carried out strictly according to the manufacturer's instructions. Gradient-diluted standards and serum samples were added to 96-well plates pre-coated with specific antibodies (100 μL per well) and incubated at room temperature for 2 h. After the plates were washed five times, enzyme-labelled secondary antibody was added at 100 μL per well, followed by another 2 h of incubation at room temperature. The plates were washed five times again, substrate solution was added, and the reaction was allowed to proceed in the dark for 20 min. Stop solution was then added to terminate the reaction. Absorbance was read at 450 nm using a SpectraMax i3x microplate reader (Molecular Devices), with 570 nm used as the reference wavelength. Standard curves were generated from standard concentrations and corresponding absorbance values, and cytokine concentrations in each sample were calculated accordingly. Each sample was tested in duplicate, and a concentration difference of less than 10% between duplicate wells was considered acceptable.

Conventional serum tumour marker measurement

To provide routine laboratory comparators for the circRNA/cytokine panel, pretreatment carcinoembryonic antigen (CEA), cytokeratin 19 fragment antigen 21-1 (CYFRA21-1), carbohydrate antigen 125 (CA125), and neuron-specific enolase (NSE) results measured at the same clinical time point were retrieved from the hospital laboratory information system. These markers were measured by routine automated chemiluminescence immunoassays in the central laboratory using manufacturer-matched calibrators and internal quality-control materials, and only results from analytical runs that met the laboratory's acceptance criteria were included. Analyser, reagent catalogue, lot, and reference-interval metadata were not retained in the retrospective research extract; therefore, these markers were used only as contextual laboratory comparators and were not incorporated into the circRNA/cytokine models.

Analytical quality control for cytokine measurement

Analytical quality-control parameters were recorded for ELISA measurement of IL-1β, IL-6, and TNF-α. Across low, medium, and high quality-control levels, the intra-assay CVs were 4.60%–4.61% for IL-1β, 3.90%–3.92% for IL-6, and 4.80%–4.82% for TNF-α; the corresponding inter-assay CVs were 6.77%–6.82%, 6.10%–6.13%, and 7.20%–7.21%. Mean duplicate differences were 4.44%, 4.81%, and 4.54%, with maximum differences of 8.4%, 7.8%, and 8.7%, respectively. All 60 duplicate measurements met the predefined <10% criterion, and no sample was outside the validated assay range. Serum samples were assayed without predilution, and no study sample was outside the validated working range; consequently, no out-of-range imputation was required. The archived record did not preserve the catalogue or lot identifier of the quality-control material.

Preliminary analytical stability assessment of circRNA-CDR1as

To perform a preliminary analytical stability assessment of circRNA-CDR1as under predefined sample-handling conditions, serum samples from three patients in the BM group were pooled, mixed thoroughly, and aliquoted into 200 μL portions. The aliquots were exposed to the following conditions: room-temperature storage for 0, 6, 12, 24 and 48 h; repeated freeze-thaw cycles at -80 °C for 0, 1, 3, 5 and 7 cycles; storage at 4 °C, -20 °C and -80 °C for 1 month; and RNase A treatment at final concentrations of 0.1 μg/mL, 1 μg/mL and 10 μg/mL at 37 °C for 30 min. RNA was extracted after each treatment using the same protocol. Untreated pooled serum was used as the reference condition, and relative signal retention and change rates were calculated for circRNA-CDR1as and linear GAPDH. RNA integrity after RNase A treatment was not evaluated independently by agarose-gel electrophoresis; consequently, this experiment assessed target-specific qRT-PCR signal retention rather than global serum RNA integrity. The analytical validation also included inter-operator, inter-batch, and haemolysis-interference experiments. The inter-operator and inter-batch CVs were 5.8% and 7.4%, respectively; mean target-signal retention was 97.0% with mild haemolysis, 92.0% with moderate haemolysis, and

80.9% with severe haemolysis, supporting the exclusion of visibly homolysed specimens.

Statistical analysis

Statistical analyses were performed using SPSS version 26.0. Distributional assumptions were assessed using the Shapiro-Wilk test and quantile-quantile plots, while homogeneity of variance was assessed using Levene's test. Continuous variables are presented as mean±standard deviation; independent-group comparisons used the Student's t test when variances were homogeneous and Welch's t test when they were unequal, while Mann-Whitney U tests were used as sensitivity analyses for non-normally distributed variables when patient-level data were available. Paired pre- and post-treatment comparisons were performed using the paired t test after assessment of the paired differences, and categorical variables were compared using the chi-square test or Fisher's exact test, as appropriate. Benjamini-Hochberg false-discovery-rate correction was applied within each family of four biomarker comparisons. ROC curves were used to assess discrimination of BM status and poor short-term response, with AUCs and 95% confidence intervals reported; cut-off values were selected using the Youden index. Logistic regression was used to construct the cytokine-only and four-marker models. Because the models were developed and evaluated in the same cohort without reliable internal resampling, their AUCs and cut-off values were regarded as apparent exploratory estimates. All tests were two-sided, and $P<0.05$ was considered statistically significant.

Results

Reliability of serum circRNA-CDR1as and cytokine measurements

In the preliminary pooled-serum analytical stability assessment, circRNA-CDR1as retained 0.91±0.06 of the baseline target-specific signal after 48 h at room temperature and 0.92±0.05 after seven freeze-thaw cycles, while 86% of the target-specific signal remained after exposure to 10 μg/mL RNase A for 30 min. The qRT-PCR assay showed a linear dynamic range of 10^1–10^3 copies/μL, a standard-curve R^2 of 0.998, amplification efficiency of 96.5%, intra-assay CVs of 2.8%–4.2% and inter-assay CVs of 3.5%–5.1%. ELISA measurements for IL-1β, IL-6 and TNF-α met predefined quality-control criteria (*Supplementary Tables S1–S4*).

Comparison of baseline characteristics

Age, sex, disease course, and other baseline characteristics were first compared between the BM and nonBM groups. No statistically significant differences were observed in the reported baseline variables between the two groups ($P>0.05$; *Table II*).

Comparison with conventional serum tumour markers

Compared with the nonBM group, patients with BM had higher serum levels of CEA (17.40±12.29 vs. 8.59±5.70 ng/mL, $P<0.001$), CYFRA21-1 (5.90±3.40 vs. 4.21±2.10 ng/mL, $P=0.003$), CA125 (42.70±24.80 vs. 26.80±15.49 U/mL,

Table II Baseline clinical characteristics of patients in the nonBM and BM groups.

	nonBM group (n=35)	BM group (n=65)	t or χ^2	P value
Age	57.11±7.54	59.11±9.89	1.040	0.301
Sex (male/female)	21/14	38/27	0.022	0.881
ECOG score			0.966	0.069
0 points	12	24		
1 point	18	32		
2 points	5	9		
Pathological type			0.01	0.92
Adenocarcinoma	31	58		
Large cell carcinoma	4	7		
Disease duration (months)	4.11±1.41	4.22±1.69	0.302	0.764

Table III Conventional serum tumour markers in patients with and without brain metastasis.

Marker	nonBM group (n=35)	BM group (n=65)	t value	P value
CEA (ng/mL)	8.59±5.70	17.40±12.29	4.887	<0.001
CYFRA21-1 (ng/mL)	4.21±2.10	5.90±3.40	3.073	0.003
CA125 (U/mL)	26.80±15.49	42.70±24.80	3.935	<0.001
NSE (ng/mL)	12.80±4.59	19.60±8.09	5.362	<0.001

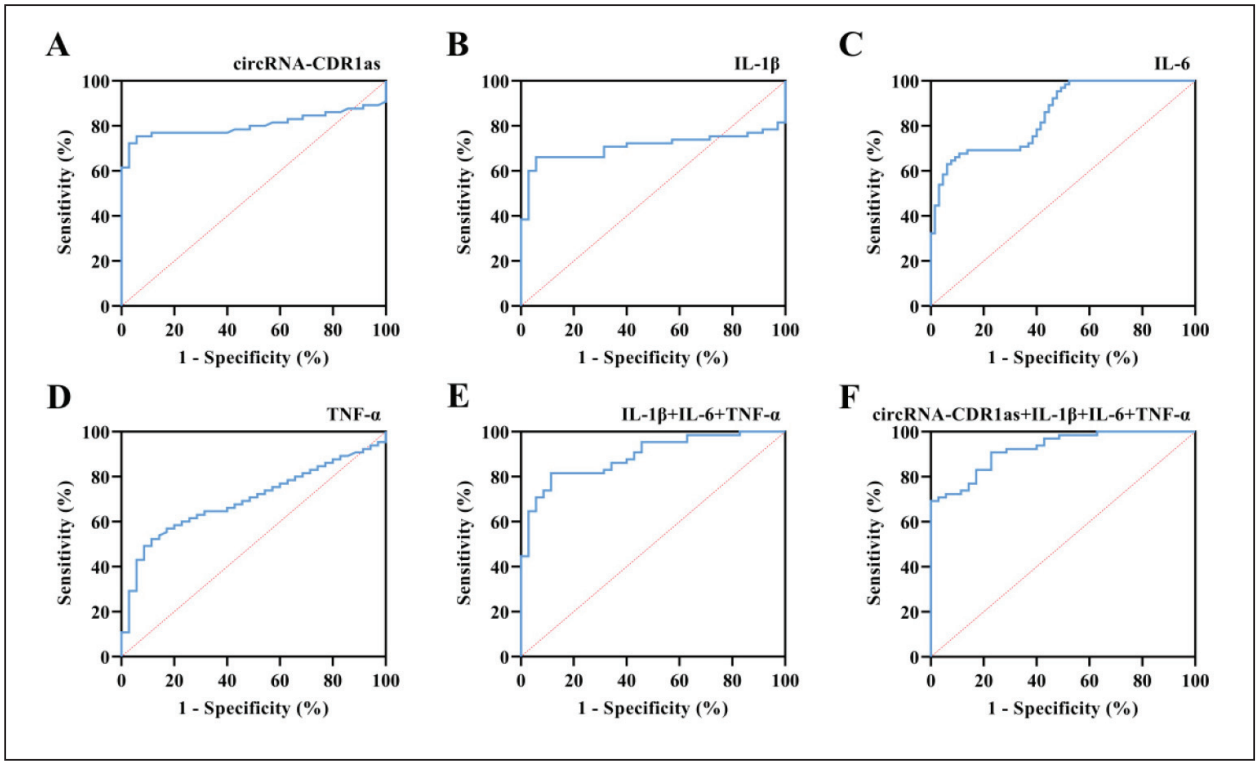

Figure 1 Receiver operating characteristic curve analysis of serum circular RNA CDR1as and inflammatory cytokines for discriminating BM status in non-small cell lung cancer

(A) Discriminatory performance of circular RNA CDR1as; (B) discriminatory performance of IL-1β; (C) discriminatory performance of IL-6; (D) discriminatory performance of TNF-α; (E) discriminatory performance of the combined inflammatory cytokine panel including IL-1β, IL-6, and TNF-α; (F) discriminatory performance of the four-marker model combining circular RNA CDR1as, IL-1β, IL-6, and TNF-α.

P<0.001) and NSE (19.60±8.09 vs. 12.80±4.59 ng/mL, P<0.001). Among these routine markers, NSE and CEA showed the most pronounced between-group differences, providing a clinically familiar reference context for evaluating the circRNA/cytokine panel (*Table III*).

Association of serum circRNA-CDR1as and inflammatory cytokines with BM status

The BM group showed higher serum circRNA-CDR1as than the nonBM group (3.04±0.82 vs. 1.92±0.58, P<0.001), as well as higher IL-1β (16.80±5.20 vs. 13.20±4.60 pg/mL, P=0.001), IL-6 (30.60±9.40 vs. 24.50±8.10 pg/mL, P=0.002) and TNF-α (38.50±11.80 vs. 31.40±10.20 pg/mL, P=0.003). ROC analysis showed that circRNA-CDR1as had the best single-marker performance for discriminating BM status, with an AUC of 0.804 (95% CI: 0.715–0.894), sensitivity of 75.4%, and specificity of 94.3%. The cytokine-only model improved the AUC to 0.891 (95%CI: 0.830–0.953), while the four-marker model combining

Table IV Discriminatory value of serum circRNA-CDR1as and inflammatory cytokines for BM status.

	Groups	n	circRNA-CDR1as	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)
Expression comparison	nonBM group	35	1.92±0.58	13.20±4.60	24.50±8.10	31.40±10.20
	BM group	65	3.04±0.82	16.80±5.20	30.60±9.40	38.50±11.80
	t	-	7.165	3.434	3.243	3.005
	P	-	0	0.001	0.002	0.003
ROC analysis	Indicator	AUC	95%CI	cut-off	Sensitivity	Specificity
	circRNA-CDR1as	0.804	0.715–0.894	>3.104	0.754	0.943
	IL-1β	0.708	0.603–0.813	>20.177	0.662	0.943
	IL-6	0.722	0.622–0.822	>35.437	0.631	0.886
	TNF-α	0.697	0.594–0.799	>45.1	0.523	0.886
	IL-1β+IL-6+TNF-α	0.891	0.830–0.953	>0.755	0.815	0.886
	circRNA-CDR1as+IL-1β+IL-6+TNF-α	0.924	0.875–0.972	>0.906	0.692	1

Table V Treatment-related changes in serum circRNA-CDR1as and inflammatory cytokines in patients with brain metastasis.

Timing	n	circRNA-CDR1as	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)
Before treatment	65	3.04±0.82	16.80±5.20	30.60±9.40	38.50±11.80
After treatment	65	2.45±0.78	14.94±4.98	27.17±9.12	34.55±11.17
t	-	5.945	2.797	3.201	2.642
P	-	<0.001	0.007	0.002	0.010

circRNA-CDR1as, IL-1β, IL-6 and TNF-α showed the highest apparent AUC of 0.924 (95%CI: 0.875–0.972), with sensitivity of 69.2% and specificity of 100.0% (*Figure 1, Table IV*).

Dynamic changes in serum circRNA-CDR1as and inflammatory cytokines after treatment

In patients with BM, paired serum samples were used to evaluate short-term changes in the serum biomarker panel after treatment. Among patients with BM, serum circRNA-CDR1as, IL-1β, IL-6, and TNF-α levels were lower after two treatment cycles than at pretreatment (Table V). In a secondary comparison with the pretreatment nonBM group, post-treatment circRNA-CDR1as remained higher in the BM group (2.45±0.78 vs 1.92±0.58, P<0.001), whereas the corresponding differences in IL-1β, IL-6, and TNF-α were not statistically significant (P=0.084, P=0.137, and P=0.159, respectively).

Association of post-treatment serum biomarkers with short-term response

ROC analysis showed that circRNA-CDR1as had the strongest single-marker performance for distinguishing poor short-term response, with an AUC of 0.812 (95% CI: 0.698–0.925), sensitivity of 87.0%, and specificity of 76.2%. The cytokine-only model yielded an AUC of 0.880 (95% CI: 0.800–0.960), whereas the four-marker model combining circRNA-CDR1as, IL-1β, IL-6, and TNF-α showed the highest apparent AUC of 0.918 (95% CI: 0.854–0.983), with sensitivity of 91.3% and specificity of 81.0%. These findings suggest that persistent elevation of circRNA-CDR1as and inflammatory cytokines after treatment may be associated with an unfavourable early clinical outcome (*Figure 2, Table VI*).

Discussion

This exploratory study evaluated serum circRNA-CDR1as combined with IL-1β, IL-6, and

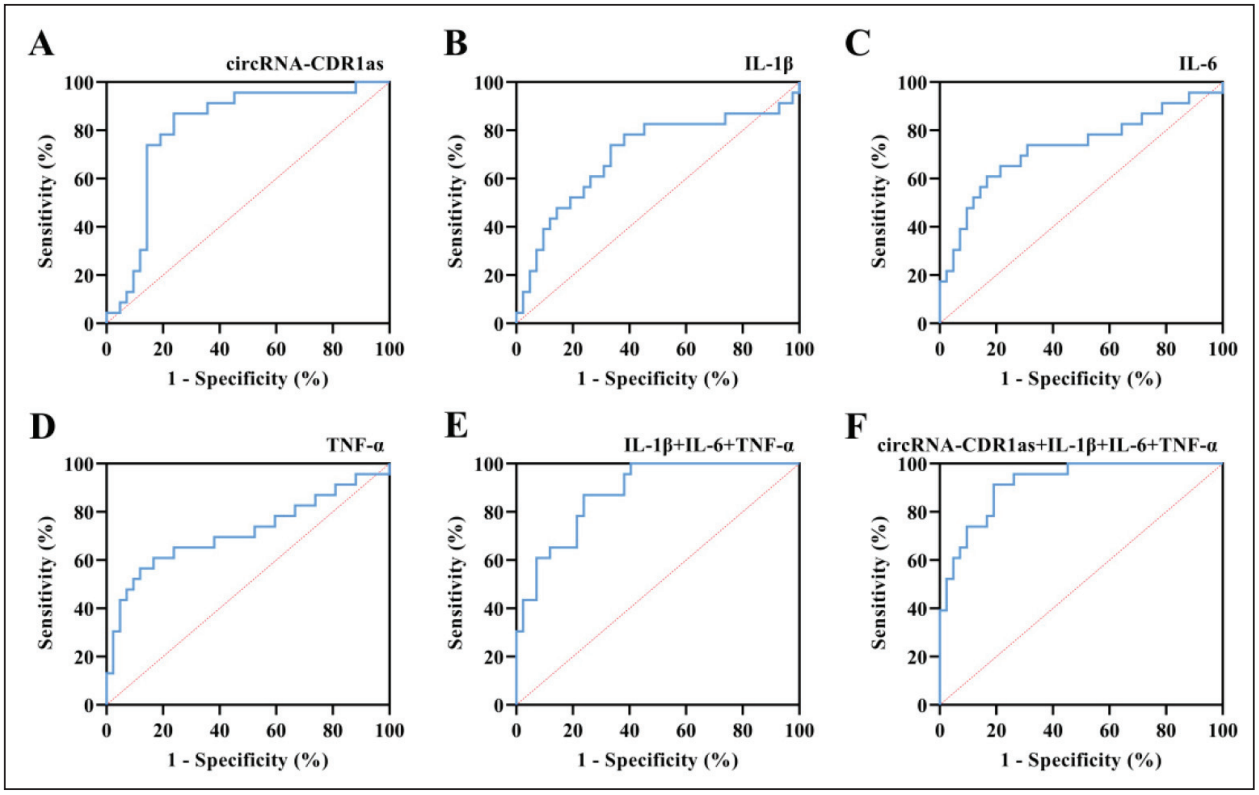

Figure 2 Receiver operating characteristic curve analysis of serum circRNA-CDR1as and inflammatory cytokines for distinguishing poor short-term response in patients with brain metastasis.

(A) Predictive performance of circular RNA CDR1as; (B) predictive performance of IL-1β; (C) predictive performance of IL-6; (D) predictive performance of TNF-α; (E) predictive performance of the combined inflammatory cytokine panel including IL-1β, IL-6, and TNF-α; (F) predictive performance of the four-marker model combining circular RNA CDR1as, IL-1β, IL-6, and TNF-α.

Table VI Predictive value of post-treatment serum circRNA-CDR1as and inflammatory cytokines for poor short-term response in patients with brain metastasis.

	Groups	n	cir-cRNA-CDR1as	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)
Expression comparison	Disease control	42	2.19±0.66	13.70±4.50	24.80±8.20	31.90±10.10
	Poor short-term response	23	2.92±0.78	17.20±5.10	31.50±9.30	39.40±11.60
	t	-	3.996	2.86	3.003	2.715
	P	—	<0.001	0.006	0.004	0.009
ROC analysis	Indicator	AUC	95%CI	cut-off	Sensitivity	Specificity
	circRNA-CDR1as	0.812	0.698–0.925	2.478	0.87	0.762
	IL-1α	0.701	0.555–0.847	13.573	0.739	0.667
	IL-6	0.729	0.586–0.871	35.053	0.609	0.833
	TNF-α	0.716	0.569–0.864	45.309	0.565	0.881
	IL-1β+IL-6+TNF-α	0.88	0.800–0.960	0.292	0.87	0.762
	circRNA-CDR1as+IL-1β+IL-6+TNF-α	0.918	0.854–0.983	0.258	0.913	0.810

TNF-α in NSCLC; the principal findings were that these circulating biomarkers were associated with BM status, decreased after two treatment cycles in the BM group, and were higher after treatment among BM patients with poor short-term response. The accompanying stability and quality-control data support the reliability of these serum measurements. Still, the clinical relevance of the study is mainly reflected in their associations with metastatic status and short-term treatment response.

The analytical findings support the feasibility of measuring serum circRNA-CDR1as under the standardised assay conditions used in this study. The qRT-PCR workflow demonstrated acceptable linearity, amplification efficiency, precision, analytical sensitivity, spike-in recovery, operator and batch reproducibility, and defined susceptibility to severe haemolysis (19, 20); however, the pooled-serum stability experiment assessed target-specific signal retention and does not constitute comprehensive clinical laboratory validation (21). The ELISA measurements also met the predefined analytical-range, precision, and duplicate-agreement criteria, supporting the internal reliability of the cytokine measurements.

For BM assessment, circRNA-CDR1as showed better single-marker discrimination than IL-1β, IL-6, or TNF-α. Although this pattern may reflect complementary information from a circulating RNA analyte and nonspecific inflammatory markers, the present observational study did not investigate the underlying biological mechanism. The moderate single-marker performance of the cytokines also indicates that these measurements should be interpreted together with clinical findings and analytical quality-control information rather than as disease-specific signals (22, 23). Their combined use improved discriminatory performance, suggesting that multiple immunochemical markers may better represent the inflammatory background than a single cytokine (24). After circRNA-CDR1as was added, the four-marker model showed the strongest performance, supporting the complementary value of serum RNA and cytokine measurements as a laboratory biomarker panel.

In addition, the inclusion of conventional tumour markers also provides a clinically familiar laboratory context for interpreting the circRNA/cytokine panel: CEA, CYFRA21-1, CA125, and NSE were higher in patients with BM, confirming that routine serum tumour markers carry disease-related information in this cohort. However, the aim of the present study was not to replace established laboratory tests with a single molecular marker, but to evaluate whether a serum RNA measurand combined with immunochemical cytokine testing could provide complementary laboratory information. This framework supports the interpretation of serum circRNA-CDR1as and inflammatory cytokines as circulating biomarkers with potential relevance to metastatic burden and early treatment response in NSCLC. The apparent performance of the present panel may also be considered in relation to other liquid-biopsy approaches. Serum exosomal lipopolysaccharide-binding protein distinguished metastatic from nonmetastatic NSCLC with an AUC of 0.803, a sensitivity of 83.1%, and a specificity of 67.0%, although that study was not specific to brain metastasis. A more recent plasma exosomal miRNA-metabolite model developed for lung-cancer brain metastasis achieved an AUC of 0.927. The apparent AUC of 0.924 in the present cohort was numerically similar to the latter result; nevertheless, differences in study populations, analytes, outcome definitions, and validation strategies preclude direct performance comparisons, and the lower sensitivity and absence of external validation limit the present panel as a stand-alone screening test.

Several limitations should be acknowledged. First, this was a single-centre retrospective study with a limited sample size; a complete source-population screening log, the exact MRI-to-sampling interval, independent dual-reader adjudication, patient-level platinum-agent selection and dose modification, concurrent brain-directed therapy, and several preanalytical timing variables were not uniformly retained. Second, GAPDH was used as the endogenous reference. Still, group-level GAPDH Ct stability was unavailable, while the pooled-serum stability experiment assessed target-specific signal retention without an independent global RNA-integrity test. Third, the combined models and Youden-derived cut-off values were developed and evaluated in the same cohort, and neither internal resampling nor external validation was available; consequently, the apparent AUCs may be optimistically biased. Short-term response was assessed after only two treatment cycles, and the retained binary response dataset did not permit a CR/PR versus SD/PD sensitivity analysis. Fourth, potential confounders, including extracranial tumour burden, brain-lesion number, corticosteroid use, and other inflammatory conditions, were not fully controlled. Finally, the panel cannot replace contrast-enhanced cranial MRI, establish clinically validated decision limits, or independently guide treatment; prospective multicentre validation, external model assessment, and broader interlaboratory analytical verification are required.

Conclusion

Serum circRNA-CDR1as, IL-1β, IL-6, and TNF-α were associated with BM status in this exploratory NSCLC cohort; among patients with BM, these markers decreased after two treatment cycles and were higher after treatment in those with poor

short-term response. The combined panel showed stronger apparent discrimination than the individual markers. Still, it cannot replace contrast-enhanced cranial MRI or serve as an independent basis for treatment decisions. It may provide auxiliary laboratory information for BM risk stratification and short-term response assessment, subject to prospective multicentre validation, external model validation, and further interlaboratory analytical verification.

Consent to publish

All authors gave final approval of the version to be published.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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