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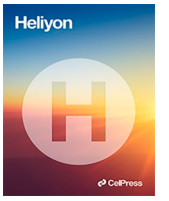

Research article

Plasma-based transcriptomic non-coding signature for predicting relapse in pediatric acute lymphoblastic leukemia

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A R T I C L E I N F O

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Recurrence
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Risk score
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A B S T R A C T

Pediatric Acute Lymphoblastic Leukemia (ALL) is the most common malignant tumor of the hematological system in children, and its relapse after treatment has consistently been a significant factor hindering prognosis. This study aimed to develop a blood-based non-invasive method for predicting relapse in children with ALL. Two cohorts of pediatric ALL patients were analyzed. Through high-throughput profiling, three miRNAs and three circRNAs were identified as potential biological markers, exhibiting a gradient increase in expression from healthy controls to the relapsed group. Logistic regression analysis revealed the superior predictive ability of the combined non-coding RNA panel compared to individual groups. A nomogram incorporating the non-coding RNA panel and other clinical risk features was developed. Combining the non-coding RNA panel with relevant risk features could enhance predictive accuracy. The non-coding RNA panel remained an independent predictor of relapse in the validation cohort, and its combination with clinical features formed a superior risk stratification model. In conclusion, this blood-based non-invasive method holds promise for predicting relapse in pediatric ALL patients at the time of initial diagnosis. The non-coding RNA panel, along with clinical risk features, may significantly impact patient care and outcomes.

1. Background

Acute lymphoblastic leukemia (ALL) is a cancer that affects the blood and bone marrow [1]. This type of cancer is the most prevalent among children, constituting roughly 25 % of all pediatric cancer cases [2,3]. Plasma diagnostic markers are substances

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present in blood that can be used to diagnose and monitor disease progression of a disease [4]. The development of plasma diagnostic markers has been an important area of research in children with ALL.

Minimal residual disease (MRD) is one of the most promising plasma diagnostic markers for ALL [5]. MRD refers to the small number of cancer cells that remain in the body after treatment. MRD can be detected using sensitive laboratory techniques such as polymerase chain reaction (PCR) or flow cytometry. Studies have shown that monitoring MRD levels in children with ALL can help to predict the likelihood of relapse and guide treatment decisions [6]. Cytokines are other plasma diagnostic markers that have shown promise in children with ALL. Cytokines are small proteins that play a role in the immune response to infections and inflammation. In children with ALL, changes in cytokine levels are associated with disease progression and treatment response. For example, studies have shown that elevated levels of certain cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are associated with poorer prognosis [7]. Other plasma diagnostic markers that have been studied in children with ALL include soluble adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2) [8]. These markers have been shown to be associated with disease progression and response to treatment in some studies; however, further research is needed to determine their clinical usefulness. Finally, if the marker is clinically useful, it can be incorporated into clinical practice for ALL diagnosis and monitoring. This could improve the accuracy and efficiency of diagnosis and treatment monitoring, leading to better outcomes in children with ALL.

MRD enables the identification of a small number of leukemic cells remaining after therapy, indicating incomplete remission and an increased risk of relapse [9]. However, challenges persist in the standardization of MRD assays across different laboratories, affecting the reliability and accuracy of the results. Another vital area of research focuses on cytogenetic abnormalities, such as Philadelphia chromosome (Ph+), MLL gene rearrangements, and hypodiploidy, which have been associated with a higher risk of relapse in pediatric ALL [10]. Although these genetic markers have contributed to risk stratification and targeted treatments, other cytogenetic subgroups remain poorly characterized, limiting their full prognostic relevance. Immunophenotypic markers, particularly those detected using flow cytometry-based immunophenotyping, offer an alternative approach to MRD detection. By identifying leukemic cells based on specific surface markers, these markers provide valuable information on treatment response and risk of relapse. Functional and proteomic markers have also been explored to identify drug resistance-associated proteins and understand their role in chemoresistance in ALL. Alterations in drug transporters and efflux pumps, such as P-glycoprotein (P-gp), have been implicated in drug resistance, and identification of these markers may help develop personalized therapeutic approaches [11]. However, functional studies and validation in larger patient cohorts are essential for effectively translating these findings into clinical practice. Omics technologies, including genomics, transcriptomics, and proteomics, have emerged as powerful tools for the comprehensive profiling of childhood ALL patients. Using these approaches, novel biomarkers and potential therapeutic targets have been identified. Nevertheless, the integration of omics data and their validation in independent cohorts remain challenging, hindering their clinical implementation.

The bone marrow microenvironment is essential for leukemic cell survival and chemoresistance in pediatric ALL, with interactions among stromal cells, extracellular matrix, and soluble factors influencing disease progression and relapse [12]. The role of tumor-infiltrating lymphocytes (TILs) in modulating this environment is a growing research area, with potential implications for immune-based therapies to prevent relapse [13]. While MRD and cytogenetic markers are established in clinical practice, standardization challenges remain. Emerging markers and proteomic approaches show promise but require further validation for clinical use.

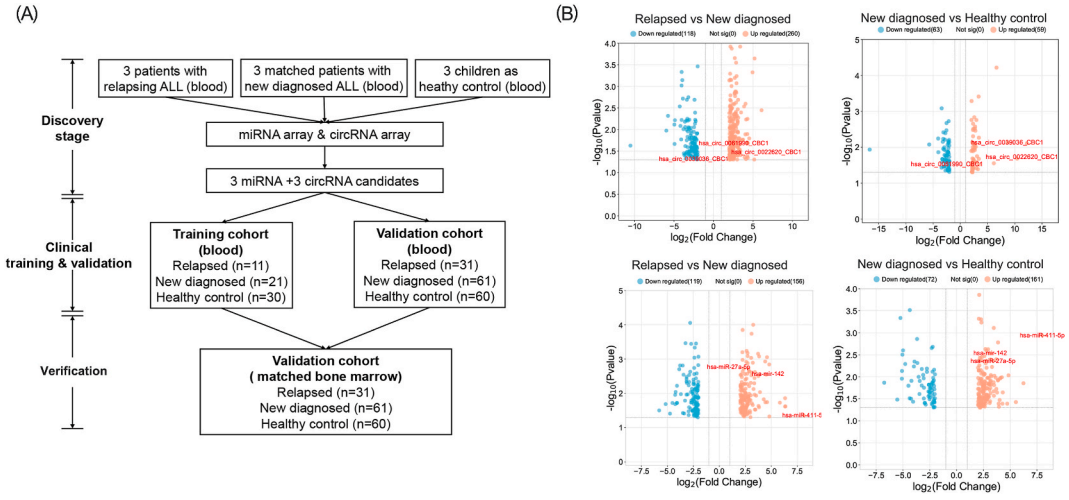

Fig. 1. Research Strategy and High-Throughput Chip Screening. (A) Overview of the research strategy. **(B)** Differential expression levels detected in peripheral blood plasma of relapsed ALL patients, newly diagnosed ALL patients, and healthy children using miRNA and circRNA microarray. The upper panel represents miRNA, while the lower panel represents circRNA. Genes with high expression are labeled in red, and those with low expression are labeled in blue.

Advancing our understanding of these factors may lead to targeted therapies to reduce relapse and improve outcomes in pediatric ALL [14].

To bridge this knowledge gap, we assessed the feasibility of converting our previously identified transcriptomic non-coding biomarkers (miRNAs and circRNAs) into a blood-based, noninvasive assay. This was accomplished by systematically analyzing blood samples from multiple cohorts of children with ALL. In this study, we propose the use of high-throughput chip technology to screen for abnormally elevated plasma non-coding RNAs (ncRNAs) in patients with relapsed ALL. The identified ncRNAs will be further validated through multiple assessments to evaluate their capacity to predict leukemia relapse in pediatric patients. Additionally, we aimed to transform bone marrow-based biomarkers into blood-based detection biomarkers by conducting supplementary validation with bone marrow samples. The ultimate goal is to develop a clinically attractive non-invasive diagnostic method for ALL relapse detection in children.

2. Methods

2.1. Patient cohorts

The study included 172 pediatric samples, including peripheral blood and bone marrow specimens. Of these, 90 cases were healthy children with matched serum and bone marrow samples, whereas 82 cases were children with acute lymphoblastic leukemia (ALL) with matched serum and bone marrow samples. A total of 113 samples were excluded due to sample quality issues or the inability to obtain matched samples. The samples were randomly divided into two independent clinical cohorts, a training set and a validation set. The validation set comprised plasma sample validation and matched bone marrow sample validation (Fig. 1A). All children with ALL included in the study were aged 14 years or younger and treated at the Hematology-Oncology Department of the Affiliated Children's Hospital of Nanjing Medical University between January 2011 and January 2020. The diagnosis of ALL was based on the CCLG-2008 and 2015 protocol, which involves immunophenotypic, morphological, cytogenetic, and molecular biology examinations. Patients with Down syndrome, those who died within 15 days of induction chemotherapy, non-compliant patients, and those with irregular chemotherapy were excluded from the study. All enrolled patients were followed-up until January 2023, with a follow-up duration of at least 6 months. Relapse in Acute Lymphoblastic Leukemia (ALL) was characterized based on the criteria outlined in the NCCN Clinical Practice Guidelines in Oncology, Pediatric Acute Lymphoblastic Leukemia, Version 2.2020 [15], and 2022 CSCO Pediatric and Adolescent Leukemia Diagnosis and Treatment Guidelines. All relapse cases included isolated bone marrow relapse, extramedullary relapse, and bone marrow combined with extramedullary relapse. In brief, isolated bone marrow relapse was defined as having one of the following criteria with no evidence of leukemia infiltration at other sites: (1) $\geq$20 % primitive/immature lymphoblasts in the bone marrow (by morphology or flow cytometry); (2) 5%–20 % primitive/immature lymphoblasts in the bone marrow (by morphology or flow cytometry) with evidence of molecular biology positivity; (3) 5%–20 % primitive/immature lymphoblasts in the bone marrow (by morphology or flow cytometry) without molecular biology positivity, but with at least two consecutive results separated by 10–14 days and tested in different laboratories. Isolated extramedullary relapse refers to relapse in the central nervous system and/or testes with no evidence of relapse in the bone marrow. The study was approved by the Ethics Committee of the Affiliated Children's Hospital of Nanjing Medical University (IRB-2023-012) and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from the parents of all children with ALL. All patients received standardized treatment according to the CCLG-2008 and 2015 protocol [16].

2.2. Clinical data collection and analysis

On the 33rd day of remission induction, minimal residual disease (MRD) was measured by multi-parameter flow cytometry in bone marrow samples. Fusion genes, including ETV6-RUNXl, BCR-ABLl, TCF3-PBXl, and MLL gene rearrangement, were detected using fluorescence in situ hybridization (FISH) or reverse transcription-polymerase chain reaction (RT-PCR) techniques. The clinical risk classification of patients with ALL was categorized into regular, intermediate, or high-risk groups.

2.3. RNA extraction

The Qiagen miRNeasy kit was utilized for RNA extraction from all serum samples. In detail, 200 μL of either serum or bone marrow sample was initially thawed on ice and then centrifuged at 3000 g for 5 min to remove cellular debris. Subsequently, 200 μL of the supernatant was combined with five times its volume of QIAzol solution (Qiagen) for lysis. To control for any variations between samples during RNA extraction, 3.5 μL of synthetic C. elegans miRNA (cel-mir-39) was introduced to each sample post-denaturation. The total RNA was then isolated and purified according to the manufacturer's guidelines. Prior to conducting polymerase chain reaction (PCR), RNA was extracted from both serum and bone marrow samples for the creation of cDNA.

2.4. High-throughput and real-time quantitative reverse transcription PCR

The Agilent Human miRNA Microarray Release 14.0, 8 $\times$ 15 K platform was used for miRNA and the Agilent-069978 Arraystar Human CircRNA microarray platform was used for circRNA. Differential data screening was based on a fold change of $>$2 and $p < 0.05$. For miRNA, cDNA was synthesized from total RNA using a TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). For circRNAs, reverse transcription was performed using random primers. Real-time PCR analysis was conducted using

the SensiFAST Probe Lo-ROX Kit (Bioline, London, UK) on a QuantStudio 7 Flex real-time PCR system (Applied Biosystems, Foster City, CA). To quantify the target transcripts, their relative levels were assessed and standardized using miR-16 as a reference for miRNA and β-actin for circRNA. Additionally, cel-mir-39 was used as an external reference control. Normalized expression values were log10-transformed for downstream statistical analysis.

2.5. Statistical analysis

The detailed clinical characteristics of all enrolled patients are presented in Table 1, and categorical data were compared between the training and validation sets using the chi-square or Mann-Whitney *U* test. Independent weights were calculated based on miRNA and circRNA expression levels using risk stratification. Briefly, the upper 95 % confidence interval (CI) of each non-coding RNA value in the control group was used as the cutoff for the expression level of a particular non-coding RNA. If the expression of a non-coding RNA in a sample was higher than the 95 % CI, it was assigned a value of 1; if it was lower than the 95 % CI, it was assigned a value of 0. The risk score was defined based on a linear combination of the expression levels of each noncoding RNA. For example, the risk score (RSF) of sample i, using information from six circRNAs, was calculated as follows: RSFi = $\sum$6j-1Wj. sij. In this equation, sij represents the risk score of non-coding RNA j in sample i and Wj represents the weight of the risk score of non-coding RNA j. The risk scores of the six ncRNAs were calculated using the weights obtained from the univariate logistic regression analysis for each non-coding RNA. The samples were then ranked based on their RSF and divided into high- and low-risk groups, with risk analysis determined using the Youden index. Receiver operating characteristic (ROC) curves and area under the curve (AUC) values were used to evaluate the performance of non-coding RNAs in predicting childhood ALL relapse in all cohorts. Logistic regression risk models were used for univariate or multivariate analysis to evaluate the 95 % confidence interval (CI) and odds ratios (OR). In predictive models, we utilized the DeLong test to determine statistically significance difference. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using Medcalc version 16.2.0 (Medcalc Software bvba, Ostend, Belgium). Visualizations were created with GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) and R version 3.5.0 (R Development Core Team, https://cran.r-project.org/).

3. Results

3.1. Analysis clinical characteristics in training cohort and validation cohort

To develop a blood-based non-invasive method to predict the likelihood of relapse in children with ALL, we first confirmed that the two groups of pediatric ALL patients exhibited similar clinical and pathological characteristics. The training cohort was comprised of 20 patients, including 11 (55 %) with relapsed childhood ALL. The validation cohort included 60 ALL patients, of whom 31 (52 %) were in a relapsed state. Table 1 presents the detailed clinical features of the patients in both the cohorts. We observed no statistically significant differences between the two cohorts in terms of the ALL relapse rate or any other clinical and pathological characteristics, thereby eliminating any unintentional bias among the patient cohorts examined in our study.

3.2. Plasma non-coding Transcriptome risk assessment identifies childhood ALL relapse

Through high-throughput profiling, we identified three miRNAs (hsa-miR-27a-5p, hsa-mir-142, and hsa-miR-411-5p) and three

Table 1
Clinicopathologic characteristics of clinical cohorts.

Characteristics	Training cohort (n = 20)	Validation cohort (n = 61)	*P* value
Age			0.849
<1 year	10	32	
≥1 year	10	29	
Gender			0.651
Male	9	31	
Female	11	30	
Fusion gene			0.738
Negative	12	34	
Positive	8	27	
WBC			0.219
$\leq 100 \times 10^9$/L	7	31	
$>100 \times 10^9$/L	13	30	
MRDb			0.745
Negative	11	31	
Positive	9	30	
Risk subgroup			0.732
Standard	8	26	
Intermediate	5	19	
High	7	16	

WBC count in newly diagnosed peripheral blood.
MRD at 33rd day.

circRNAs (hsa_circ_0022620, hsa_circ_0061990, and hsa_circ_0039036) as potential biological markers (Fig. 1B), exhibiting a gradient increase in expression among healthy controls, the initial diagnosis group, and the relapsed group. Following this, we assessed the expression of the identified non-coding RNA panel in a randomly chosen training cohort consisting of 11 relapsed ALL patients, 20 newly diagnosed patients, and 30 healthy children using real-time quantitative reverse transcription PCR (RT-qPCR). The results indicated that the non-coding RNA panel comprising three miRNAs and three circRNAs exhibited the highest expression in the relapsed group and the lowest expression in healthy children (Figure S1). Next, we systematically explored the diagnostic accuracy of the non-coding RNA panel in detecting relapse in childhood ALL. We developed a risk prediction model via logistic regression in the training set of patients, enabling the detection of relapse in ALL cases through a panel of three microRNAs (AUC = 0.89; 95 % CI, 0.22–39.44) or a distinct set of three circular RNAs (AUC = 0.96; 95 % CI, 0.31–96.51). When combining all three miRNAs and three circRNAs into a joint non-coding RNA panel, the predictive ability for relapse significantly surpassed that of the other groups (AUC, 0.97; 95 % CI, 1.23–115.21) (Figure S2A and B). Furthermore, univariate analysis suggested consistent predictive capabilities for relapse in both the miRNA and circRNA groups (miRNA group: odds ratio (OR), 3.44; P = 0.035; circRNA group: OR, 8.12; P = 0.018). The combined non-coding RNA group demonstrated a significantly superior predictive ability compared to the individual groups (OR, 9.22; P = 0.014). These results indicate that the non-coding RNA panel has excellent predictive potential for identifying high-risk relapses in childhood ALL at the time of initial diagnosis (Figure S2C).

3.3. Validation of nomogram-based combined non-coding RNA panel for predicting childhood ALL relapse

Following the promising results of our blood-based non-coding RNA panel in predicting childhood ALL relapse, we validated its robustness and accuracy in a larger independent validation cohort comprising 31 relapsed ALL patients, 61 newly diagnosed ALL patients, and 60 healthy children. RT-PCR analysis confirmed consistent expression of the non-coding RNA panel (Fig. 2). The diagnostic accuracy in matched bone marrow samples was consistent with that of tissue samples (AUC, 0.95; 95 % CI, 1.31–91.55) (Fig. 3A and B), further highlighting the clinical significance of our simple non-coding RNA panel for identifying relapse in childhood ALL patients. However, the sensitivity and specificity in the plasma samples of the validation cohort were slightly lower than those in the bone marrow samples and training cohort (AUC, 0.89; 95 % CI, 1.11–75.33), suggesting a potential decrease in efficacy with an

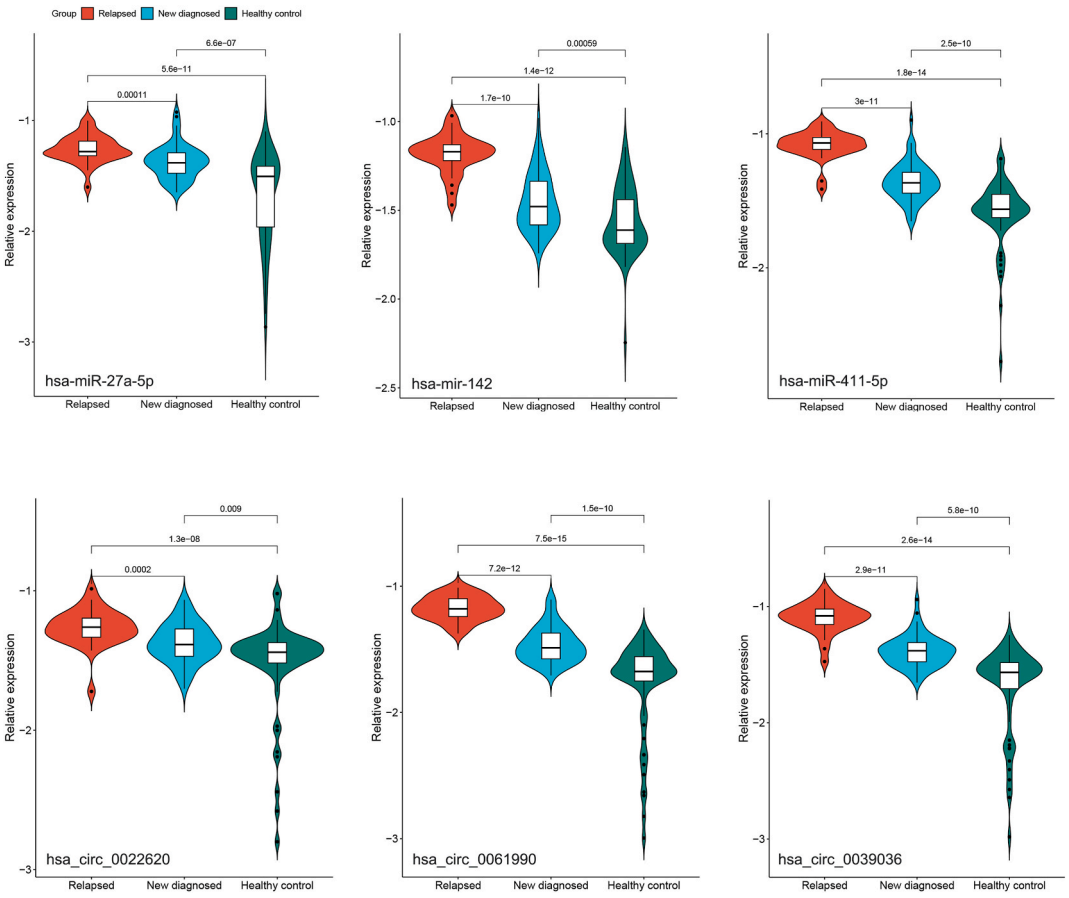

Fig. 2. Candidate targets were identified in the validation set. Data is presented in the format of mean ± standard deviation and has been log10-transformed.

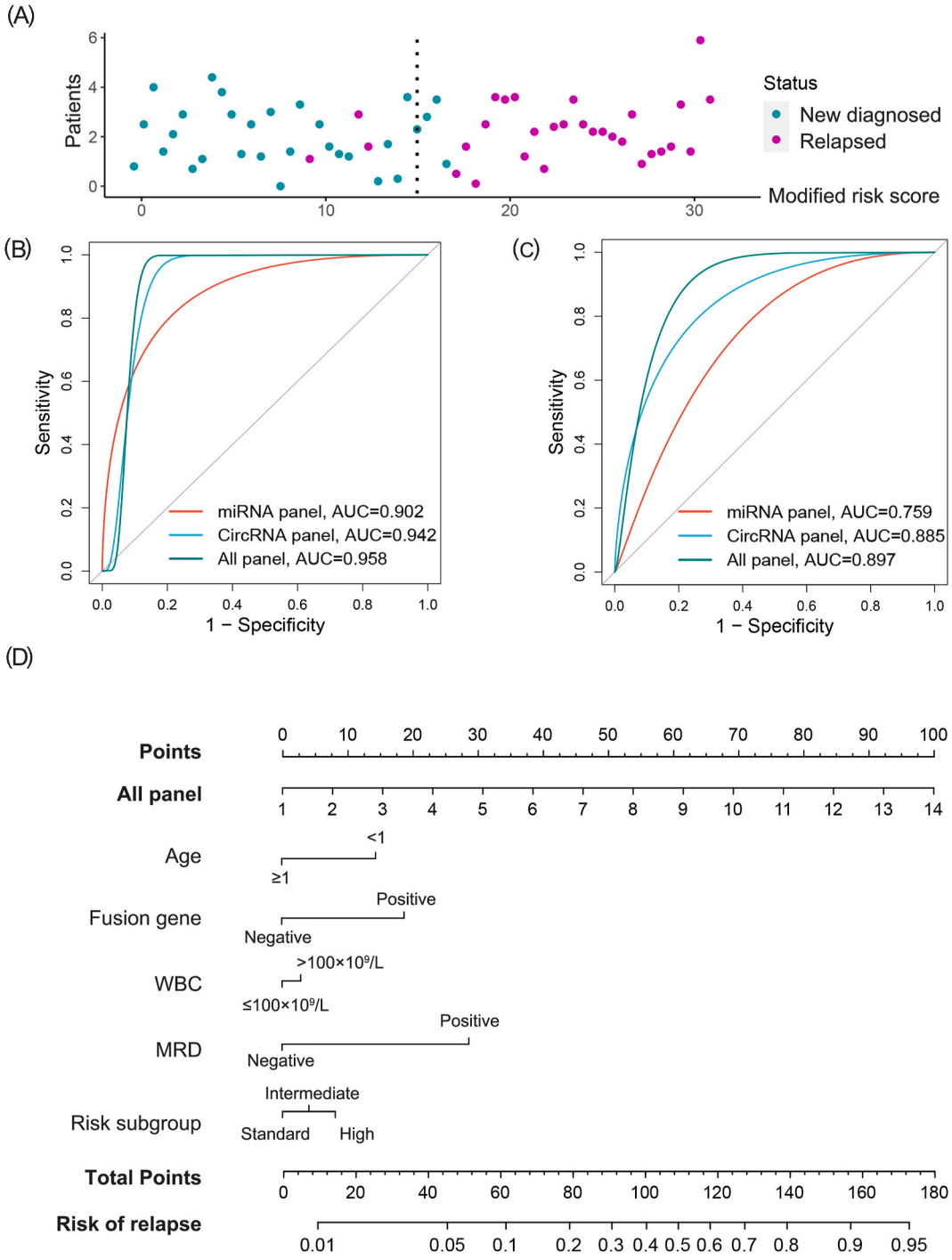

Fig. 3. Validation of candidate targets' predictive effectiveness for relapse in an independent cohort. **(A)** Individual risk scores were calibrated to derive composite risk scores utilizing the Youden index cutoffs derived from the non-coding RNA expression-based risk model. The discriminatory capability of this scoring system for predicting relapse in the separate validation cohort is visually depicted through a scatter diagram. **(B)** Receiver operating characteristic curves for plasma miRNA panel and circRNA panel in pediatric patients from the independent validation cohort. **(C)** Performance of the bone marrow-derived miRNA panel and circRNA panel in predicting relapse among pediatric patients within the independent validation set, as depicted by ROC curves. **(D)** A nomogram has been constructed to illustrate the likelihood of relapse risk, where, in a clinical setting, assigning scores to each variable leads to a cumulative total, which is then located on the axis representing total points.

increase in sample size, necessitating further validation through combined analyses(Fig. 3C).

3.4. Predicting childhood ALL relapse using the nomogram-based combined non-coding RNA panel and clinical features

To facilitate the clinical translation of the biomarker panel and ensure its reliability, we evaluated its performance along with other clinical risk features associated with childhood ALL relapse, including age (<1 year and ≥1 year), fusion genes (including but not limited to ETV6-RUNX1, BCR-ABL1, TCF3-PBX1, and MLL gene rearrangement), initial white blood cell count ($\leq 100 \times 10^9$/L and $>100 \times 10^9$/L), MRD status after 33 days of treatment, and risk stratification (Standard, Intermediate, High). We developed a nomogram to predict the probability of relapse in the validation cohort, based on the impact estimates of each risk factor. In the risk assessment process, individual risk elements were assigned scores, and the aggregate of these scores for each patient correlated with their estimated likelihood of disease recurrence. Our approach integrated both histopathological and molecular risk attributes. While acknowledging that additional pathologically derived risk evaluation parameters imparted a degree of influence to the predictive model, it was our panel of non-coding RNA markers that emerged as the predominant factor, carrying the utmost weight within the model's architecture. This panel proved to be the most autonomous and statistically momentous indicator for predicting relapse in pediatric ALL cases (Fig. 3D).

Currently, markers for predicting relapse in childhood ALL at the time of the initial diagnosis are still in the exploratory phase. Based on our results, we speculate that combining the non-coding RNA panel with clinically relevant risk features highly associated with relapse can further enhance the accuracy of predicting relapse risk in childhood ALL patients. Assessment of the validation cohort's plasma specimens yielded notable enhancements in both diagnostic sensitivity and specificity for relapse identification (AUC =

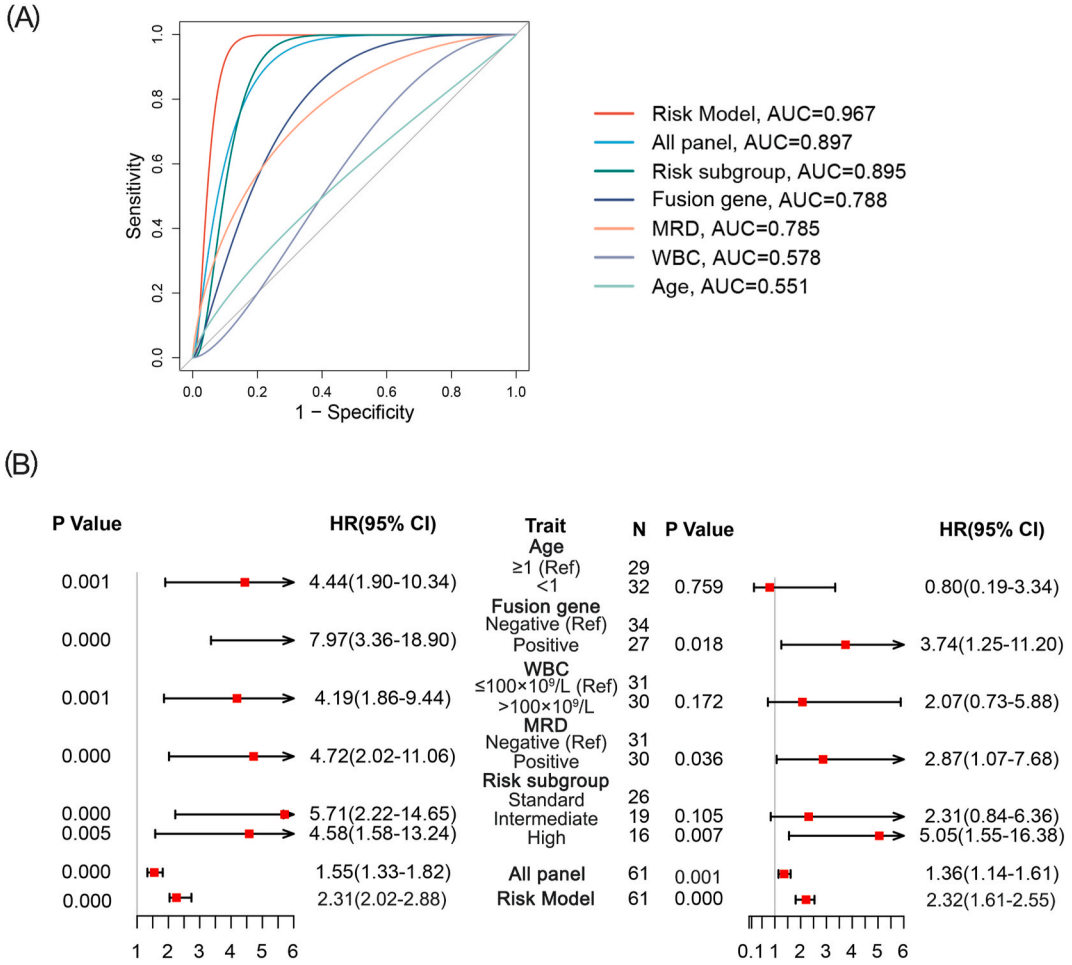

Fig. 4. Validation of the Childhood ALL Relapse Risk Prediction Model. (A) In the plasma samples of patients from the validation cohort, the risk stratification model combining non-coding RNA groups and pathological risk factors showed higher detection accuracy compared to detection using the non-coding RNA group alone or risk factors alone (AUC, 0.90). **(B)** Forest plot of univariate logistic regression analysis for patients from the validation cohort. **(C)** Forest plot of multivariate logistic regression analysis for patients from the validation cohort, including clinical-pathological variables, non-coding RNA groups, and the risk stratification model.

0.96; 95 % CI, 1.35–95.29, p = 0.012) (Fig. 4A). We subsequently used the cutoff thresholds derived from Cox regression to classify all patients into high- and low-risk groups based on the six miRNA and circRNA biomarkers. Subsequently, the univariate logistic regression examination established that our panel of non-coding RNAs retained its position as an autonomous prognosticator of ALL recurrence within the validation group, surpassing any singular clinical risk factor (OR, 1.55; 95 % CI, 1.33–1.82; P ≤ 0.001). Expanding upon this, both univariate and multivariate logistic regression assessments validated that the integration of our non-coding RNA panel with clinical characteristics constituted a more proficient risk classification schema compared to the panel alone. This combined approach served as an independent forecaster of relapse, demonstrating elevated predictive power (univariate: OR, 2.31; 95 % CI, 2.02–2.88; P ≤ 0.001; in the multivariate context: OR, 2.32; 95 % CI, 1.61–2.55; P ≤ 0.001) (Fig. 4B). Overall, these data highlight the predictive significance of our risk stratification model in forecasting relapse risk in childhood ALL patients at the time of the initial diagnosis.

4. Discussion

Acute Lymphoblastic Leukemia (ALL) holds significant promise in improving patient outcomes and guiding treatment strategies [17]. This study aimed to identify potential biomarkers and assess their predictive ability for relapse in pediatric patients with ALL. The research process involved the confirmation of similar clinical and pathological characteristics in two patient cohorts, identification of non-coding RNA biomarkers through high-throughput profiling, and validation of their diagnostic accuracy. Additionally, the study evaluated the performance of the biomarker panel along with other clinical risk features to create a comprehensive risk stratification model for predicting relapse in childhood ALL.

The initial step was to ensure that the training and validation cohorts exhibited comparable clinical and pathological characteristics. Both cohorts included pediatric ALL patients, with the training cohort consisting of 20 patients, 55 % of whom had relapsed childhood ALL, and the validation cohort comprising 60 ALL patients, 52 % of whom were in a relapsed state. The absence of statistically significant differences in ALL relapse rates and other clinical and pathological characteristics between the two cohorts helped eliminate any unintentional bias, bolstering the study's credibility.

Currently, the known biomarkers for predicting childhood ALL relapse have several limitations. Traditional prognostic factors such as MRD and specific genetic mutations have shown predictive value [18,19]; however, they lack the desired sensitivity and specificity [20]. Additionally, these markers may not capture dynamic changes in the leukemic microenvironment during relapse, hindering their ability to detect subtle alterations in disease progression. miRNAs are a class of small non-coding RNAs that have emerged as potential biomarkers for childhood ALL relapse prediction [21]. These molecules are involved in gene regulation and play crucial roles in cancer pathogenesis. Dysregulation of specific miRNAs has been associated with tumor initiation, progression, and metastasis, making them attractive candidates for biomarker development [22]. CircRNAs are another class of non-coding RNAs that exhibit unique characteristics such as resistance to exonucleases and increased stability, making them potential biomarker candidates [23]. CircRNAs may provide additional insights into the intricate regulatory networks underlying leukemia relapse. Despite their potential, circRNAs also face challenges as biomarkers [24,25]. Technical issues related to their detection and quantification, such as PCR primer design and potential contamination, may introduce variability into the results. Moreover, our understanding of the functional roles of circRNAs in leukemia pathogenesis is still evolving, necessitating further research to elucidate their exact contributions.

Potential biomarkers were identified using high-throughput profiling, which revealed three miRNAs (hsa-miR-27a-5p, hsa-mir-142, and hsa-miR-411-5p) and three circRNAs (hsa_circ_0022620, hsa_circ_0061990, and hsa_circ_0039036) as potential biological markers. These ncRNAs exhibited a gradient increase in expression from healthy controls to the initial diagnosis group and further to the relapsed group, suggesting their potential relevance in identifying disease progression. To validate these findings, real-time quantitative reverse transcription PCR was performed on a randomly selected training set comprising 11 patients with relapsed ALL, 20 newly diagnosed patients, and 30 healthy children. The results confirmed that the non-coding RNA panel had the highest expression in the relapsed group and the lowest expression in healthy children, further supporting their potential as predictive biomarkers for relapse in childhood ALL.

Logistic regression analysis was then employed to train a risk assessment model in the training cohort, allowing for the identification of relapse in ALL patients using either the three miRNAs or the three circRNAs separately. Interestingly, when combined into a joint non-coding RNA panel, the predictive ability for relapse significantly surpassed that of individual groups, highlighting the importance of considering multiple biomarkers. The combined non-coding RNA group demonstrated a significantly superior predictive ability compared to the individual groups, indicating the potential synergistic effect of these biomarkers in identifying high-risk relapse in childhood ALL at the time of initial diagnosis.

The promising results from the training cohort prompted validation of the biomarker panel in a larger, independent validation cohort consisting of 31 relapsed ALL patients, 61 newly diagnosed ALL patients, and 60 healthy children. The consistent expression of the non-coding RNA panel, as confirmed by RT-PCR analysis, reinforced the clinical significance of these biomarkers in identifying relapse in childhood ALL patients. However, the slightly lower sensitivity and specificity observed in plasma samples than in bone marrow samples and the training cohort indicated the need for further validation through combined analyses to ensure the reliability of the biomarker panel.

To enhance the clinical translation of the biomarker panel and ensure its practicality, this study evaluated its performance along with other clinical risk features associated with childhood ALL relapse, including age, fusion genes, initial white blood cell count, MRD status after 33 days of treatment, and risk stratification. A nomogram was developed to predict the probability of relapse in the validation cohort based on the impact estimates of each risk factor, and the noncoding RNA panel was found to possess the highest weight in the model. This underscores the significance of non-coding RNA biomarkers as the most independent and significant

predictors of relapse in childhood ALL patients.

The current exploratory phase of markers for predicting relapse in childhood ALL highlights the potential implications of integrating a non-coding RNA panel with clinically relevant risk features. Examination of plasma specimens from the validation group yielded marked advancements in both the sensitivity and specificity for relapse diagnosis, thereby reinforcing the pivotal role of non-coding RNA-based biomarkers. The classification of patients into high- and low-risk groups based on the six miRNA and circRNA biomarkers demonstrated the independent predictive ability of the non-coding RNA panel, even when compared to single clinical risk factors.

5. Conclusion

Taken together, the results of this study led to the development of a comprehensive risk stratification model that incorporates the non-coding RNA panel and clinical features. This model exhibited superior predictive accuracy for predicting relapse risk in childhood ALL patients at the time of initial diagnosis compared to using the non-coding RNA panel or individual clinical risk factors alone. Overall, these findings have significant implications for the management and prognosis of childhood ALL and offer a promising avenue for improving patient outcomes through early and accurate relapse prediction. However, further validation studies and prospective investigations are warranted to establish the clinical utility of these biomarkers and to facilitate their integration into routine clinical practice. Ultimately, the successful implementation of a blood-based non-invasive method for predicting relapse in childhood ALL can be a groundbreaking advancement in pediatric oncology, potentially transforming patient care and survival rates.

Data availab ility statement

The original data presented in the study are included in the article, which are available from the corresponding author upon reasonable request. RNA sequencing data reported in this paper are available in the ArrayExpress database (accession numbers E-MTAB-13176 and E-MTAB-13177).

CRediT authorship contribution statement

Yaping Wang: Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Funding acquisition. **Xiaopeng Ma:** Validation, Methodology, Investigation. **Huimin Li:** Visualization, Validation, Supervision. **Ji'ou Zhao:** Writing – original draft, Validation. **Meiyun Kang:** Resources, Project administration. **Liucheng Rong:** Methodology, Formal analysis. **Yao Xue:** Visualization, Software. **Jiali Wang:** Investigation, Formal analysis. **Junwei Tang:** Writing – review & editing, Data curation, Conceptualization. **Yongjun Fang:** Project administration, Validation, Writing – review & editing.

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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