Circulating Exosomal MicroRNAs as Diagnostic and Prognostic Biomarkers in Patients with Diffuse Large B-cell Lymphoma

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Research

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Title page

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Abstract

Background: Exosomal microRNAs (miRNAs) are potential biomarkers for a variety of tumors but have not yet been tested in diffuse large B-cell lymphoma (DLBCL). Here we sought to determine whether circulating exosomal miRNAs are differentially expressed in the blood of DLBCL patients compared with those in the blood of non-DLBCL patients with lymphoma and healthy subjects. In addition, we sought to explore whether circulating exosomal miRNAs could be used as prognostic predictors for DLBCL patients.

Methods: Circulating exosomal miRNAs were isolated and used to perform miRNA expression profiling using quantitative reverse transcription polymerase chain reaction (qRT-PCR) based on Exiqon microarray from 10 DLBCL patients and 5 healthy subjects. Using qRT-PCR, miRNA candidates were first evaluated in the testing stage (24 DLBCL patients vs. 24 healthy subjects) and further confirmed in validation stage(99 DLBCL patients vs. 65 healthy subjects). Meanwhile, we also explored miRNAs

concentrations in 29 non-DLBCL lymphoma cases, which were enrolled as concurrent control in this study. And lastly, relationships between miRNA level and patient outcomes including overall survival (OS) and progression-free survival (PFS) were studied.

Results: Five circulating exosomal miRNAs (miR-379-5p, miR-135a-3p, miR-4476, miR-483-3p and miR-451a) were differently expressed in DLBCL patients compared with healthy controls. The 5-miRNA panel enabled us to predicted the probability of a diagnosis of DLBCL with an area under curve(AUC) of 0.863. Four circulating exosomal miRNAs (miR-379-5p, miR-135a-3p, miR-155-5p and miR-451a) were differently expressed between DLBCL patients and concurrent controls, the AUC of this 4-miRNA panel in distinguishing DLBCL patients from concurrent controls was 0.775. One miRNA, namely miR-451a, was significantly associated with both PFS and OS of DLBCL patients in the univariate analysis, and still statistically significant after adjusting for the International Prognostic Index(IPI) in the multivariate analysis. The combination of circulating miR-451a with IPI had a better predication for PFS and OS.

Conclusions: Our study suggested subsets of circulating exosomal miRNAs could be useful noninvasive biomarkers for the diagnosis of DLBCL and the use of circulating exosomal miRNA improves the identification of patients with newly diagnosed DLBCL with poor outcomes.

Keywords

Diffuse large B-cell lymphoma, exosome, microRNA, diagnosis, prognosis, biomarker

Background

Diffuse large B-cell lymphoma (DLBCL) is a lymphoproliferative disorder originating in B-lymphocytes. The most common subtype of non-Hodgkin Lymphoma, it accounts for 30% to 40% of all cases in different geographic regions (1), and for more than 15,000 deaths in China every year (2). Currently, the diagnosis of DLBCL depends largely on pathologic analysis of biopsy tissue (3), which is an invasive procedure and often poses some risk to patents. Moreover, because most patients with DLBCL lack specific, apparent symptoms, many patients are diagnosed at late, even incurable stage (4), which greatly impair the outcomes of DLBCL patients. Although the International Prognostic Index(IPI) has been

widely used as prognostic factors (5), patients in similar prognostic groups often have heterogeneous outcomes (6), suggesting certain shortcomings with the current prognostic evaluation system. Thus, new and noninvasive biomarkers of the diagnosis and prognosis of DLBCL are urgently needed.

Exosomes are tiny vesicles (30 to 100nm in diameter) wrapped in cup-shaped lipid bilayers that are released into body fluids by many kinds of cells (7). Exosomes are stable in peripheral blood and encapsulate many bioactive molecules (7, 8), such as signal proteins, enzymes, and nucleic acids, including microRNA (miRNA)(7). These miRNAs are small (21 to 23 nucleotides), noncoding RNAs that help regulate gene expression through interaction with mRNA at the post-transcriptional level (9). Several studies have suggested that exsomal miRNAs isolated from peripheral blood could be noninvasive biomarkers for detecting tumor, or for monitoring disease progress and treatment efficacy (10-12). Although Circulating exosomal miRNA has been studied in many malignant cancers, their value in assessing patients with DLBCL has not yet been determined. Thus, we sought to determine whether circulating exosomal miRNAs could serve as biomarkers for the diagnosis and prognosis of DLBCL.

Methods

Study Design and Participants

The study was designed as a prospective, explorative one. To determine the value of serum exosomal miRNAs in diagnosing DLBCL, we conducted a rigorous three-stage study, consisting of a screening stage to identify candidate miRNAs, a testing stage to select likely predictors, and a validation stage to verify their predictive abilities. An additional figure shows the flowchart of our study in more detail [see Supplemental Figure 1].

The cohort in screening stage included 10 DLBCL patients and 5 healthy controls. They were 9 males and 6 females from ages 30 to 75 years, with no significant age and gender difference between DLBCL group and healthy control group. We prospectively interrogated a testing stage of 48 individuals and another independent validation stage of 164 individuals. The two stage were as follows:(1) The cohort in testing stage included 24 DLBCL patients and 24 healthy controls; (2) The cohort in validation stage consisted of 99 DLBCL patients and 65 healthy controls. Beyond that, 29 patients who had 1 of 3

lymphoma subtypes served as concurrent controls. Among these concurrent controls, 19 had natural killer/T-cell lymphoma, 4 had follicular lymphoma and 6 had Hodgkin lymphoma.

The lymphoma cases were hospitalized patients recruited at the Department of Hematology and the Department of Oncology in West China Hospital who didn't receive any specific treatment (including surgical treatment, chemotherapy and radiotherapy), without other systemic malignancies and serious infections, between August 2016 and December 2017. The diagnosis of lymphoma was established using National Comprehensive Cancer Center(NCCN) guideline (13). Histopathological examination and other laboratory tests were conducted by the College of American Pathologist (CAP) certified central lab of West China Hospital. Healthy controls were people who had no history of malignancy and whose health status were confirmed by routine physical examinations at West China Hospital. Post-treatment surveillance evaluation was conducted through outpatient follow-up or telephone interview every 3 months for the first years and every 6 months for next years. The Cut-off date for follow-up was May 31st, 2020.

Exosome Isolation and Characterization

A 6 ml peripheral blood sample was collected within 48 hours of admission from each participant and separated for serum within 4 hours in a centrifuge at 3,000×g for 15 minutes at room temperature and at 10,000×g for 30 minutes at 4°C. ExoQuick Exosome Precipitation Solution (System Biosciences, USA), an isolation kit for sensitive downstream application (10), was used to extract exosome from serum. Details regarding the process of exosome extraction are presented in Supplemental Figure 2A. Isolated exosomes were identified by Nanoparticle tracking analysis (NTA), Transmission Electron Microscopy (TEM) and Western blot (WB). To be more specific, the size and concentration of the particles were examined by nanoparticle tracking with a Nanosight NS300 (Malvern Instruments, UK). The morphology of the particle was determined with TEM (FEI Tecnai™ G2 Spirit, Czech Republic) at 80 KV. Exosome-specific marker proteins CD63, CD9, CD81 and HSP70 were adequately detected by Western blot, probed with corresponding antibodies (System Biosciences, USA). GAPDH (Beyotime, China) was used as internal controls.

Exosomal RNAs Isolation and cDNA Preparation

Total RNA was extracted by using an miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany) according to manufacturer's instructions. Ce_miR-39_1 (Qiagen, Germany) was chosen as a "Spike-In" normalization control for qRT-PCR quantification (14, 15) and added to the reaction system when the exosome pellets were resuspended in Qiazol. RNA quality and yield were measured with an ND-1000 Nanodrop Spectrophotometer (Thermo Fisher Scientfic, MA). RNA samples with a 260/280-nm absorbance ratio great than 1.8 and a 260/230-nm absorbance ratio great than 2.0 were considered to be acceptable for subsequent analysis. An miScript II RT Kit (Qiagen, Germany) was used to reverse transcription for cDNA. The obtained cDNA was diluted into 100 μl of RNase-free water and stored at -80 °C until use.

Exosomal RNA Profiling

RNA labeling, and array hybridization was performed by the KangChen Bio-tech Company (Shanghai, China) with the instruction of Exiqon's manual. Replicated miRNAs were averaged, and miRNAs with intensities of 30 or greater in all samples were chosen for calculating the normalization factor. Expressed data were normalized using Median normalization, and differentially expressed exosomal miRNAs with statistical significance were selected based on the following criteria: an absolute expression fold changes greater than 1.5 and a false discovery rate (FDR) value less than 0.05. Real-time PCR was used for conformation. Differentially expressed miRNAs were identified through volcano plot screening. Cluster analysis was carried out by hierarchical clustering to show distinguishable miRNA expression profiling among samples.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) of miRNA

miRNA was quantified with a miScript SYBR® Green PCR Kit and miRNA-specific primers (Qiagen, Germany). A no-cDNA template control and a negative control were included in each plate. All miRNAs were measured in a blinded fashion and all samples were analyzed in triplicate. The average cycle threshold (Ct) was recorded, to be acceptable, the $Ct_{(miRNA)}$ had to range between 10 and 34 and be 6 lower than the negative control. The relative concentration of miRNA were calculated using the comparative $2^{-\Delta\Delta Ct}$ method as: $\Delta Ct = Ct_{(miRNA)} - Ct_{(Ce\_miR\text{-}39\_1)}$, $\Delta\Delta Ct = \Delta Ct - \text{average } Ct_{(\text{healthy control})}$.

Statistical Methods

Relative expression concentrations of miRNA were compared between different groups with nonparametric Mann-Whitney and Kruskal-Wallis tests. MiRNA species were considered to be differently expressed if the fold-change was greater than 1.5 and P-value was less than 0.05.

To determine the effect of miRNAs on progression-free survival(PFS) and overall survival(OS), the Kaplan-Meier method and log-rank test were performed to plotted PFS and OS based on the dichotomy of median of miRNA. A Cox proportional hazard model was employed to compute the hazard ratio (HR). The multivariate Cox proportional hazard modeling was used to identify independent outcome predictors after adjusting for IPI values, which was one accepted confounding factor in DLBCL prognosis (5).

Receiver operating characteristic (ROC) curves and logistic regression were used to estimate the diagnostic and prognostic value of the candidate miRNAs. The area under curve (AUC) of a single candidate miRNA and of combined panels were compared to determine their diagnostic performance. The predictive values of miRNA expression for PFS and OS were also calculated from ROC curves with R-language and cross-validation was applied to this analysis to avoid overfitting (10, 16). We compared the curves of the miRNA expressions alone, of IPI, and both to evaluate the predictive value of the miRNA expressions.

Data were analyzed with R version 3.6.2, SPSS software version 22.0 and GraphPad Prism software version 7.0. All statistical tests were 2-sided, alpha was set to 0.05 and 95% confidence intervals (CI) were calculated as needed.

Results

Characteristics of Enrolled Participants

We prospectively enrolled a final number of 256 individuals, including 15 subjects in testing stage (10 DLBCL patients and 5 healthy controls), 48 subjects in testing stage (24 DLBCL patients and 24 healthy controls), 164 subjects in validation stage (99 DLBCL patients and 65 healthy controls) and 29 non-DLBCL lymphoma cases that worked as concurrent controls between August 2016 and December 2017

at West China Hospital, Sichuan University. Baseline characteristics of DLBCL patients were presented in Supplemental Table 1.

The median follow-up time of DLBCL case was 36 (ranged from 1 to 45) months. Overall 123 included DLBCL patients (10 DLBCL patients in screening stage were excluded to avoid repeated detection), 12 DLBCL patients were lost to follow-up and another 2 who transferred to local hospital cannot be able to tell their disease states clearly. Finally, 111 DLBCL cases were included for OS analysis while 109 DLBCL cases for PFS analysis. Further investigation suggested 36.0%(40/111) DLBCL patients dead during this study period (August 2016 to May 2020) and 62.5%(25/40) deaths occurred within 12 months of diagnosis. Meanwhile, 48.6%(53/109) DLBCL cases suffered from progression between August 2016 to May 2020 and 71.7%(38/53) patients relapsed within one year.

Exosome Characterization

Nanoparticle tracking and electron microscopy showed that exosomes appeared as cup-shaped vesicles between 80 and 95nm in diameter, which is consistent with previous studies (7, 17). Immunoblot analysis detected exosomal marker proteins CD63, CD9, CD81, and HSP70 in the extract. For more details about the results of exosome characterization, please refer to Supplemental Figure 2B, 2C and 2D.

Identification of Differently Expressed Circulating Exosomal miRNAs

In the screening stage, exosomal RNAs from 10 DLBCL patients and 5 healthy controls were analyzed with microarrays. Among 3100 miRNAs detected by microarray, profiling data analysis predicted that 157 were high expression and 175 were low expression in DLBCL group (Clustering analysis of microarrays is presented in Supplemental Figure 3). After result confirmation by RT-PCR to remove false positives, 20 top microRNAs exhibiting the largest changes (Table 1), together with other two miRNAs, miR-155-5p(18, 19) and miR-21-5p(18, 19), which were identified in tissue sample as diagnostic biomarkers for DLBCL by many previous studies, were subsequently analyzed in the testing stage.

In the testing stage, we further quantified the expression of the above 22 miRNAs by qRT-PCR in 24 DLBCL patients and 24 healthy controls. Analysis identified 8 individual miRNAs (miR-379-5p, miR-

135a-3p, miR-4476, miR-483-3p, miR-451a, miR-551a, miR-135b-5p and miR-155-5p) that meet the predetermined criteria ($Ct_{(miRNA)}$ value ranging from 10 to 34 and 6 lower than the negative control), and therefore were included in the next analysis. The remaining 14 miRNAs were excluded because of their extremely low expression. 5 miRNAs, consisting of 3 high-expression miRNAs (miR-379-5p, miR-135a-3p and miR-4476) and 2 low-expression miRNAs (miR-483-3p and miR-451a) among the above 8 miRNAs differently expressed as defined by fold changes greater than 1.5 and a P value less than 0.05. Relative expression of the above 8 circulating exosomal miRNAs is presented in Supplemental Figure 4A.

In the validation stage, we tested the above 5 miRNAs in 99 DLBCL patients and 65 healthy controls. Compared with the controls group, miR-379-5p, miR-135a-3p, and miR-4476 in the DLBCL patients were upregulated, and miR-483-3p and miR-451a were down downregulated (see Supplemental Figure 4B).

Similarly, these 5 miRNAs were tested in lymphoma subtype controls. The result suggested miR-379-5p, miR-135a-3p, miR-4476 and miR-451a were differently expressed($P<0.05$) when compared to DLBCL patients, whereas only miR-451a was differently expressed when compared to healthy controls. (see Supplemental Figure 4C).

Diagnostic Value of Circulating Exosomal miRNAs

To determine the diagnostic performance characteristics of the 5 miRNAs in distinguishing DLBCL patients from healthy controls, we plotted ROC curves for each miRNA in the testing and validation stages, as well as for all miRNAs combined. The result suggested the combined panel of 5 miRNAs showed better performance in diagnosing DLBCL with an AUC of 0.863 (95% CI 0.811-0.915) than single miRNA (see Figure 1A, 1B, 1C and Table 2).

We also plotted ROC curves for the four miRNAs with differential expression between DLBCL patients and lymphoma subtype controls, the AUC (95% CI) for miR-379-5p was 0.732 (0.615 to 0.849); for miR-135a-3p; 0.668 (95%CI 0.559 to 0.777); for miR-4476, 0.635 (0.518 to 0.753); and for miR-451a, 0.671 (0.574 to 0.768). The AUC (95% CI) for the four combined miRNAs was 0.775 (95%CI, 0.692 to 0.859) (see Supplemental Figure 5A)

We estimated the diagnostic value of miR-451a for distinguishing the lymphoma subtype controls from the healthy controls as well. Unexpectedly, the single miR-451a showed excellent performance, with an AUC of 0.765(95% CI 0.681-0.849) (see Supplemental Figure 5B).

Association between the Exosomal miRNAs Abundance and Clinical Characteristics

We next explored the expression of exosomal miRNAs in DLBCL patients grouped by clinical features. The results suggested that miR-451a was expressed in lower concentration in patients with late-stage (III-IV) DLBCL (see Supplemental Figure 6).

Prognostic significance of Circulating Exosomal miRNAs in DLBCL

Given that exosomal miR-451a were differentially expressed in different DLBCL groups, we hypothesized that these miRNAs, especially miR-451a, may predict the prognosis of the disease. The association between the miRNAs and patient outcomes were first tested with Log-rank tests to eliminate those that were not associated with PFS or OS. Multivariate Cox regression adjusting for IPI showed that only miR-451a independently predicted both PFS and OS in multivariate analyses (see Supplemental Table 2).

The relationship between miR-451a on PFS and OS was determined by Kaplan-Meier curves with miRNA expression dichotomized at the median value. The result suggested that lower concentration miR-451a were associated with poor outcomes in patients newly diagnosed with DLBCL (see Figure 2).

We further assessed the utility of circulating exosomal miR-451a as prognostic biomarkers for DLBCL. Overall, the signature of miR-451a plus IPI had a higher AUC than when the miRNA signature was excluded (see Figure 3).

Discussion

With the emerging concept of "liquid biopsy", we finally have a non-invasive alternative to conventional tissue biopsy or radiologic tests for different pathologic conditions, including tumors. Among various biomolecules associated with tumors, the exosomal miRNAs are the most promising. Circulating exosomal miRNAs can be relatively easily isolated from peripheral blood and tested by qRT-PCR,

creating a useful tool for diagnosis and prognosis in cancer (10, 12, 20, 21). Indeed, miRNAs in circulating exosomes have been reported to be superior to exosomal glypican-1^{+} concentrations and to carbohydrate antigen 19-9 (the accepted diagnostic biomarker for pancreas cancer) when used to detect pancreatic ductal adenocarcinoma or to differentiate between ductal adenocarcinoma and chronic pancreatitis (20). Further, plasma vesicle miRNA concentrations can be used to monitor therapeutic response in Hodgkin lymphoma (12), whereas circulating exosomal let-7b and miR-18a have been associated with the prognosis in multiple myeloma (10).

Compared with other noninvasive biomarkers, such as circulating miRNAs (14, 22), circulating tumor cells and cell-free DNA (23), exosomal miRNAs have protective lipid bilayer membrane and therefore more stable. In addition, these above biomarkers complicate making diagnoses and prognoses because they are passively released by apoptotic and necrotic cells (24). Exosomes, however, are intact vesicles that are actively secreted by living cells (11, 25), rendering a more reliable and accurate body status for predicting diagnosis or prognosis.

To our knowledge, this is the first large study that proves the clinical significance of circulating exosomal miRNAs in newly diagnosed DLBCL cohort. In this study, we designed a rigorous three-stage study to determine the value of serum exosomal miRNAs in diagnosing patients with DLBCL. Five miRNAs (miR-135a-3p, miR-379-5p, miR-4476, miR-483-3p, and miR-451a) were identified through microarray analysis and qRT-PCR. The combined panel of all 5 miRNAs had an excellent ability in diagnosing DLBCL with a AUC of 0.863 (95%CI 0.811-0.915). However, the panel did not perform well in discriminating between patients with DLBCL from concurrent controls with lymphoma subtypes. We speculated that this poor ability was principally caused by the complex composition of the current controls, which consisted of patients with 3 different lymphoma subtypes.

miR-451a, plus the established prognosis factors IPI, had an even greater AUC than when the miRNA signature was excluded for predicting PFS and OS. Although IPI has been established as prognostic markers for patients with DLBCL for many years and is easy to determine and is clinically useful (5), it may not be specific enough to predict the heterogeneous outcomes of patients with DLBCL because it mainly relies on clinical features and does not account biological heterogeneity. Therefore, it is still of great significances to explore noninvasive biomarkers that reflect the molecular aspects of DLBCL, and

our study indeed proved that circulating exosomal miRNAs could improve the prognostic stratification in patients with DLBCL.

The 5 identified exosomal miRNAs have been reported in many types of tumors and may be related to tumor generation and development. Lower concentration of miR-451a was reported to be associated with poorer pathologic stage in patients with lung cancer and promoted cell survival by targeting c-MYC in patients with prostate cancer (26, 27). Our previous research also indicated miR-451a was a potential biomarker for therapy response monitoring of DLBCL patients and its expression level gradually increased in patients that achieve remission (28). This result was also validated by other researchers (29). Compared with the other 4 validated miRNAs included in our study, miR-451a was greatly enriched in the circulating exosome (see Supplemental Figure 7). This result is similar to that in other studies of cell lines (30), and a recent study has suggested that the interaction between RNA-binding ubiquitin E3 ligase (MEX3C) and adaptor-related protein complex 2(AP-2) may contribute to the enrichment phenomenon (31). miR-483-5p has been identified as an anti-tumor miRNA in breast cancer by targeting histone deacetylase 8 or Cyclin E1, which suggested the effects of miR-483-3p on cell growth and apoptosis (32, 33).

MiR-379-5p was reported to be down-regulated in many kinds cancer tissue. However, overexpression of miR-379-5p was found in DLBCL patients in our study. We assumed this was because miRNAs could be selectively secreted from their original cancer cells by exosomes. Previous study indicated that miR-379-5p was down-regulated in gastric cancer tissue sample and functioned as a tumor suppressor in cancer development. Interestingly, researchers also found exosomal miR-379-5p was higher in circulating exosomal samples of gastric cancer patients with poor outcomes. Scholars further validated the up-regulated expression level of miR-379-5p in cell line model and speculated the translocation of miR-379-5p from cancer cells to circulation might contribute to the high-regulation of miR-379-5p in circulation exosomal samples (34). Similar conditions were also reported in lung cancer (35, 36). This suggested the profiling of miRNAs in circulating exosome may have a great difference with those in tissue or blood.

Both of miR-135a-3p and miR-4476 were reported as upregulated miRNAs that promoted cell proliferation, migration and invasion in CNS tumors (37, 38). Further mechanistic analyses indicated the adenomatous polyposis coli(APC), a negative regulator of the Wnt/β-catenin signaling pathway, is a

direct target of miR-4476 and mediated the oncogenic effect of miR-4476 in glioma (38). This may explain the high concentrations of this two miRNAs in DLBCL cases.

Another challenge in our study was how to choose appropriate reference for data normalization in qRT-PCR. As far as we know, there is no consensus regarding reference genes for measuring serum miRNAs. Ce_miR-39_1 is a C. elegans miR-39 mimic that has been commonly used as a spike-in in blood (14, 15). Therefore, we added an equal amount to the working solution for Ce_miR-39_1 ($1.6X10^8$ copies/μl) at the beginning of RNA extraction to normalize data and a high concordance across plates was observed.

Conclusions

In conclusion, we identified a panel of 5-miRNAs (miR-379-5p, miR-135a-3p, miR-4476, miR-483-3p and miR-451a) in the circulating exosome that can be used as noninvasive biomarkers for diagnosing DLBCL, and circulating exosomal miR-451a was with prognostic value in these patients. The combination of miR-451a with IPI could serve as a better indicator for prediction of PFS and OS.

List of Abbreviations

miRNA: microRNAs

DLBCL: diffuse large B-cell lymphoma

qRT-PCR: quantitative reverse transcription polymerase chain reaction

IPI: International Prognostic Index

OS: overall survival

PFS: progression-free survival

NCCN: National Comprehensive Cancer Center(NCCN)

CAP: College of American Pathologist

NTA: nanoparticle tracking analysis

TEM: transmission electron microscopy

WB: western blot

FDR: false discovery rate

Ct: cycle threshold

ROC: receiver operating characteristic

AUC: area under curve (AUC)

CI: confidence intervals

GCB: germinal center B-cell

MEX3C: RNA-binding ubiquitin E3 ligase

AP-2: adaptor-related protein complex 2(AP-2)

GI: gastrointestinal;

CNS: central nervous system

LDH: lactate dehydrogenase

β_2-MG: β_2-microglobulin

Declarations

Ethics Approval and Consent to Participate

The protocol, procedures, and materials of this study were approved by the Institutional Review Board of West China Hospital [NO.2016(302), NO. 2017(380), NO.2019(217), we had 3 approval documents

for this study since the validation period for every document was one year]. All participants provided written informed consent for the blood draws and follow-up.

Consent for Publication

Not applicable.

Availability of Data and Materials

The microarray profile data will be submitted to NCBI Gene Expression Omnibus once manuscript was accepted.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contribution

Di Cao, Xia Cao and Yu Jiang searched literature, performed the experiments, analyzed the data and wrote the initial manuscript. Juan Xu, Caixia Jing and Yu Feng oversaw participant enrollment and acquisition of clinical and laboratory data. Mao Li and Xia Cao collected patients' follow-up data. Deying Kang provided expert advice for statistics, and Yuhuan Zheng provided administrative, technical, and material support. Caigang Xu supervised the whole project, conceptualized and designed the study, and revised the article. All authors have reviewed the manuscript.

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References

1. Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. Blood. 2011;117(19):5019-32.

2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. CA: a cancer journal for clinicians. 2016;66(2):115-32.

3. Liu Y, Barta SK. Diffuse large B-cell lymphoma: 2019 update on diagnosis, risk stratification, and treatment. American journal of hematology. 2019;94(5):604-16.

4. Cheson BD, Pfistner B, Juweid ME, Gascoyne RD, Specht L, Horning SJ, et al. Revised response criteria for malignant lymphoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007;25(5):579-86.

5. International Non-Hodgkin's Lymphoma Prognostic Factors P. A predictive model for aggressive non-Hodgkin's lymphoma. The New England journal of medicine. 1993;329(14):987-94.

6. Gisselbrecht C, Glass B, Mounier N, Singh Gill D, Linch DC, Trneny M, et al. Salvage regimens with autologous transplantation for relapsed large B-cell lymphoma in the rituximab era. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010;28(27):4184-90.

7. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nature reviews Immunology. 2002;2(8):569-79.

8. Boukouris S, Mathivanan S. Exosomes in bodily fluids are a highly stable resource of disease biomarkers. Proteomics Clinical applications. 2015;9(3-4):358-67.

9. Kong YW, Ferland-McCollough D, Jackson TJ, Bushell M. microRNAs in cancer management. The Lancet Oncology. 2012;13(6):e249-58.

10. Manier S, Liu CJ, Avet-Loiseau H, Park J, Shi J, Campigotto F, et al. Prognostic role of circulating exosomal miRNAs in multiple myeloma. Blood. 2017;129(17):2429-36.

11. Melo SA, Sugimoto H, O'Connell JT, Kato N, Villanueva A, Vidal A, et al. Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. Cancer cell. 2014;26(5):707-21.

12. van Eijndhoven MA, Zijlstra JM, Groenewegen NJ, Drees EE, van Niele S, Baglio SR, et al. Plasma vesicle miRNAs for therapy response monitoring in Hodgkin lymphoma patients. JCI insight. 2016;1(19):e89631.

13. Zelenetz AD, Gordon LI, Wierda WG, Abramson JS, Advani RH, Andreadis CB, et al. Diffuse Large B-Cell Lymphoma Version 1.2016. J Natl Compr Canc Netw. 2016;14(2):196-231.

14. Hosokawa K, Kajigaya S, Feng X, Desierto MJ, Fernandez Ibanez Mdel P, Rios O, et al. A plasma microRNA signature as a biomarker for acquired aplastic anemia. Haematologica. 2017;102(1):69-78.

15. Farina NH, Wood ME, Perrapato SD, Francklyn CS, Stein GS, Stein JL, et al. Standardizing Analysis of Circulating MicroRNA: Clinical and Biological Relevance. Journal of cellular biochemistry. 2014;115(5):805-11.

16. Heagerty PJ, Zheng Y. Survival model predictive accuracy and ROC curves. Biometrics. 2005;61(1):92-105.

17. Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. BMC genomics. 2013;14:319.

18. Lim EL, Trinh DL, Scott DW, Chu A, Krzywinski M, Zhao Y, et al. Comprehensive miRNA sequence analysis reveals survival differences in diffuse large B-cell lymphoma patients. Genome Biol. 2015;16(1):18-.

19. Lawrie CH, Soneji S, Marafioti T, Cooper CDO, Palazzo S, Paterson JC, et al. MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. Int J Cancer. 2007;121(5):1156-61.

20. Lai X, Wang M, McElyea SD, Sherman S, House M, Korc M. A microRNA signature in circulating exosomes is superior to exosomal glypican-1 levels for diagnosing pancreatic cancer. Cancer letters. 2017;393:86-93.

21. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature. 2015;523(7559):177-82.

22. Malumbres R, Sarosiek KA, Cubedo E, Ruiz JW, Jiang X, Gascoyne RD, et al. Differentiation stage-specific expression of microRNAs in B lymphocytes and diffuse large B-cell lymphomas. Blood. 2009;113(16):3754-64.

23. Li M, Jia Y, Xu J, Cheng X, Xu C. Assessment of the circulating cell-free DNA marker association with diagnosis and prognostic prediction in patients with lymphoma: a single-center experience. Annals of hematology. 2017;96(8):1343-51.

24. Mohrmann L, Huang H, Hong DS, Tsimberidou AM, Fu S, Piha-Paul S, et al. Liquid Biopsies Using Plasma Exosomal Nucleic Acids and Plasma Cell-Free DNA compared with Clinical Outcomes of Patients with Advanced Cancers. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017.

25. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecologic oncology. 2008;110(1):13-21.

26. Chen Q, Hu H, Jiao D, Yan J, Xu W, Tang X, et al. miR-126-3p and miR-451a correlate with clinicopathological features of lung adenocarcinoma: The underlying molecular mechanisms. Oncology reports. 2016;36(2):909-17.

27. Chang C, Liu J, He W, Qu M, Huang X, Deng Y, et al. A regulatory circuit HP1gamma/miR-451a/c-Myc promotes prostate cancer progression. Oncogene. 2017.

28. Cao D, Jiang Y, Feng Y, Jing CX, Xu J, Xu CG. [The Value of Circulating Exsomal miR-451a to Monitor Therapy Response in Diffuse Large B Cell Lymphoma]. Sichuan da xue xue bao Yi xue ban = Journal of Sichuan University Medical science edition. 2018;49(3):399-403.

29. Xiao XB, Gu Y, Sun DL, Ding LY, Yuan XG, Jiang HW, et al. Effect of rituximab combined with chemotherapy on the expression of serum exosome miR-451a in patients with diffuse large b-cell lymphoma. Eur Rev Med Pharmacol Sci. 2019;23(4):1620-5.

30. Guduric-Fuchs J, O'Connor A, Camp B, O'Neill CL, Medina RJ, Simpson DA. Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. BMC genomics. 2012;13:357.

31. Lu P, Li H, Li N, Singh RN, Bishop CE, Chen X, et al. MEX3C interacts with adaptor-related protein complex 2 and involves in miR-451a exosomal sorting. PloS one. 2017;12(10):e0185992.

32. Huang X, Lyu J. Tumor suppressor function of miR-483-3p on breast cancer via targeting of the cyclin E1 gene. Exp Ther Med. 2018;16(3):2615-20.

33. Menbari MN, Rahimi K, Ahmadi A, Mohammadi-Yeganeh S, Elyasi A, Darvishi N, et al. miR-483-3p suppresses the proliferation and progression of human triple negative breast cancer cells by targeting the HDAC8>oncogene. Journal of cellular physiology. 2020;235(3):2631-42.

34. Liu X, Chu KM. Exosomal miRNAs as circulating biomarkers for prediction of development of haematogenous metastasis after surgery for stage II/III gastric cancer. J Cell Mol Med. 2020;24(11):6220-32.

35. Zhou F, Nie L, Feng D, Guo S, Luo R. MicroRNA-379 acts as a tumor suppressor in non-small cell lung cancer by targeting the IGF-1R-mediated AKT and ERK pathways. Oncology reports. 2017;38(3):1857-66.

36. Cazzoli R, Buttitta F, Di Nicola M, Malatesta S, Marchetti A, Rom WN, et al. microRNAs derived from circulating exosomes as noninvasive biomarkers for screening and diagnosing lung cancer. J Thorac Oncol. 2013;8(9):1156-62.

37. Cipro Š, Belhajová M, Eckschlager T, Zámečník J. MicroRNA expression in pediatric intracranial ependymomas and their potential value for tumor grading. Oncol Lett. 2019;17(1):1379-83.

38. Lin J, Ding S, Xie C, Yi R, Wu Z, Luo J, et al. MicroRNA-4476 promotes glioma progression through a miR-4476/APC/β-catenin/c-Jun positive feedback loop. Cell death & disease. 2020;11(4):269.

Figure Legends

Figure 1. The performance characteristics of circulating exosomal miRNAs in diagnosing DLBCL. Panels A, B, and C show the ROC curves of individual miRNAs and for the combined

457 miRNA panel in the testing, validation and combined phase. A) Results for 24 patients and 24 healthy
458 controls in the testing stage of study. B) Results for 99 DLBCL patients and 65 healthy controls in the
459 validation stage of study. C) Results for the miRNAs combined in the testing and validation stages
460 from 123 DLBCL patients and 99 healthy controls.

461 **Figure 2. Survival analysis of different concentrations of circulating exosomal miR-451a in**
462 **DLBCL patients.** Kaplan-Meier survival curves of A) predicting progression-free survival(PFS) and
463 B) overall survival(OS) in patients with DLBCL. miRNAs expression was dichotomized as high or low
464 according to the median value.

465 **Figure 3. The prognostic performance characteristics of circulating exosomal miR-451a in**
466 **DLBCL patients.** The AUC of IPI, miR-451a, and the combined indicator miR-451a and IPI are
467 expressed as blue, green and red curve. A is for progression free survival, and B is for overall survival.
468 Cross-validation has been applied to this analysis to avoid overfitting.

469 **Supplemental Figure 1. The process of identifying miRNAs with diagnostic value in patients with**
470 **diffuse large B-cell lymphoma.** 332 miRNAs were detected to be differently expressed between 10
471 DLBCL cases and 5 Heathy subjects by microarray and 5 miRNAs were identified to be with statistical
472 significance with qRT-PCR test.

473 **Supplemental Figure 2. Isolation and validation of exosomes from serum of patients with diffuse**
474 **large B-cell lymphoma.** A) The process of exosome extraction. Exosomes isolated from 200 ml of
475 serum were enough for transmission electron microscopy or Nanosight or western blot analysis,
476 whereas 800 ml of serum was enough to identify exosomal miRNAs with qRT-PCR tests. B)
477 Nanosight analysis determined that exosome sizes ranged from about 80 to 95 nm in diameter. C)
478 Transmission electron microscopy showed that serum exosomes were characterized by cup-shaped
479 nanovesicles with a diameter of about 90 nm. D) Western blot showed that the extractions were
480 equipped with exosomal-specific marker proteins CD63, CD9, CD81, and HSP70. Samples 1, 2, 3
481 were blood samples from three patients in the screening stage of analysis.

482 **Supplemental Figure 3. Clustering analysis of microarrays between patients with the disease**
483 **(Group 2, red bar) and healthy controls (Group 1, blue bar).** Colors on heatmap indicates relative

miRNA expression concentrations: red suggests upregulated expression and green suggests downregulated expression.

Supplemental Figure 4. Expression concentrations of miRNAs in the circulating exosome. The Y-axis represents the relative expression of miRNAs normalized to Ce_miR-39_1. Data are presented as means and standard error of mean(mean±SEM). A) Relative expression of 8 miRNAs (miR-379-5p, miR-135a-3p, miR-4476, miR-483-3p, miR-451a, miR-551a, miR-135b-5p, and miR-155-5p) in the serum exosome of 24 DLBCL patients and 24 healthy controls in the testing stage of analysis. B) Relative expression of 5 miRNAs (miR-135a-3p, miR-379-5p, miR-4476, miR-483-3p, and miR-451a) in the serum exosomes of 99 DLBCL patients and 65 healthy controls in the validation stage of analysis. C) Relative expression of the 5 miRNAs in the serum exosomes in 123 DLBCL patients, 29 controls with lymphoma subtypes(concurrent control), and 89 healthy controls when the results of the testing and validation stages were combined.

Supplemental Figure 5. Performance of circulating exosomal miRNAs in diagnosis and differential diagnosis of lymphoma subtypes. A) The ability of miR-379-5p, miR-135a-3p, miR-4476, miR-451a to discriminate DLBCL patients from controls with lymphoma subtypes. B) Performance of miR-451a in discriminating between healthy controls with lymphoma subtypes.

Supplemental Figure 6. Association between the abundance of exosomal miRNAs and clinical characteristics. The Y-axis represents the relative expression of miRNAs normalized to Ce_miR-39_1. Data are presented as means and standard error of mean(mean±SEM). The results show that only miR-451a concentrations were inversely correlated with disease stage. Patients with late-stage (III-IV) had relative lower expression level of miR-451a.

Supplemental Figure 7. Comparisons of relative expression concentrations of 5 identified miRNAs. The Y-axis represents the relative expression of miRNAs normalized to Ce_miR-39_1. Data are presented as means and standard error of mean(mean±SEM). The results suggest miR-451a was highly enriched in circulating exosome when compared to the other 4 miRNAs.

Table

510 **Table 1. 20 miRNAs Exhibiting the Largest Changes in the Screening Stage**

Expression	MiRNA	Fold change	False discovery rate
Up Expression	has-miR-4476	12.43074478	3.30E-07
	has-miR-4464	12.35507967	0.005060152
	has-miR-200c-3p	10.89192057	3.35E-08
	has-miR-5187-5p	10.40018529	5.45E-09
	has-miR-1244	8.849505258	0.000116896
	has-miR-379-5p	7.799123979	0.000120611
	has-miR-450a-5p	6.906357877	2.10E-07
	has-miR-135a-3p	6.682322073	1.86E-06
	has-miR-30b-3p	6.681591434	0.000402273
Down Expression	has-miR-1204	0.030985756	5.03E-07
	has-miR-135b-5p	0.041376179	3.81E-06
	has-miR-551a	0.043168624	3.35E-08
	has-miR-451a	0.06927249	1.32E-06
	has-miR-5193	0.072698935	3.72E-06
	has-miR-576-5p	0.076929174	3.56E-06
	has-miR-647	0.079285483	9.62E-07
	has-miR-1255b-2-3p	0.116410637	1.45E-06
	has-miR-885-5p	0.140525814	5.23E-07
	has-miR-152-5p	0.145584992	1.71E-08
	has-miR-483-3p	0.159971047	3.75E-06

511

512 **Table 2. Performance Characteristics of microRNAs for Diagnosis in 133 Diffuse Large B-Cell**

513 **Lymphoma Patients**

	microRNA, area under the ROC curve (95% CI)					
Stage	**miR-379-5p**	**miR-135a-3p**	**miR-4476**	**miR-483-3p**	**miR-451a**	**panel**
Testing	0.795	0.819	0.689	0.719	0.677	0.934
	0.665~0.925	0.700~0.939	0.535~0.843	0.568~0.870	0.512~0.842	0.869~0.999
Validation	0.756	0.731	0.644	0.714	0.638	0.878
	0.681~0.832	0.648~0.813	0.558~0.730	0.633~0.795	0.549~0.727	0.821~0.934
Combined	0.766	0.750	0.653	0.711	0.646	0.863
	0.701~0.830	0.682~0.819	0.579~0.727	0.639~0.782	0.568~0.724	0.811~0.915

514

Figures

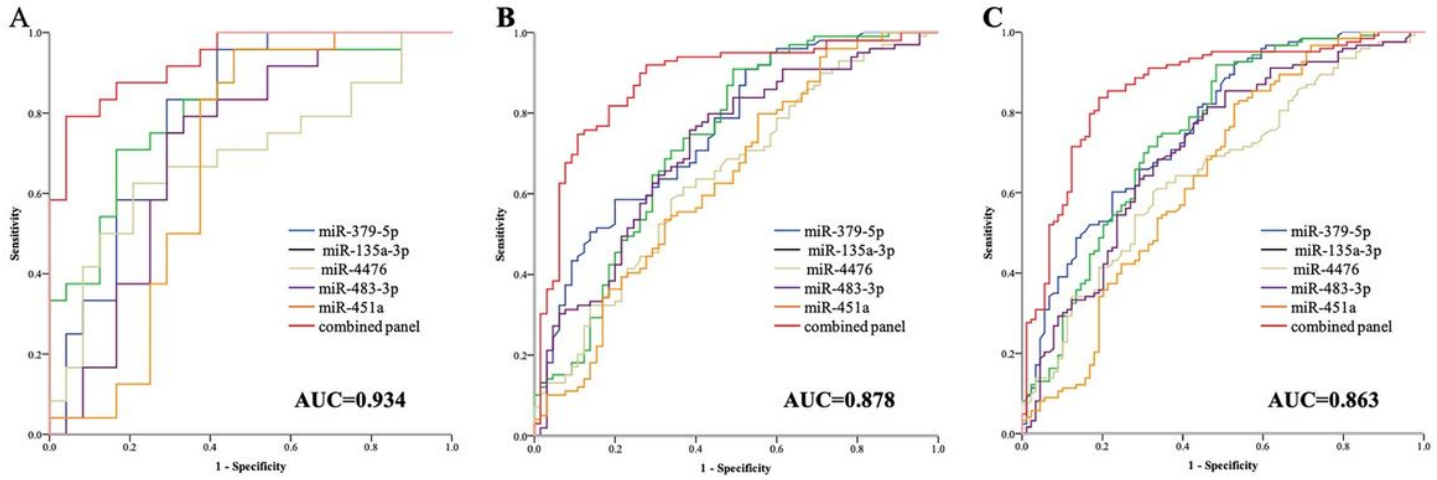

Figure 1

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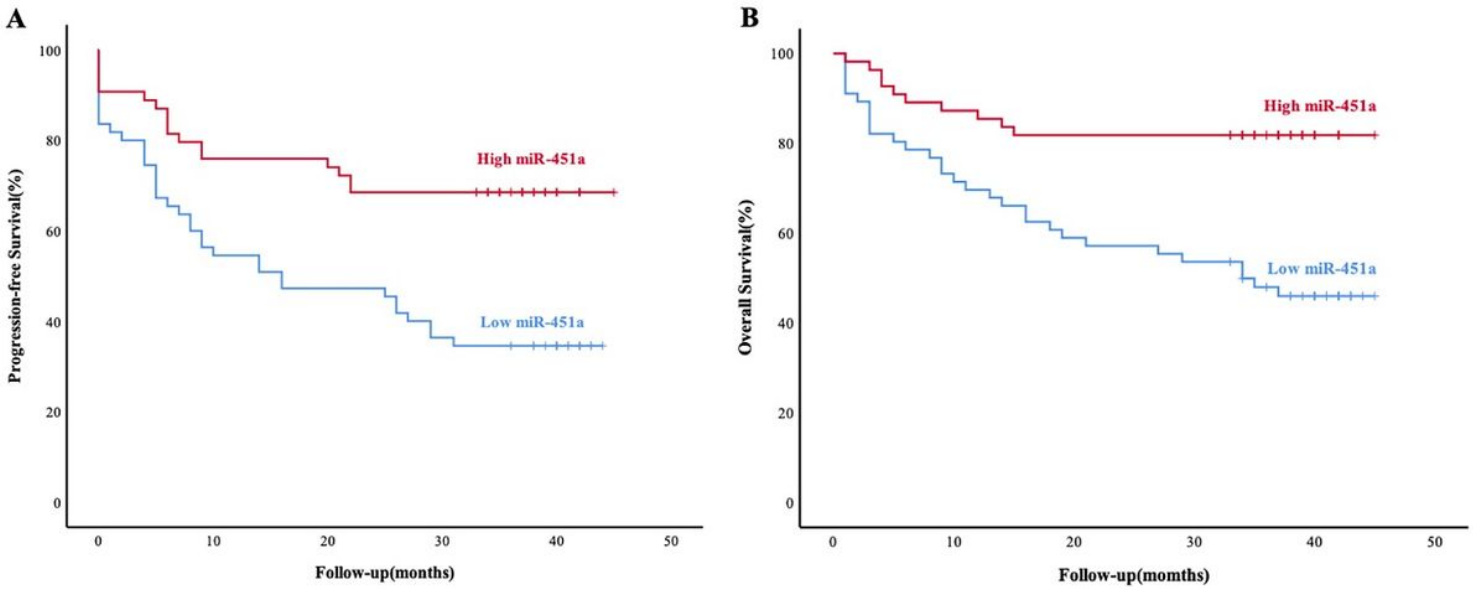

Figure 2

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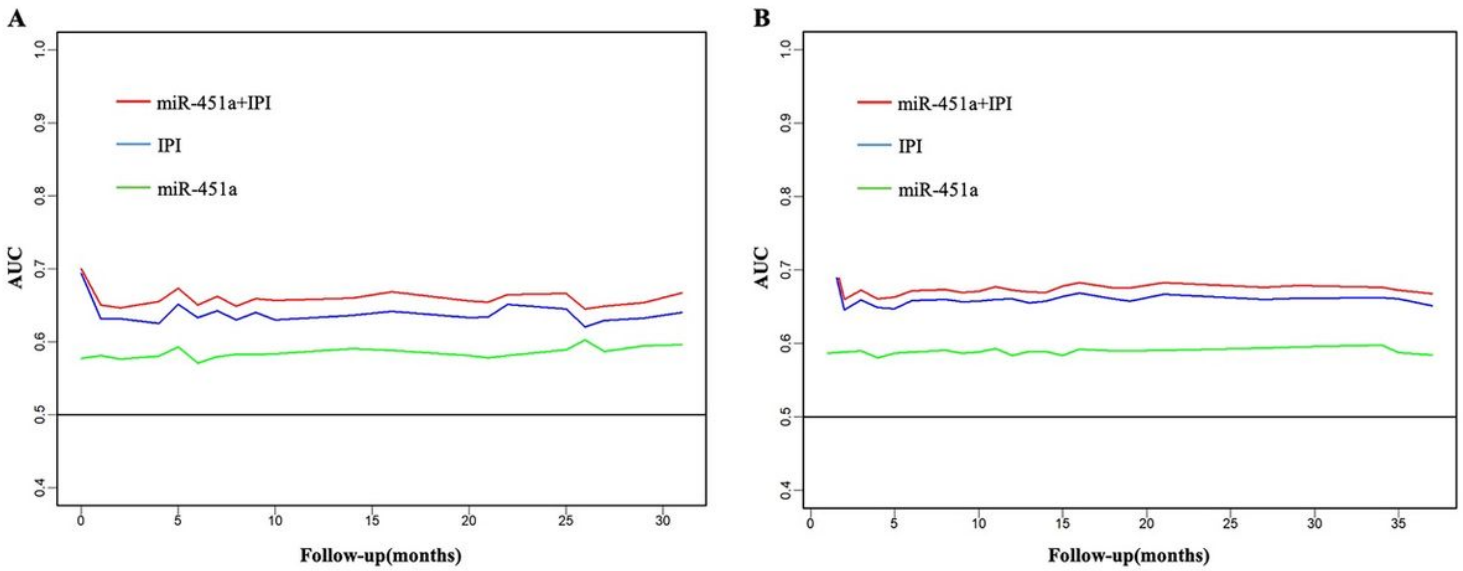

Figure 3

The prognostic performance characteristics of circulating exosomal miR-451a in DLBCL patients. The AUC of IPI, miR-451a, and the combined indicator miR-451a and IPI are expressed as blue, green and red curve. A is for progression free survival, and B is for overall survival. Cross-validation has been applied to this analysis to avoid overfitting.

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