Metabolomics Profiling in Prediction of Immunotherapy Efficacy in Advanced Non-small Cell Lung Cancer

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Research Article

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Abstract

Objective: To explore potential metabolomics biomarker in predicting the efficacy and the survival outcomes after the first-line programmed death-1 (PD-1) immunotherapy in the patients with advanced non-small cell lung cancer (NSCLC).

Methods: A total of 46 consecutive eligible patients were assigned to receive first-line PD-1 immunotherapy. Serum samples were prospectively collected before initial treatment to perform metabolomics profiling analyses under the application of gas chromatography mass spectrometry (GC-MS). The metabolomics signatures were extracted by using a binary least absolute shrinkage and selection operator (LASSO) logistic regression and calculated as a metabolomics score by liner fit for each patients. The metabolomics score was further combined with the clinical predictors to build a metabolomics nomogram for predicting the immunotherapy efficacy in advanced NSCLC patients. The ROC curves were used to evaluate the predicting performance of the metabolomics score, the clinical predictors and the metabolomics nomogram.

Results: Seven metabolites including Urea, Tyrosine, L-threonine, Xylitol, Thymol, Linoleic acid and DL-isoleucine were identified associated with the immunotherapy response. Age was identified as the clinical predictor by the logistic regression. The receiver operating characteristic curve (AUC) was 0.96 (95% CI: 0.92-1.00) for metabolomics score, 0.72 (95% CI: 0.53-0.91) for the clinical predictor and 0.97 (95% CI: 0.93-1.00) for the metabolomics nomogram in differentiating progressive disease (PD) groups from disease control (DC) groups. The median progression-free survival (PFS) after immunotherapy in patients with low risk was significantly longer than those with high risk in the metabolomics nomogram (11.8 vs.2.8 months, P < .001).

Conclusion: This study developed an effective and convenient discriminant metabolomics nomogram that can predict the efficacy and the survival outcomes of PD-1 immunotherapy in advanced NSCLC.

Introduction

The incidence rate of lung cancer is the highest worldwide, and its five-year survival rate is lower than 20% [1]. Non-small cell lung cancer (NSCLC) including lung adenocarcinoma, squamous cell carcinoma and large cell carcinoma etc. accounting for about 40% of lung malignancies [2]. Options for the treatment of NSCLC include surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapy, or a combination of these treatments [3]. Recently, immune checkpoint inhibitor antibodies against programmed death-1 (PD-1) or programmed death-ligand 1 (PD-L1) have been approved for the precise treatment of advanced NSCLC without targetable genetic alterations [4]. However, the efficacy of immunotherapy treatment in advanced NSCLC is very limited with response rates of 20%-40% [5].

Several methods have been developed to evaluate immunotherapy responses including PD-L1 expression [6], DNA methylation [7], tumor mutational burden [8], and T cell proliferation [9]. However, to select the

optimal patient for immunotherapy remains a challenge because most of the available biomarkers such as PD-L1 expression are insufficient to predict the immunotherapy efficacy accurately. Recently, the metabolism have been proposed as a mechanism potentially affecting the response and the toxicity to immunotherapy [10]. Thus, potential metabolism biomarkers that related to drug resistance and can predict the efficacy of immunotherapy in advanced NSCLC are needed.

Metabolomics is an omics technology that provides information, including amino acids and fatty acids, which can be found in the serum, urine, and other body fluids, and tissues [11]. The abundance of certain metabolites can reflect a disease state, which makes metabolomics a valuable field for monitoring disease status and exploring biomarkers to predict the efficacy of immunotherapy.

We hypothesize that specific serum metabolic phenotype could be used to predict the respond to immunotherapy in advanced NSCLC, and can therefore be extrapolated from analysis of the metabolic profiles of pre-immunotherapy serum. To explore this hypothesis, we used gas chromatography mass spectrometry (GC-MS) to perform the metabolomics profiling analysis to identified potential metabolic biomarkers to predict the efficacy of immunotherapy in patients with advanced NSCLC.

Methods

Study design and patient population

This prospective study was reviewed by the Institutional Review Board of Jinshan Hospital, Fudan University (No.JIEC2021S47). All patients signed the informed consent. All the methods were carried out in accordance with relevant guidelines and regulations.

From December 2018 to October 2021, 60 consecutive patients were enrolled in this study. All the patients were pathological confirmed NSCLC with advanced stage IV. And they received a first-line immunotherapy of Toripalimab (200 mg/time), Camrelizumab (200 mg/time) or Sintilimab (200 mg/time) intravenously every 21 days for 4-6 cycles until disease progression. Exclusion criteria: (1) patients with symptoms associated with infection, such as increased leukocytes and neutrophils, or inflammation indicated by lung CT (n=5); (2) patients not completing two cycles of immunotherapy (n=4); (3) patients lost of follow-up (n=5). Finally, 46 patients were enrolled in the metabolomics analysis. They were deviled into a disease control (DC) group (including partial response [PR] and stable disease [SD]) and a progressive disease (PD) group. All the serum samples were collected before the first-cycle of the immunotherapy treatment. The tumor responses to the immunotherapy were evaluated by Response Evaluation Criteria in Solid Tumors, version 1.1 (RECIST v1.1).

Clinical information and serum sample collection

Clinical information including age, gender, tumor position, metastases position, pathological subtype, disease stage and metabolic syndrome situations (clinically diagnosed diabetes, hypertension, or hyperlipidemia) were collected. Fasting peripheral blood (2-4 mL) was collected with a serum separator

tube within one week before the first cycle of immunotherapy. The blood was centrifuged at 1,200 g for 10 min at 4°C within 30 min after collection, and stored at -80°C.

Metabolite extraction and profiling analysis

Serum samples were thawed and vortexed thoroughly on ice. For hydrophilic metabolites extraction, 100 μL of serum sample was mixed with 400 μL methanol. The mixture was vortexed for 30 s and incubated for 6-8 hours at -80°C. After centrifugation at 14,000 g at 4°C for 15 min, 250 μL supernatant was transferred to a fresh tube and lyophilized under vacuum. The dried samples were reconstituted in 50 μL 80% methanol, vortexed for 60 s, and incubated at 4°C for 15 min. The samples were centrifuged at 12,000 g at 4°C for 30 min. Finally, 60 μL of supernatant was used for GC-MS analysis. For hydrophobic metabolite extraction, 100 μL of serum sample was mixed with 400 μL of chloroform/methanol (2:1, vol/vol). The mixture was vortexed for 30 s and centrifuged at 10,000 g for 10 min at room temperature. Then, the lower organic-phase (200 μL) was transferred to a fresh tube and lyophilized under vacuum. The dried samples were dissolved in 150 μL of dichloromethane/methanol (2:1, vol/vol), vortexed for 30 s, and then centrifuged at 12,000 g for 15 min at room temperature. Finally, 60 μL of supernatant was used for GC-MS analysis[12].

The analyses were performed on GC-MS (7890B-5977A, Agilent Technologies, Waldrom, Germany). Metabolite identification ID was carried out by using retention times (Rt) compared to pure compounds Rt (Sigma-Aldrich, Shanghai, China). Pooled quality control (QC) samples were analyzed at the beginning of the sample queue followed by one QC sample inserted for every ten samples.

Data processing

Peak extraction and alignment was performed using Seahorse Analytics (Agilent). Features that existed in at least 80% samples in one group were retained. First, the metabolomics features was normalized to the sum of the peak area of a sample. Then metabolomics features with Pearson correlation coefficients greater than 0.9 were identified as redundant features. If two features had a Pearson correlation coefficient > 0.9, the feature with the largest mean absolute correlation was removed. Third, A binary least absolute shrinkage and selection operator (LASSO) logistic regression analysis with 10-fold cross validation was used to select the metabolomics features to generate metabolomics signatures. A metabolomics score for each patient was calculated using a linear combination of metabolomics signatures weighted by their respective coefficients derived from linear regression[13].

Multivariate binary logistic regression analysis was used to select were significant predictors for response of immunotherapy from the collected clinical information.

Metabolomics nomogram discrimination

The metabolomics nomogram was developed by combining metabolomics score with independent clinical predictors by using a multivariable logistic regression method. To select the optimal combination,

the Akaike Information Criterion (AIC) score were used. The model with the lowest AIC score was selected as a metabolomics nomogram. A heatmap was computed to analyze the correlation between the metabolomics features and the independent clinical predictors. The receiver operator characteristic (ROC) and area under the ROC curve (AUC) were employed to evaluate the accuracy of the metabolomics score, clinical predictors and metabolomics nomogram in predicting the efficiency of immunotherapy in NSCLC.

Statistical analysis

All statistical analyses were performed in R (Version 4.0.2; http://www.r-project.org/). Sample size estimating was calculated by using between-group and within-group standard deviations of metabolomics score. Shapiro-Wilk test and Bartlett test were used to assess the normality and variance homogeneity. metabolomics score and clinical parameter were compared by one-way ANOVA followed by Bonferroni correction (met normality and variance homogeneity) or Mann-Whitney U test (if not met normality or variance homogeneity). Survival analysis was assessed with Kaplan-Meier curves via log-rank tests. The ROC and AUC were employed to evaluate the predictive accuracy of the metabolomics features and clinical predictors. The "caret" package was used for redundant features elimination; the "glmnet" package was used for binary LASSO logistic regression, linear regression, and multivariate binary logistic regression; the "rms" package was used for nomogram and calibration curve plotting; the "pROC" package was used for AUC calculation; the "FELLA" package was used for enrichment and pathway analyses. The "pwr" package was used for sample size estimating. All the statistical tests were two-sided and considered statistically significant at $P < .05$, unless otherwise stated.

Results

Patient baseline characteristics

A sample size of 20.5 in each group was achieved with Type II error of 0.001 and Type II error of 0.1. In total, 46 serum samples were collected from the patients with histologically confirmed NSCLC. The work flow of this study is shown in Figure 1. All the patients received immunotherapy as the first-line therapy. Most patients achieved disease control, including those with a confirmed PR or SD (n = 7, 15.2% and n = 28, 60.8%). The clinical characteristics are shown in Table 1.

Table 1
Comparison of clinicopathologic characteristics between DC and PD patients

	DC (n = 35)	PD (n = 11)	P-value
Gender			
Female	7 (20.0%)	3 (27.3%)	.927
Male	28 (80.0%)	8 (72.7%)	
Age	59.8 (8.62)	64.8 (6.90)	.050
Metabolomics score	0.52 (0.04)	0.66 (0.05)	< .001
Overall survival	11.8 [9.3, 30.9]	11.8 [9.2, 31.6]	.300
Progression-free survival	11.8 [9.3, 30.9]	2.8 [0.30, 10.3]	< .001
Tumor position			
Central type	24 (68.6%)	7 (63.6%)	1
Peripheral type	11 (31.4%)	4 (36.4%)	
Lung metastases			
Negative	19 (54.3%)	5 (45.5%)	.869
Positive	16 (45.7%)	6 (54.5%)	
Brain metastases			
Negative	23 (65.7%)	6 (54.5%)	.756
Positive	12 (34.3%)	5 (45.5%)	
Bone metastases			
Negative	22 (62.9%)	7 (63.6%)	1
Positive	13 (37.1%)	4 (36.4%)	
Liver metastases			
Negative	23 (65.7%)	9 (81.8%)	.524
Positive	12 (34.3%)	2 (18.2%)	
Other site metastases			
Negative	28 (80.0%)	10 (90.9%)	.706
Positive	7 (20.0%)	1 (9.1%)	

DC, disease control; PD, progressive disease. Data presented as mean (SD), Median [Min, Max] or N (ratio).

	DC (n = 35)	PD (n = 11)	P-value
Pathological subtype			
Adenocarcinoma	22 (62.9%)	8 (72.7%)	.178
Squamous cell carcinoma	8 (22.9%)	0 (0%)	
Other NSCLC	5 (14.3%)	3 (27.3%)	
Metabolic syndrome			
Negative	23 (65.7%)	9 (81.8%)	.524
Positive	12 (34.3%)	2 (18.2%)	
DC, disease control; PD, progressive disease. Data presented as mean (SD), Median [Min, Max] or N (ratio).			

No significant differences of the gender, tumor position, metastases position, pathological subtype, disease stage and metabolic syndrome and overall survival (OS) were shown between CD and PD groups. Higher metabolomics score, younger age, and longer time to progression) were shown in CD group than PD group (Table 1). The multivariate binary logistic regression analysis showed that age were the clinical predictor for PD in response of immunotherapy of NSCLC (P = .050).

Metabolomics profiling of pre-immunotherapy serum samples

The 64 pre-immunotherapy serum samples were analysis by using GC-MS. After peak alignment and removal of missing values, 57 metabolomics features were obtained (Supplementary Figure 1). After removing redundant features, 52 metabolomics features were entered in to a LASSO regression (Figure 2A, B). Finally, 7 metabolomics features that exhibited significant differences between the DC group and the PD group were selected as the metabolomics signatures. These metabolomics signatures were considered as the subsequent identification of potential metabolite biomarkers for predictive of immunotherapy response in advanced NSCLC (Figure 2C). In these metabolites, 5 (Urea, Tyrosine, L-threonine, Xylitol, Thymol) with lower levels, and 2 (Linoleic acid and DL-isoleucine) with elevated levels were found in the DC group compared to the PD group (Supplementary Table 1). A heatmap shows the correlation of the clinical information and the metabolomics signatures (Figure 2D).

Metabolomics nomogram development and performance assessment

A metabolomics nomogram by combining the metabolomics signatures and the clinical predictor (age) was developed for predicting the immunotherapy response in advanced NSCLC patients (Figure 3). The sensitivity, specificity, negative predictive value, positive predictive value and AUC of ROC were 0.80, 1.00, 1.00, 0.61, 0.96 (95% CI: 0.92-1.00) for the clinical predictor; and were 0.83, 0.64, 0.88, 0.54, 0.72 (95% CI: 0.53-0.91) for the metabolomics signatures; and were 0.89, 1.00, 1.00, 0.73, 0.97 (95% CI: 0.93-1.00) for the metabolomics nomogram.

Enrichment and pathway analysis

The pathway analysis were analyzed and queried in the following databases: The Human Metabolome Database (HMDB, http://www.hmdb.ca/), Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/). Results show pentose and glucuronate interconversions, glycine, serine and threonine metabolism, linoleic acid metabolism, and gentral carbon metabolism in cancer are evolved in immunotherapy response in NSCLC patients (Figure 4).

Prognostic prediction

We further analyzed the potential prediction ability of this metabolomics nomogram on progression-free survival (PFS). The median PFS of patients who were classified as DC cases was 11.8 (95% CI: 9.3-30.9) month, significantly longer than 2.8 (95% CI: 0.3-10.3) month of PD cases (log-rank, P < .001). The overall survival (OS) of patients predicted as DC cases was similar with the overall survival of patients of PD cases (median OS, 11.8 month versus 11.8 month, log-rank, P = .30) (Table 1 and Figure 5).

Discussion

In this study, a metabolomics and clinical based nomogram was developed to predict the efficacy of PD-1 immunotherapy in patients with advanced NSCLC before treatment delivery. By using metabolomics analysis, this metabolomics nomogram provides a promising strategy to personalize immunotherapy, which could be easily applied by physicians to select patients who might benefit from PD-1 immunotherapy.

Metabolites are low molecular weight products of the cellular processes, which are fundamental to understand the functional status of cells [14].As the ‘downstream’ omics of genomic, transcriptomic and proteomics, metabolomics is functional readouts of a cellular state and therefore more closely linked to phenotype than genes and proteins, which can reflect alterations of biological states, even when no measurable changes of genes and proteins are detected [15]. Thus, metabolomics is more likely to provide candidates of potential biomarkers [16]. Moreover, metabolomics studies can be assessed noninvasively by using blood and urine, which are less expensive than other omics approaches, and are easier to be used in clinical practice. Cancer cells metabolism is profoundly altered and, therefore, produces molecules that are specific and typical of non-physiological conditions [17]. In consonance with peptide expression as an indicator of disease, the abundance of cancer-driven metabolic abnormalities may be representative of a disease state or be indicative of the cancer’s pathogenesis, allowing for improved diagnostics and disease monitoring.

The property of metabolites interacting with and targeting of therapeutics makes metabolomics a valuable field for advancements in immunotherapy [18]. In this study, a total of 7 metabolites were selected as the predictor of PD-1 immunotherapy efficiency in NSCLC. These metabolites are mainly

involved in the metabolism of pyrimidine, carbohydrate, amino acids, and fatty acids which are closely associated with cancer progression.

Urea is largely derived from the urea cycle reactions through hepatic detoxification of free ammonia and cleared by urination. Recently, study reveals that an intriguing link between serum urea and cancer risks [19]. Serine/threonine kinase 2 promotes proliferation can be used to predict metastasis and poor prognosis in NSCLC [20]. The efficacy of epidermal growth factor receptor tyrosine kinase inhibitors for EGFR gene is also well established in NSCLC [21]. Xylitol is a widely used anti-caries agent that has anti-inflammatory effects. Previous studies indicate that xylitol has potential in therapy against lung cancer by inhibiting cell proliferation and inducing autophagy of A549 cells [22]. Thymol can act as a safe and potent therapeutic agent to treat NSCLC by inducing mitochondrial pathway-mediated apoptosis via ROS generation, macromolecular damage and SOD diminution in A549 cells [23].

Previous studies showed that conjugated linoleic acid was able to induce apoptosis, up-regulate PPARG gene expression and activate PPARγ protein in certain human cancer cell lines, in which, conjugated linoleic acid has the ability to activate pathways that lead to cell death in lung cancer [24]. Plasma free amino acid, profiles are altered in cancer patients. Increases in proline, isoleucine, phenylalanine and ornithine were observed in the early detection of lung cancers [25].

This study had several limitations. First, the metabolite is highly dynamic and sensitive to a wide range of factors, the result of this study needs to be validated to warrant the consistency and reproducibility. Second, some of the patients were not able for surgical management before the immunotherapy, which may have potential influence on the results due to selection bias. Furthermore, larger sample, multi-center and prospective studies should be carried out for validating the metabolomics nomogram to provide reliable evidence for further clinical application.

Conclusions

The discriminant metabolomics nomogram developed in this study offers a feasible and convenient strategy to personalize treatment. The high accuracy of metabolomics nomogram provides the possibility of predicting the effect of immunotherapy in NSCLC patients.

Abbreviations

AIC, Akaike Information Criterion; AUC, area under the ROC curve; LASSO, binary least absolute shrinkage and selection operator; GC-MS, gas chromatography mass spectrometry; NSCLC, Non-small cell lung cancer; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; QC quality control; Rt, retention times

Declarations

Ethics approval and consent to participate

This prospective study was reviewed by the Institutional Review Board of Jinshan Hospital, Fudan University (No.JIEC2021S47). All patients signed the informed consent. All the methods were carried out in accordance with relevant guidelines and regulations.

Availability of data and material

The datasets generated and/or analysed during the current study are available in the lungmeta repository, https://github.com/loopnownow/lungmeta

Consent for publication

Not Applicable

Competing interests

The authors declare no conflict of interest

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Authors' contributions

YL and RXQ designed the research study. YL ZHZ and RXQ performed the research. XSL and YY provided help and advice on acquisition of data. YL analyzed the data. YL and RXQ wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Not Applicable

Statement

All the methods were carried out in accordance with relevant guidelines and regulations.

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Figures

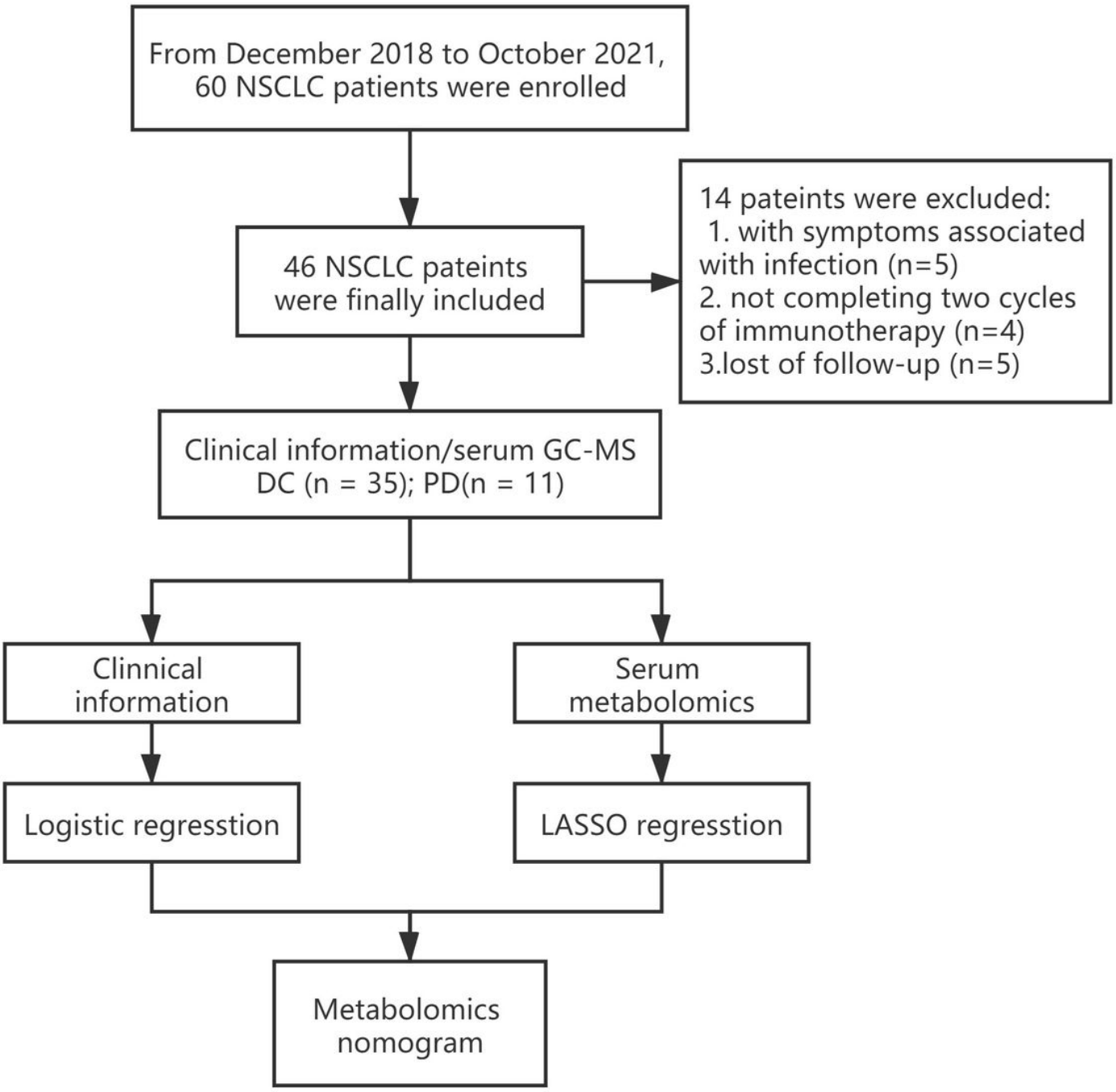

Figure 1

Work flow of this study. DC, disease control; PD, progressive disease.

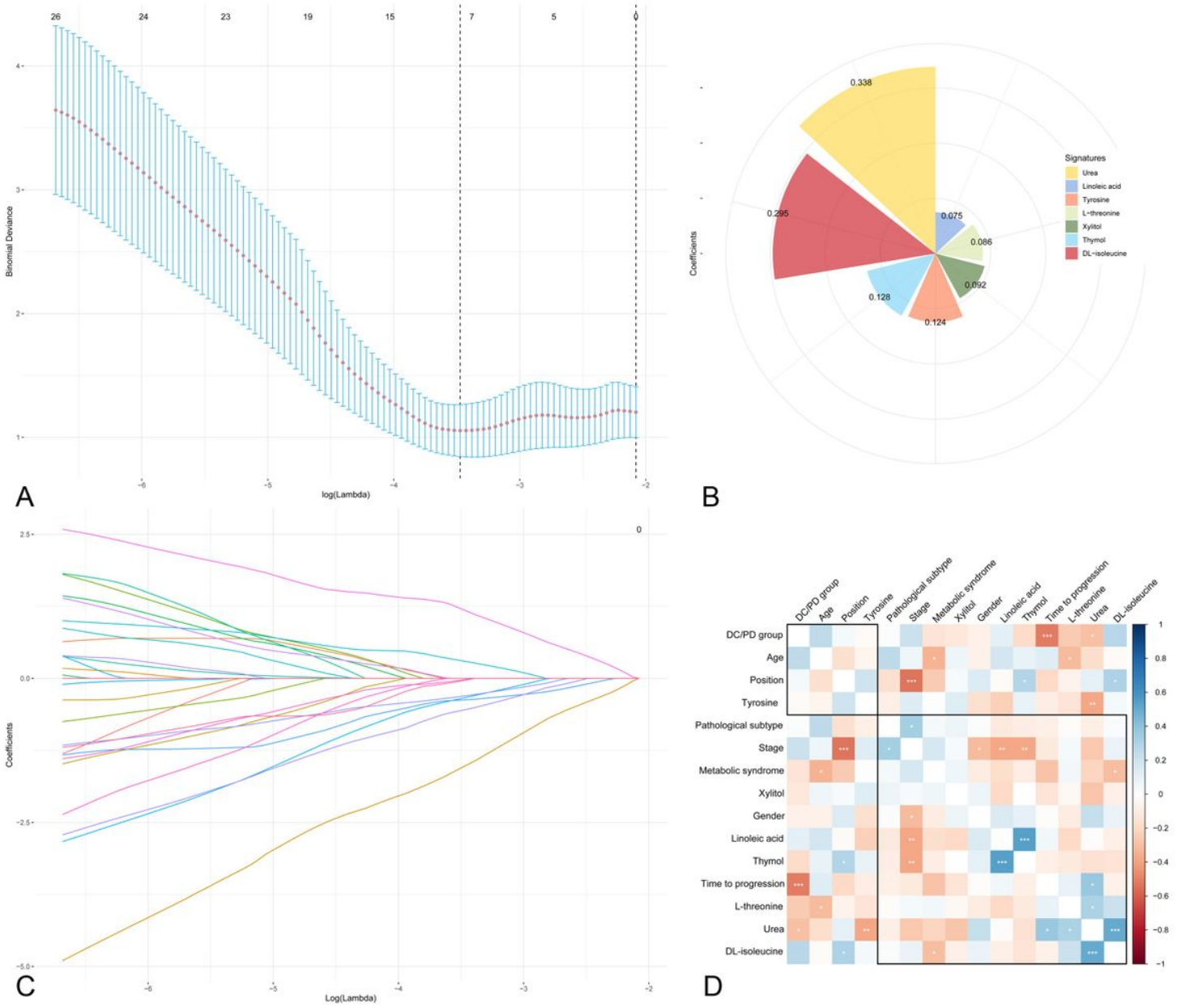

Figure 2

The selection process of the metabolomics features and the correlation of each metabolomics features and clinical information. Selection of the tuning parameter lambda (lambda) using 10-fold cross validation (A). Binomial deviances from the LASSO regression cross validation model are plotted as a function of log (lambda) (B). Seven metabolomics signatures selected and its corresponding coefficients by a liner fitting (C). The correlations of the patients clinical information and metabolomics features. The blue/red line indicates a positive/negative correlation (*P < .05; **P < .01; ***P < .001).

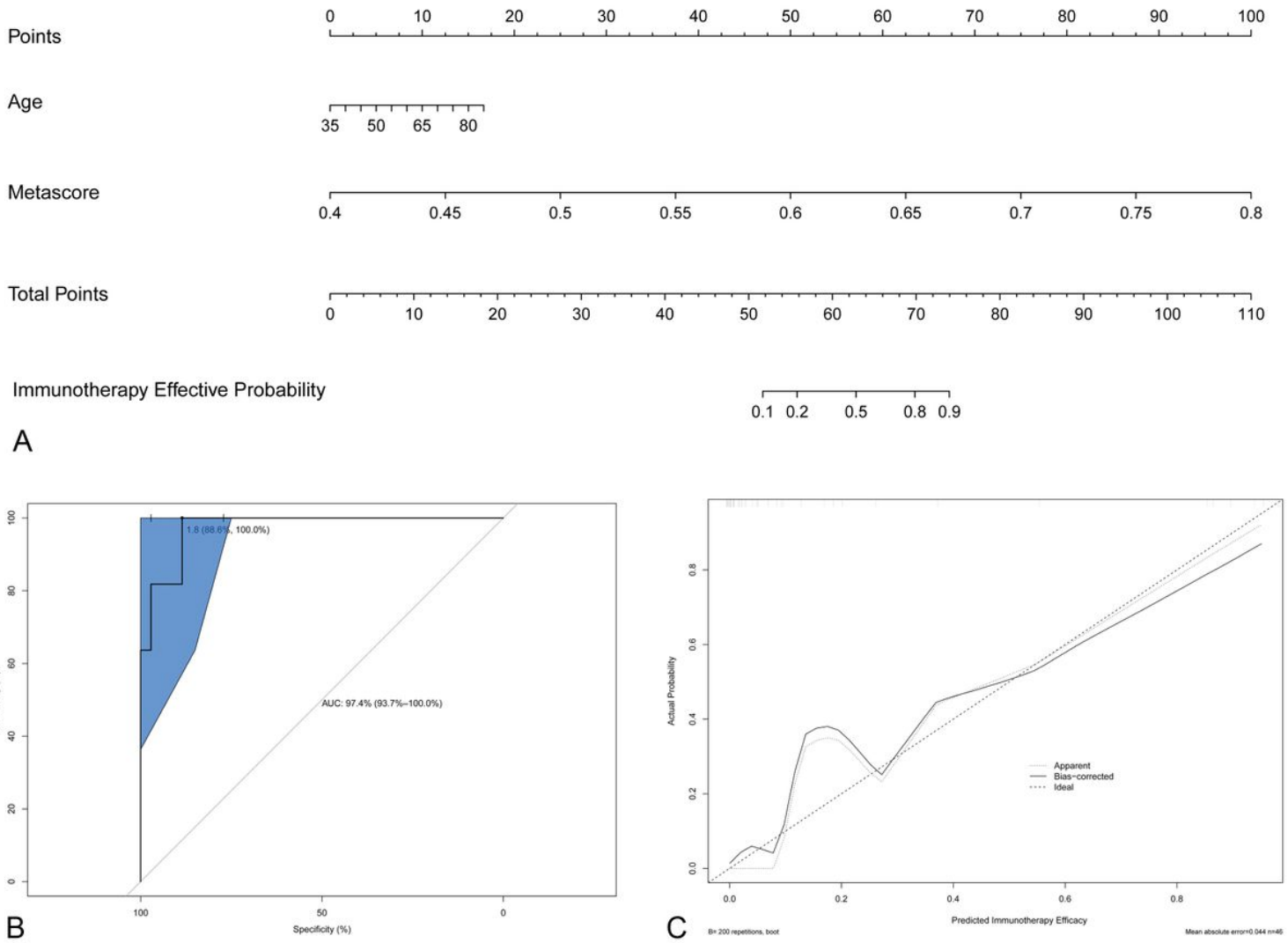

Figure 3

The metabolomics nomogram. The metabolomics nomogram is constructed by integrating the metabolomics score with patient's ages (A). The ROC curves of the metabolomics nomogram (B). The calibration curves of the metabolomics nomogram (C).

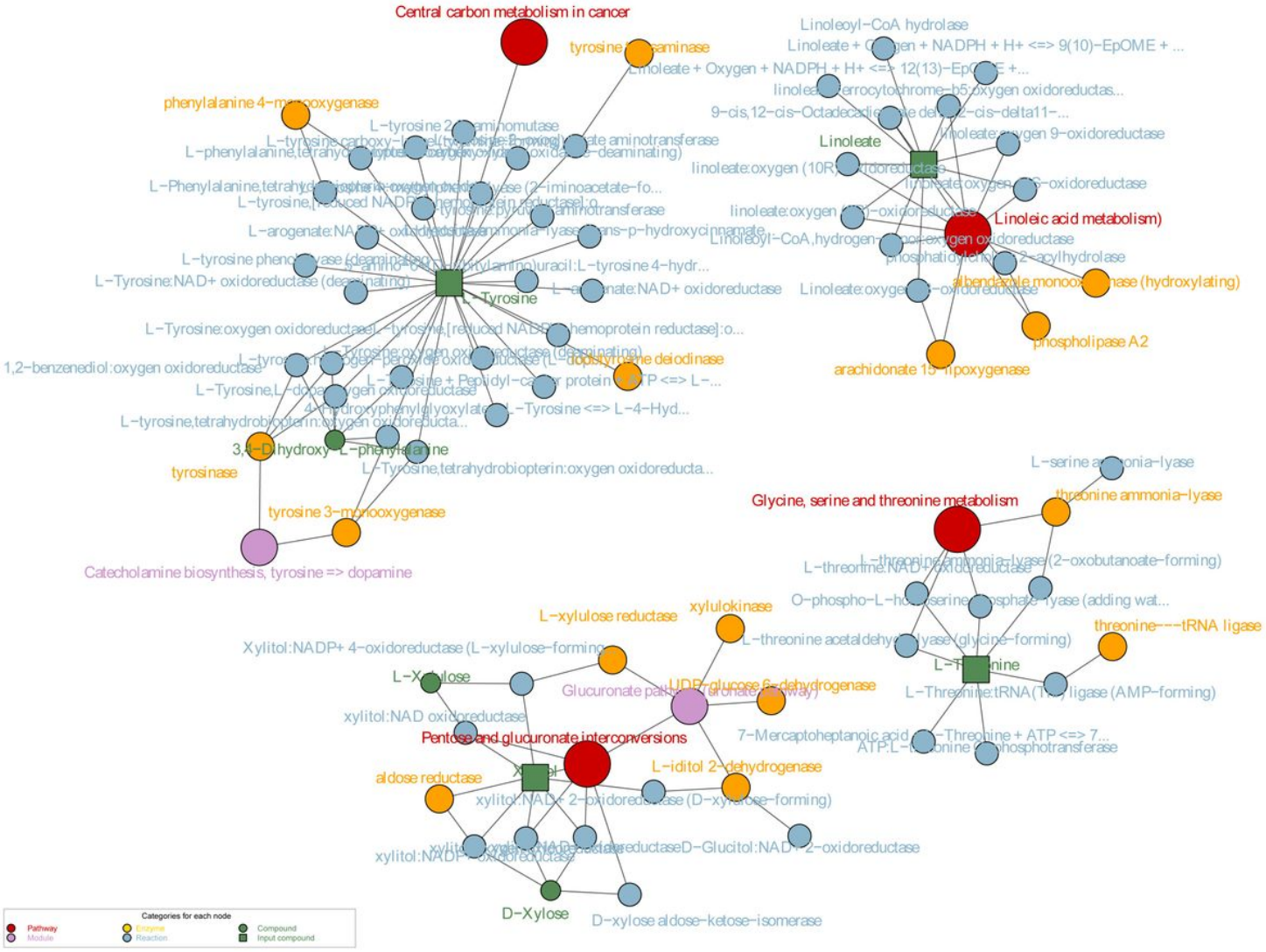

Figure 4

The metabolic pathways of the metabolomics signatures involved in predicting the immunotherapy efficacy in advanced non-small cell lung cancer.

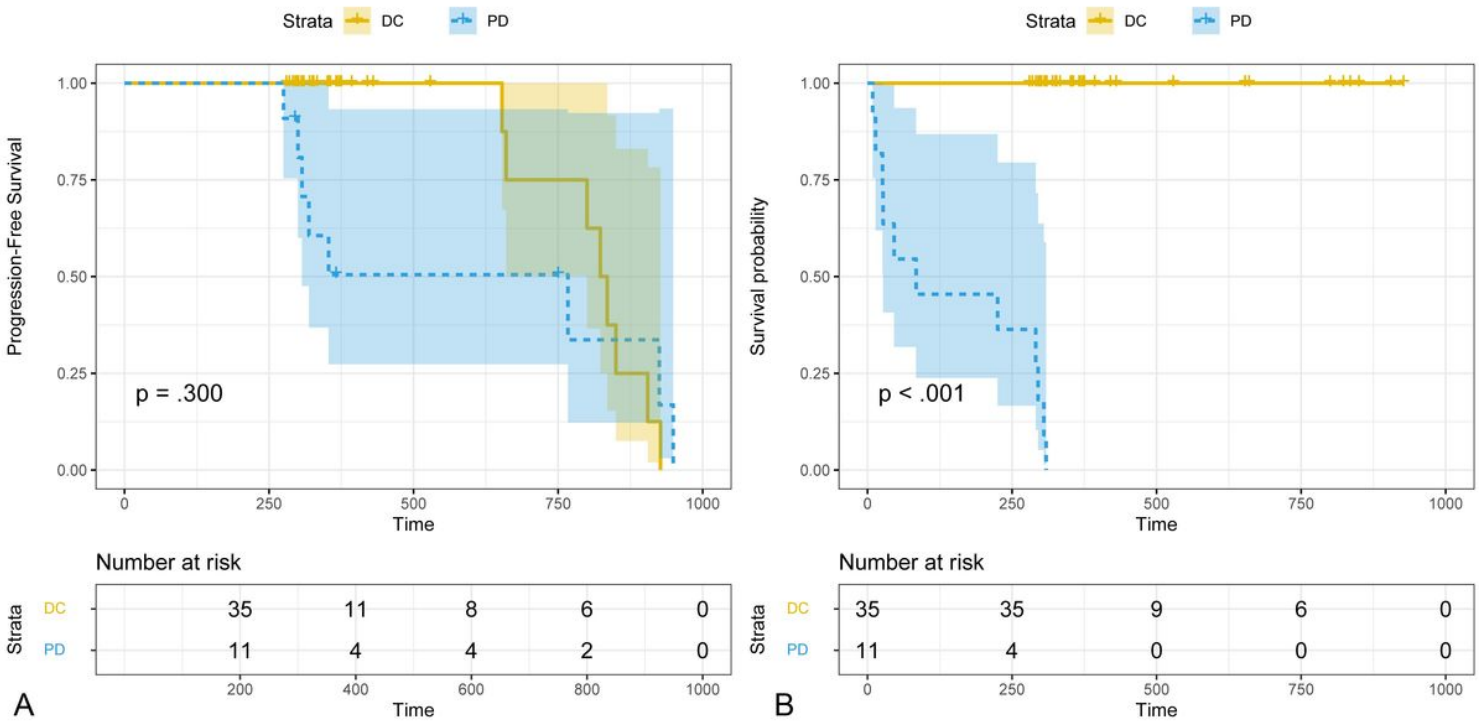

Figure 5

The Kaplan-Meier analysis of prognostic prediction in DC group and PD group. Shown are the analysis of the progression-free survival (A), and the overall survival (B). P values were calculated by log-rank test. DC, disease control; PD, progressive disease.

Supplementary Files

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