Original Article

# Single nucleotide polymorphisms of casitas B-lineage lymphoma proto-oncogene-b predict outcomes of patients with advanced non-small cell lung cancer after first-line platinum based doublet chemotherapy

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**Background:** Casitas B-lineage lymphoma proto-oncogene-b (CBLB) influences the threshold of T cell activation and controlling peripheral T cell tolerance. In the present study, we hypothesize that potentially functional single nucleotide polymorphisms (SNPs) in CBLB are associated with clinical outcomes in patients advanced non-small cell lung cancer (NSCLC) treated with the first-line chemotherapy.

**Methods:** We genotyped three SNPs (rs2305035, rs3772534 and rs9657904) at CBLB in 116 advanced NSCLC patients with progression free survival (PFS) data and 133 advanced NSCLC patients with overall survival (OS) data, and we assessed their associations, 95% confidence interval (CI), with clinical outcomes by using Cox proportional hazards regression analyses. In silico functional analysis was also performed for the SNPs under investigation.

**Results:** We found that associations between the three SNPs and PFS/OS were not significant in the overall NSCLC patients. The rs2305035 AA genotype was associated with a worse PFS in female patients and those of non-smokers or light smokers (95% CI, 1.14–11.81, P=0.030; 95% CI, 1.42–10.24, P=0.008; and 95% CI, 1.39–9.93, P=0.009; respectively), compared with the GG+AA genotypes. We also found that the rs9657904 CC genotype was significantly associated with a worse OS than TT + TC genotypes in male advanced NSCLC patients. Further in silico functional analysis revealed that the rs965704 T allele was significantly associated with lower mRNA expression levels of the CBLB gene.

**Conclusions:** Our findings identified two CBLB SNPs (rs2305035 and rs9657904) that were significantly associated with PFS and OS in several subgroups of Chinese advanced NSCLC patients after the first-line chemotherapy.

**Keywords:** Non-small cell lung cancer (NSCLC); single nucleotide polymorphism (SNP); casitas B-lineage lymphoma proto-oncogene-b (CBLB); first-line platinum based doublet chemotherapy

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## Introduction

The platinum-based doublet chemotherapy as the first-line treatment is a critical option for patients with advanced non-small cell lung cancer (NSCLC). However, the short term and long-term efficacies of chemotherapy have not been satisfying in clinical practice. Currently, the promising treatment for the advanced NSCLC is immunotherapy by modulating the host immune response, local immune effects and tumor microenvironment (1-3).

Accumulating results from published studies had illuminated the association of local immune status with chemotherapy or immunotherapy responses and clinical outcomes in various types of cancer (4-7). The capacity of lymphocytes, particularly the T cells that recognize tumor antigens, provides a potential actionable treatment by fighting against cancer (8). The proportions of various T cells (for examples, effecting T cells and regulating T cells) could be changed by chemotherapy (4,9). Therefore, the functions of T cells may be altered by chemotherapy, influencing on the tumor and thus patients' prognosis.

The E3 ligase Cbl-b is one member of the Cbl family, which is vital in post-transcriptional protein modification by ubiquitination and also regulate several signaling pathways in various immune cells (10). Up-regulating Cbl-b in gastric cells can reduce the expression of p-glycoprotein (P-gp) and partially reverse chemotherapeutics resistance (11). Over expression of Cbl-b could enhance the efficacy of VP-16 (12) and promote drug sensitivity of anthracyclines (13). Conversely, many studies have suggested that deactivation of Cbl-b enhances CD8+ T cells' capacity of killing tumor cells (14,15), reduces Foxo3a phosphorylation in regulatory T cells (16) and rejects cancer metastasis via natural killer cells (17). On the other hand, the dead tumor cells caused by chemotherapy could provide antigen to dendritic cells (DCs) and play vital role in anti-tumor immune (18). Furthermore, chemotherapeutics can decrease regulatory T cells and enhance anticancer immune responses in mice and humans (19,20). So Cbl-b was involved in modulation of chemotherapeutics sensitivity and anti-tumor immune responses. Previously, we reported that genotypes of rs2305035 in casitas B-lineage lymphoma proto-oncogene-b (CBLB) in 393 NSCLC Caucasian patients treated with chemoradiation predicted clinical outcomes and risk of radiation-induced pneumonitis (21). In the present study, we hypothesized that rs2305035 and other functional single nucleotide polymorphisms (SNPs) in CBLB might predict clinical outcomes in Chinese patients with advanced NSCLC treated with the platinum-based doublet chemotherapy as the first-line therapy.

## Methods

### *Patient population*

The present study initially included 200 patients with advanced NSCLC treated with the first-line platinum-based doublet chemotherapy at Henan Tumor Hospital (Zhengzhou, China) between October 2013 and April 2015, from whom DNA samples were available. The main chemotherapy regimens were pemetrexed plus platinum, gemcitabine plus platinum and taxanes plus platinum with 21 days per cycle. All patients were staged according to the 7$^{th}$ edition of the American Joint Committee on Cancer (AJCC) staging system. Patients who had completed 4 cycles of the first-line combined chemotherapy were included in the final analysis. Totally, there were 116 patients remained for the analysis of progression free survival (PFS) and 133 patients for the analysis of overall survival (OS) after excluding patients with missing follow-up information. According to RECIST 1.1, we evaluated tumor response by computed tomography (CT) at the time of every follow-up visit at the hospital.

Blood samples from all enrolled subjects were collected before treatment. Genomic DNA was extracted from peripheral blood leukocytes using a DNA extracting Kit (item No: SK8224, Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. DNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm. Smoking index defined as the number of cigarettes smoked per day multiplied by the number of years smoked (22) was used to quantitate smoking. All subjects underwent complete evaluation of blood, differential white blood cell counts, coagulation function and D dimer level before any treatment. The neutrophil-lymphocyte ratio (NLR) was defined as the neutrophil counts divided by the lymphocyte counts, and the platelet-lymphocyte ratio (PLR) was defined as the platelet counts divided by the lymphocyte counts (23,24). NLR, PLR and D dimer were classified as patients' characteristics that may have an effect on survival. The present study was approved by Henan Tumor Hospital's institutional review board (No. 20120755) in compliance with Helsinki Declaration. And in the retrospective study, patients' consents were waived.

 

### *SNP selection and genotyping*

We searched the online SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP; https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html; http://regulomedb.org/) and related literature to identify all potentially functional SNPs of CBLB with a minor allele frequency (MAF) ≥0.05 among subjects of Asian descent. We found six potentially functional SNPs (rs3772534, rs7649466, rs2305037, rs2305036, rs2305035 and rs1042852) with MAF ≥0.05 within the region of 20-kb up- and downstream of the CBLB gene. Linkage disequilibrium (LD) analysis was used to optimize SNP selection to reduce redundant SNPs. Having removed SNPs in high LD ($r^2$ >0.6), we finally selected three SNPs for genotyping: rs3772534 C>T; rs2305035 G>A; rs9657904 C>T.

The three SNPs in CBLB (rs3772534, rs2305035 and rs9657904) were genotyped using direct DNA sequencing method. The used sequencing primers including: rs2305035 (forward 5'-CTATTTGTTTAGGAGTCGGATGG-3', reverse 5'-CATCATCAAGGACTCCTCACG-3', amplifying a fragment of 192 bps); rs3772534 (forward 5'-GCCAATGCCTCTTGAAGC-3', reverse, 5'-GCTTTGCGTATTTCTTACCTTA-3', amplifying a fragment of 220 bps); rs9657904 (forward, 5'-ACAGTAGTTTTAAGAGCAGTGATCC-3', reverse, 5'-TGAATTGGAATTAAGGCAGG-3', amplifying a fragment of 204 bps). The PCR amplification system was performed in a total volume of 50 uL. The amplification conditions were used as follows: 94 ℃ for 10 min, followed by 35 cycles of 30 s at 94 ℃, 30 s at 57 ℃, and 30 s at 72 ℃, and ending with a single 10-min extension step at 72 ℃. In the genotyping experiments for the three SNPs, the digestion products were resolved by electrophoresis in 1.5% agarose gel.

### *Statistical analysis*

PFS was calculated from the date that treatment began to the first time of tumor progression, or date of death of any cause before tumor progression, or the last contact date. OS was calculated from the date of diagnosis until death or the date of last contact. The median survival time was estimated by using Kaplan-Meier method, and the survival curves were compared by using log-rank test. Multivariate Cox proportional hazards regression models were used to estimate the effect of each genotype [in terms of hazards ratios (HRs) and their corresponding 95% confidence intervals (CIs)] on PFS and OS with or without adjustment for selected factors by using the stepwise selection method in SAS (for PFS, two variables were adjusted: visceral metastasis number and PLR; for OS, the adjusted factor are smoking status and stage). The proportional hazard assumption was met for all clinical variables and SNPs (*Table S1*). All tests were two-sided, and a P value <0.05 was considered statistically significant. We also used the false-positive report probability (FPRP) to test for false positive associations. For all significant genetic effects observed in the present study, we calculated FPRP levels at 0.0001, 0.001, 0.01, 0.1, and 0.25. The HR was set close to 0.67 (protection) or 1.50 (risk) in the present study, and a probability value <0.2 was considered noteworthy. All data were analyzed with SAS 9.2 software (SAS Institute Inc., Cary, NC, USA), if not mentioned elsewhere.

## Results

### *Patient characteristics*

As shown in *Table 1*, in PFS analysis, there were 75 males and 41 females with a median age of 58 years (range, 23–82 years). There were 75 patients (64.7%) with stage IV, and 41 patients 35.3% had stage II–III (most of these patients received radiotherapy after or concurrent chemotherapy) diseases, all of whom received at least 4 cycles the first-line chemotherapy or induction chemotherapy. The adenocarcinoma accounted for 63.8% and squamous 30.2%, and most patients (88.8%) had one visceral metastasis (lung, bone or brain). Based on a previous study (24), 5 and 180 were defined by the threshold value for NLR and PLR, respectively, 92 patients had normal D-2-dimer, and 24 patients had elevated D-2-dimer. The overall median PFS for 116 patients was of 7.02 months.

*Table 1* also shows the characteristics of 133 patients treated with the first-line chemotherapy for OS analysis. In this OS dataset, there were 85 males and 48 females, the median age was 59 years (range, 25–76 years), 88 patients (66.2%) had stage IV diseases, and 45 patients (33.8%) patients had stage II or III diseases. Other clinical characteristics were similar to the PFS dataset, and the overall median OS was of 24 months.

We found that patients with visceral metastasis number >1 and PLR ≥180 had a worse PFS in both univariate and multivariate Cox models. In the OS analysis, patients with never smoking or early stage had a longer OS in multivariate Cox models (*Table 1*). The adjustments for

 

**Table 1** Univariate and multivariate analysis of characteristics of NSCLC patients and their associations with clinical outcomes

| Patients' characteristics | Progress free survival (PFS) | | | | | | | Overall survival (OS) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total No. (%) | Event No. (%) | Median PFS (months) | P | Adj P | Adj HR | Adj 95% CI | Total No. (%) | Event No. (%) | Median OS (months) | P | Adj[a] P | Adj[a] HR | Adj[a] 95% CI |
| Sex | | | | | | | | | | | | | | |
| Male | 75 (64.7) | 50 (66.7) | 6.77 | – | – | 1.00 | – | 85 (63.9) | 50 (58.8) | 23.30 | – | – | 1.00 | – |
| Female | 41 (35.3) | 26 (63.4) | 8.73 | 0.899 | 0.807 | 0.94 | 0.57–1.54 | 48 (36.1) | 28 (58.3) | 24.63 | 0.920 | 0.408 | 1.35 | 0.67–2.72 |
| Age (years) | | | | | | | | | | | | | | |
| <65 | 86 (74.1) | 55 (64.0) | 8.13 | – | – | 1.00 | – | 95 (71.4) | 52 (54.7) | 25.53 | – | – | 1.00 | – |
| ≥65 | 30 (25.9) | 21 (70.0) | 6.70 | 0.319 | 0.454 | 1.22 | 0.73–2.03 | 38 (28.6) | 26 (68.4) | 24.57 | 0.458 | 0.696 | 1.10 | 0.68–1.77 |
| Smoking status | | | | | | | | | | | | | | |
| Ever | 48 (41.4) | 30 (62.5) | 6.37 | – | – | 1.00 | – | 62 (46.6) | 40 (64.5) | 19.63 | – | – | 1.00 | – |
| Never | 68 (58.6) | 46 (67.7) | 8.13 | 0.867 | 0.614 | 0.88 | 0.55–1.43 | 71 (53.4) | 38 (53.5) | 25.53 | 0.214 | 0.017 | 0.57 | 0.36–0.91 |
| Smoking index | | | | | | | | | | | | | | |
| <400 | 73 (62.9) | 48 (65.8) | 8.47 | – | – | 1.00 | – | 78 (58.6) | 42 (53.9) | 25.53 | – | – | 1.00 | – |
| ≥400 | 43 (37.1) | 28 (65.1) | 6.37 | 0.682 | 0.575 | 1.15 | 0.71–1.87 | 55 (41.4) | 36 (65.5) | 16.90 | 0.275 | 0.697 | 1.23 | 0.43–3.50 |
| Stage | | | | | | | | | | | | | | |
| IV | 75 (64.7) | 56 (74.7) | 7.13 | – | – | 1.00 | – | 88 (66.2) | 60 (68.2) | 22.10 | – | – | 1.00 | – |
| II–III | 41 (35.3) | 20 (48.8) | 13.07 | 0.053 | 0.242 | 0.73 | 0.43–1.24 | 45 (33.8) | 18 (40.0) | – | 0.004 | 0.017 | 0.57 | 0.36–0.91 |
| Histology | | | | | | | | | | | | | | |
| Adeno | 74 (63.8) | 45 (60.8) | 8.53 | – | – | 1.00 | – | 86 (64.7) | 48 (55.8) | 30.27 | – | – | 1.00 | – |
| Squamous | 35 (30.2) | 24 (68.6) | 5.93 | 0.243 | 0.203 | 1.39 | 0.84–2.30 | 43 (32.3) | 28 (65.1) | 18.07 | 0.190 | 0.069 | 1.61 | 0.96–2.70 |
| Others | 7 (6.0) | 7 (100.0) | 7.13 | 0.901 | 0.467 | 1.36 | 0.60–3.07 | 4 (3.0) | 2 (50.0) | – | 0.856 | 0.842 | 0.87 | 0.21–3.56 |
| Visceral metastasis number | | | | | | | | | | | | | | |
| 1 | 103 (88.8) | 66 (64.0) | 8.13 | – | – | 1.00 | – | 121 (91.0) | 69 (57.0) | 25.23 | – | – | 1.00 | – |
| >1 | 13 (11.2) | 10 (76.9) | 5.93 | 0.028 | 0.016 | 2.33 | 1.17–4.61 | 12 (9.0) | 9 (75.0) | 14.60 | 0.041 | 0.100 | 1.84 | 0.89–3.82 |
| NLR | | | | | | | | | | | | | | |
| <5 | 102 (87.9) | 65 (63.7) | 8.13 | – | – | 1.00 | – | 120 (90.2) | 67 (55.8) | 25.23 | – | – | 1.00 | – |
| ≥5 | 14 (12.1) | 11 (78.6) | 4.88 | 0.112 | 0.578 | 1.22 | 0.61–2.41 | 13 (9.8) | 11 (84.6) | 8.93 | 0.056 | 0.070 | 1.82 | 0.95–3.48 |
| PLR | | | | | | | | | | | | | | |
| <180 | 65 (56.0) | 33 (50.8) | 10.50 | – | – | 1.00 | – | 75 (56.4) | 40 (53.3) | 34.87 | – | – | 1.00 | – |
| ≥180 | 51 (44.0) | 43 (84.3) | 5.70 | 0.001 | 0.000 | 2.40 | 1.48–3.90 | 58 (43.6) | 38 (65.5) | 17.53 | 0.034 | 0.098 | 1.47 | 0.93–2.31 |
| D-2-dimer | | | | | | | | | | | | | | |
| <3 | 92 (79.3) | 59 (64.1) | 8.53 | – | – | 1.00 | – | 110 (82.7) | 64 (58.2) | 25.23 | – | – | 1.00 | – |
| ≥3 | 24 (20.7) | 17 (70.8) | 6.77 | 0.291 | 0.513 | 1.21 | 0.69–2.13 | 23 (17.3) | 14 (60.9) | 22.10 | 0.752 | 0.251 | 1.43 | 0.78–2.61 |

P values were calculated by univariate and multivariate Cox proportional hazards models. Adj, adjusted by visceral metastasis number and PLR for PFS analysis; adj[a], adjusted by smoking status and stage for OS analysis. NSCLC, non-small cell lung cancer; NLR, neutrophil-to-lymphocyte ration; PLR, platelet-to-lymphocyte ratio; Adeno, adenocarcinoma; CI, confidence interval; HR, hazards ratio.

other factors were accomplished by a Cox stepwise model with all known clinical characteristics (visceral metastasis number and PLR for PFS, smoking status and stage for OS).

### *Associations of CBLB SNPs with PFS and OS*

Associations of the three SNPs (rs3772534, rs2305035 and rs9657904) with PFS and OS were evaluated by using multivariate Cox models with adjustment for other selected factors (as shown in *Table 2*). However, we did not find any significant associations of either SNPs with PFS and OS (in a dominant genetic model for PFS, P=0.133, 0.498, and 0.935 for rs3772534, rs2305035, and rs9657904, respectively; in a dominant genetic model for OS, P=0.872, 0.741, and 0.780, respectively; in recessive model for PFS, P=0.266, 0.118, and 0.229, respectively; in a recessive genetic model for OS, P=0.403, 0.905, and 0.123, respectively).

### *Stratification analysis*

For assessing subgroup effects, we performed stratification analysis by clinical characteristics under both dominant and recessive genetic models. In the dominant genetic model, the results were shown in *Tables S2* and *S3*. Only the rs2305035 AG + AA genotype was associated with OS in age ≥65 patients adjusted by smoking status and stage. While in the recessive genetic model, the rs2305035 AA genotype was more prominently associated with PFS in females, never smoking, smoking index <400 or PLR <180 (*Table 3*). Furthermore, the associations between the rs9657904 CC genotype and PFS by age ≥65 or NLR ≥5 were more remarkable (*Table 3*) (*Figure 1A*). In OS subgroup analysis, the rs9657904 CC genotype was significantly associated with OS in male patients (adjusted HR =2.03, 95% CI, 1.04–3.98, P=0.038) (*Table 4*) (*Figure 1B*).

To provide functional evidence for the associations, we further evaluated the correlation between the potentially functional SNPs (rs9657904 and rs2305035) and CBLB mRNA expression levels using the public available mRNA expression data of 90 lymphoblastoid cell lines derived from eastern Asian population in HapMap (25). Consistent with the association results, the CC genotype of rs9657904 was shown to be associated with a relatively higher level of mRNA expression of CBLB, compared with the TT + TC genotypes (P=0.029) (*Figure 2A*). However, the genotype of rs2305035 was not associated with the CBLB mRNA expression (P=0.176) (*Figure 2B*).

### *FPRP analysis*

The FPRP values (26) were calculated at different prior probability levels for all significant findings (*Table 5*). As the assumption of prior probability was 0.25, the association of PFS with the AA genotype of rs2305035 was still noteworthy for patients with never smoking or smoking index <400 (FPRP =0.189, or 0.196). Meanwhile, the CC genotype of rs9657904 were also statistically associated with OS in male patients (FPRP =0.196).

## Discussion

In the present study, we investigated the associations between potential functional SNPs in CBLB and the OS and PFS of patients with advanced NSCLC treated with the first-line combined chemotherapy. We found that the rs2305035 AA genotype was associated with a worse PFS in patients who were female, never smoking, had a smoking index <400 or PLR <400; and the rs9657904 CC genotype was associated with a worse PFS in patients with age ≥65 or NLR ≥5. In OS analysis, males' patents with the rs9657904 CC genotype had a worse OS, compared with those males with rs9657904 TT + TC genotypes. The present study suggests that SNPs in CBLB might influence the PFS and OS of NSCLC patients with particular characteristics after the first-line combined chemotherapy in Chinese.

In our previously published study (21), we found that patients with AA/AG genotypes of rs2305035 had better clinical outcomes than GG carriers in Caucasian populations. However, such correlations were not found in the present study with Chinese patients. Such discrepancy might be due to ethnic difference, which had been reported to be one important confounder factor in association analysis (27,28). However, both studies suggested that SNPs in CBLB were associated with clinical outcomes in advanced NSCLC patients. In the present study, we had shown that the CC genotype of rs9657904 was associated with a higher level of CBLB mRNA and worse clinical outcomes in Chinese NSCLC patients with the first-line chemotherapy, which suggests that up-regulation of CBLB might influence the prognosis of NSCLC. Additional functional analyses are required to investigate the relationship between the prognosis of advanced NSCLC and Cbl-b expressions in tumor tissues.

 

**Table 2** Associations between the genotypes of CBLB and progress free survival (PFS)/overall survival (OS) in NSCLC patients

| Genotypes | PFS | | | | | | OS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total No. | Event No. (%) | P | Adj[a] P | Adj[a] HR (95% CI) | Median PFS (months) | No. of patients | No. of events (%) | P | Adj[b] P | Adj[b] HR (95% CI) | Median OS (months) |
| Overall | 115 | 75 (65.2) | – | – | – | – | 132 | 77 (58.3) | – | – | – | – |
| CBLB (rs3772534 G>A) | | | | | | | | | | | | |
| GG | 61 | 36 (59.0) | – | – | 1.00 (reference) | 6.70 | 85 | 51 (60.0) | – | – | 1.00 (reference) | 23.70 |
| AG | 52 | 38 (73.1) | 0.177 | 0.107 | 0.67 (0.41–1.09) | 8.53 | 44 | 25 (56.8) | 0.962 | 0.805 | 1.06 (0.66–1.72) | 25.23 |
| AA | 2 | 1 (50.0) | 0.192 | 0.302 | 0.34 (0.04–2.66) | 22.33 | 3 | 1 (33.3) | 0.407 | 0.387 | 0.42 (0.06–3.05) | – |
| P trend test | – | – | 0.080 | 0.069 | 0.66 (0.42–1.03) | – | – | – | 0.681 | 0.780 | 0.94 (0.62–1.44) | – |
| AG/AA | 54 | 39 (72.2) | 0.133 | 0.088 | 0.66 (0.41–1.06) | 8.73 | 47 | 26 (55.3) | 0.872 | 0.993 | 1.00 (0.62–1.61) | 25.23 |
| GG + AG | 113 | 74 (65.5) | 0.266 | 0.420 | 0.43 (0.06–3.31) | 7.57 | 129 | 76 (58.9) | 0.403 | 0.376 | 0.41 (0.06–2.98) | 24.63 |
| CBLB (rs2305035 G>A) | | | | | | | | | | | | |
| GG | 71 | 42 (59.2) | – | – | 1.00 (reference) | 8.13 | 72 | 41 (56.9) | – | – | 1.00 (reference) | 24.63 |
| AG | 37 | 26 (70.3) | 0.793 | 0.907 | 0.97 (0.58–1.62) | 7.13 | 51 | 31 (60.8) | 0.703 | 0.963 | 1.01 (0.63–1.62) | 22.53 |
| AA | 7 | 7 (100.0) | 0.114 | 0.072 | 2.22 (0.93–5.31) | 4.25 | 9 | 5 (55.6) | 0.971 | 0.655 | 1.24 (0.48–3.23) | 37.47 |
| P trend test | – | – | 0.256 | 0.332 | 1.22 (0.82–1.81) | – | – | – | 0.830 | 0.765 | 1.06 (0.73–1.54) | – |
| AA/AG | 44 | 33 (75.0) | 0.498 | 0.723 | 1.09 (0.67–1.76) | 7.07 | 60 | 36 (60.0) | 0.741 | 0.875 | 1.04 (0.66–1.63) | 25.23 |
| GG + AG | 108 | 68 (63.0) | 0.118 | 0.061 | 2.25 (0.96–5.25) | 7.87 | 123 | 72 (58.5) | 0.905 | 0.655 | 1.24 (0.49–3.14) | 23.70 |
| CBLB (rs9657904 C>G) | | | | | | | | | | | | |
| TT | 22 | 14 (63.6) | – | – | 1.00 (reference) | 7.07 | 29 | 18 (62.1) | – | – | 1.00 (reference) | 30.67 |
| CT | 61 | 39 (63.9) | 0.667 | 0.107 | 0.67 (0.41–1.09) | 8.47 | 76 | 41 (54.0) | 0.901 | 0.963 | 0.99 (0.76–2.86) | 24.63 |
| CC | 32 | 22 (68.8) | 0.542 | 0.302 | 0.34 (0.04–2.66) | 6.63 | 27 | 18 (66.7) | 0.240 | 0.254 | 1.47 (0.76–2.86) | 22.23 |
| P trend test | – | – | 0.460 | 0.069 | 0.66 (0.42–1.03) | – | – | – | 0.271 | 0.285 | 1.21 (0.85–1.72) | – |
| CT/CC | 93 | 61 (65.6) | 0.935 | 0.088 | 0.66 (0.41–1.06) | 7.87 | 103 | 59 (57.3) | 0.780 | 0.724 | 1.10 (0.65–1.88) | 23.30 |
| TT + CT | 83 | 53 (63.9) | 0.229 | 0.560 | 1.18 (0.67–2.07) | 8.13 | 105 | 59 (56.2) | 0.123 | 0.153 | 1.49 (0.86–2.56) | 26.33 |

P values were calculated by univariate and multivariate Cox proportional hazards models. [a], adjusted by visceral metastasis number and PLR for PFS analysis; [b], adjusted by smoking status and stage for OS analysis. NSCLC, non-small cell lung cancer; CBLB, casitas B-lineage lymphoma proto-oncogene-b; CI, confidence interval; HR, hazards ratio; PLR, platelet-lymphocyte ratio.

**Table 3** Stratification analysis of rs2305035 and rs9657904 associated with progress free survival (PFS) under recessive models by patients' characteristics

| Patients' characteristics | Event/total No. | rs2305035 | | | | | | rs9657904 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (GG + AG) event No. | AA event No. | P | Adj P | Adj HR | Adj 95% CI | (TT + CT) event No. | CC event No. | P | Adj P | Adj HR | Adj 95% CI |
| Overall | 76/116 | 68 | 7 | 0.118 | 0.061 | 2.25 | 0.96–5.25 | 55 | 19 | 0.229 | 0.560 | 1.18 | 0.67–2.07 |
| Sex | | | | | | | | | | | | | |
| Male | 50/75 | 46 | 3 | 0.583 | 0.214 | 2.61 | 0.58–11.80 | 36 | 13 | 0.149 | 0.138 | 1.76 | 0.84–3.69 |
| Female | 26/41 | 22 | 4 | 0.027 | 0.030 | 3.66 | 1.14–11.81 | 17 | 9 | 0.626 | 0.987 | 1.01 | 0.39–2.65 |
| Age (years) | | | | | | | | | | | | | |
| <65 | 55/86 | 50 | 4 | 0.459 | 0.087 | 2.98 | 0.85–10.43 | 40 | 14 | 0.752 | 0.748 | 0.89 | 0.45–1.78 |
| ≥65 | 21/30 | 18 | 3 | 0.163 | 0.219 | 2.31 | 0.61–8.81 | 13 | 8 | 0.056 | 0.037 | 3.27 | 1.07–9.98 |
| Smoking status | | | | | | | | | | | | | |
| Ever | 30/48 | 27 | 2 | 0.696 | 0.921 | 2.33 | 1.01–7.28 | 21 | 8 | 0.279 | 0.356 | 1.57 | 0.60–4.07 |
| Never | 46/68 | 41 | 5 | 0.006 | 0.008 | 3.82 | 1.42–10.24 | 32 | 14 | 0.536 | 0.968 | 0.99 | 0.48–2.01 |
| Smoking index | | | | | | | | | | | | | |
| <400 | 48/73 | 27 | 2 | 0.005 | 0.009 | 3.72 | 1.39–9.93 | 34 | 14 | 0.500 | 0.903 | 0.96 | 0.48–1.93 |
| ≥400 | 28/43 | 41 | 5 | 0.653 | 0.817 | 0.78 | 0.10–6.30 | 19 | 8 | 0.320 | 0.251 | 1.750 | 0.67–4.56 |
| Stage | | | | | | | | | | | | | |
| IV | 56/75 | 51 | 4 | 0.256 | 0.376 | 1.72 | 0.52–5.67 | 35 | 20 | 0.567 | 0.682 | 1.14 | 0.61–2.13 |
| II–III | 20/41 | 17 | 3 | 0.104 | 0.045 | 4.22 | 1.04–17.17 | 18 | 2 | 0.716 | 0.949 | 1.050 | 0.23–4.87 |
| Histology | | | | | | | | | | | | | |
| Adeno | 45/74 | 42 | 2 | 0.167 | 0.363 | 1.97 | 0.46–8.50 | 30 | 14 | 0.890 | 0.550 | 0.79 | 0.36–1.73 |
| Squamous | 11/35 | 19 | 5 | 0.802 | 0.438 | 1.61 | 0.48–5.40 | 18 | 6 | 0.162 | 0.212 | 1.97 | 0.68–5.74 |
| Others | 7/7 | 7 | 0 | – | – | – | – | 5 | 2 | 0.890 | 0.428 | 2.67 | 0.24–30.20 |
| Visceral metastasis number | | | | | | | | | | | | | |
| 1 | 66/103 | 59 | 7 | 0.085 | 0.062 | 2.24 | 0.96–5.24 | 50 | 16 | 0.423 | 0.392 | 1.29 | 0.72–2.31 |
| >1 | 10/13 | 9 | 0 | – | – | – | – | 3 | 6 | 0.698 | 0.390 | 0.500 | 0.10–2.43 |
| NLR | | | | | | | | | | | | | |
| <5 | 65/102 | 58 | 7 | 0.093 | 0.063 | 2.25 | 0.96–5.30 | 48 | 17 | 0.746 | 0.762 | 0.91 | 0.48–1.71 |
| ≥5 | 11/14 | 10 | 0 | – | – | – | – | 5 | 5 | 0.015 | 0.030 | 11.74 | 1.26–108.95 |
| PLR | | | | | | | | | | | | | |
| <180 | 33/65 | 29 | 4 | 0.044 | 0.033 | 3.27 | 1.10–9.69 | 26 | 7 | 0.874 | 0.629 | 0.80 | 0.33–1.96 |
| ≥180 | 43/51 | 39 | 3 | 0.723 | 0.645 | 1.40 | 0.33–5.89 | 27 | 15 | 0.071 | 0.208 | 1.61 | 0.77–3.39 |
| D-2-dimer | | | | | | | | | | | | | |
| <3 | 59/92 | 53 | 6 | 0.101 | 0.058 | 2.30 | 0.97–5.44 | 42 | 17 | 0.314 | 0.529 | 1.22 | 0.66–2.27 |
| ≥3 | 17/24 | 15 | 1 | – | – | – | – | 11 | 5 | 0.631 | 0.796 | 0.84 | 0.23–3.10 |

P values were calculated by univariate and multivariate Cox proportional hazards models. Adj, adjusted by visceral metastasis number and PLR for PFS analysis. NLR, neutrophil-to-lymphocyte ration; PLR, platelet-to-lymphocyte ratio; Adeno, adenocarcinoma; PFS, progression free survival; CI, confidence interval; HR, hazards ratio.

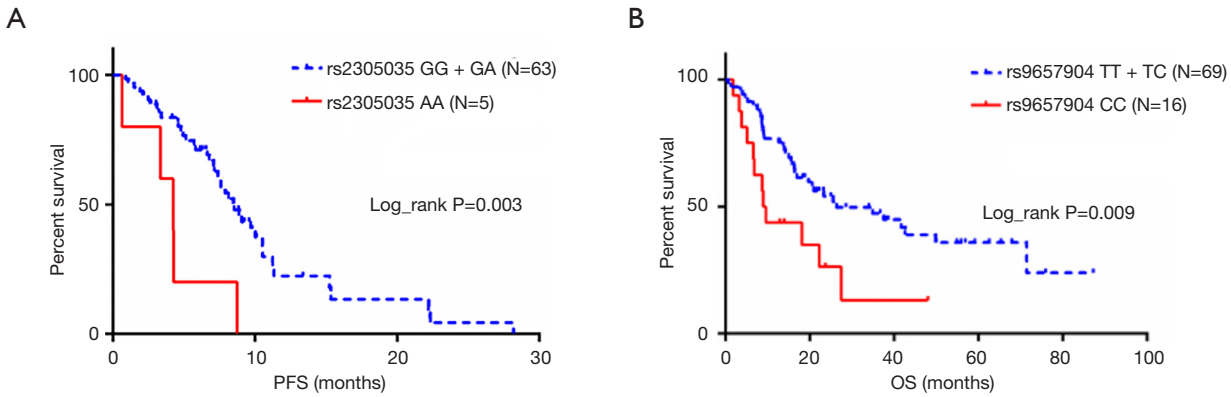


**Figure 1** Survival analyses by genotypes of rs9657904 and rs2305035. (A) PFS by genotypes of rs2305035 in never smoking patients; (B) OS by genotypes of rs9657904 in male patients. PFS, progression free survival; OS, overall survival.

Accumulating evidence has showed the prognosis of cancer patients and chemotherapy efficacy can be predicted by tumor immune micro-environment disparities, such as tumor-infiltrating lymphocytes (TILs) and T cells subsets. Adams *et al.* reported that stromal lymphocytic infiltration constituted a robust prognostic factor in triple-negative breast cancer; and higher stromal TIL scores were associated with better prognosis in multivariable analysis (29). Asano *et al.* found that tumor-infiltrating CD8 to FOXP3 lymphocyte ratio predicted treatment responses to neoadjuvant chemotherapy of aggressive breast cancer (4). Furthermore, maintenance of immune tolerance is a critical hallmark of the immune system. So far, CBLB appears to be a central player in balance activating and inhibitory inputs to immune cells (10). Several studies suggested silencing CBLB *in vivo* as a therapeutic strategy to target cancer (30,31). Besides, CBLB encodes a multifunctional adaptor protein in RING-family E3 ubiquitin ligase to negatively modulate the activation of T cell receptor (TCR) and B cell receptor (BCR) (32,33).

The identified SNP rs9657904 was reported to be associated with multiple sclerosis (MS), an auto-immune disease, in several genome wide association studies (GWAS) (34-36). In our findings, in male subgroup, the rs9657904 CC genotype of CBLB was associated with a worse OS, compared with the T genotypes, which was in consistence with studies of MS. In the previous studies (34), the allele T in rs9657904 (which is associated with an increased MS risk) was strongly associated with decreased expression of 234112_at, a probe that overlaps CBLB but is not part of its isoforms. From the above-mentioned evidence, it might be reasonable to hypothesize that decreased CBLB expression will enhance immune response by activating TCR and BCR. As the allele T of rs9657904 in the 5' upstream of CBLB was associated with lower CBLB expression possibly, it would influence the immune system by enhancing anti-tumor immune response. Noticeably, downregulation of Cbl-b in gastric cancer cells and leukemia cells can reduce the sensitivity of chemotherapeutics by activation of PI3K/Akt pathway (11-13). While, it is unclear that the effect of Cbl-b on the sensitivity of chemotherapy in lung cancer cells. Nevertheless, down-regulation of Cbl-b can strengthen T cell anti-tumor immune response (14,15,31,37). PI3K was identified as a substrate for Cbl-b. The activation of Cbl-b (–/–) T cells can be suppressed by PI3K inhibitor (38). It is inconsistent that Cbl-b has different role in tumor cells and T cells without clarification. Thereby, the functions of Cbl-b research focusing on tumor cells, anti-tumor immune system and chemotherapeutics' sensitivity should be conducted.

Similar to Cbl-b acting as a negative regulator in immune system, programmed death 1 (PD-1) on T cell surface and its ligand PD-L1 on tumor cell surface play a vital role in tumor immune escape. A recent study showed that overexpression of PD-L1 in NSCLC patients with surgery was associated with worse neoadjuvant chemotherapy efficacy. Furthermore, *in vitro*, the expression of PD-L1 was upregulated in cisplatin-resistance lung cancer cells by activation of the PI3K/Akt pathway; and knockdown of PD-L1 significantly reduced cisplatin resistances (39). Black *et al.* found that PD-1/PD-L1 axis can cause tumor cell resistance to chemotherapeutics. And inhibition of PD-1/PD-L1 by anti-PD-1 antibody strengthen doxorubicin

 

**Table 4** Stratification analysis of rs2305035 and rs9657904 associated with OS under recessive models by patients' characteristics

| Patients' characteristics | Event/ total No. | rs2305035 | | | | | | rs9657904 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (GG + AG) event No. | AA event No. | P | Adj P | Adj HR | Adj 95% CI | (TT + CT) event No. | CC event No. | P | Adj P | Adj HR | Adj 95% CI |
| Overall | 78/133 | 76 | 1 | 0.905 | 0.655 | 1.24 | 0.49–3.14 | 59 | 18 | 0.123 | 0.153 | 1.49 | 0.86–2.56 |
| Sex | | | | | | | | | | | | | |
| Male | 50/85 | 47 | 3 | 0.431 | 0.191 | 2.27 | 0.66–7.77 | 38 | 12 | 0.011 | 0.038 | 2.03 | 1.04–3.98 |
| Female | 28/48 | 25 | 2 | 0.521 | 0.712 | 0.76 | 0.17–3.32 | 21 | 6 | 0.555 | 0.626 | 0.80 | 0.32–1.99 |
| Age (years) | | | | | | | | | | | | | |
| <65 | 52/95 | 49 | 3 | 0.950 | 0.980 | 1.02 | 0.31–3.35 | 41 | 11 | 0.251 | 0.372 | 1.37 | 0.69–2.75 |
| ≥65 | 26/38 | 23 | 2 | 0.734 | 0.461 | 1.86 | 0.36–9.60 | 18 | 7 | 0.351 | 0.429 | 1.44 | 0.58–3.59 |
| Smoking status | | | | | | | | | | | | | |
| Ever | 40/62 | 39 | 1 | 0.904 | 0.453 | 2.20 | 0.28–17.21 | 32 | 8 | 0.064 | 0.190 | 1.71 | 0.77–3.79 |
| Never | 38/71 | 33 | 4 | 0.975 | 0.958 | 1.03 | 0.36–2.92 | 27 | 10 | 0.471 | 0.528 | 1.27 | 0.61–2.63 |
| Smoking index | | | | | | | | | | | | | |
| <400 | 42/78 | 37 | 4 | 0.924 | 0.940 | 1.040 | 0.37–2.96 | 31 | 10 | 0.567 | 0.543 | 1.25 | 0.60–2.61 |
| ≥400 | 36/55 | 35 | 1 | 0.927 | 0.517 | 1.97 | 0.25–15.43 | 28 | 8 | 0.079 | 0.243 | 1.630 | 0.72–3.68 |
| Stage | | | | | | | | | | | | | |
| IV | 60/88 | 58 | 2 | 0.307 | 0.537 | 0.64 | 0.15–2.68 | 45 | 15 | 0.453 | 0.270 | 1.40 | 0.77–2.53 |
| II–III | 18/45 | 14 | 3 | 0.185 | 0.131 | 2.730 | 0.74–10.01 | 14 | 3 | 0.383 | 0.300 | 1.990 | 0.54–7.27 |
| Histology | | | | | | | | | | | | | |
| Adeno | 48/86 | 43 | 4 | 0.567 | 0.433 | 1.53 | 0.53–4.43 | 36 | 11 | 0.442 | 0.302 | 1.45 | 0.72–2.95 |
| Squamous | 28/43 | 27 | 1 | 0.249 | 0.419 | 0.43 | 0.06–3.35 | 22 | 6 | 0.230 | 0.821 | 0.89 | 0.33–2.43 |
| Others | 2/4 | 2 | – | – | – | – | – | 1 | 1 | 0.999 | 1.000 | – | – |
| Visceral metastasis number | | | | | | | | | | | | | |
| 1 | 69/121 | 63 | 5 | 0.964 | 0.549 | 1.33 | 0.52–3.42 | 53 | 15 | 0.148 | 0.141 | 1.56 | 0.86–2.82 |
| >1 | 9/12 | 9 | – | – | – | – | – | 6 | 3 | 0.826 | 0.712 | 0.720 | 0.13–4.03 |
| NLR | | | | | | | | | | | | | |
| <5 | 67/120 | 62 | 4 | 0.878 | 0.421 | 1.53 | 0.54–4.34 | 52 | 14 | 0.286 | 0.229 | 1.46 | 0.79–2.70 |
| ≥5 | 11/13 | 10 | 1 | 0.565 | 0.481 | 0.39 | 0.03–5.31 | 7 | 4 | 0.438 | 0.470 | 0.560 | 0.11–2.72 |
| PLR | | | | | | | | | | | | | |
| <180 | 40/75 | 37 | 3 | 0.588 | 0.143 | 2.570 | 0.73–9.09 | 33 | 7 | 0.769 | 0.641 | 1.23 | 0.52–2.92 |
| ≥180 | 38/58 | 35 | 2 | 0.517 | 0.579 | 0.65 | 0.14–2.96 | 26 | 11 | 0.148 | 0.295 | 1.47 | 0.71–3.04 |
| D-2-dimer | | | | | | | | | | | | | |
| <3 | 64/110 | 58 | 5 | 0.961 | 0.578 | 1.31 | 0.51–3.39 | 49 | 14 | 0.383 | 0.342 | 1.35 | 0.73–2.48 |
| ≥3 | 14/23 | 14 | – | – | – | – | – | 10 | 4 | 0.015 | 0.106 | 3.17 | 0.78–12.81 |

P values were calculated by univariate and multivariate Cox proportional hazards models. Adj, adjusted by smoking status and stage. Adeno, adenocarcinoma; OS, overall survival; NLR, neutrophil-to-lymphocyte ration; PLR, platelet-to-lymphocyte ratio; CI, confidence interval; HR, hazards ratio.

 

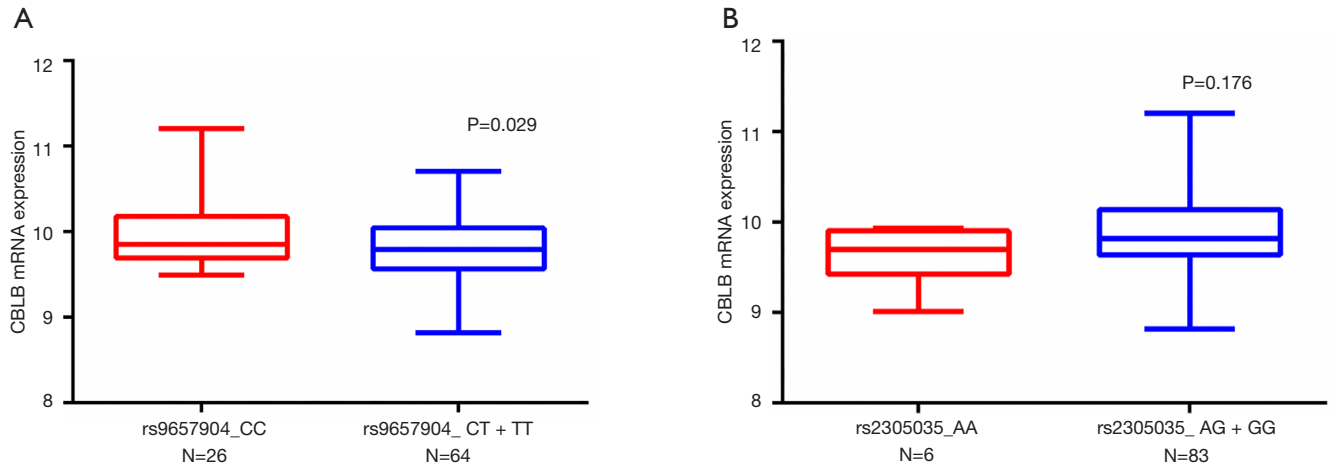


**Figure 2** Analyses of CBLB mRNA expression by genotypes of rs9657904 and rs2305035. (A) Associations between rs9657904 genotypes and CBLB mRNA expression under recessive model; (B) associations between rs2305035 genotypes and CBLB mRNA expression under recessive model. CBLB, casitas B-lineage lymphoma proto-oncogene-b.

**Table 5** False-positive report probability values for associations between the association of clinical outcomes in NSCLC and the frequency of genotypes

| Genotype | Positive HR (95% CI) | P | Statistical power | Prior probability | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 0.25 | 0.1 | 0.01 | 0.001 | 0.0001 |
| AA *vs.* GG + GA (rs2305035 recessive model) | | | | | | | | |
| PFS | | | | | | | | |
| Female | 3.66 (1.14–11.81) | 0.030 | 0.156 | 0.365 | 0.633 | 0.950 | 0.995 | 0.999 |
| Never smoking | 3.82 (1.42–10.24) | 0.008 | 0.099 | 0.189 | 0.412 | 0.885 | 0.987 | 0.999 |
| Smoking index <400 | 3.72 (1.39–9.93) | 0.009 | 0.108 | 0.196 | 0.422 | 0.889 | 0.988 | 0.999 |
| PLR <180 | 3.27 (1.10–9.69) | 0.033 | 0.188 | 0.342 | 0.610 | 0.945 | 0.994 | 0.999 |
| GG *vs.* AG + AA (rs2305035 dominant model) | | | | | | | | |
| OS | | | | | | | | |
| Age ≥65 | 3.08 (1.13–8.41) | 0.028 | 0.200 | 0.297 | 0.559 | 0.933 | 0.993 | 0.999 |
| CC *vs.* TT + TC (rs9657904 recessive model) | | | | | | | | |
| PFS | | | | | | | | |
| Age ≥65 | 3.27 (1.07–9.98) | 0.037 | 0.194 | 0.367 | 0.635 | 0.950 | 0.995 | 0.999 |
| NLR ≥5 | 11.74 (1.26–108.95) | 0.030 | 0.060 | 0.603 | 0.820 | 0.980 | 0.998 | 1.000 |
| OS | | | | | | | | |
| Male | 2.03 (1.04–3.98) | 0.038 | 0.483 | 0.196 | 0.423 | 0.890 | 0.988 | 0.999 |

NSCLC, non-small cell lung cancer; NLR, neutrophil-to-lymphocyte ration; PLR, platelet-to-lymphocyte ratio; PFS, progression free survival; OS, overall survival; CI, confidence interval; HR, hazards ratio.

to inhibit metastasis *in vivo* (40). According to the above evidences, immunotherapy targeting the immune response modulating genes might have synergistic effect with chemotherapy to eliminate tumor cells.

The present study has several limitations. First, this was a retrospective study and corresponding tumor tissues were not available for correlative studies of tumor-infiltrating immune cells. Secondly, it is still unclear about the underlying molecular mechanism of the association of rs2305035 and rs9657904 with clinical outcomes. Thirdly, the sample size of this study was relatively small, which might limit its power to detect other moderate effects and interaction effects on clinical outcomes, or lead to overestimation of the effect sizes of SNPs. Further larger and independent studies are warranted to verify our findings.

In conclusion, we found that the rs2305035 AA genotype and rs9657904 CC genotype of CBLB appeared to predict a shorter PFS in Chinese patients with some special characteristics and with advanced NSCLC treated with the first-line chemotherapy. In addition, the rs965790 CC genotype was associated with a worse OS in male patients. Finally, our findings suggest that CBLB has the potential to be a target of immunotherapy in future studies. It will be valuable and meaningful to clarify the comprehensive effects of CBLB on immune cells, tumor cells and chemotherapeutics' sensitivity.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The present study was approved by Henan Tumor Hospital's institutional review board (No. 20120755) in compliance with Helsinki Declaration. And in the retrospective study, patients' consents were waived.

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