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Prognostic utility of Multiplex ligation-dependent probe amplification in Acute T cell lymphoblastic leukemia /T lymphoblastic lymphoma

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Article

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Abstract

The clinical management of T-ALL/LBL remains challenging due to its genetic heterogeneity and variable outcomes. Integrating comprehensive genetic profiling, including CNAs and NGS mutations, is crucial for refining risk stratification.We retrospectively analyzed 66 adult T-ALL/LBL patients diagnosed between 2017 and 2021. Genetic profiling was performed using MLPA to detect key CNAs and NGS gene mutations. Clinical characteristics, OS and EFS were assessed.NGS mutations were identified in 90.9% (60/66) of patients, with NOTCH1 (46.96%), PHF6 (25.76%), and NRAS (18.18%) being the most frequent. MLPA revealed CNAs in 68.3% (41/60) of sequenced patients, with Del10q (22.73%) and Del17q (15.15%) being the most common. Multivariate analyses identified mutations in TET2 and NRAS, along with Del17q, as independent prognostic factors. A novel prognostic model was constructed by assigning one point for each of these risk factors, stratifying patients into low- (0 points), intermediate- (1 point), and high-risk (≥ 2 points) groups. This model significantly discriminated outcomes, with progressively worse OS and EFS across groups ($p < 0.05$). Subgroup analysis of 41 patients who underwent allogeneic hematopoietic stem cell transplantation confirmed that HSCT failed to improve the poor prognosis of the high-risk group defined by this model.Our study demonstrated the synergistic value of integrating MLPA and NGS for risk stratification in adult T-ALL/LBL.The developed prognostic model, based on TET2, NRAS, and del17q, effectively identifies a very-high-risk patient population with dismal outcomes even after allogeneic HSCT, facilitating early intervention and guiding novel therapeutic strategies.

Introduction

Acute T cell lymphoblastic leukemia /T lymphoblastic lymphoma(T-ALL/LBL)

- is an aggressive malignancy that accounts for 10%-15% of pediatric and 25% of adult ALL/LBL cases[1, 2].Management of T-ALL/LBL is clinically challenging as relapse is common and fatal. Prognosis in adult patients is inferior to pediatric patients with frequent relapse and poor response to therapy[3].Therefore, it is necessary to identify biomarkers for the efficient identification of high-risk patients and to design novel therapies. T-ALL/LBL is a highly heterogeneous disease, and molecular features such as mutation patterns, DNA copy number, cytogenetics, epigenetics, and gene expression vary widely among individuals. While extensive heterogeneity in molecular alterations in T-ALL/LBL makes it challenging for researchers and clinicians to effectively utilize molecular features in clinics, nevertheless, some major subgroups have been identified and efforts are underway for clinical implementation[4].
- Distinct subtypes of T-ALL/LBL have been identified based on immunophenotype, which can also be determined using gene expression profiling (GEP)[5–6].Recent studies have shown that copy number alteration (CNA) are common in all types of leukemia including T-ALL/LBL, especially the genes involved in transcription, cell cycle regulations, and T-cell differentiation.[7–10] Previous studies have suggested that the T-ALL/LBL genome exhibits frequent mutations and CNA in CDKN2A/2B cell cycle regulators and NOTCH1[11].CNAs could be helpful in refining the T-ALL risk stratification in future. In addition, the presence of other factors such as additional molecular

alterations including epigenetic modifications, and apoptosis or other cytogenetic aberrations may contribute to complicating the patient prognosis. This reinforces the need to analyze the combined possible alterations in a single assay. The aim of our study was to evaluate the utility of Multiplex Ligation Dependent Probe Amplification (MLPA) in the determination of key CNA in T-ALL/LBL. Further, we assessed prognostic significance of these CNAs and determinedtheir mutual associations, which highlighted their utility in risk stratification of T-ALL.

Methods

Patients and samples

A total of 93 patients from January 2017 to December 2021 were retrospectively enrolled and were diagnosed with T-ALL/T-LBL according to the World Health Organization(WHO) Criterion 2016. Bone marrow samples at initial diagnosis of these patients were obtained.After excluding patients with incomplete clinical data or insufficient biological samples, 66 patients were included in the final statistical analysis. Clinical characteristics, including age, gender, white blood cell count at diagnosis, immunophenotype, and treatment regimen were collected from medical records. Patients were given the informed consent to genetic analyses and laboratory data for research studies. The study was approved by the Medical Ethics Committee of Tianjin Cancer Institute and Hospital.

Cytogenetic analysis

Cytogenetic studies using standard G-banding techniques on heparinized BM samples were performed as part of the diagnostic work-up. At least 20 metaphase cells were analyzed whenever possible. Clonal abnormalities were defined as 2 or more cells with the same chromosomal gain or structural rearrangement or at least 3 cells with the same chromosomal deletion. Chromosome identification and karyotype descriptors used the International System for Human Cytogenetic Nomenclature (ISCN)

Targeted next generation sequencing and data analysis

The targeted sequencing panel covered 267 common genes in hematologic malignancies and the sequencing data was processed into vcf files with the reference genome hg19 in the Clinical Testing Center. ANNOVAR software was utilized for variants annotation. Gene mutations were filtered based on the following criteria: a. sequencing depth greater than or equal to 150×; b. mutant reads greater than or equal to 15×; c. mutation frequency greater than or equal to 1%; d. mutations located in exons or splicing sites; e. mutation frequency in healthy populations [The 1000 Genomes Project (1000G), The Genome Aggregation Database (gnomAD)] less than 0.005. All the above candidate mutation sites were then filtered using Integrative Genomics Viewer (IGV) to eliminate false positives. Mutation spectrum was conducted using the R package Maftools.

Multiplex ligation-dependent probe amplification (MLPA)

Bone marrow specimens were collected from patients at diagnosis. Genomic DNA was extracted using the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen Biosciences, cat no. AP-MN-BL-GDNA-250 Union city, CA, USA). Fifty nanograms of gDNA were subjected to MLPA analysis by using SALSA MLPA P414-A1 T-LBL/ALL Aprobe mix (MRC-Holland, Amsterdam, Netherlands). The probe mix contained 46 probes targeting at chromosomal regions of interest in T-LBL/ALL and 12 internal reference gene probes targeting at regions that are generally unchanged in T-LBL/ALL.MLPA reactions including internal quality controls and negative controls were performed according to the manufacturer instructions. The PCR products were analyzed using ABI 3130XL Genetic analyzer (Applied Biosystems, Foster City, CA, USA) and Coffalyser.net software (MRC Holland, Amsterdam, Netherlands) according to the manufacture instruction. In addition, 10 DNA samples derived from the peripheral blood of healthy donors were subjected to MLPA analysis. The “Mean±2SD” (95% CI, P = 0.05) and “Mean±3SD” (95% CI, P = 0.01) values for each individual probe. To improve the evaluation of the results with a larger CI, the “Mean±3SD” reference range was used as the cutoff value for CNA determination in our study.

Statistical analysis

Categorical variables were compared using the Chi-square test or Fisher's exact test, as appropriate. Continuous variables were compared using the Student's t-test or Mann-Whitney U test. Overall survival (OS) and event-free survival (EFS) were estimated using the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate Cox proportional hazards models were used to identify prognostic factors. All statistical analyses were performed using SPSS Statistics 21, and a two-sided p-value < 0.05 was considered statistically significant.

Results

Characterization of patients

- As shown in Fig. 1, among the 93 enrolled T-ALL/LBL patients, 66 were eligible for statistical analysis. Regarding NGS, mutations were detected in 60 patients (90.9%, 60/66,) and MLPA identifies copy number changes in 41 (68.3%, 41/60) patients, while the remaining 19 showed no CNAs. Conversely, among the 6 mutation-negative patients, 2 were found to have CNAs via MLPA.Characteristics of 66 patients were showed in Table 1, there were 44 males and 22 females,and the most common subtype is T-LBL.We calculated the International Prognostic Index (IPI) and confirmed that 42 patients were high risk, disease.Forty-one patients underwent allogeneic hematopoietic stem cell transplantation.

Table 1
Characteristics of T-ALL/LBL patients in our study (n = 66)

Sex	**Total n(%)**
male female	44 (66.7%)
	22 (33.3%)
Age at diagnosis (years)	
median range	32
	19–74
Immunophenotype	
pro-T	23 (34.9%)
pre-T cortical	12 (18.2%)
LBL	1 (1.5%)
Mixed phenotype	27 (40.9%)
	3(4.5%)
Risk group	
no data	3 (4.5%)
HR	21 (31.9%)
non-HR	42 (63.6%)
WBC (10^9/L)	
median range	73.5
	3.9–765.1
Mediastinal tumour	36 (54.5%)
Bone marrow involvement	100 (100%)
Central nervous system involvement	16 (24.2%)
HSCT	
No	24(36.4%)
ASCT	1(1.5%)

HR: high risk; nonHR: non-high risk; WBC: white blood cell count; CR1-first complete remission; CR2-second complete remission

Sex	Total n(%)
Allo-SCT	41(62.1%)
Outcome	
CR1	29 (43.9%)
CR2	3 (4.5%)
Exitus	34 (51.5%)
Follow up (months)	
median range	57.6
	40.5–90.8
HR: high risk; nonHR: non-high risk; WBC: white blood cell count; CR1-first complete remission; CR2-second complete remission	

Frequency distribution and survival analysis of NGS in T-ALL/LBL

- Survival analyses were performed for all patients, with a follow-up period ranging from 46 to 89 months (mean follow-up: 56.3 months). The median OS was 48 months, while the median EFS was not reached. The 5-year OS and EFS rates were 46.64% and 52.65%, respectively(Fig. 1).

Next-generation sequencing (NGS) results from 66 patients revealed the most frequently mutated genes were NOTCH1 (31/66,46.96%), PHF6(17/66,25.76%), and NRAS(12/66, 18.18%)(Fig. 2A).Comparative survival analysis between patients with and without detected mutations demonstrated that those lacking mutations had a significantly better prognosis.As shown in Fig. 2B and 2C, patients with mutations (n = 60) had significantly inferior OS and EFS compared to those without(n = 6) (median OS: 35 months vs not reached, log-rank P < 0.05;median EFS: 35 months vs not reached, log-rank P < 0.05). Our analysis of the effect of mutation count on survival outcomes revealed no significant association between the number of mutations and patient prognosis, with no statistical significance observed as shown in Fig. 2D and 2E. Patients with different numbers of mutations (no mutation, n = 6，1 mutation,n = 4，2 mutation,n = 8，≥3 mutation, n = 48)

Copy number variations and survival analysis of MLPA in T-ALL/LBL

MLPA results from 66 patients, del 10q(15/66, 22.73%), and del 17q(10/66, 15.15%). Statistical analysis of CNAs demonstrated that among the cohort, 22 patients (33.3%) showed no genomic alterations,

whereas 18 (27.3%) and 15 (22.7%) patients harbored one and two CNA abnormalities, respectively（Fig. 3).

Survival analysis revealed that while the presence of CNA showed no statistically significant correlation with prognosis, a trend toward better outcomes was observed in patients without detectable CNA. As illustrated in Fig. 3, patients with CNAs (n = 22) demonstrated significantly inferior OS and EFS compared to those without CNAs (n = 44) (median OS: 29 months vs not reached; median EFS: 22 months vs not reached). Regarding the impact of CNA burden on survival outcomes, our analysis indicated no significant association between the number of CNAs and patient prognosis (EFS/OS) (Fig. 3). No statistically significant differences were observed among groups with varying CNA loads (no CNA, n = 22; 1 CNA, n = 18; 2 CNAs, n = 15; ≥3 CNAs, n = 11).

Survival analysis of MLPA and NGS

- Survival analysis of individual mutations revealed that mutations TET2、NRAS and KMT2D were significantly associated with patient prognosis in our cohort (P < 0.05).Survival analysis of individual CNAs revealed that Del 17q significantly associated with patient prognosis in our cohort (P < 0.05,Fig. 4) .

Both NGS and MLPA results were included in our analysis, and we also performed a multivariable analysis(Table 2),demonstrating significant associations between factors TET2,NRAS,Del 17q and clinical outcomes (all P < 0.05). Based on these findings,we established a prognostic scoring system assigning 1 point for each risk factor, categorizing patients into:Low-risk (0 points), Intermediate-risk (1 point), High-risk (≥ 2 points).Survival analysis validated this stratification, showing statistically significant separation between risk groups (P < 0.05) with progressively worse outcomes across groups(Fig. 5A,B).

Table 2
Multivariate analysis of OS and EFSin 66 T-ALL/LBL patients

	OS			**EFS**		
	Multivariate			**Multivariate**		
	HR	**95%CI**	***P***	**HR**	**95%CI**	***P***
NRAS	29.35	3.385 to 55.31	0.028	34.52	6.682 to 62.35	0.0167
KMT2D	10.94	-10.25 to 32.13	0.301	10.65	-12.07 to 33.37	0.3469
TET2	20.61	-6.06 to 50.29	0.039	22.23	-5.51 to 58.97	0.0394
Del 17q	21.65	-4.071 to 41.38	0.043	23.65	-5.715 to 43.01	0.0288

Subgroup Analysis of Allogeneic stem cell Transplantation Patients

Our subgroup analysis of patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT) demonstrated that HSCT significantly improved clinical outcomes. Among the 66 patients, we conducted subgroup analyses stratified by transplantation status (transplanted vs. non-transplanted). The median follow-up duration was 57.6 months (range: 40.5–90.8months). The 5-year OS rate was 69.3%, and the 5-year EFS rate was 69%. The transplantation group (n = 42) showed superior results compared to the non-transplantation group (n = 24), with median OS: 29 months vs not reached (P<0.001) and median EFS: 22 months vs not reached (P<0.001)(Fig. 5C□5D).

Among the 42 transplanted patients, 41 underwent allo-HSCT. When applying our prognostic stratification system to the HSCT subgroup, we observed that HSCT failed to improve outcomes in patients presenting with more than two risk factors(Fig. 5E□5F).

Discussion

In this study, we comprehensively analyze different molecular methods for prognosis guidance of T-ALL/LBL patients at different stages based on clinical features and genetic abnormality. Additionally, we establish an MLPA and NGS score model that assists the prognosis of T-ALL/LBL patients by molecular characteristics.The molecular landscape of T-ALL/LBL is characterized by remarkable heterogeneity, driven by a complex interplay of chromosomal structural variations, CNAs, and pointmutations affecting key signaling pathways and transcriptional regulators.While conventional cytogenetics and FISH have been foundational, their resolution is limited[12].The integration of MLPA and NGS has therefore become instrumental in advancing the field, with each technology offering unique and complementary strengths[13–14].

MLPA maintains a vital role in the diagnostic workup of T-ALL/LBL.Liu Y, et al [15] showed that the most frequent CNA is the 9p21.3 locus, and this CNA encompassed the CDKN2A/CDKN2B genes. and its homozygous deletion is a strong marker of poor prognosis, enabling refined risk stratification from diagnosis.The value of MLPA in detecting deletions in key tumor suppressor genes like PTEN(10q23.31), and RB1(13q14.2) is well-documented and provides rapid prognostic stratification For instance, the co-occurrence of NOTCH1(9q34.3)/FBXW 7 mutations with CDKN2A (9p21.3) deletion is linked to a favorable prognosis[16], while PTEN (10q23.31) deletions may indicate a more aggressive disease and resistance to therapy[17]A unique strength of MLPA is its ability to detect therapeutically relevant amplifications. The NUP214-ABL1fusion gene, present in 4–6% of T-ALL/LBL cases, often exists on amplified episomes. MLPA probes for the ABL1 gene readily identify this amplification, serving as a surrogate marker. This is clinically paramount, as it identifies patients who may benefit from adding tyrosine kinase inhibitors (e.g., dasatinib) to their chemotherapy, significantly improving outcomes[18].T-ALL is classified into subtypes based on oncogenic transcription factors (e.g., TAL1, TLX1). These subtypes are frequently associated with characteristic secondary CNAs. By corroborating this CNA profile, MLPA provides supporting evidence for molecular subtyping, which carries intrinsic prognostic significance and can guide therapeutic intensity [19].

NGS technologies provide an unbiased, comprehensive view of the T-ALL/LBL genome, overcoming the limitations of targeted approaches. The most significant recent contribution of NGS lies in refined risk stratification. A pivotal 2023 study by the GRAALL group defined a novel molecular classification by sequencing 72 genes in adult T-ALL patients.This model identified a very-high-risk subgroup characterized by mutations in RAS/PTEN or JAK/STAT pathways, which had a staggering 5-year relapse rate of 51%. Crucially, when this NGS-based classification was combined with MRD and WBC count, it stratified patients into groups with dramatically different outcomes: a favorable group with 93% overall survival and an adverse group with only 53% survival[20]. Beyond risk stratification, NGS is critical for identifying therapeutically actionable lesions. It reliably detects fusions involving ABL-class genes (ABL1, ABL2, CSF1R, PDGFRB), JAK2, and FLT3, enabling the use of tyrosine kinase inhibitors. Furthermore, NGS reveals mutations targetable by experimental agents, such as those in NOTCH1 (amenable to γ-secretase inhibitors) and the PI3K/AKT/mTOR pathway[21].NGS also illuminates the dynamic process of clonal evolution driving relapse. By comparing diagnostic and relapsed samples, NGS uncovers the emergence of resistant subclones with mutations in NT5C2 or PRPS1, providing insights for designing salvage therapies[22].

In our study, for the results of MLPA,the most frequent CNA in recent stduy is the 9p21.3 locus, which was consistent with our study.While our result revealed the most frequently CNA proportion were 22.73%,lower than their result(70%), Perhaps it was related to the small sample size we included in our study. Moreover, there were no significant difference in prognosis between patients with the CDKN2A /CDKN2B (9p21.3) genes, PTEN(10q23.31) ,and RB1(13q14.2) and those without them and this might be because allogeneic transplantation has improved the adverse prognosis caused by this indicator. Survival analysis of individual CNAs revealed that Del 17q significantly associated with patient prognosis in our cohort. For the results of NGS, the most frequently mutated genes in big study[20] were NOTCH1(77%), CDKN2A(66%), PHF6(50%), BCL11B(22%), DNM2 (20%), and FBXW7(19%).While in our study, the most frequent mutations were NOTCH1 (46.96%), PHF6(25.76%), and NRAS(18.18%), Perhaps this is because our study participants were mainly adults, while the main participants of the research were children, because our results were consistent with research on adults [23] .Survival analysis of individual mutations revealed that mutations TET2,NRAS, and KMT2D were significantly associated with patient prognosis in our cohort. However, the 2023 European Leukemia Net ALL guidelines propose that in adult T-ALL, unfavorable prognostic factors include the absence of NOTCH1 and FBXW7 mutations, as well as the presence of RAS or PTEN abnormalities. Our study results indicated that while the survival analysis for PTEN did not reach statistical significance, the curves showed a distinguishable separation. This suggests that with a larger sample size, statistical significance might be achieved. Additionally, no statistically significant difference in survival was observed between patients with NOTCH1/FBXW7 mutations and those without these mutations. This may indicate that allogeneic hematopoietic stem cell transplantation could mitigate the poor prognosis associated with the absence of NOTCH1 and FBXW7 mutations to some extent.

Even more encouragingly, we have developed a prognostic model that can effectively stratified patients into distinct risk groups. Subsequent statistical analysis of the patient subgroup undergoing allogeneic

transplantation revealed that those classified as high-risk by our model continued to demonstrate poor prognosis. This indicates that allogeneic transplantation may not overcome the unfavorable outcomes predicted for this high-risk group, thereby enabling earlier clinical identification of patients with particularly dismal prognosis.

The transition of these advanced diagnostics into clinical practice is already shaping a new era of precision medicine for T-ALL/LBL. The ability to identify high-risk patients at diagnosis allows for the upfront allocation of intensified or novel therapies.

In modern diagnostic laboratories, MLPA and NGS are not competitors but essential, synergistic partners. A proposed integrated workflow is highly effective: Rapid First-Line Screening with MLPA: MLPA can be used as a rapid, cost-effective first-line screen for the most prognostically significant CNAs and, with emerging MS-MLPA kits, for defining epigenetic subgroups.Comprehensive Profiling with NGS: In parallel, NGS (using targeted panels, whole exome, or whole genome sequencing) provides a comprehensive mutational profile, identifying cooperative mutations and novel fusions.

Declarations

Ethics Statement

We have strictly adhered to the ethical principles concerning human participants in this research endeavor. Before their engagement in the study, all participants were comprehensively briefed about its objectives, methodologies, potential risks, and possible benefits. Moreover, they were duly apprised of their right to voluntary participation and the liberty to withdraw from the study at any given time without encountering any adverse repercussions.subsequently, following the assurance of complete comprehension from each participant, we obtained written informed consent documents duly signed by all individuals involved. Furthermore, we shall be unwavering in upholding the confidentiality and privacy of the participants, utilizing the data exclusively for research purposes, thus ensuring the impervious protection of their personal identity information against any potential disclosure. this study has garnered approval from Medical Ethics Committee of Tianjin Cancer Hospital Airport Hospital on 19 December 2025 (IREC number: LWK-2025-0248).

Conflicts of Interest

Jing Ma,Juan Sun, Qian Li, Chen Tian, Hongliang Yang, Yong Yu, Xiao fang Wang, Haifeng Zhao, Zeng Cao, Xiaofei Ai, Zhigang Zhao, and YafeiWang declare that they have no conflict of interest.

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Author Contribution

MJ and ZZG were involved in creating the study protocol; MJ and SJ were involved in the process of the data collection. MJ、TC、YHL and YY performed statistical analysis and data interpretation; MJ、LQ、CZ and ZZG involved in preliminary data design and assessment, and literature review. WXF and ZHF drafted the manuscript. CZ and ZZG provided a critical revision and regular feedback of the manuscript. All authors contributed to the refinement of the study protocol. All authors have read and approved the final manuscript.

Data Availability

Anonymized data are available upon reasonable request.

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Figures

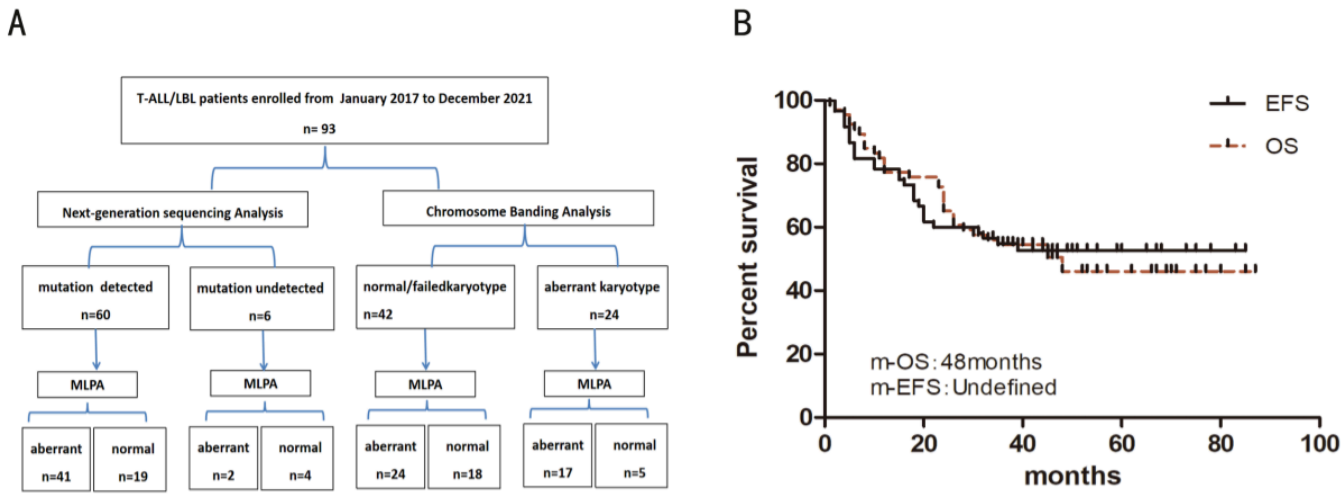


Figure 1

A.Distribution of patient cases categorized by various detection methodologies. B.Survival analysis of all patients enrolled in our study.

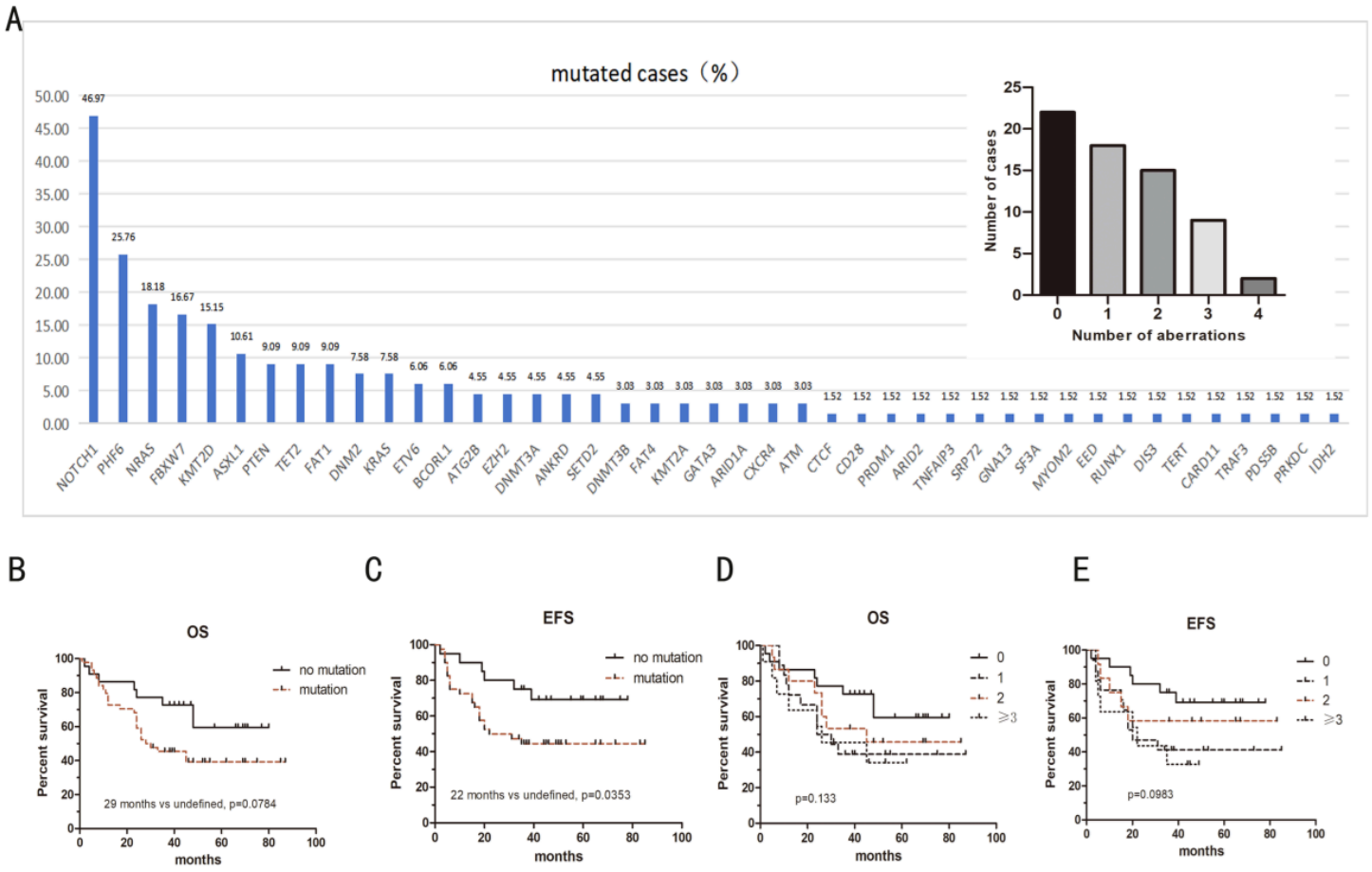


Figure 2

A.Next-generation sequencing (NGS) results of 66 patients.Overall Survival（B）and Event Free Survival（C） of patients with mutations and no-mutations.Overall Survival（D）and Event Free Survival（E）of patients with different numbers of mutations .

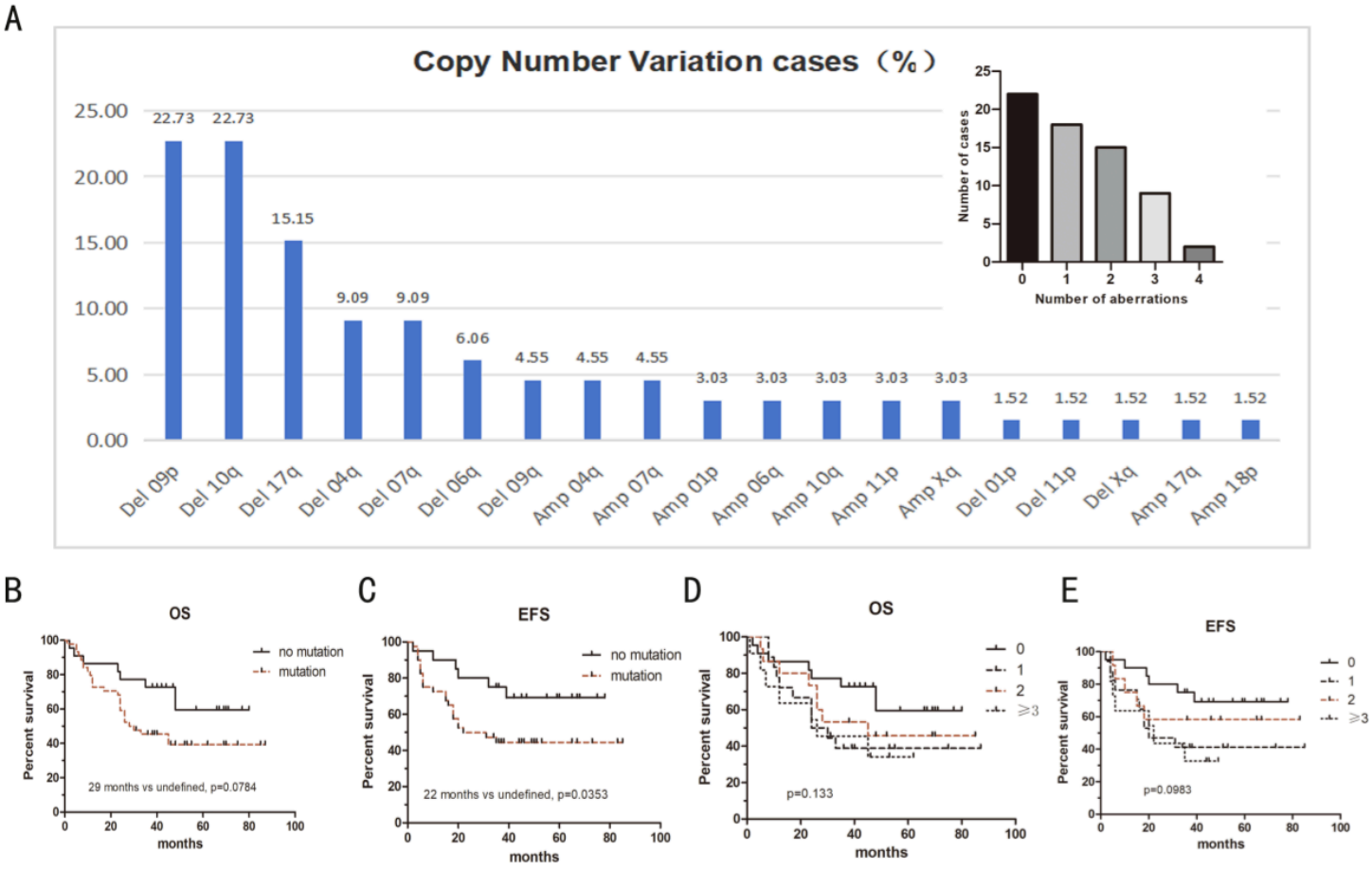


Figure 3

A.Copy number alteration results of 66 patients.Overall Survival（B）and Event Free Survival（C）of patients with copy number variations and no-copy number variations.Overall Survival（D）and Event Free Survival（E）of patients with different numbers of copy number variations.

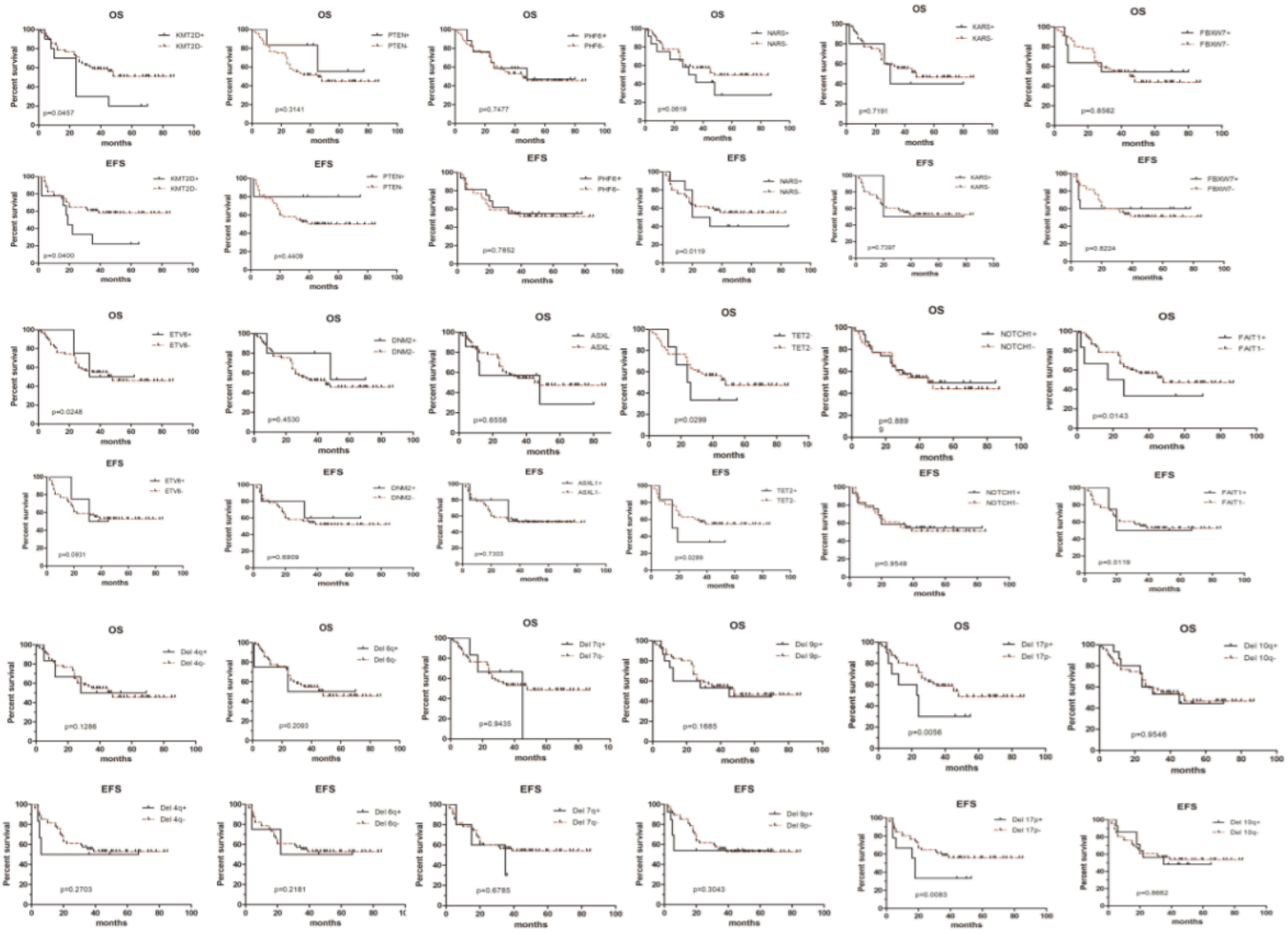


Figure 4

Survival analysis of individual mutations and CNAs.

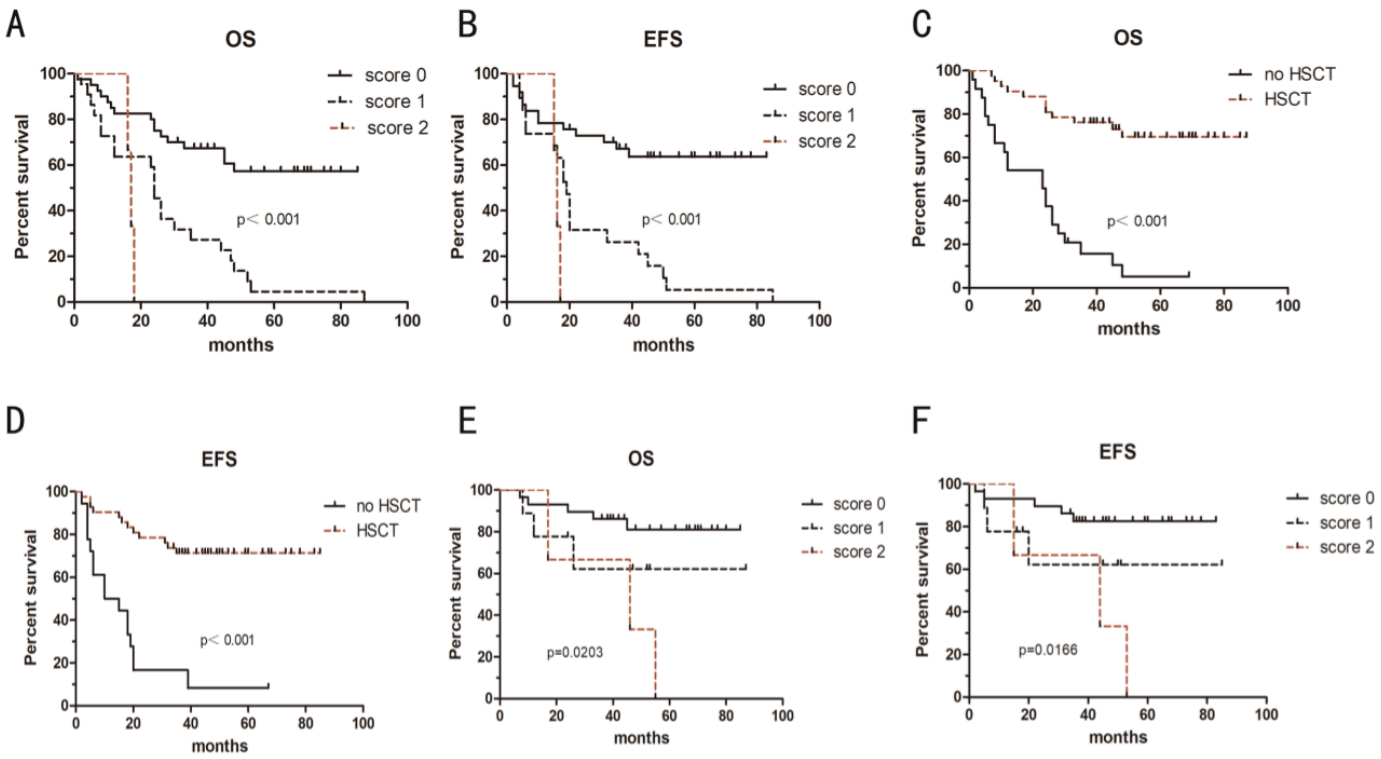


Figure 5

Sixty-six patients were stratified according to the results (TET2,NRAS,Del 17q) and we acquired by prognostic analysis and each index was one score, OS（A）and EFS（B）of different risk groups.Survival analysis（OS （C）（EFS（D）） of patients with versus without allogeneic transplantation. Forty-two patients with allogeneic stem cell transplantation were stratified according to the results (TET2,NRAS,Del 17q) and we acquired by prognostic analysis and each index was one score.OS（E）and EFS（F）of different risk groups.