Comparison of multi-omics results between patients with acute myeloid leukemia with long-term survival and healthy controls

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Background: Acute myeloid leukemia (AML) is a group of highly heterogeneous diseases, for which approximately 35–40% of patients younger than 60 years old can be cured. However, the multi-omics characteristics and immune cell infiltration (ICI) status of adult long-term survival patients with AML patients compared with healthy controls are still relatively under-explored.
Methods: A total of 10 healthy transplant donors (control group) and 11 long-term survival patients with AML with *de novo* sampling from 2019 to 2020 at the Institute of Hematology in the Hospital of Blood Diseases were enrolled. We simultaneously performed 850 K methylation and bulk RNA-seq on these 21 patients for comparing the differential gene methylation and expression levels between the two groups. The analysis of immune cell gene expression was based on 4 algorithms single sample gene set enrichment analysis (ssGSEA), EPIC, ESTIMATE and immunophenotype score (IPS) on the bulk RNA-seq data.
Results: The differential methylation positions (DMPs) of the control group was significantly higher than that of the long-term survival group (P<0.01). The hypomethylated probes for Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment is summarized as follows: the significant pathway was related to NK-cell-mediated cytotoxicity and amino acid metabolism. We also found the Differential expression genes (DEGs) of long-term survival AML were roughly similar, and the DEGs were highly relevant to the cellular amino acid metabolic process pathway by gene set enrichment analysis (GSEA). Based on the further univariate and multivariate Cox survival analyses in GSE37642, genes crosslinked of DEGs and DMPs: LOXL1 and PDZRN4, which characterized as hypomethylated and upregulated, may become an AML prognostic marker (P<0.05). Besides, compared with the long-term-survival AML patients who discontinued chemotherapy after >3 years and the healthy donors, T cell-, natural killer cell-, MHC- and effector cell (EC)-related genes were downregulated; suppressor cells (SC) and checkpoint (CP) cells were significantly upregulated in the long-term-survival AML patients who discontinued chemotherapy after <3 years.
Conclusions: In terms of DNA methylation, RNA expression and ICI, AML patients with long-term survival were slightly different than that of healthy people. The profile of long-term AML survivors, especially those who discontinued chemotherapy less than 3 years, still differed from that of healthy people.

Keywords: Acute myeloid leukemia (AML); immune cell infiltration (ICI); long-term survival; RNA-seq

Introduction

Acute myeloid leukemia (AML) is a group of highly heterogeneous diseases, for which approximately 35–40% of patients younger than 60 years old can be cured (1). Clinically, patients with AML in complete remission (CR) for >3 years and measurable/minimal residual disease (MRD)-negative can be considered as 'long-term survival patients' (1).

The concept of immune cell infiltration (ICI) was derived from quantitative assessment of increased tumor-infiltrating lymphocytes (TILs), which is highly related to the tumorigenesis, development, metastasis and mortality of solid tumors (2).

Great effort has been made to explore the significant markers associated with an unfavorable prognosis of AML patients (3,4). Recently, Wang *et al.* provided an immune risk score model for predicting the survival of patients with AML. The immune cells enumerated by CIBERSORT algorithm: monocytes, resting mast cells, eosinophils, CD8 + positive T cells, resting NK cells, resting dendritic cells and resting memory CD4 + T cells were the most representative cells in the bone marrow microenvironment of AML (3). Dong *et al.* integrated the multi-omics in terms of drug sensitivity, signaling pathways and ICI in AML from the viewpoint of public transcriptional profile mining (4).

However, no one has evaluated the immune cell proportions in different AML status. To improve knowledge of the characteristics of adult long-term survival patients with AML, we mainly focused on the multi-omics (DNA methylation and gene expression) difference of long-term survival AML population and healthy donors. In addition, the ICI status of AML patients compared with healthy controls were explored.

We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6681/rc).

Methods

Patients

A total of 10 healthy transplant donors (control group) and 11 long-term survival patients with AML with *de novo* sampling from 2019 to 2020 at the Institute of Hematology in the Hospital of Blood Diseases were enrolled. The long-term survival patients were divided into two groups: those who discontinued chemotherapy after >3 or <3 years.

Written informed consent was given by all patients, who were in sustained remission for >3 years until the last follow-up on August 30, 2021. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional committee of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences (No. KT2018091-EC-2).

DNA methylation and RNA-seq analysis

We simultaneously performed 850 K methylation and bulk RNA-seq for these 21 study subjects. R packages 'ChAMP' and 'DEseq2' were used for differential analysis. We defined genes with |log FC| ≥1 and adjusted P<0.05 as differential expression genes (DEG). Moreover, genes with |delta β| ≥0.1 and adjusted P<0.05 were designated differential methylation positions (DMP).

ICI analysis

Based on the bulk RNA-seq data, a reference was made based on the definition of 27 immune cell types in solid tumor (2). The single sample gene set enrichment analysis (ssGSEA) (4), CIBERSORT (5) and EPIC (6) algorithms were used to quantify the immune cell-related gene expression in the bone marrow microenvironment, and cell types irrelevant to AML immunity (i.e., "Blood vessels", "Normal mucosa", "SW480 cancer cells" and "Lymph vessels") were excluded. ESTIMATE (7) was used to evaluate the immune and stromal contents of AML bone marrow environment.

The immunophenotype score (IPS) algorithm (8) is a panel of immune cell-related genes used to predict the immunotherapy response and consists of four clusters: major histocompatibility complex (MHC)-related molecules, effector cells (EC), suppressor cells (SC) and checkpoint cells (CP). The z-score for each sample was calculated by the weighted averaged z-score of four cluster values requiring the corresponding gene expressions and their weights.

Gene Expression Omnibus (GEO) dataset mining

GEO database RNA-seq expression data (accession No. GSE 37642) through PubMed was used for selected gene survival analysis. Totally, 531 AML patients with clinical information and RNA expression were extracted. The expression profile fragments per kilobase per million was

Table 1 Clinical characteristics of study cohort

No.	Sex	Age (years)	FAB	Outcome	Stop chemo till sampling time (months)	DFS till sampling time (months)	OS (months) until now
1	M	39	M2	Alive	36	42	68
2	F	61	M2	Alive	36	42	67
3	M	52	M5	Alive	36	42	76
4	F	39	M4	Alive	45	54	79
5	F	26	M1	Alive	48	56	80
6	M	58	M5	Alive	50	58	83
7	F	40	M2	Alive	37	43	71
8	M	67	M5	Alive	31	38	63
9	M	34	M2	Alive	29	31	57
10	F	43	M4	Alive	18	21	47
11	M	57	M2	Alive	15	19	46

F, female; M, male; FAB, French-American-British classification; OS, overall survival; DFS, disease-free survival.

converted into transcripts per kilobase million and then "Combat" was used to remove the batch effect.

Statistical analysis

The statistical data analysis in this study was performed using R version 3.6.2. Significance was determined using the Mann-Whitney test, and all P values were two-sided and considered statistically significant when <0.05.
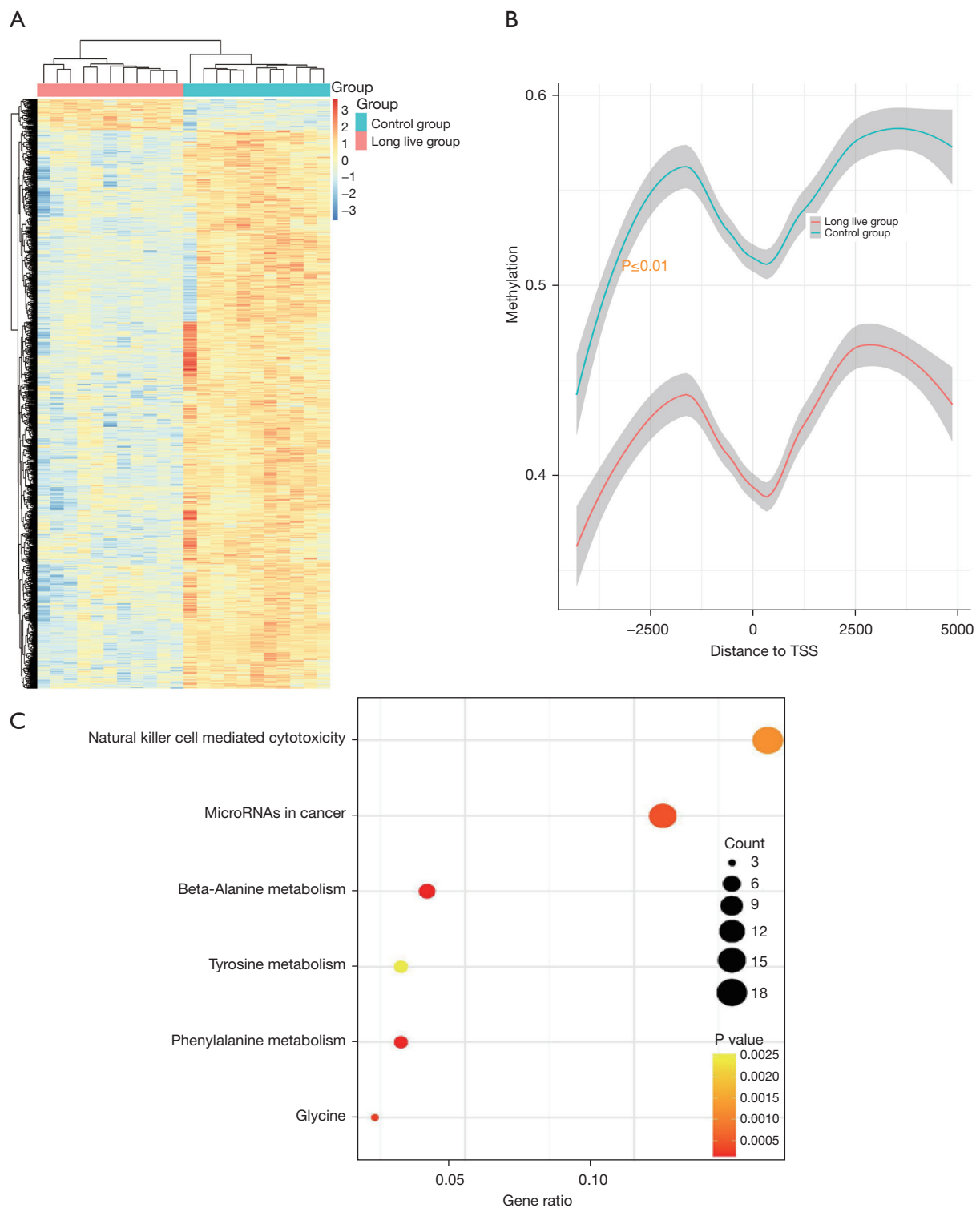
Results

Clinical data

The 11 patients with AML with long-term survival comprised 6 men and 5 women, with a median age of 43 [26–67] years. Among them, 7 patients completed chemotherapy for at least 3 years (discontinued chemotherapy after >3 years) and 4 completed chemotherapy in <3 years (discontinued chemotherapy after <3 years). All patients achieved CR after one course of induction chemotherapy, followed by 3–6 courses of consolidation cycle treatment. None of them received allogeneic hematopoietic stem cell transplantation. The median overall survival (OS) and disease-free survival (DFS) till sampling time were 42 [19–58] and 36 [15–50] months, respectively (*Table 1*). A total of 10 healthy people were transplant donors (7 men, 3 women), with a median age of 43 [22–56] years.

Multi-omics differences

We detected 1,152 DMPs: 1,092 hypomethylated probes and 60 hypermethylated probes (*Figure 1A*). The methylation level of the control group was significantly higher than that of the long-term survival group (P<0.01) (*Figure 1B*). The hypomethylated probes for Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment are summarized as follows: the significant pathway was related to NK-cell-mediated cytotoxicity and amino acid metabolism (*Figure 1C*), whereas no signaling pathway was found to be enriched by hypermethylated probes.

Next, we analyzed the DEGs between these two groups: 651 genes upregulated and 142 genes downregulated in the long-term survival AML group (*Figure 2A*). We found the DEGs of long-term survival AML were roughly similar, and the DEGs were highly relevant to the cellular amino acid metabolic process pathway by GSEA (*Figure 2B*).

We crosslinked the methylation data and RNA profiles of the controls and patients. Comparing with healthy people, 8 genes (*LOXL1*, *CFI*, *RHBDF1*, *RFPL2*, *NCR3*, *DNM1*, *ARHGAP6* and *PDZRN4*) were hypomethylated and upregulated, gene CRISP2 was hypermethylated and downregulated, 3 genes (*PDGFRA*, *CHST1*, and *MMRN1*) were hypomethylated and downregulated, CCR10 was hypermethylated and upregulated in the long-term survival AML patients with a threshold of |delta beta| >0.1, and |log FC| >1 (*Figure 2C*).

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Figure 1 The comparison of long-term survival acute myeloid leukemia (AML) group and healthy Control group in DNA level. (A) The differential methylation position (DMP) heatmap of long-term survival (Long Live) Group and Control Group comparison; (B) extracting DMPs from each CpG island for the long-term survival (Long Live) group and methylation comparison. X: distance to the transcription start site (TSS); Y: methylation level ranging [0–1]. (C) KEGG pathway enrichment for the long-term survival group's upregulated genes.
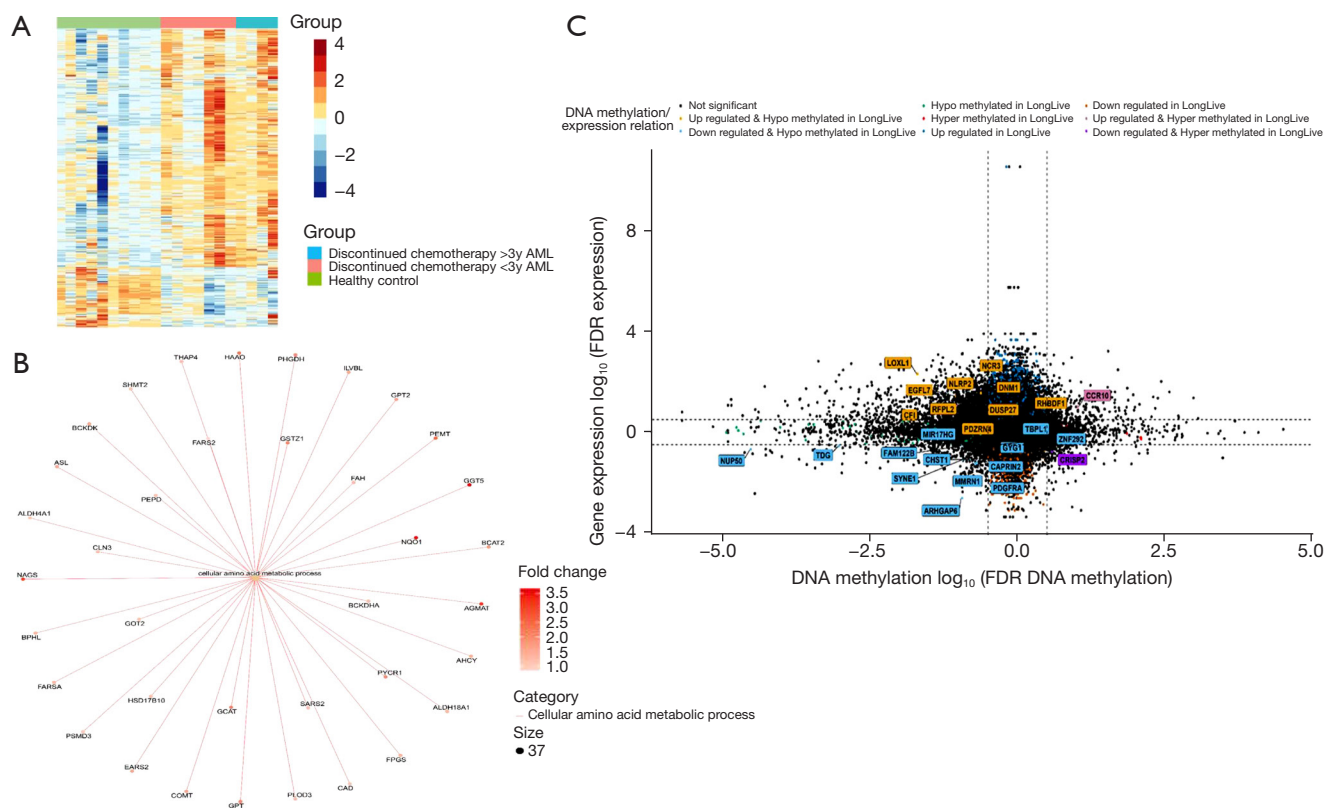
Figure 2 The crosslink analysis of long-term survival acute myeloid leukemia (AML) group and healthy Control group. (A) RNA-seq gene expression heatmap of the long-term survival AML group and healthy Control group comparison. (B) Enrichment Gene Ontology (GO) plot for the cellular amino acid metabolic process and its related genes. (C) Starburst plot showing the crosslink analysis of DNA methylation and RNA expression relation (healthy people *vs.* long-term survival AML patients).

To identify the significance of DEGs and DMPs between the groups, we put these crosslink genes into the GEO database for further exploration of the prognostic significance of the genes.

The univariate Cox analysis results of the signature for GEO: hazard ratio (HR) of LOXL1 was 1.12 with 95% confidence interval (CI) from 1.024 to 1.217 (P=0.011848); HR of PDZRN4 was 1.34 with 95% CI from 1.05 to 1.7 (P=0.017353); HR of MMRN1 was 1.08 with 95% CI from 1.00 to 1.16 (P=0.036575); HR of CFI was 1.20 with 95% CI from 1.01 to 1.44 (P=0.042251).

The multivariate Cox analysis results of the signature for GEO: HR of LOXL1 was 1.140 with 95% CI from 1.045 to 1.245 (P=0.003); HR of PDZRN4 was 1.246 with 95% CI from 1.009 to 1.582 (P=0.028).

Based on the univariate and multivariate Cox analyses, two crosslinked genes LOXL1 and PDZRN4 (*Table 2*) characterized as hypomethylated and upregulated, may

become an AML prognostic marker (P<0.05).

ICI landscape

To investigate the ICI difference between the healthy controls and long-term survival patients with AML, we separately calculated the immune cell gene expression by ssGSEA, EPIC, ESTIMATE and IPS algorithms (*Figure 3A*).

The results of ssGSEA analysis suggested that the immune cell proportion and related gene expression status of patients discontinuing chemotherapy for >3 years were extremely similar to those of healthy people, except for the NK-CD56 bright cells (P=0.016), which were highly downregulated in patients with AML who completed chemotherapy for >3 years. In contrast, compared with the healthy controls, there was more infiltration of NK cells, gamma delta T cell and CD8 T cells, and less of

Table 2 Univariate and multivariate Cox analyses of crosslinked genes in GEO cohort

Gene	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
LOXL1	1.12 (1.024–1.217)	0.011848	1.14 (1.045–1.245)	0.003
PDZRN4	1.34 (1.05–1.70)	0.017353	1.246 (1.009–1.582)	0.028
MMRN1	1.08 (1.00–1.16)	0.036575	1.085 (0.982–1.168)	0.107
CFI	1.20 (1.01–1.44)	0.042251	1.166 (0.969–1.402)	0.103
DNM1	0.92 (0.85–1.01)	0.080694	–	–
NCR3	0.77 (0.55–1.08)	0.127044	–	–
PDGFRA	1.17 (0.78–1.77)	0.438077	–	–
CRISP2	1.06 (0.90–1.25)	0.485599	–	–
CHST1	1.12 (0.76–1.65)	0.568466	–	–
ARHGAP6	1.12 (0.74–1.71)	0.577477	–	–
CCR10	1.05 (0.80–1.38)	0.710786	–	–
RHBDF1	0.97 (0.80–1.17)	0.755652	–	–
RFPL2	0.98 (0.76–1.25)	0.848194	–	–

GEO, Gene Expression Omnibus; HR, hazard ratio; CI, confidence interval.

eosinophils, neutrophils, Tem (effector memory T cells), B cells and macrophages in patients who discontinued chemotherapy after <3 years.

Then, the IPS score quantitatively evaluated the immunotherapeutic response of these two patient groups applying the four main broadly similar clusters of MHC, SC, CP and EC (*Figure 3B*). The immune cell-related gene expression profile of patients with AML who discontinued chemotherapy after >3 years was roughly similar to that of the normal controls. Statistical differences were only observed in immune CPs PD-1 (P=0.025) and LAG3 (P=0.025) and ECs CD8 (P=0.01) and temCD4 (P=0.025). Generally, the long-term survival AML patients and the controls showed different immune cell patterns (*Figure 3C*).

Furthermore, we separately analyzed the relationship between crosslinked prognostic genes and the immune cell proportion. Our results suggested the two groups presented different infiltration patterns of crosslinked genes (*Figure 4A,4B*). It worth noting that *PDZRN4* tended to have a negative correlation with immune cells, especially the B cell and T cell family (P<0.01), in the long-term-survival AML patients who discontinued chemotherapy after <3 years, in contrast to no significant relationship in long-term-survival AML patients who discontinued chemotherapy after >3 years and the healthy controls.

Discussion

Currently, an explanation for the difference between long-term survival patients with AML and healthy people remains unclear. One way to examine this problem is to compare the similarities and differences of bone marrow hematopoietic cells and immune microenvironments between these two groups of patients.

The immune cell subpopulation estimation was horizontally contrasted in our long-term survival AML cohort based on different algorithms (2,5-9). The whole-immune phenotype results roughly agreed with these algorithms.

The functional roles of bone marrow cells show various capabilities: cytotoxic lymphocytes and NK cells have antitumor effects; macrophages and neutrophils possess both tumor-promoting and antitumor effects; myeloid derived SCs, regulatory T cells and mast cells are recognized as tumor-promoting. Interestingly, there was less infiltration of both T cells and NK cells in patients with AML who discontinued chemotherapy after <3 years. DMP hypomethylation probe enrichment was also consistently associated with the NK-cell cytotoxicity pathway.

The IPS score was utilized to evaluate the immunotherapeutic response. MHC, EC, SC and CP
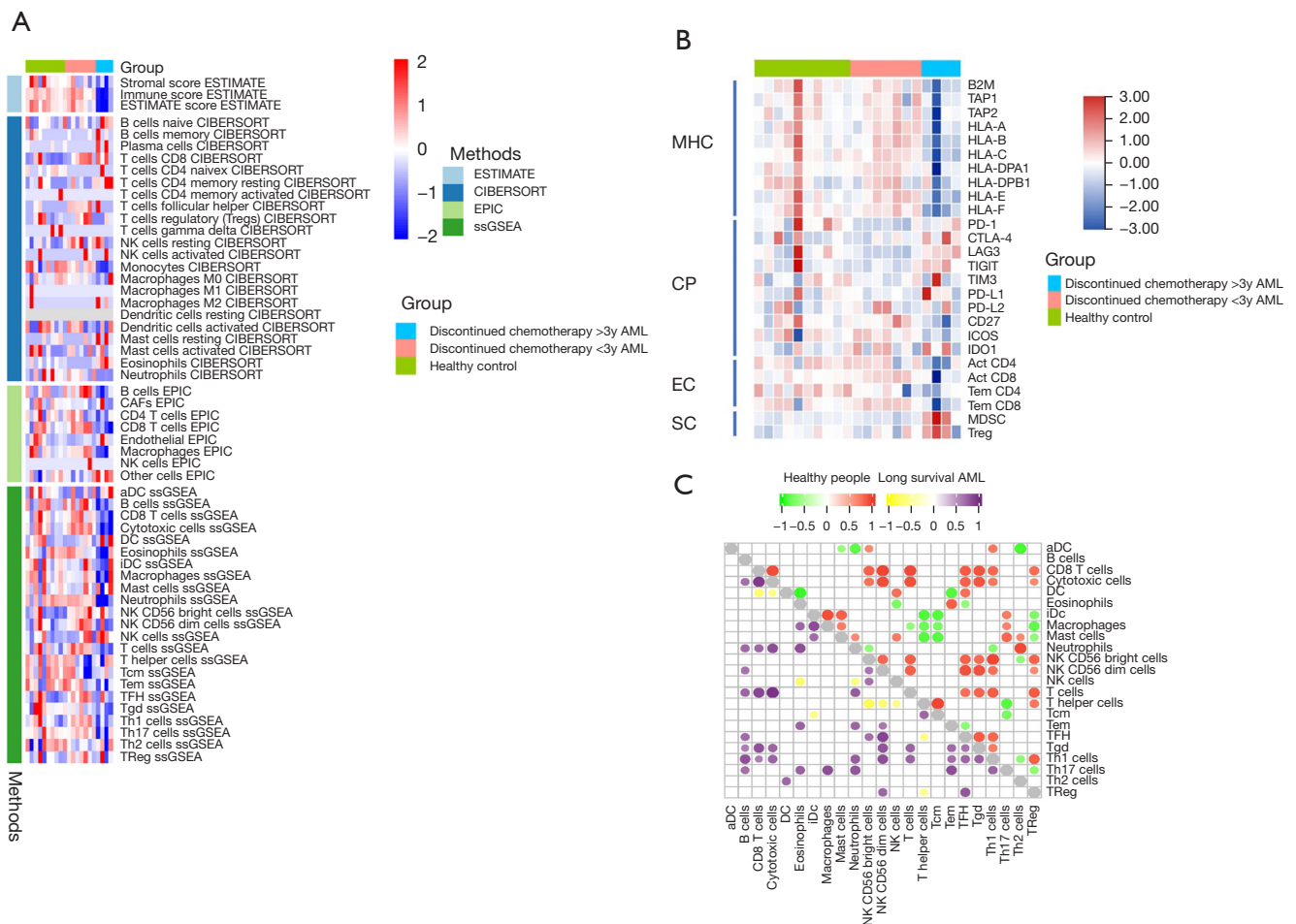
Figure 3 Immune cell analysis of two study groups. (A) Immune cell infiltration of long-term survival acute myeloid leukemia (AML) patients (discontinued chemotherapy <3 or >3 years) and healthy controls calculated by ESTIMATE, CIBERSORT, EPIC and single sample gene set enrichment analysis (ssGSEA) algorithms. (B) Immunophenotype score (IPS) of long-term survival AML patients (discontinued chemotherapy <3 or >3 years) and healthy controls. (C) Comparison of immune cell types between long-term survival AML patients (long survival AML) and healthy controls based on ssGSEA. CP, checkpoints; EC, effector cells; MHC, major histocompatibility complex; SC, suppressor cells; ssGSEA, single sample gene set enrichment analysis.

were four cluster gene expressions in AML patients with long-term survival that were highly similar to those in the healthy controls, but distinct from patients with long-term AML survival who discontinued chemotherapy after <3 years. Although a minority of patients with leukemia benefit from immune CP blockade therapy, a study proved that blocking the immunosuppressive microenvironment by targeting immune inhibitory receptors may hinder AML development (10). Also, a previous study suggested that the development and function of NK cells and cytotoxic T cells were inhibited in patients with AML (11). The specific mechanism remains to be elucidated.

The clinical response was found to correlate with proportions of immune cells in solid malignancies (12). AML survival was strongly associated with lymphocytes and T-lymphocyte proportion in the bone marrow (13). Reconstitution of the lymphocyte population after chemotherapy may reduce the risk of AML relapse (14).

Considering that the immune cell gene expression of patients with AML who discontinued chemotherapy after <3 years was different from that of the healthy controls, the antitumor cell populations in the long-term survival AML patients, such as T cells and NK cells, were assumed to have rebuilt after chemotherapy for 3 years. A further

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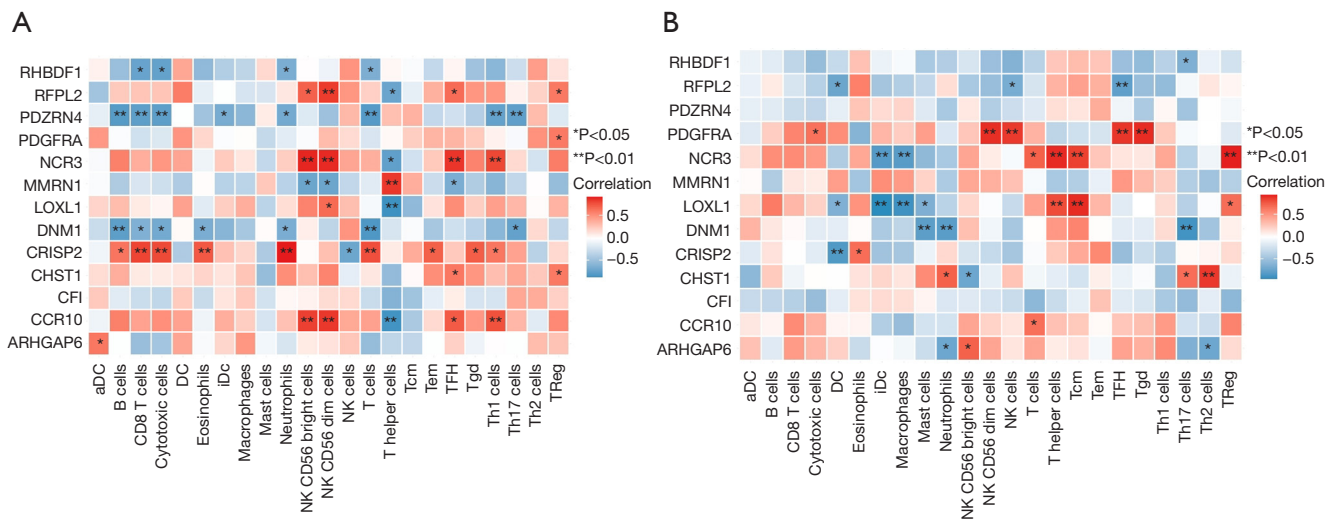
Figure 4 Relationship between immune cells and crosslinked genes of the different groups of patients. (A) Patients who discontinued chemotherapy after <3 years. (B) Patients who discontinued chemotherapy after >3 years and the healthy controls.

mechanism of immune cell reconstruction and influence on AML prognosis remains to be explored.

In summary, DNA methylation, RNA expression and TILs in patients with AML with long-term survival were slightly different than in healthy people. The profile of long-term AML survivors, especially those who discontinued chemotherapy after <3 years, still differed from that of healthy people. Therefore, these patients still need clinical follow-up until complete restitution of immune function is achieved.

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups. com/article/view/10.21037/atm-21-6681/rc

Data Sharing Statement: Available at https://atm.amegroups. com/article/view/10.21037/atm-21-6681/dss

Conflicts of Interest: All authors have completed the

ICMJE uniform disclosure form (available at https://atm. amegroups.com/article/view/10.21037/atm-21-6681/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional committee of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences (No. KT2018091-EC-2) and written informed consent was given by all patients.

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