Small bone marrow adipocytes predict poor prognosis in acute myeloid leukemia

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Supplementary Materials and methods

Subjects

Bone marrow (BM) trephine biopsy specimens of 70 patients at diagnosis with acute myeloid leukemia (AML) and 70 controls with lymphoma or solid tumors without BM infiltration were retrospectively analyzed in this study. The treatment protocols were previously described.[1] Patients were excluded from this study if they were treated less than 6 months, voluntarily withdrew from therapy, had any prior history of bone or BM disease or their disease transformed from myelodysplastic syndrome. Patients with diabetes or antidiabetic drug history were also excluded. Patients diagnosed with acute promyelocytic leukemia were excluded in our study because these patients had a better prognosis, which would affect the survival assessment for other subtypes of leukemia. The cases of AML at diagnosis were classified according to FAB criteria.[2] This study was approved by the Medical Ethical Committee of our institute.

Adipocyte measurements

BM trephine biopsies were obtained from the posterior iliac crest, and BM tissue paraffin or plastic-embedded slices were made after fixation and decalcification according to conventional methods. BM sections were observed using an optical microscope imaging system (Olympus BX41, Tokyo, Japan) and adipocyte number (Ad. N; per square millimeter), adipocyte area (Ad. Ar; square

micrometers), adipocyte diameter (Ad. Dm; micrometers) and the percentage of adipocyte volume per tissue volume (Ad. V/TV) were measured by tracing every individual adipocyte and analyzed with Image-Pro Plus 5.1.[3] Ten fields were randomly selected at x100 magnification and analyzed at x400 magnification. The fields were selected as close to the whole biopsy specimen as possible. The graticule-crossing point overlaying the cytoplasm of fat cells or border was considered as a fat cell positive point. All biopsies were read in a blinded manner, without knowledge of the group, to avoid any bias in the final analysis.

Cell culture and regents

The leukaemic cell lines THP-1 and K562 (American Type Culture Collection, Manassas, VA, USA) were cultured in 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Gibco, Grand Island, NY, USA) at 37°C in 5% CO2. The HL-60 cell line was cultured in IMEM supplemented with 20% FBS. Primary AML blasts and mesenchymal stem cells in BM (BMSCs) were isolated by Ficoll-Hypaque (Axis-Shield Diagnostics, Dundee, Scotland, UK) density-gradient centrifugation after informed consent. BMSCs were differentiated into adipocytes as previously described.[4] Co-culture of THP-1, K562, HL-60 cells (5×10^5) or primary AML blasts (1×10^6) with adipocytes was carried out using a Transwell culture system (0.4μm pore size; Millipore) as previously described.[5] Conditioned medium from the leukemic cell lines was obtained from cells cultured with high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% FBS.

Cell proliferation assay

A CCK8 kit (Dojindo, Japan) was used for the evaluation of cell proliferation. For CCK8 assay, cells (3×10^3) were seeded into 96-well plates, cultured for 5d, and CCK8 reagent was then added to each well and incubated for 2 h at 37°C. The measurement of absorption was performed using microplate reader at 450 nm (Multiscan FC, Thermo Fisher, USA).

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using Trizol (Invitrogen, Paisley, UK), and the RNA was converted into cDNA using the PrimeScript™ RT reagent Kit (Takara Bio Inc, Shiga, Japan) for RT-qPCR. All RT-qPCR reactions were performed using an ABI 7500 system (Biosystems, Foster City, CA, USA) and the SYBR Premix Ex Taq reagent kit (Takara Bio Inc, Shiga, Japan). The sequences of primers are as followings: *HSL* forward primer 5'-GACCCCTGCACAACATGATG-3', reverse primer 5'-

TGAGCAGCACCCTTTG GATG-3'; *ATGL* forward primer 5'-GGCTTCCTCGGCGTCTACTA-3', reverse primer 5'-TTTACCAGGTTGAAGGAGGGG-3'; *GAPDH* forward primer 5'- AATG GACAACTGGTCGTGGAC-3', reverse primer 5'- CCCTCCAGGGGATCTGTTTG-3'.

Free fatty acids (FFA) detection

Adipocytes co-cultured in the presence or absence of leukemic cells in high glucose DMEM supplemented with 10% FBS for 5d through transwell co-culture system. Then the supernatant of adipocytes was collected and stored in -80°C.The content of FFA was quantified using a colorimetric method via a commercial kit (Sigma Aldrich, St. Louis, Missouri). The assay was carried out according to manufacturer's specifications.

Immunohistochemistry

The staining procedure was done in accordance with the instructions provided with an UltraVision Quanto Detection System HRP DAB kit's (Thermo Scientific, TL-060-QHD, CA, USA). In brief, sections were deparaffinized in xylene and rehydrated by exposure of the specimens to graded ethanol. Antigen retrieval was performed by sodium citrate buffer with pH 6.0 at 90°C for 15 min. Anti-PERILIPIN 1 (PLIN 1) monoclonal antibody (1:500 diluted, 4854,VALA Science, USA) was incubated at 4°C overnight. Horseradish peroxidase (HRP) labelled secondary antibody and the substrate diaminiobenzidine (DAB) were used according to the kit's instructions. Then sections were viewed under the light microscope (Olympus, Tokyo, Japan).

Statistical analysis

Values were calculated as mean ± SD. The Mann-Whitney U test and χ^2 test were applied for continuous characteristics and categorical variables between the remission group and the refractory group, respectively. The Spearman's correlation test was used to analyze the correlation between adipocytes and BM blast percentage. Hazard ratios (HR) and 95% confidence intervals (CI) between the different groups were estimated by Cox regression analysis. Difference in overall survival (OS) and relapse-free survival (RFS) between different groups was assayed with the log-rank test and survival curves plotted using the Kaplan-Meier method. For all analyses, $P<0.05$ was considered to be significant. All statistical analyses were performed using SPSS 20.0 software program (Statistical Package for Social Science, SPSS Inc. Chicago, IL., USA).

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Supplementary Table S1. Clinical characteristics of AML patients

	Remission	Refractory	*P* -value
N	32	38	
Age (years)	57(23-80)	54.5(21-82)	.77
Sex (male/female)	17/15	19/19	.79
BMI (kg/m^2)	22.4±9.1	22.4±3.4	.66
Weight (kg)	62.8±12.0	63.0±8.7	.64
pWBC ($10^9/l$)	27.6±20.5	33.2±27.6	.74
BM blast%	63.7±22.2	65.5±23.3	.65
FAB subtype			.99
M0	1	1	
M1	6	6	
M2	7	9	
M4	11	12	

M5	6	6
M6	1	2

Abbreviations: N, number; BMI, body mass index; pWBC, white blood cell count in periphery; BM blast%, bone marrow blast percentage; FAB, French–American–British classification systems.

Supplementary Table S2. Univariate Cox regression for the OS and RFS of AML patients

	OS (n=70)			RFS (n=32)		
Variable	HR	95%CI	*P*-value	HR	95%CI	*P*-value
Age	1.042	1.014-1.070	.003	3.612	1.365-9.558	.010
pWBC (10^9/L)	1.003	.989-1.017	.709	.989	.965-1.015	.414
BM blast %	1.003	.984-1.021	.782	.980	.958-1.003	.081
FAB subtype	.973	.801-1.183	.785	1.113	.867-1.429	.402
Ad. N (/mm^2)	.992	.984-1.001	.068	1.003	.993-1.013	.523
Ad. Ar (μm^2)	.950	.910-.992	.020	1.000	.999-1.001	.629
AV/TV (%)	.999	.998-1.000	.002	.994	.946-1.044	.804
Ad. Dm (μm)	.974	.952-.997	.024	1.000	.976-1.025	.994
Small Ad. N (/mm^2)	1.510	1.321-1.725	.000	1.349	1.184-1.537	.000
Small Ad. V (%)	2.008	1.608-2.506	.000	4.066	2.297-7.195	.000

Abbreviations: OS, overall survival; RFS, relapse free survival; HR, hazard ratio; CI, confidence interval; pWBC, white blood cell count in periphery; BM blast%, bone marrow blast percentage; FAB, French–American–British classification systems; Ad. N, adipocyte number; Ad. Ar, adipocyte area; AV/TV, the percentage of adipocyte volume per tissue volume; Ad. Dm, adipocyte diameter; Ad. V, adipocyte volume.

Supplementary Table S3. Multivariate Cox regression for the OS and RFS of AML patients

	OS (n=70)			RFS (n=32)		
Variable	HR	95%CI	*P*-value	HR	95%CI	*P*-value
Age	.65	.23-1.9	.65	.48	.11-2.13	.33
Small Ad. N (/mm^2)	1.53	1.31-1.78	.000	1.18	1.01-1.38	.036

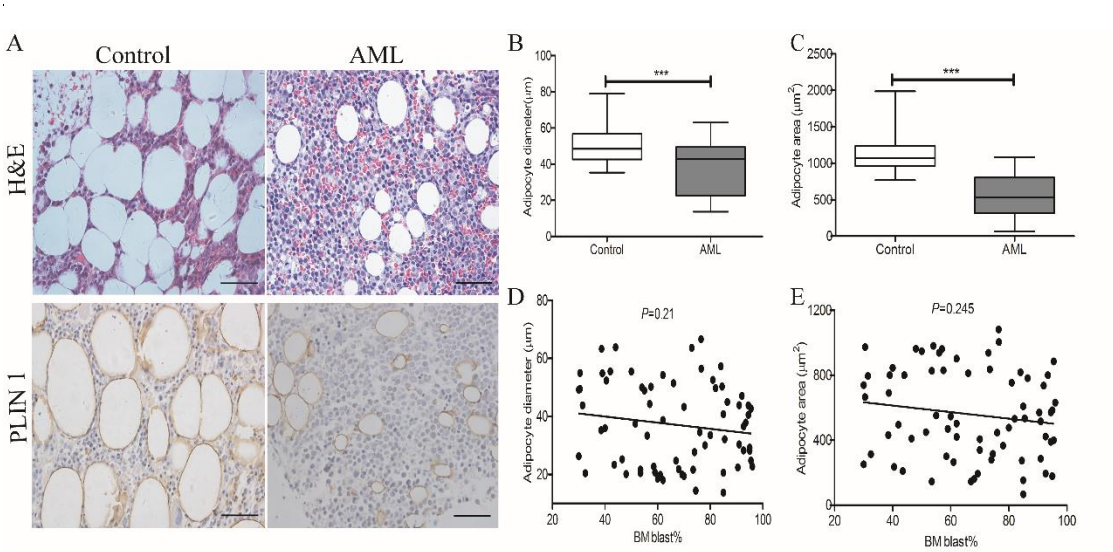
Small Ad. V (%)	1.65	1.19-2.29	.003	3.37	1.89-6.00	.000

Abbreviations: OS, overall survival; RFS, relapse free survival; HR, hazard ratio; CI, confidence interval; Ad. N, adipocyte number; Ad. V, adipocyte volume

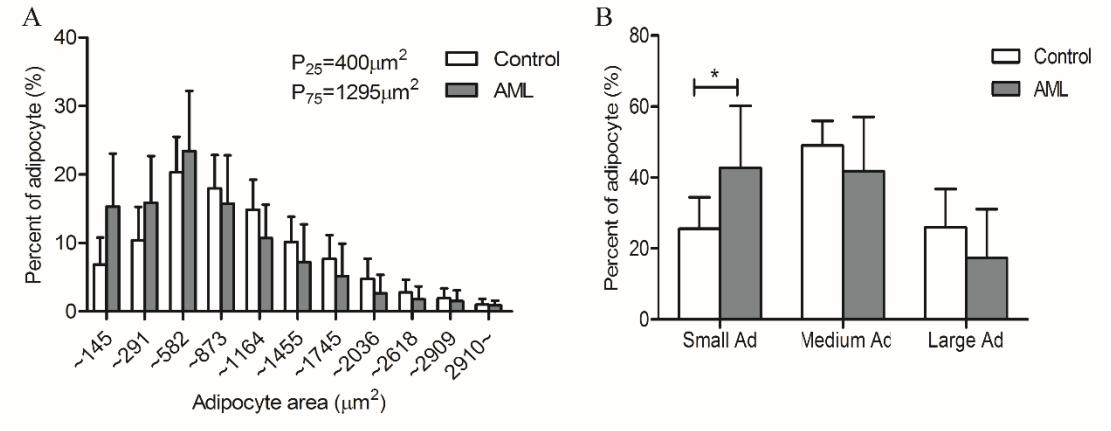
Supplementary Table S4. Univariate Cox regression of the quartiles of the small adipocyte volume and number for the survival of AML patients

OS (n=70)					RFS (n=32)				
Variable	Cut-off value	HR	95%CI	*P*-value	Variable	Cut-off value	HR	95%CI	*P*-value
Small Ad. V (%)	2.3	6.072	2.298-16.046	.000	Small Ad. V (%)	1.4	25.75	.987-150.515	.051
	4.5	4.150	1.911-9.013	.000		2.5	30.233	3.901-234.30	.001
	8.0	3.804	.000-9.702	.440		4.7	29.410	5.774-149.793	.000
Small Ad. N (/mm^2)	10.6	35.413	4.402-284.890	.001	Small Ad. N (/mm^2)	4.5	6.128	1.361-27.591	.018
	17.0	50.236	10.446-241.586	.000		9.2	14.302	3.131-65.325	.001
	34.4	32.093	7.393-139.320	.000		15.5	8.985	2.836-28.461	.000

Abbreviations: OS, overall survival; RFS, relapse free survival; HR, hazard ratio; CI, confidence interval; Ad. N, adipocyte number; Ad. V, adipocyte volume.



Supplementary Figure S1. Adipocytes are small in AML patients and not linearly correlative with BM blasts. A, H&E staining and IHC with PERILIPIN 1 (PLIN 1) antibody in bone marrow sections from the controls and primary AML patients. Scale bar represents 25μm. B-C, Box-plots show adipocyte diameter (B) or adipocyte area (C) in 70 samples of all AML patients and the controls using Mann-Whitney U test. ****P<0.001*. D-E, Scatter plot shows no linear correlation of adipocyte diameter (D) or adipocyte area (E) with the blasts in bone marrow of AML patients (*n*=70, *R*=-0.15, *P*=0.21, or *R=-0.15, P=0.245,* Spearman correlation test).



Supplementary Figure S2. Small adipocytes increase in AML patients. A, Frequency distribution of adipocyte area in the controls (*n=70*) and AML patients (*n=70*). The 25th percentile (P_{25}) and the 75th percentile (P_{75}) of adipocyte area in the controls were 400μm^2 and 1295μm^2, respectively. B, The proportion of adipocytes with different sizes classified by adipocyte area determined by Mann-Whitney U test in AML patients (*n=70*) and the controls (*n=70*).* *P<0.05.*

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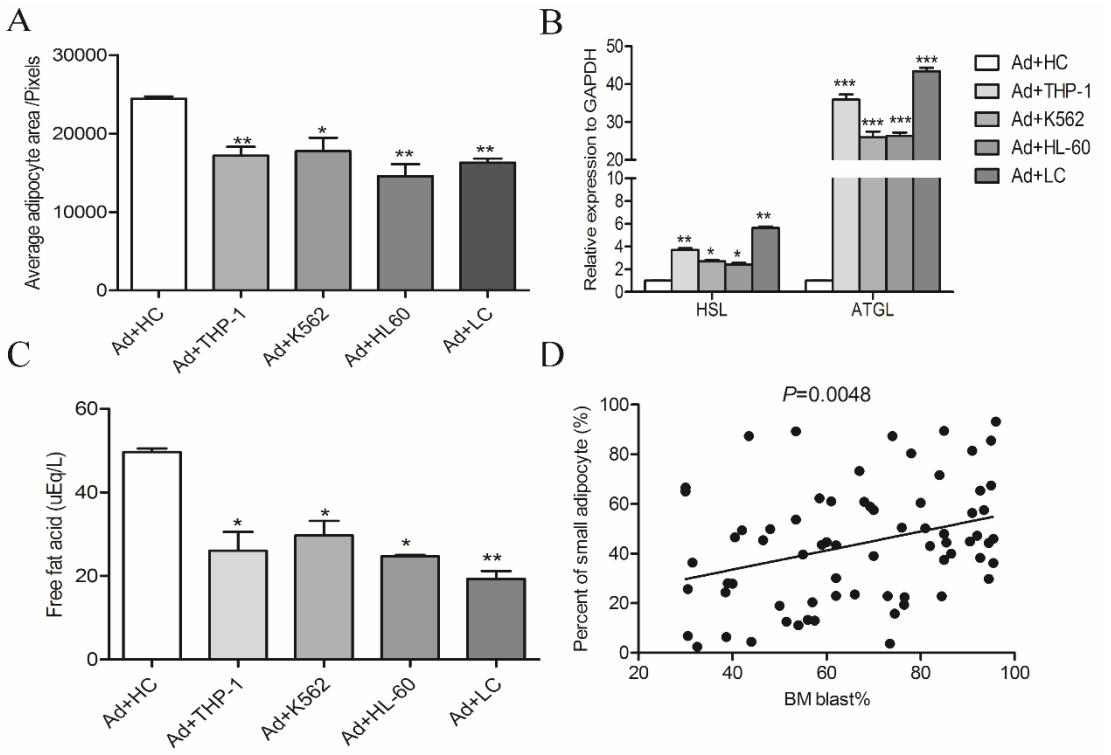

Supplementary Figure S3. The interaction between small adipocytes and leukemic cells.

A, The average area of adipocytes co-cultured with THP-1 cell line (Ad+THP-1), K562 cell line (Ad+K562), HL-60 cell line (Ad+HL-60) and primary AML blasts (Ad+LC) was compared with that of mature adipocytes co-cultured with primary hematopoietic cells (Ad+HC) by using Image Pro-Plus 5.1. *$P<0.05$, **$P<0.01$. B, RT-qPCR analysis of lipolytic genes (*HSL* and *ATGL*) in mature adipocytes from these five groups. *$P<0.05$, **$P<0.01$, ***$P<0.001$. C, The content of free fat acid (FFA) in the supernatant of adipocytes from indicated groups was detected by colorimetric method. *$P<0.05$, **$P<0.01$. Values shown were the mean ± s.e.m. D, Scatter plot showed positive linear correlation between small adipocyte content and the blasts in bone marrow of AML patients (n=70, R=0.33, P=0.0048, Spearman correlation test).