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Research paper

Genetic variations of bone marrow mesenchymal stromal cells derived from acute leukemia and myelodysplastic syndrome by targeted deep sequencing

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ABSTRACT

Bone marrow mesenchymal stromal cells (MSCs), which support proliferation and differentiation of hematopoietic stem cells, may play a crucial role in the pathogenesis of myeloid neoplasms. To determine whether MSCs in myeloid neoplasms harbor distinct somatic mutations that may affect their function, we used a targeted gene sequencing panel containing 50 myeloid neoplasm-associated genes with coverage of ≥500. We compared the genetic alterations between MSCs and bone marrow hematopoietic (BM) cells from patients with acute leukemia (n = 5) or myelodysplastic syndrome (MDS, n = 5). Non-synonymous somatic mutations, such as DNMT3A-R882H and FLT3-D835Y, were only detected in BM cells with high allelic frequency. We found several non-synonymous genetic variants overlapping BM cells and MSCs, including TP53 and ASXL1, partially owing to the heterogenous cell fraction of MSC samples and lineage fidelity. We also found MSC-specific genetic variants with very low allelic frequency (7% to 8%), such as NF1-G2114D and NF1-G140. Further studies in large cohorts are needed to clarify the molecular properties of MSCs including age-related genetic alterations by targeted deep sequencing.

1. Introduction

The bone marrow mesenchymal stromal cell (MSC) population, the major component of hematopoietic microenvironment [1,2], is comprised of a mixture of several adherent cell types including fibroblasts, endothelial cells, macrophages, osteoclasts [3,4], and mesenchymal stem cells that can differentiate into adipocytes, astrocytes, cardiomyocytes, chondrocytes, hepatocytes, muscles, neurons, and osteoblasts [5]. The definition of MSC is therefore somewhat obscure owing to the complexity of cells in the stromal cell fraction. The hematopoietic microenvironment is involved in the pathogenesis of acute myeloid leukemia (AML) as well as myelodysplastic syndrome (MDS) [6,7]. Accordingly, dysfunction of MSCs leading to insufficient stromal support, impaired osteogenic differentiation activity, and increased IL-6 secretion has been reported in AML and MDS [8–10]. In addition to the functional abnormalities of MSCs, a distinct gene expression profile in MSCs from AML and MDS has also been reported using either microarray or RNA sequencing [11–13].

Several reports demonstrated cytogenetic abnormalities in stromal cell fractions derived from AML and MDS patients [14–19]. More recent report using whole exome sequencing demonstrated that genetic alterations in the stromal compartment in 16 AML patients were non-specific [13], while other studies using mouse models suggested niche-induced oncogenesis [20,21]. Therefore, whether MSCs derived from hematologic disorders harbor specific somatic mutations that may play a key role in the pathogenesis of myeloid neoplasms remains unclear. In practical terms, genetic analysis of MSCs is challenging because it is difficult to exclude genetic polymorphisms by simultaneous study of germinal cells, in addition to lineage fidelity of MSCs. To address these questions and to gain more insight into genetic properties of MSCs derived from patients with acute leukemia and MDS, we analyzed genetic variants in 50 major genes associated with myeloid malignancies by a targeted deep sequencing.

2. Materials and methods

2.1. Patients

Ten consecutive patients with myeloid malignancies (five with acute leukemia and five with MDS) were included in this study

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Table 1
Patient characteristics and cytogenetic results.

UPN	Age (years)	Gender	Diagnosis	Karyotypes of BM cells	Karyotypes of BMSCs
Acute leukemia					
36	74	Male	AML	45,XY,-18[6]/46,XY[27]	47,XY,+7[2]/46,XY,-15,der(1)t(1;15)(q24;q13)[1]/46,XY[22]
37	55	Female	AML	Multiple missing chromosomes[a] [11]/46,XX[7]	46,XX,t(3;12)(q24;q13),t(1;8)(p24;q22)[2]/47,XX,+8[1]/46,XX,-7,+der(7)t(1;7)(q11;p21)[1]/45,XX,-5,-6,-12,+2mar[1]/46,XX[21]
38	77	Male	AML	46,XY,inv(9)(p12q13)[15]/46,XY[6]	46,XY[6]
39	61	Male	BAL	46,XY,-4,add(5)(q31),-13, add(14)(q32),+2 ~ 4mar[19]/46,XY,-4,add(5)(q31),add(14)(q32),-20,+2mar[1]/46,XY,-8,-8,+2mar[1]	46,XY[1]
40	42	Female	t-MDS/AML	46,XX,inv(16)(p13q22)[4]/47,XX,idem,+22[18]	46, XX[5]
Myelodysplastic syndrome					
10	60	Male	RA (intermediate)	46,XY,del(20)(q11)[20]/46,XY[1]	46,XY[2]
18	75	Male	RCMD (very high)	41-44,X,-Y,del(5)(q13), del(17)(p12)[16]/-18[14]/t(1;3)(p22;q22),del(7)(p15)[11]/add(2)(q37),-13[10]/-22,+mar[5]/-20[4]/del(11)(q23)[3]	46,XY[4]
25	72	Male	RCMD (intermediate)	46,XY,-7,+mar[21]	45,XY,del(1)(p12),-15[2]/46,XY[13]
13	70	Male	RAEB-2 (intermediate)	45,X,-Y[6]/46,XY[16]	No metaphase
35	67	Male	RAEB-2 (high)	46,XY[10]	46,XY,del(3)(p21),add(4)(p15)[8]/multiple deletion

UPN, unique patient number; BM, bone marrow; BMSC, bone marrow stromal cell; RA, refractory anemia; RCMD, refractory cytopenias with multilineage dysplasia; RAEB-2, refractory anemia with excess blasts-2; AML, acute myeloid leukemia; BAL, biphenotypic acute leukemia; MDS, myelodysplastic syndrome; (x), IPSS-R; [n], number of metaphases.

[a] multiple missing chromosomes: representative karyotype = 42,XX,-5,-7,-8,-10, 43,XX,-6,-15,-17; missing 17 in 6 cells, missing 5 in 4 cells, missing 11 in 3 cells, missing 11b in 3 cells, missing 19 in 3 cells, missing 6 in 23 cells, and missing 7 in 3 cells.

(Table 1). All patients were treated at Tokyo Medical University, Tokyo, Japan from April 2011 to September 2015. The patients' mean age was 65.3 years (range 42–77 years). The diagnoses were established according to the 2008 World Health Organization criteria: three patients were diagnosed with *de novo* AML, one patients with biphenotypic acute leukemia (BAL), one patient with therapy-related myelodysplastic syndrome/acute myeloid leukemia (t-MDS/AML), one patient with refractory anemia (RA), two patients with refractory anemia with excess blasts (RAEB), and two patients with refractory cytopenia with multilineage dysplasia (RCMD). Cytogenetic analyses of both bone marrow and MSCs were performed as reported previously [22]. Bone marrow and MSCs obtained from three patients with non-Hodgkin's lymphoma (NHL) without bone marrow invasion were also used as non-myeloid malignancy controls for deep sequencing. Written informed consent was obtained from all patients. The study was validated by the internal review boards of Tokyo Medical University and followed both the Declaration of Helsinki and "Guidelines for Genetic Tests and Diagnoses in Medical Practice" by The Japanese Association of Medical Sciences.

2.2. Primary MSC culture

MSCs were obtained by the classical adhesion method with a minor modification [23,24]. Briefly, 0.5–1 mL of freshly obtained bone marrow aspirates were cultured in an equivalent volume of RPMI1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% of fetal bovine serum (FBS; GE Healthcare UK, Buckinghamshire, England), 1% of penicillin streptomycin (P/S; Thermo Fisher Scientific), and Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) containing 10% of FBS (GE Healthcare, UK), 1% of P/S, and 1% of non-essential amino acids (NEAA; Thermo Fisher Scientific). Primary cultured cells were cultured for 3–5 days, and medium was exchanged to DMEM (with 10% of FBS, 1% of P/S, 1% of NEAA) for non-hematopoietic expansion, after removing non-adherent cells. Over a period of 1–2 weeks, the adherent cells cultured with DMEM were split by trypsinization and harvested for cryopreservation with Cell Banker 1 (ZENOAQ, Fukushima, Japan) until use.

The cultured MSC population was identified as CD73+, CD90+, CD105+, CD34-, CD45- and HLA-DR- by flow cytometry with < 5% CD34+ and CD45+. Data for all cell surface markers were obtained using a BD Accuri C6 (Becton Dickinson, Franklin Lakes, NJ, USA), with monoclonal antibodies from BD Pharmingen (San Jose, CA, USA). MSCs differentiated to adipocytes and osteoblasts after exposure to MSC differentiation media (PromoCell, Heidelberg, Germany) for at least one week. Adipocyte and osteoblast differentiation was determined by staining with Oil Red (Sigma, St. Louis, MO, USA) and Alizarin Red (Wako, Osaka, Japan), respectively (Supplementary Fig. 1).

2.3. Genomic DNA extraction

MSCs were collected for DNA extraction after one passage of culture. Bone marrow mononuclear cells containing hematopoietic cells (hereafter BM cells) were separated using Ficoll-Hypaque gradients as reported previously [25]. Buccal mucosa cells were also collected from three patients (from whom informed consent was obtained) by gently stroking the intraoral cavity three times using sterile foam tipped applicators (GE Healthcare, UK). Genomic DNA was extracted from BMSCs, BM cells and buccal mucosa cells using a Genta PureGene Cell Kit (QIAGEN, Hilden, Germany), according to the suppliers' instruction.

2.4. GeneRead DNAseq library preparation

The targeted gene fragments were amplified from 40 ng of genomic DNA from each patient using the GeneRead DNAseq Panel PCR Kit V2 (QIAGEN) and GeneRead DNAseq Targeted Panel V2 (QIAGEN), resulting in an average amplicon size of 150 bp. Amplicons were purified using Agecourt AMP XP Beads (BECKMAN COULTER, Brea, CA, USA). Following library construction, purified amplicons were end-repaired (GeneRead DNA Library I Core Kit; QIAGEN) and patient-specific barcode adapters were ligated (GeneRead Adapter I Set 12-plex; 12 different ones in total; QIAGEN). Constructed libraries were purified using the GeneRead Size Selection Kit (QIAGEN) and Agecourt AMP XP Beads (BECKMAN COULTER). Purified libraries were then amplified using the GeneRead DNA I Amp Kit (QIAGEN), and purified using the QIAquick PCR Purification Kit (QIAGEN), resulting in an average amplicon size of 280 bp. Each library was eventually pooled equimolarly in one tube for DNA sequencing. The quality of libraries was confirmed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific) with the Molecular Probes Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).