

Downregulation of extracellular vesicle microRNA-101 derived from bone marrow mesenchymal stromal cells in myelodysplastic syndrome with disease progression

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Received July 4, 2019; Accepted November 11, 2019

DOI: 10.3892/ol.2020.11282

Abstract. To evaluate the mechanism underlying the communication between myeloid malignant and bone marrow (BM) microenvironment cells in disease progression, the current study established BM mesenchymal stromal cells (MSCs) and assessed extracellular vesicle (EV) microRNA (miR) expression in 22 patients with myelodysplastic syndrome (MDS) and 7 patients with acute myeloid leukemia and myelodysplasia-related changes (AML/MRC). Patients with MDS were separated into two categories based on the revised International Prognostic Scoring System (IPSS-R), and EV-miR expression in BM-MSCs was evaluated using a TaqMan low-density array. The selected miRs were evaluated using reverse transcription-quantitative PCR. The current study demonstrated that the expression of BM-MSC-derived EV-miR was heterogenous and based on MDS severity, the expression of EV-miR-101 was lower in high-risk group and patients with AML/MRC compared with the control and low-risk groups. This reversibly correlated with BM blast percentage, with which the cellular miR-101 from BM-MSCs

or serum EV-miR-101 expression exhibited no association. Database analyses indicated that miR-101 negatively regulated cell proliferation and epigenetic gene expression. The downregulation of BM-MSC-derived EV-miR-101 may be associated with cell-to-cell communication and may accelerate the malignant process in MDS cells.

Introduction

The hematopoietic microenvironment comprises a mixture of bone marrow mesenchymal stromal cells (BM-MSCs), fibroblasts, vascular endothelial cells, etc. BM-MSCs support the proliferation and differentiation of hematopoietic stem cells, and themselves undergo differentiation into various functional cells, including adipocytes, osteocytes, and cells from the chondrogenic lineage (1,2). Studies on hematologic malignancies have demonstrated genetic abnormalities in neoplastic cells; however, the role of the hematopoietic microenvironment in the pathogenesis of human myeloid neoplasia is still unclear, therefore we attempted to assess BM-MSCs. Considering the anatomical architecture, BM-MSCs are components of the hematopoietic niche, and hematopoietic cells lie in close proximity to the bone endosteal surface, which is in direct contact with endothelial cells (1) and hence linked to drug sensitivity (3). BM-MSCs also act as immunosuppressive agents by inhibiting the proliferation of immune cells (4,5). Therefore, BM-MSCs may have an important effect on adjacent hematopoietic cells and immune control system in hematologic neoplasia.

Recent studies have demonstrated that neoplastic cells shed extracellular vesicles (EVs), including exosomes, and endow BM-MSCs to facilitate progression of neoplastic cells (6,7). Exosomes are EVs, about 50-150 nm in size, and contain genetic elements such as messenger RNA, microRNA (miR), and DNA (8-10). EVs act as cargos involved in cell-to-cell communication and are associated with the transformation of adjacent fibroblasts into cancer-associated fibroblasts (CAFs), which are involved in cancer progression (11,12). Tumor cell-derived EVs, including exosomes, also act as angiogenesis

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Abbreviations: BM, bone marrow; BM-MSC, bone marrow mesenchymal stromal cell; miR, microRNA; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; AML/MRC, acute myeloid leukemia with myelodysplasia-related changes; TLDA, TaqMan-low density array; CAF, cancer-associated fibroblast; IPSS-R, revised International Prognostic Scoring System

Key words: myelodysplastic syndrome, bone marrow mesenchymal stroma cells, extracellular vesicle, microRNA-101, disease progression

agents in the cancer microenvironment (10). Thus, certain types of tumors have the ability to reconstruct the surrounding environment to facilitate the progression of neoplastic cells. On the contrary, BM-MSCs affect functions of neoplastic cells associated with therapy resistance and disease progression (4). Therefore, cell-to-cell communication may exist between tumor cells and the surrounding environment, including fibroblasts, microvessels, and other adjacent tissues.

miRs are very short non-coding RNAs around 20 bp in length and are detected in various cell types and body fluids, including serum, urine, and saliva (9,10). miRs are considered as the candidates of liquid biopsy. In the body fluids, miRs are usually encapsulated in EVs in the stable forms and are transported to other cells, wherein they control cellular functions. As BM-MSCs are major players in the hematopoietic tissue and their genetic alterations in myelodysplastic syndrome (MDS) is plausible, we attempted to search EV-miRs in the BM-MSCs obtained from patients with MDS and acute myeloid leukemia with myelodysplasia-related changes (AML/MRC). We found that the expression of EV-miR-101 in BM-MSCs was down-regulated and related to MDS progression.

Materials and methods

Patients. Twenty-nine consecutive patients with myeloid malignancies (22 with MDS and 7 with AML/MRC) were enrolled in this study (Table I). The diagnoses were established as per the World Health Organization criteria, and included three patients with MDS with single lineage dysplasia (SLD), seven with multilineage dysplasia (MLD), two with unclassifiable MDS, one with 5q-syndrome, five with excess blast (EB)-1, and four with EB-2. Risk analysis was carried out based on the revised International Prognostic Scoring System (IPSS-R) (13) and risk categorization was performed as per the NCCN guideline (14). Four patients with MDS with intermediate risk were tentatively included in the low-risk MDS group. Written informed consents were obtained from all patients before the collection of specimens according to the Declaration of Helsinki. The present study was validated by the internal review boards of Tokyo Medical University (no. 2648, approved 22 April, 2014). As controls, human BM-MSCs from healthy donors (normal BM-MSCs) were purchased from Lonza Inc. Control serum was obtained from age-matched healthy individuals.

Culture of BM-MSCs. BM-MSCs from patients with MDS and AML/MRC were isolated using the conventional plastic adhesion method with a minor modification (15). Briefly, 0.5 to 1 ml of freshly obtained heparinized BM aspirates were cultured in equivalent volumes of Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% penicillin-streptomycin (P/S; Thermo Fisher Scientific, Inc.) and Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific) containing 10% FBS (GE Healthcare), 1% of P/S, and 1% non-essential amino acids (NEAAs; Thermo Fisher Scientific, Inc.). Cells were cultured for 3 to 5 days, and the medium was changed to DMEM (with 10% of FBS, 1% of P/S and 1% of NEAAs) for non-hematopoietic expansion after the removal of non-adherent cells. Cultured BM-MSC popula-

tion was identified as CD34⁻/CD45⁻/CD73⁺/CD90⁺/CD105⁺ with flow cytometry with <5% CD34⁺ and CD45⁺ (15).

Isolation and miR profiling of BM-MSCs. BM-MSCs (4x10⁴ cells/cm²) were cultured in 5 ml of DMEM and the culture supernatants were harvested after 48 h of incubation. EV fraction was purified with Exoquick-TC reagent (System Biosciences) according to the manufacturer's instructions. EVs were quantitated with a nanoparticle tracking analysis (NanoSight LM10; Malven) (16-18). Isolation of BM-MSC-derived EV-miRs or cellular-miR and serum EV-miR was performed using the miRNeasy kit (Qiagen). Two-hundred microliters of EV were diluted with 700 μ l of QIAzol (Qiagen). After 5 min incubation, 1 nM ath-miR-159 (Hokkaido System Science) was added [18]; the mixture was vortexed for 30 sec and incubated on ice for 10 min. Phenol extraction and cartridge filtration were subsequently performed according to the manufacturer's instruction.

Screening of candidate miRNA. miRNA profiling was carried out using a TaqMan low-density miRNA array (TLDA; Thermo Fisher Scientific, Inc.), as previously reported (16,17). PCR was done on a Applied Biosystem 7900 HT thermocycler (Carlsberg) according to the manufacturer's recommended program (the reaction was first incubated at 95°C for 2 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min) using SDS2.2 software and Data Assist (Thermo Fisher Sciences, Inc.). The expression of miRNAs was calculated based on cycle threshold (Ct) values using the 2^{- $\Delta\Delta$ C_q} method (19) normalized to those of ath-miR-159, which was spiked in each sample. Data analysis was carried out using GeneSpring software (Agilent Technologies). The Benjamin-Hochberg algorithm was used for the estimation of false discovery rates, as previously reported (16,17).

Validation of candidate miRNA expression by qRT-PCR. To compare selected miRNA expression in various fractions (EV-miRNA, cellular miRNA, and serum EV-miRNA), quantitative real-time PCR was performed by TaqMan[®] MicorRNA Assays (ThermoFisher Scientific, Inc.) using an ABI Prism 7900 sequence detection system (Applied Biosystem) according to the manufacturer's instruction.

The microRNA specific stem-loop primers (has-miR-101, cat. no. 000438; ath-miR-159, cat. no. 000338) were purchased from ThermoFisher Scientific, Inc. The reaction was first incubated at 95°C for 2 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min, as we have reported previously (18).

Statistical analysis and evaluation of miR-101 targets. Data were expressed as means \pm standard deviation (SD). Mann-Whitney U and chi-square tests were used to determine statistical significance for comparisons between the control and test groups. Multiple groups were compared with one-way analysis of variance (ANOVA). Statistical analysis was carried out using R and GraphPad Prism software (v. 5c for Macintosh; GraphPad Software Inc.).

miRs with a Δ Ct value >1.0 or <-1.0, and P-values <0.05 were considered to exhibit differential expression. Following identification of differentially expressed miRs, the predicted target genes for these altered miRs were subjected to

Table I. Hematologic features of myelodysplastic syndrome patients of whom bone marrow mesenchymal stroma cells were obtained.

UPN	Age (Year)	M/F	Dx (WHO)	Date of material obtained	Transition to acute leukemia	Outcome	IPSS	IPSS-R	WBC	Neutrophils	Hb	Plt	PB-blasts (%)	BM-blasts (%)	Karyotype
33	51	M	MDS-SLD	2014/10/10	No	Alive	Int-1	Int	5200	4030	7.6	135	0	0.8	complex
54	58	F	MDS-EB1	2014/12/16	No	Alive	Int-1	Int	2400	1080	9.3	74	0	5.6	46,XX
59	55	M	MDS-MLD	2015/1/27	No	Alive	Low	Very low	4700	2961	13.7	56	0	0	46,XY
82	78	M	MDS-MLD	2015/4/30	No	Unknown	Int-1	Very low	1900	1267	10.6	57	0	2	45,X,-Y
95	68	M	MDS-SLD	2015/8/20	No	Unknown	Low	Low	10200	7905	12.1	18	0	1.2	46,XY
93	82	M	MDS-SLD	2015/8/11	No	Dead	Low	Very low	2200	1606	10.4	105	0	0	46,XY,del(20)(q11)
101	77	M	MDS-U based on defining cytogenetic abnormality	2015/9/17	No	Alive	Low	Very low	4600	2852	10	30	0	0	45,X,-Y
102	47	F	MDS-MLD	2015/9/30	No	Alive	Int-1	Low	2800	1274	8.3	15	0	0	46,XX
103	22	M	MDS-MLD	2015/10/9	No	Unknown	Low	Int	2600	845	6.3	21	0	0	46,XY
107	82	F	MDS-MLD	2015/10/22	No	Alive	Low	Low	6100	4148	11.2	187	0.5	0.4	47,XX,+8
64_2	46	F	MDS-MLD	2016/3/8	No	Alive	Low	Very low	4500	2835	13.1	299	0	2	46,XX
141	84	M	MDS with isolated del(5q)	2016/5/19	No	Alive	Int-1	Int	3500	1295	9.5	344	0	0.5	46,XY,del(5q)
115	50	M	MDS-MLD	2016/10/11	No	Unknown	Int-1	Low	6600	5214	4.3	42	0.5	2	46,XY,del(11)(q14)
8	72	M	MDS-EB1	2014/8/8	No	Unknown	Int-1	High	1700	408	9	78	0	5.2	46,XY
12	38	M	MDS-EB2	2014/8/26	No	Unknown	Int-2	High	3000	1410	10.3	95	0.5	10	45,XY,-7
58	71	M	MDS-EB1	2015/1/22	No	Unknown	Int-2	Very high	2900	2233	8.3	31	0	9.2	complex
22	66	M	MDS-EB1	2014/11/27	Yes	Unknown	Int-1	High	2000	910	7.6	16	0	7.6	46,XY
44	72	M	MDS-EB2	2015/1/22	Yes	Dead	High	Very high	16300	2853	9.1	97	5	16.8	46,XY,-7,+m/46,XY,idem,del(12)(p13)
38	56	M	MDS-EB2	2014/10/14	No	Dead	Int-2	High	5500	4895	8.3	164	0	12.4	46,XY
120	68	F	MDS-EB2	2016/3/10	Yes	Dead	High	Very high	1100	374	7.8	19	0	17.2	complex
108	87	M	MDS-U with > 1% PB blasts	2015/10/26	No	Dead	in-2	High	11400	10864	8.1	65	1.5	4.8	46,XY,del(20)(q11)/46,XY, idem,-7,+8
175	68	M	MDS-EB1	2016/2/3	Yes	Dead	Int-2	High	2000	1110	8.8	30	2	4.4	complex
4	77	M	AML with MRC	2014/4/2		Dead			4500	393	8.9	23	17	49.5	complex
30	72	M	AML with MRC	2014/10/8		Unknown			800	248	7.7	50	2	21	45,X,-Y
13	61	M	AML (M6A) with MRC	2014/9/10		Dead			1300	338	7.6	17	12	12.8	complex

Table I. Continued. Hematologic features of myelodysplastic syndrome patients of whom bone marrow mesenchymal stroma cells were obtained.

UPN	Age (Year)	M/F	Dx (WHO)	Date of material obtained	Transition to acute leukemia	Outcome	IPSS	IPSS-R	WBC	Neutrophils	Hb	Plt	PB-blasts (%)	BM-blasts (%)	Karyotype
18	67	F	AML with MRC	2014/9/16		Dead			2000	520	7.9	7	2.5	66	45,XX,-7
127	64	M	AML with MRC	2016/4/11		Alive			1300	240	9.3	78	0	23.2	46,XY
133	67	F	AML with MRC	2016/4/26		Dead			1400	400	7.3	25	8	34	45,XX,-18
98	42	F	AML with MRC	2015/9/9		Dead			2000	270	11.3	175	1.5	21	46,XX,inv(16)(p13;q22)/47,XX,idem+22

UPN, unique patient number; M/F, male/female; Dx, diagnosis; IPSS, International Prognostic Scoring System; IPSS-R, revised IPSS; WBC, white blood cell count (x10⁹/l); Hb, hemoglobin g/dl; Plt, platelets (x10¹²/l); PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndrome; AML with MRC, acute myeloid leukemia with myelodysplasia-related changes; Unknown, due to transfer to another hospital.

experimental validation using miR-target interaction database MiRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>). In addition, functional annotation of target genes was carried out with Database for Annotation, Visualization, and Integrated Discovery (DAVID Bioinformatics tools v6.7) (<http://david.abcc.ncifcrf.gov>).

Results

Downregulation of BM-MSC-derived EV miR-101 in patients with high-risk MDS. To identify the association between EV-miRs from BM-MSCs and risk in patients with MDS, we used TLDA to screen the miR expression profile between low-risk group (n=13: Very low, low, and intermediate risk; 9 males/4 females, mean age 61.5 years (range 22 to 84 years old)) and high-risk group (n=9: High and very high risk; 8 males/1 females, mean age 66.4 years (range 38 to 87 years old)) separated by the IPSS-R. Seven patients with AML-MRC included 4 males and 3 females, mean age 64.3 years with ranging 42 to 77 years old). We extracted nine miRs (has-miR-375, has-miR-101, has-miR-424, has-miR-548c-3p, has-miR-15b, has-miR-485-3p, has-miR-579, has-miR-195, and has-miR-369-3p) that were differentially expressed between low-risk and high-risk groups using R software-T test (Figs. 1 and S1) (GSE133276). We then compared the expression patterns of BM-MSC-derived EV-miRs detected with TLDA between normal controls (Lonza) and low-risk group or high-risk group. Of the nine EV-miRs, EV-miR-101 showed no significant difference between normal control and low-risk groups (P=0.5824), while its expression was downregulated in the high-risk group (P=0.0012). We separated 13 MDS patients as low-risk group, including 5 with very low MDS by IPSS-R with marrow blasts <2%, therefore, we considered the character of BM-MSC may overlap of those from normal control. On the other hand, EV-miR-15b expression was downregulated in both the low-risk (P=0.0004) and high-risk (P=0.0001) groups as compared with that in the control BM-MSCs, suggesting that EV-miR-15b could serve as a biomarker for MDS rather than that for disease progression (Fig. 1).

To confirm the observation of low expression level of BM-MSC-derived EV-miR-101 in high-risk group and patients with AML/MRC, we quantified the expression of EV-miR-101 with qRT-PCR. As a result, we found no significant difference in EV-miR-101 expression level in BM-MSCs from normal control and low-risk groups (P=0.2579). In contrast, EV-miR-101 expression significantly decreased in the high-risk group (P<0.0001) and patients with AML/MRC (P<0.0001) (Fig. 2A), indicating that BM-MSC-derived EV-miR-101 expression level was associated with disease severity in patients with MDS.

Expression pattern of miR-101 in BM-MSCs and serum EV-miR-101. We assessed miR-101 level in BM-MSCs using qRT-PCR, as the pattern of BM-MSC-derived EV-miR-101 was related to MDS disease progression. The relative expression level of miR-101 in control BM-MSCs (Lonza) was lower than that reported in the BM-MSCs from the high-risk group (P=0.0058) and patients with AML/MRC (P=0.0095). No significant difference was reported between normal control and low-risk groups (P=0.8713). Therefore, the expression

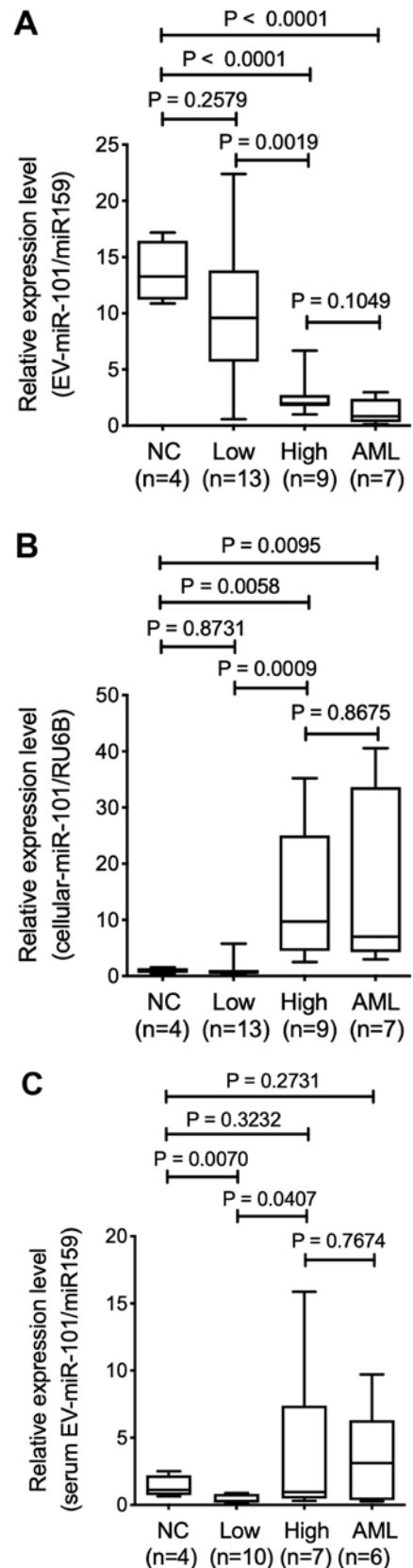
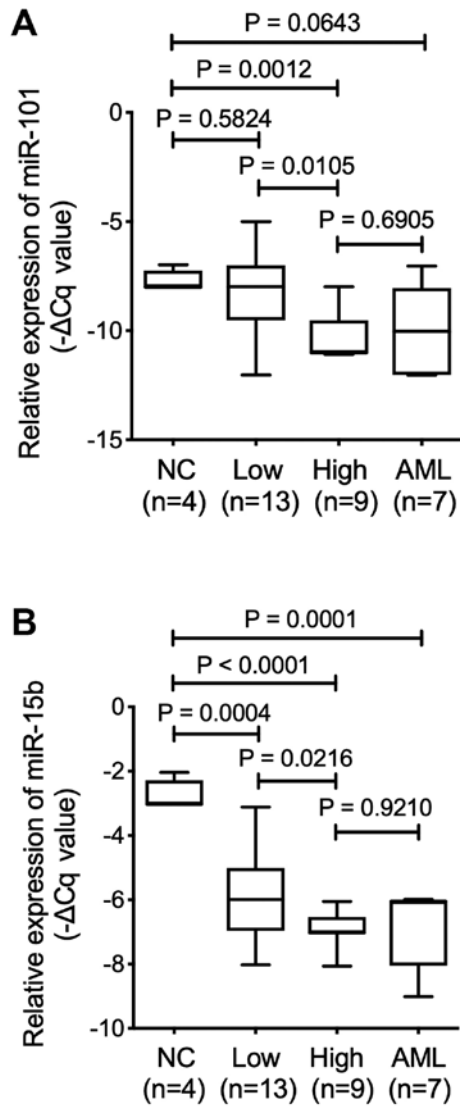


Figure 1. Expression of extracellular vesicle miR derived from BM-MSCs using TaqMan low-density Array (Thermo Fisher Scientific, Inc.). miR expression according to normal control (NC) vs. low-risk MDS group (Low), high-risk MDS group (High) and AML/MRC group (AML). Box plots depict the 75th percentile, median and 25th percentile values; and whisker plots represent minimum to maximum expression. P values were calculated using the independent two-sample *t*-test. (A) The expression of EV-miR-101 was significantly higher in the low-risk group compared with the high-risk group. The expression of miR-101 in the low-risk group indicated no significant difference compared with the normal controls. (B) Significant differences in the expression of miR-15b were observed between low-risk and normal control groups. miR, microRNA; BM-MSCs, bone marrow mesenchymal stromal cells; NC, negative control; MDS, myelodysplastic syndrome; AML/MRC, acute myeloid leukemia with myelodysplasia-related changes.

profile of cellular miR-101 seemed to be a mirror image of the EV-miR-101 obtained from BM-MSCs (Fig. 2B).

We next assessed EV-miR-101 level obtained from the serum using qRT-PCR. Serum was obtained from identical patients with MDS assessed by TLDA, and normal control serum was obtained from age-matched healthy individuals (n=4). The expression level of serum EV-miR-101 was significantly downregulated in low-risk group (n=10) as compared with that in healthy control (P=0.0070), while no significant difference was observed in high-risk group (n=7) (P=0.3232) or patients with AML/MRC (n=6) (P=0.2731) as

Figure 2. EV miR101 obtained from BM-MSCs. (A) Expression of miR-101 derived from BM-MSCs (B) or serum EV-miR-101 expression (C) as assessed using reverse transcription-quantitative PCR. The expression of EV-miR-101 in the BM-MSCs was significantly higher in the low-risk MDS group compared with the high-risk MDS group, while cellular miR-101 expression was significantly increased in the high-risk group compared with the low-risk group or normal controls. EV, extracellular vesicle; miR, microRNA; BM-MSCs, bone marrow mesenchymal stromal cells; MDS, myelodysplastic syndrome.

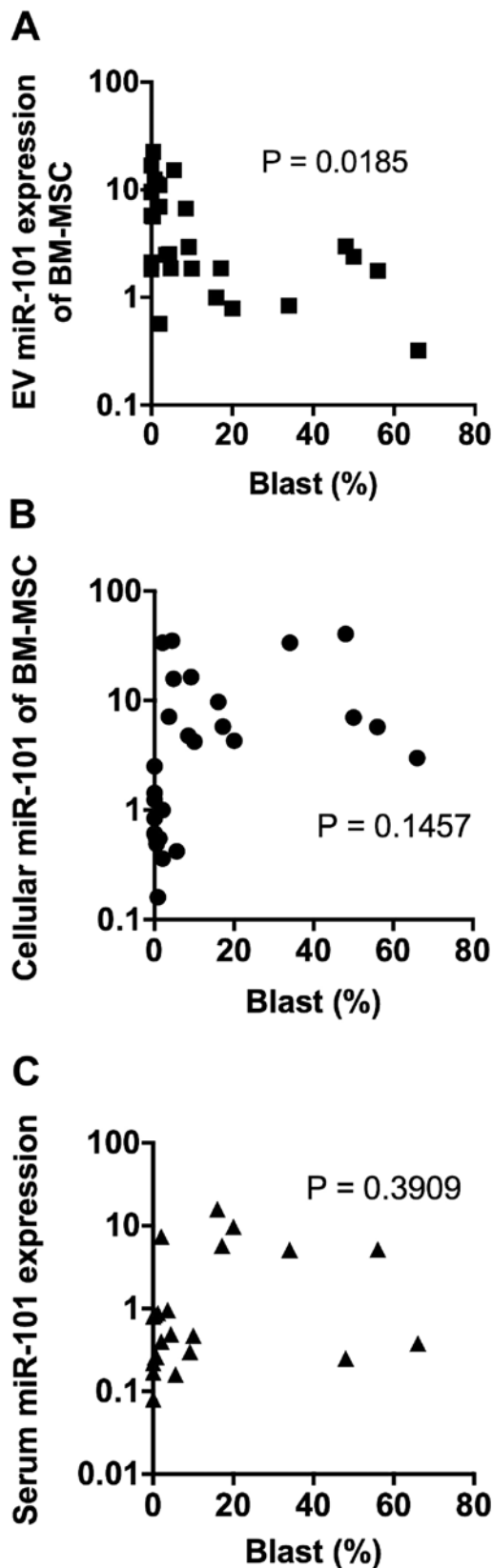


Figure 3. Correlation between BM blast percentage and (A) BM-MSC-derived EV-miR-101, (B) cellular miR-101 from BM-MSCs or serum (C) EV-miR-101. BM, bone marrow; BM-MSCs, bone marrow mesenchymal stromal cells; miR, microRNA.

compared with healthy subjects (Fig. 2C). Serum EV-miR-101 expression pattern correlated with neither BM-MSC-derived

EV-miR-101 nor BM-MSC-derived cellular-miR-101 level (data not shown).

MiR-101 expression level and BM blast percentage. We assessed the association between miR-101 expression levels and percentage of blasts in the BM. The BM-MSC-derived EV-miR-101 level negatively correlated with the occupancy of blasts in the BM ($P=0.0185$) (Fig. 3A). No significant correlations were observed between BM blast percentage and BM-MSC-derived cellular miR-101 level ($P=0.1457$) (Fig. 3B) or serum EV-miR-101 level ($P=0.3909$) (Fig. 3C).

Target for miR-101. MiRTarBase was used to identify the predicted target genes of miR-101 to determine its biological significance. More than 50 target genes were extracted by MiRTarBase, and the target genes that showed a strong evidence are summarized in Supplementary Table I. The epigenetic regulator genes, including enhancer of zester homolog 2 (*EZH2*), part of polycomb repressive complex subunit (*SUZ12*), ten-eleven translocation oncogene family member 2 (*TET2*), and proto-oncogenes (*c-FOS* and *c-MYC*), were experimentally validated with a luciferase reporter assay in the literature. Functional annotation analysis with DAVID revealed the most affected biological pathways of these genes downregulated in the high-risk group, including cell-cycle related pathway and epigenetic regulation pathway (Table II).

Discussion

The present study demonstrates that EV-miR-101 expression level in BM-MSCs was different depending on MDS severity. High-risk group and patients with AML/MRC showed significantly lower expression of BM-MSC-derived EV-miR-101 than the low-risk group or normal controls. In contrast, the expression of miR-101 derived from BM-MSCs was upregulated in patients from the high-risk group, indicative of the dissociation between miR-101 expression level and EV from BM-MSCs in patients with MDS. Although no relationship was observed between BM blast percentage and BM-MSC-derived cellular-miR-101 or serum EV-miR-101 expression level, EV-miR-101 level from BM-MSCs exhibited reverse correlation with BM blast percentage. These findings suggest the possibility that some specific miRs from BM-MSCs may be interrupted from translocation into EVs (or intra-cellular accumulation) and this phenomenon may be linked with the increase in the number of blasts in patients with MDS. The dissociation between cellular miR and EV-miR (or exosomal miR) has been observed in neoplastic cells (9), including leukemia cells (18). However, the current findings deny the possible utility of serum miR measurement as liquid biopsy for various diseases. The importance of BM-MSC-derived EV-miR may indicate the nature of EV-miR, which may participate in the communication between adjacent cells, especially between BM-MSCs and hematologic malignant cells.

Hematologic neoplasia-derived exosomes affect the micro-environment in the BM. For instance, chronic lymphocytic leukemia (CLL) cell-derived exosomes induce the transition of BM stroma cells into CAFs (20). These present enriched miR-150 and miR-146a in CLL-derived exosomes and an elevated miR-150 and miR-146a in BM-MSCs after co-culture

Table II. The gene ontology terms enriched in targets of miR-101. The terms with P-value <0.05 and false discovery rate <0.05 were listed.

Term	P-value	Benjamini
Positive regulation of transcription from RNA polymerase II promoter	9.90x10 ¹⁰	1.30x10 ⁶
Negative regulation of transcription from RNA polymerase II promoter	4.10x10 ⁷	2.60x10 ⁴
Positive regulation of gene expression	2.00x10 ⁶	8.70x10 ⁴
Negative regulation of cell proliferation	5.00x10 ⁶	1.60x10 ³
Positive regulation of transcription, DNA-templated	5.70x10 ⁶	1.50x10 ³
Response to estradiol	1.20x10 ⁵	2.50x10 ³
Positive regulation of neuroblast proliferation	3.60x10 ⁵	6.60x10 ³
Cochlea morphogenesis	4.90 x10 ⁵	7.80 x10 ³
Branching morphogenesis of an epithelial tube	5.60 x10 ⁵	8.00 x10 ³
Positive regulation of mesenchymal cell proliferation	8.20x10 ⁵	1.00x10 ²
Protein phosphorylation	1.10x10 ⁴	1.30x10 ²
Positive regulation of epithelial to mesenchymal transition	1.70x10 ⁴	1.80x10 ²
Epithelial to mesenchymal transition	1.90x10 ⁴	1.80x10 ²
Response to lipopolysaccharide	2.00x10 ⁴	1.80x10 ²
Coronary artery morphogenesis	2.20x10 ⁴	1.80x10 ²
Cellular response to hypoxia	2.70x10 ⁴	2.20x10 ²
Heart development	3.30x10 ⁴	2.40x10 ²
Thymus development	3.70x10 ⁴	2.60x10 ²
Positive regulation of apoptotic process	4.30x10 ⁴	2.90x10 ²
Response to drug	4.60x10 ⁴	2.90x10 ²
Positive regulation of cellular component movement	4.70x10 ⁴	2.80x10 ²
Negative regulation of epithelial cell differentiation	5.70x10 ⁴	3.30x10 ²
Negative regulation of gene expression, epigenetic	5.80x10 ⁴	3.20x10 ²
Regulation of apoptotic process	6.50x10 ⁴	3.40x10 ²
Positive regulation of protein phosphorylation	7.90x10 ⁴	4.00x10 ²
Positive regulation of epithelial cell proliferation	1.00x10 ³	4.80x10 ²

miR, microRNA.

with primary CLL cells. CLL exosomes induced the proliferation and migration of BM-MSCs with angiogenesis (20). We also reported that EV-miR-135b from multiple myeloma cells transfers and induces angiogenesis (18). In solid tumors, the phenomenon of transfiguration of the surrounding fibroblasts into CAF has been reported, and this change is known to affect cancer, leading to tumor progression and drug resistance (21). In hematologic neoplasia, the similar situation (CAF induction of BM-MSCs) could be plausible; however, such phenomenon is only limited in lymphoid neoplasia, i.e., CLL and multiple myeloma (20,22).

Ozdogan *et al* demonstrated the low expression level of *DICER1* gene and miR dysregulation in the BM-MSCs obtained from patients with MDS and AML along with the downregulated expression of miRs (miR-30d-5p, miR-222-3p and miR-30a-3p) and overexpression of miR-4426 in MDS-derived BM-MSCs (23). Downregulation of *DICER1* expression in the BM-MSCs from patients with MDS promoted cellular senescence and decreased the supportive capability of hematopoietic stem cells along with the downregulation of the expression of miR-17 family (miR-17-5p, miR-20a/b, miR-106a/b, and miR-93) (24). Studies on BM-MSC-derived EV (including exosomes) have focused on limited fields of

hematologic neoplasia. Wang *et al* demonstrated that mouse BM-MSC (5T33)-derived exosomes increased the viability and proliferation and decreased the apoptosis of mouse myeloma cells (5T33 MM model) in response to bortezomib resistance (3). Moreover, these authors showed that human myeloma BM-MSC-derived exosomes increased the viability of human myeloma cells (RPMI-8226) in a dose-dependent manner (3). Umezu *et al* also reported that the BM-MSC-derived EV-miR-340 from normal young donor (Lonza), rather than old donor, inhibited myeloma-induced angiogenesis via hepatocyte growth factor/c-MET signaling (25). In this study, no particular correlation was observed in the expression level between BM-MSC-derived EV-miR-101 and serum EV-miR-101, suggesting that the BM-MSC-derived EV-miR-101 may affect adjacent cells, including MDS cells.

miR-101 is known to exert anti-neoplastic effects and facilitate apoptosis (26). For instance, miR-101 exhibited suppressive effects on bladder cancer via c-FOS (27), colorectal cancer via EZH2 (28), and breast cancer via EYA1 (29). miR-101 is also known to exert suppressive effects on vascular endothelial growth factor-C (30,31). Recent reports found that miR-101 regulates the development and progression of myeloid- and

T-lymphoid lineage leukemia (32,33) and suppresses the proliferation of neoplastic cells by targeting genes related to epigenetic regulation (Tables II and SI). Thus, we speculate the possibility that EV-miR-101 from BM-MSCs transfer to MDS cells and control their proliferation via epigenetic regulation. In patients with high-risk MDS, the decrease in the expression level of EV-miR-101 may result in the release of the anti-proliferative effect on MDS cells. Another plausible possibility is that the more malignant clone during MDS progression may induce BM-MSCs to suppress EV-miR-101 expression. miR-101 antisense transfection assay showed enhanced proliferation of human myeloid leukemia (HL-60) cells with an increase in c-FOS expression (unpublished observation), thus suggesting that the downregulation of EV-miR-101 expression in patients with high-risk MDS may affect the proliferation of MDS cells.

Development of MDS is known to be associated with the accumulation of genetic abnormalities by aging, so-called 'Clonal Hematopoiesis of Indeterminate Potential (CHIP)', and additional mutations in critical genes, including epigenetic regulator genes, accelerate AML (34). In addition to genetic abnormalities in MDS cells, BM-MSCs are also shown to have synonymous or non-synonymous mutations (15,35,36) such as 'genetic injury.' The unresolved issue is whether demethylating agents available for patients with MDS could exert any effect on BM-MSCs, especially BM-MSC-derived EV, as miR-101 targets many epigenetic regulator genes. Poon *et al* demonstrated that a hypomethylating agent (azacytidine) could also target BM-MSCs in dysplastic MDS and contribute to the restoration of active hematopoiesis, whereas patients with MDS that failed to respond to hypomethylating treatment had disease progression (37). We could not reproduce the peculiarity of BM-MSC-derived miR concerning the anatomical architecture of MDS.

In conclusion, the current study demonstrates that the BM-MSCs obtained from patients with MDS were heterogeneous with respect to the expression of EV-miR. Patients with high-risk MDS and AML/MRC had lower expression of BM-MSC-derived EV-miR-101 than those with low-risk MDS. This tendency was reversible as compared with the miR-101 level from BM-MSCs; the serum EV-miR-101 expression showed no association. Although we failed to clarify the exact mechanism underlying the downregulation of EV-miR-101 expression from BM-MSCs in patients with high-risk MDS, targeting miR-101 may regulate neoplastic proliferation. Thus, the downregulation of EV-miR-101 expression may affect adjacent cell-to-cell communication and accelerate the malignant process in MDS cells.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Platform Project for Practical Research for Innovative Cancer Control from the Japan Agency for Medical Research and Development (grant no. 15Ack0106073h0002), the Private University Strategic Research-Based Support Project (grant no. S1311016) from the Ministry of Education, Culture, Sports, Science and Technology and JSPS KAKENHI (grant no. 19K16811).

Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the Gene Expression Omnibus repository, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133276>.

Authors' contributions

YS, MA, SY, SK, TS, HF, DA and KO treated the patients; TU and CKK performed TLDA, PCR and data analysis; SI established BM-MSCs; TU contributed essential reagents or tools; YS and KO wrote the manuscript; KO and JHO designed the research and supervised the project. All authors contributed to data analysis and drafting of the manuscript.

Ethics approval and consent to participate

Patient data was used in the present study according to the ethical principles for medical research involving human subjects of The Declaration of Helsinki (38). The study protocols were approved by The Regional Ethics Review Board of Tokyo Medical University (Tokyo, Japan), and all patients provided written informed consent prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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