Selective surface marker and miRNA profiles of CD34+ blast-derived microvesicles in chronic myelogenous leukemia

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Abstract. The present study aimed at investigating the selective enrichment of surface marker and functional microRNA (miRNA) profiles of cluster of differentiation (CD)34+ blast-derived microvesicles (MVs) from parental cells in chronic myelogenous leukemia (CML), thus providing an experimental basis for MVs to be used to predict characteristics of CD34+ blasts. Magnetic activated cell sorting and continuous differential centrifugation were used to isolate primary CML CD34+ blasts and MVs, in addition to utilizing flow cytometry to identify surface markers of CD34+ blasts and blast-derived MVs. Microarray analysis and the reverse transcription-quantitative polymerase chain reaction were performed to analyze miRNA profiles of CD34+ blasts and MVs. The results of the present study indicated that primary CML CD34+ blasts were able to release MVs, which were selectively enriched with the surface markers CD34 and CD123, and functional miRNAs from parental cells. A total of 15 miRNAs were upregulated in CD34+ blast derived-MVs compared with in CD34+ cells. Distinct Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology terms characterized by altered gene expression and potentially associated miRNA were identified. Upregulated miRNAs in MVs were associated with cell development, tumorigenesis and signaling pathways involving ErbB and phosphoinositide 3-kinase/protein kinase B. The present study provides evidence, which increases the understanding of physiological functions of cancer-derived MVs, and aids the understanding of the roles of CD34+ blast-derived MVs in CML-associated processes.

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Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder that is derived from abnormal pluripotent bone marrow hematopoietic stem cells, characterized by the Philadelphia chromosome and/or the breakpoint cluster region protein (BCR)-Abelson murine leukemia viral oncogene homolog 1 (ABL) fusion gene (1). Imatinib (Gleevec), a small-molecule BCR-ABL tyrosine kinase inhibitor (TKI), markedly improved the estimated overall survival rate in patients with chronic-phase (CP)-CML to between 80 and 85% at 7-10 years (2,3). However, although imatinib eradicated mature BCR-ABL+ CML cells, it was not able to achieve similar effects in CML leukemia stem cells (LSCs) since BCR-ABL tyrosine kinase is dispensable for CML LSC survival and maintenance, rendering these cells able to survive in the presence of TKIs and to eventually promote relapse (4,5). Therefore, the elucidation of the biological characteristics of the LSC is required.

Microvesicles (MVs), lipid-bilayer vesicles formed by directly budding off from the cell membrane, represent a heterogeneous population of vesicles with a diameter of between 100 and 1,000 nm (6,7). MVs are able to selectively package complex biological information from parental cells, including mRNA, microRNA (miRNA) and proteins, which serve an important role in intercellular communication (8). Previous studies have suggested that MVs are involved in inflammation, immune regulation, tumor metastasis and angiogenesis, and may also be indicators for the diagnosis and prognosis of disease (9-12).

miRNAs, a large family of small (between 22 and 24 nucleotides in length) non-coding RNAs, decrease gene expression levels by regulating the translation of certain mRNAs into protein and serve an important role in disease progression and carcinogenesis. An increasing number of studies have revealed that genetic exchange of miRNA between cells may be accomplished through MVs (13). For instance, MVs derived from endothelial progenitor cells protect the kidneys from acute

ischemic injury by miRNA-dependent reprogramming of resident renal cells (14). Additionally, embryonic stem cell-derived MVs may be useful therapeutic tools for transferring miRNA to cells and important mediators of signaling within stem cell niches (15). Although a number of cell-derived MVs are currently recognized and studied, MVs derived from primary CML LSCs remain unknown, and there is a lack of comprehensive information concerning miRNA from LSC-secreted MVs.

In the present study, CML LSCs were studied and MVs secreted from LSCs were characterized in terms of surface markers and miRNA profiles. Furthermore, the miRNA profiles in MVs of CML blasts were compared with those of the AML stem cell line KG-1a. The results of the present study revealed that these miRNAs were primarily associated with MVs and stem cells, and may affect characteristics of LSCs.

Materials and methods

Patient samples. Fresh peripheral blood (PB) or bone marrow (BM) samples were obtained from healthy donors and patients with CML (patient characteristics are listed in Table I). PB cells were also acquired from leftover material from PB transplant harvests of healthy donors. Written informed consent was obtained and the present study was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Mononuclear cells were extracted by Ficoll-Paque density gradient centrifugation (Lymphoprep™, 1.073 g/ml; Tianjinhaoyang Biological Manufacture Co., Ltd, Tianjin, China) according to the manufacturer's protocol.

Cluster of differentiation (CD)34+ cell isolation and cell culture. $CD34^+$ cells (>92% pure) were isolated using immunomagnetic separation by positive selection of cells (human CD34 microbead kit; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Primary $CD34^+$ cells were cultured in StemSpan serum-free medium (Stemcell Technologies, Inc., Vancouver, BC, Canada) which was supplemented with a growth factor mixture, including 100 ng/ml each of Fms-related tyrosine kinase-3 ligand and stem cell factor, and 20 ng/ml each of interleukin (IL)-3, IL-6 and granulocyte-colony stimulating factor (Peprotech, Inc., Rocky Hill, NJ, USA). Primary cells were counted using a hemocytometer and subsequently cultured at density of $5x10^4$ cells/ml. After 48 h, cells were collected for flow cytometry or miRNA experiments and culture medium was used for MV isolation. The KG-1a cells, a human acute myelogenous leukemia (AML) cell line, were stored long-term and passaged in the Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The KG-1a cell line, with ~95% CD34 expression, was routinely cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% fetal bovine serum (FBS); FBS-derived MVs were removed using a differential centrifugation method, including 750 x g for 15 min, followed by 1,500 x g for 20 min and then 16,000 x g for 45 min, at 4˚C. The cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. The culture medium was collected and replaced with fresh medium every 48 h.

Preparation of MVs. MVs were isolated from the conditioned medium of $CD34^+$ cells and KG-1a cells by continuous differential centrifugation as described previously (9). At the time of culture medium harvest, MVs were prepared by differential centrifugation as described in the previous section. Pellets of MVs were washed in ice-cold PBS (particles <0.1 μm were removed with a membrane filter prior to use) for flow cytometric analysis.

Flow cytometric analysis. To analyze CD123 expression, $CD34^+$ cells were stained with anti-CD34-phycoerythrin (PE) antibody (1:50; cat. no., 130-098-140; Miltenyi Biotec GmbH) and anti-CD123-PE-cyanine 7 antibody (1:50; cat. no., 306009; BioLegend, Inc., San Diego, CA, USA) per tube. A previously described method (16) was utilized to analyze the phenotype of MVs. In brief, standard microbeads with a diameter of 1 or 3 μm (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were used to set the upper size limit for MVs. MVs isolated from a 10 ml supernatant of $CD34^+$ cell-conditioned medium were resuspended in PBS (extra particles with 0.1 μm membrane filter were removed prior to use) and then stained with calcein-acetoxymethyl ester (AM) (5 μg/ml; cat. no., 65-0853-39; Thermo Fisher Scientific, Inc.), CD34 and CD123 for 20 min at ambient temperature. The stained MVs were diluted in 300 μl PBS and analyzed using a FACSAria II flow cytometer with FACSDiva version 6.1.3 software (BD Pharmingen, San Diego, CA, USA). MVs were defined as calcein-AM-positive events.

miRNA expression profiling. miRNA expression profiles were conducted on 4 samples [CP-CML $CD34^+$ cells (C-C); CP-CML $CD34^+$ cell-derived MVs (C-MV); KG-1a cells (K-C); KG-1a cell-derived MVs (K-MV)] using miRCURY™ locked nucleic acid (LNA) array (7th generation, version 18.0; Exiqon A/S, Vedbaek, Denmark) containing probes for 3,100 miRNAs. Total RNA was isolated using TRIzol (Thermo Fisher Scientific, Inc.) and purified using an RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA was quantified using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and RNA integrity was determined by gel electrophoresis. Following RNA extraction from the samples, miRNAs were labelled using the miRCURY™ Hy3™/Hy5™ Power Labeling kit (Exiqon A/S), according to the manufacturer's protocol. Subsequently, the Hy3™-labeled samples were hybridized on the miRCURY™ LNA array. Following hybridization, the slides were washed five times with the Wash Buffer kit (Exiqon A/S) and dried by centrifugation at 200 x g for 5 min. The slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments; Molecular Devices, LLC, Sunnyvale, CA, USA). Scanned images were then imported into GenePix Pro 6.0 software (Axon Instruments; Molecular Devices, LLC) for grid alignment and data extraction. The 4 replicated spots for each probe were averaged. Expressed results were normalized using the median normalization method (foreground-background signal intensity)/median of samples with intensity ≥30) and subsequently

Table I. Characteristics of patients with CML.

Patient	Sex	Age, years	Diagnosis	White blood cell count (g/l)	Treatment
CP-CML 1	F	45	CP-CML	111.70	*De novo*
CP-CML 2	M	27	CP-CML	262.26	*De novo*
CP-CML 3	M	39	CP-CML	289.50	*De novo*
CP-CML 4	M	50	CP-CML	236.57	*De novo*
CP-CML 5	M	81	CP-CML	366.99	*De novo*
CP-CML 6	F	29	CP-CML	196.20	*De novo*
CP-CML 7	F	47	CP-CML	269.60	*De novo*
CP-CML 8	M	53	CP-CML	198.20	*De novo*
BC-CML 1	F	56	BC-CML	16.80	IFN, IM
BC-CML 2	F	52	BC-CML	30.72	IM, Dasa
BC-CML 3	M	47	BC-CML	42.40	IM
BC-CML 4	M	43	BC-CML	35.20	*De novo*

CP, chronic-phase; CML, chronic myelogenous leukemia; BC, blast crisis; M, male; F, female; IFN, interferon α; IM, imatinib; Dasa, dasatinib.

identified differentially expressed (DE) miRNAs between two samples were filtered by fold change (threshold, ≥2.0).

Validation of microarray results. A total of 4 DE miRNAs identified by miRNA microarray were selected for further validation by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA (400 ng) was reverse transcribed with a reaction mix of of dNTPs (HyTest Ltd., Turku, Finland), RT buffer, MMLV reverse transcriptase, RNase inhibitor (all from Epicentre; Illumina, Inc., San Diego, CA, USA), specific primers (Balige Co., Shanghai, China) and RNAase-free water to 20 μl. The reaction was performed at 16˚C for 30 min, followed by a 40-min incubation at 42˚C and finally 85˚C for 5 min using an Applied Biosystems 9700 Real-Time PCR instrument (Thermo Fisher Scientific, Inc.).

SYBR Green miRNA assays with commercially available primers (Table II) for hsa-miR-627-5p, hsa-miR-483-5p, hsa-miR-638 and hsa-miR-1290 (all materials from Guangzhou RiboBio Co., Ltd., Guangzhou, China) were used according to the manufacturer's protocol. Relative expression was calculated with the $2^{-\Delta\Delta Cq}$ method with an Applied Biosystems ViiA 7 Real-Time PCR system (Thermo Fisher Scientific, Inc.) (17).

Target gene prediction. TargetScan, miRBase and miRanda databases were used to predict the target genes of DE miRNAs. Among the putative targets, the overlapping genes from these databases were determined.

Bioinformatics and statistical analysis. Gene Ontology (GO) analysis and pathway annotation were used to examine the gene pool of DE miRNAs. GO terms were analyzed using the GO database. Fisher's exact test was used to identify any overlap between the DE list and the GO annotation list greater than was expected by chance. The P-value denoted the statistical significance of GO term enrichment in the DE genes. Pathway analysis is a functional analysis mapping genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The P-value denoted the statistical significance of the pathway associated with the conditions. P<0.05 was considered to indicate a statistically significant difference.

Table II. Primers used in Real-time PCR.

Gene	Primer sequence
hsa-miR-483-5p	
F	5'-AGGGAAGACGGGAGAAGAGA-3'
R	5'-GTGCGTGTCGTGGAGTCG-3'
hsa-miR-627-5p	
F	5'-GGGGGTGAGTCTCTAAGAAA-3'
R	5'-CAGTGCGTGTCGTGGAGT-3'
hsa-miR-1290	
F	5'-GGGGTGGATTTTTGGAT-3'
R	5'-CAGTGCGTGTCGTGGAGT-3'
hsa-miR-638	
F	5'-AAGGATCGCGGGCGGGT-3'
R	5'-GTGCGTGTCGTGGAGTCG-3'

miRNA, microRNA; hsa, human; F, forward; R, reverse.

Results

CML CD34⁺ blasts and blast-derived-MVs express increased levels of CD123. A previous study demonstrated that IL-3 receptor α (CD123) expression is elevated in CML progenitor and stem cells compared with healthy donors (18), which led to the collection of samples from 4 patients with blast crisis (BC)-CML, 8 patients with CP-CML and 4 cases of healthy donor stem cell residue in the present study. Multicolor flow cytometry was used to determine the surface marker profile of CML CD34⁺ cells and cell-derived MVs. The results of the present study indicated that, compared with healthy CD34⁺ cell samples, CD123 expression levels of BC-CML CD34⁺

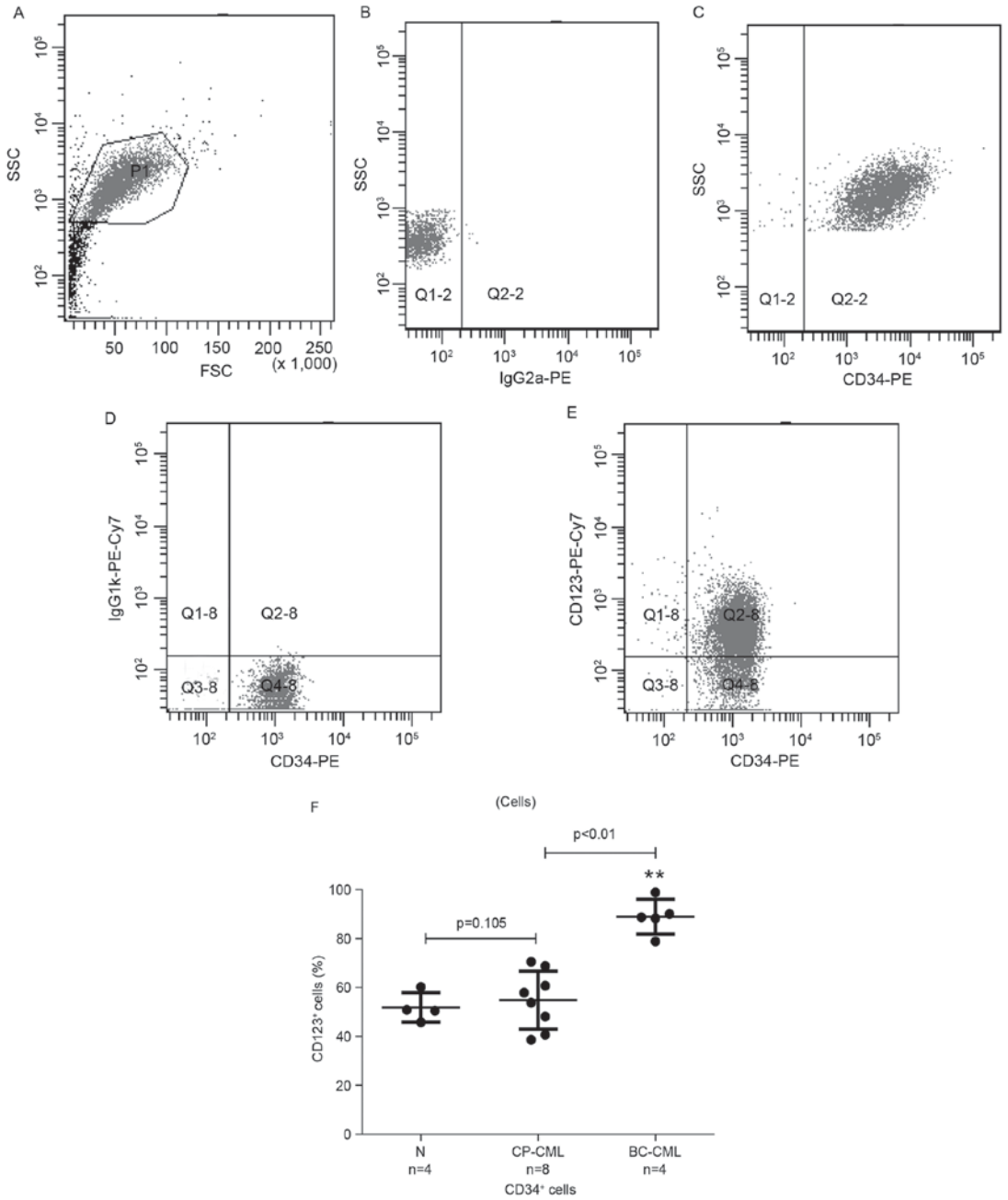

Figure 1. CD123 expression is significantly increased in CD34+ cells of patients with CP-CML and BC-CML, compared with healthy donors. The gating strategy applied to determine the proportion of CD123+ cells in CD34+ subsets was: (A) P1, active cells; (B) isotype control; (C) Q2-2, CD34+ blasts; (D) isotype control; (E) Q2-8, CD34+ CD123+ blasts. (F) Proportion of CD123+ cells within CD34+ populations from healthy donors, and patients with CP-CML and BC-CML determined using multicolor flow cytometry. Each dot represents an individual tissue sample. P=0.105, N vs. CP-CML; **P<0.01, CP-CML vs. BC-CML. CD, cluster of differentiation; CP, chronic-phase; CML, chronic myelogenous leukemia; BC, blast crisis; N, healthy donors; SSC, side scatter; FSC, forward scatter; PE, phycoerythrin; Cy7, cyanine 7; Q, quadrant; Ig, immunoglobulin.

cells were significantly increased. There was also a marked difference between BC-CML and CP-CML CD34+ cells, as presented in Fig. 1: Compared with MVs from healthy CD34+ samples, CD123 expression levels of BC-CML CD34+ cell-derived MVs were significantly increased, whereas expression levels of CP-CML CD34+ cell-derived MVs slightly increased. As presented in Fig. 2, BC-CML and CP-CML CD34+ cell-derived MVs also demonstrated significant differences. The results of the present study revealed that the expression levels of CD123 in CD34+ cells and MVs gradually increased in association with disease progression.

Comparison of MV-derived and cell-derived miRNA profiles. To validate the hypothesis that miRNAs in the MVs enabled communication and reflected genetic changes within LSCs, miRNA profiles of the MVs extracted from CML CD34+ blast cells and KG-1a were determined. It was hypothesized that there were differences in the miRNA expression levels between MVs and LSCs, therefore the RNA of CML blasts and KG-1a cells was also extracted and hybridized (CML CD34+ blast cells, CML blast-derived MV, KG-1a cells and KG-1a-derived MV, n=2). Each sample was analyzed in duplicate under similar conditions. DE miRNAs were screened using the

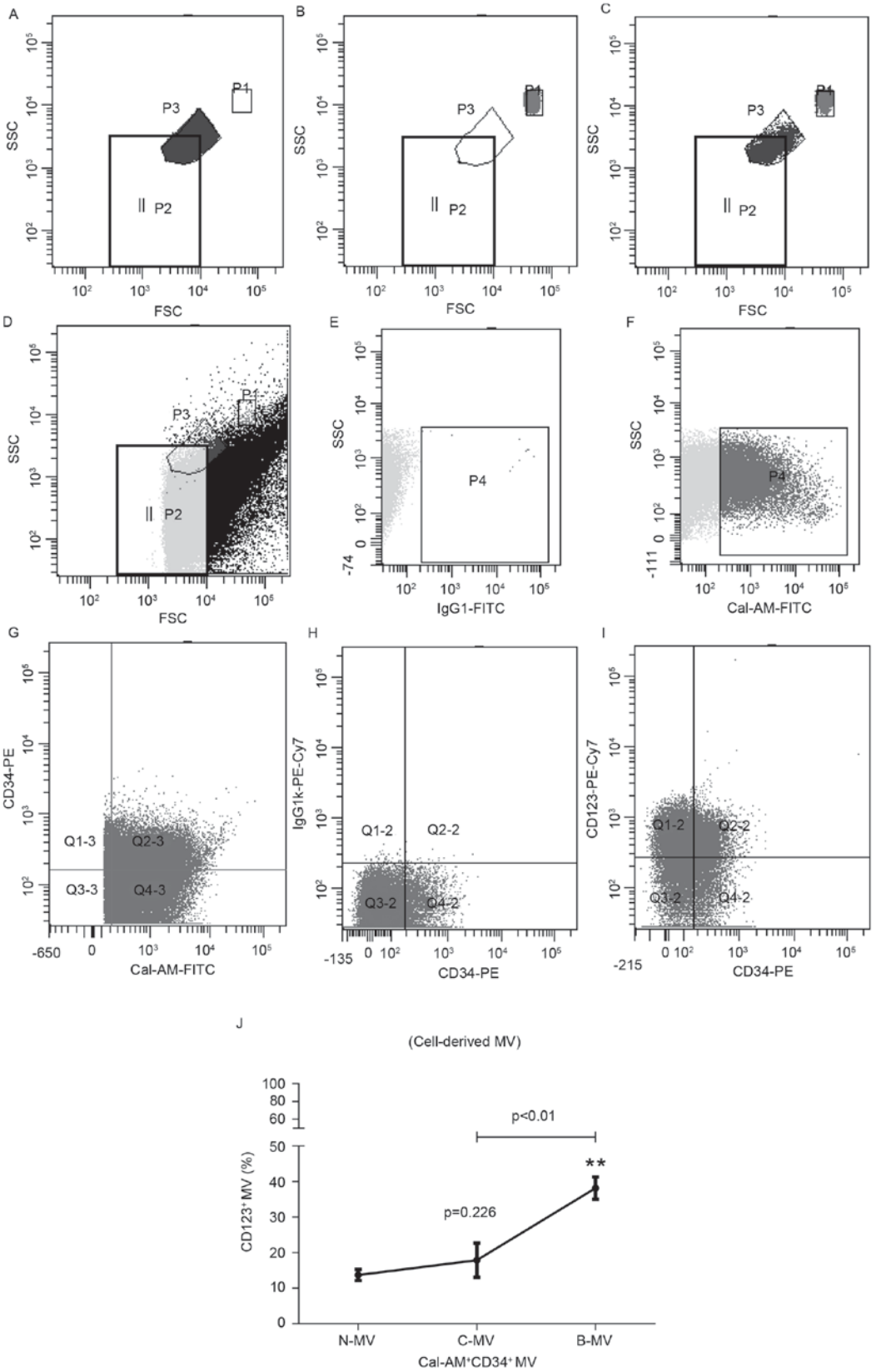

Figure 2. CD123 expression is significantly increased in CD34$^+$ blast-derived MVs of patients with CP-CML and BC-CML compared with healthy donors. Flow cytometric analysis and representative cytograms of CD123$^+$CD34$^+$ blast-derived MVs in patients with CML. (A) Fluorosphere beads of a known size were used as a standard to set the MV gate. P3 represents 1 μm beads. (B) P1 represents 3 μm beads. (C) P1 and P3 represent 3 μm and 1 μm beads, respectively. (D) Based on the calibrating beads above, P2 represents the MV gate. (E) IgG1-FITC was set as the negative control for calcein-AM$^+$ MVs. (F) Events in the MV gate (P4) were analyzed to differentiate calcein-AM$^+$ MVs from the background signal. (G) Q2-3, calcein-AM$^+$CD34$^+$ MVs; (H) CD123 isotype control, Q2-2, calcein-AM$^+$CD34$^+$CD123$^-$ MVs. (I) Q2-2, calcein-AM$^+$CD34$^+$CD123$^+$ MVs. (J) The proportion of CD123$^+$ MV within calcein-AM$^+$CD34$^+$ populations from N-MV and patients with C-MV and B-MV was determined using multicolor flow cytometry. P=0.226, N-MV vs. C-MV; **P<0.01, C-MV vs. B-MV. CD, cluster of differentiation; CP, chronic-phase; CML, chronic myelogenous leukemia; BC, blast crisis; MV, microvesicle; N-MV, healthy; C-MV, chronic-phase CML; B-MV, blast crisis CML; SSC, side scatter; FSC, forward scatter; PE, phycoerythrin; Cy7, cyanine 7; Q, quadrant; Ig, immunoglobulin; FITC, fluorescein isothiocyanate; Cal-AM, calcein acetoxymethyl ester.

Table III. miRNAs increased in CD34+ blast-derived MVs and KG-1a cell-derived MVs.

miRNA	C-MV/C-C, fold change	K-MV/K-C, fold change
hsa-miR-4732-5p	3.15	20.74
hsa-miR-1290	3.13	8.33
hsa-miR-4750-5p	2.92	5.46
hsa-miR-1908-5p	2.78	5.26
hsa-miR-483-5p	2.63	6.00
hsa-miR-638	2.58	4.65
hsa-miR-3960	2.58	3.23
hsa-miR-4516	2.44	12.97
hsa-miR-1469	2.42	5.66
hsa-miR-4467	2.41	4.12
hsa-miR-4707-5p	2.20	7.75
hsa-miR-4285	2.13	7.43
hsa-miR-4787-5p	2.12	3.90
hsa-miR-4708-3p	2.06	4.73
hsa-miR-627-5p	2.04	2.65

miRNA, microRNA; CD, cluster of differentiation; MV, microvesicle; C-C, chronic-phase chronic myelogenous leukemia CD34+ cells; C-MV, chronic-phase chronic myelogenous leukemia CD34+ blast-derived MV; K-C, KG-1a cells; K-MV, KG-1a cell-derived MV; hsa, human.

following criteria: Normalized intensity of each miRNA >30, fold changes of C-MV/CD34+ cells and K-MV/KG-1a >2.0. The present study identified that 15 miRNAs were elevated in C-MV and K-MV by setting screening criteria (Table III). The 5 most significantly upregulated miRNAs were human (hsa)-miR-4732-5p, miR-1290, hsa-miR-4750-5p, hsa-miR-1908-5p and hsa-miR-483-5p, suggesting that these MV miRNAs serve an important role in CD34+ blasts.

miRNA-target genes and GO terms. In order to investigate the significantly dysregulated MV miRNAs derived from CD34+ blasts, potential target analysis was performed. Using TargetScan, miRanda and miRBase, it was determined that 9,036, 2,412 and 2,291 target gene prediction records were identified, respectively, for the 15 overexpressed miRNAs. A total of 27 target gene results were screened through overlapping each dataset. These results suggested that these miRNAs affect various cellular biological processes including transcription, metabolism, molecular signaling pathway, proliferation, differentiation and apoptosis via the regulation of these target genes. These miRNAs may additionally be involved in tumorigenesis. Fig. 3A presents a list of associated genes targeted by DE miRNAs.

GO terms may be assigned to the potential targets. In order to understand the function of the involved genes, GO terms were divided into three groups including molecular function (MF), biological process (BP) and cellular component (CC). In the present study, focus was placed on the BP function of the involved genes. Fig. 3B presents significantly distinct GO terms of DE gene-related biological processes. The results suggested that the miRNAs of blast-derived MVs were associated with cell metabolism, cell cycle and cell adhesion.

KEGG pathway. The activation and inactivation of certain intracellular signaling pathways serve critical roles in tumor stem cell biology. To better understand the function of potential targets, signaling pathways were analyzed by KEGG (26 signaling pathways; Fig. 4). A total of 65 distinct pathways with enrichment test $P<0.01$ were identified according to the KEGG pathway database. The pathways associated with the upregulated miRNAs in MVs ($P<0.05$) included those in cancer, CML and AML, in addition to ErbB signaling and the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. The signaling pathways identified were primarily associated with regulation of cell viability, apoptosis, metabolism and tumorigenesis.

RT-qPCR. In order to validate the microarray results, RT-qPCR was performed to determine the upregulated miRNAs. A total of 4 miRNAs were selected for RT-qPCR: Hsa-miR-627-5p, hsa-miR-483-5p, hsa-miR-638 and hsa-miR-1290 (Fig. 5). Similar results were revealed in the DE miRNAs as in the miRNA microarray.

Discussion

Despite the second-generation TKIs markedly improving the outcome of patients with CML, the remaining LSCs cause disease recurrence in patients with CML (19). The biological characteristics of LSCs are quiescence, multi-differentiation and self-renewal (3); however, current strategies have difficulty accessing and eradicating LSCs (20). Similarly to the results of the present study, it had been previously demonstrated that cells may selectively package miRNAs into MVs, which are secreted under various pathophysiological conditions, and the content of MVs is considerable (21,22). Our previous study identified that BCR-ABL1 mRNA remains detectable within MVs, although the intracellular copy is 0 (strict complete molecular remission) (23). It was revealed that when maternal cells remain in the bone marrow niche, MVs can be detected and may magnify characteristics of maternal cells in the peripheral blood. Consequently, the aim of the present study was to elucidate the characteristics and molecular profiles of MVs released from specific CD34+ blasts in patients with CML.

By optimizing flow cytometric analysis, the results of the present study demonstrated that CD34 and CD123 were selectively packaged on MVs from CD34+ blasts. In addition, the expression of CD123 in CD34+ cells and MVs increased gradually along with disease progression. Thus, CD34+ cell-derived MVs may be a predictor of disease progression and may be used as a minimal residual disease marker. A previous study has revealed that CD123 is a specific surface marker of acute and chronic leukemia stem and progenitor cells (18). The present study may provide novel perspectives for the study of the biological characteristics of LSCs and the surface marker of MVs may provide the basis for the sorting of the MV subpopulation.

In the present study, it was hypothesized that miRNAs were contained in MVs, which enabled communication and

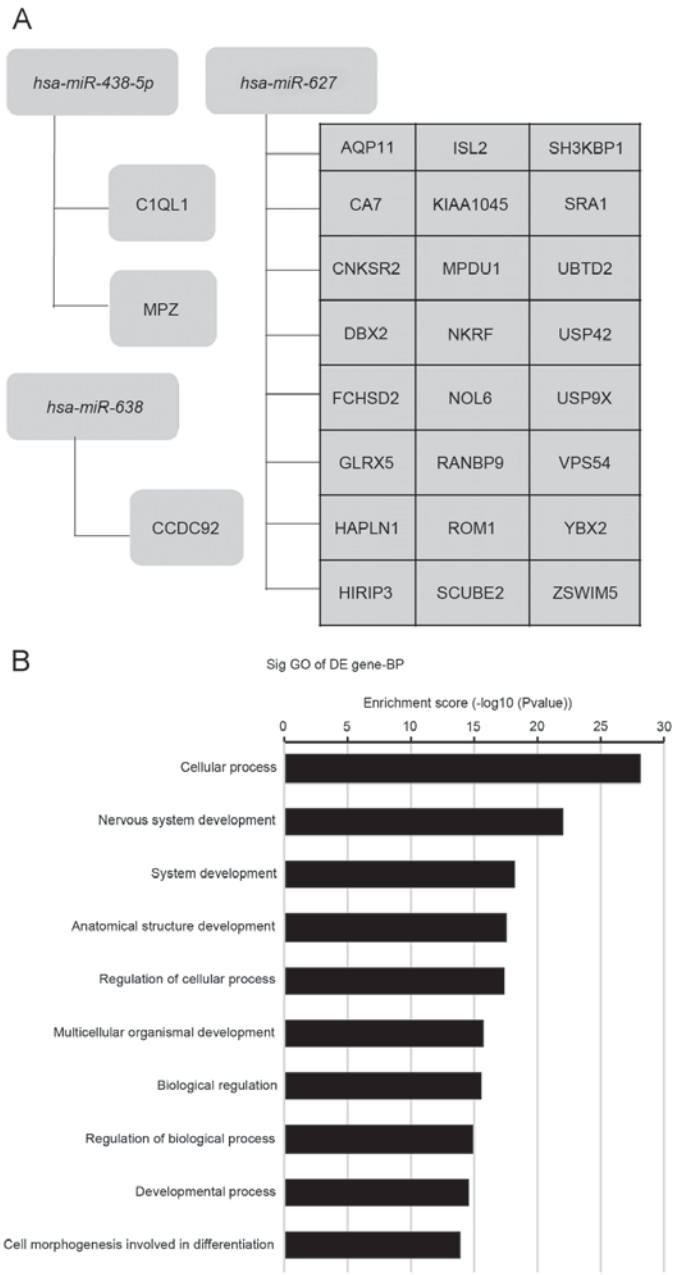

Figure 3. Analysis of microarray results. (A) Genes predicted to be targeted by DE miRNAs and (B) GO analysis. GO, Gene Ontology; Sig, significantly; DE, differentially expressed; BP, biological process.

influenced genetic changes within patients with CML. miRNA expression patterns were determined in primary CD34$^+$ cells and MV purified from CD34$^+$ cells from patients with CML. miRNAs in KG-1a cells and the associated MVs were also determined, for comparison. The results of the present study revealed that 15 miRNAs were significantly increased in MVs with respect to corresponding cells. Target gene prediction was performed to further determine the function of miRNAs. GO and pathway analysis demonstrated that the upregulated miRNAs in MVs may be associated with cell development, morphology, differentiation, metabolism and cell cycle of CD34$^+$ blasts. KEGG pathway demonstrated that upregulated miRNAs may be involved in tumorigenesis, chronic and acute myeloid leukemia and signaling pathways including ErbB, PI3K/Akt and forkhead box O (FOXO).

Of the 15 upregulated miRNAs in MVs, miR-1290 was the primary modulator and modulates 4,019 target genes. The targets of miR-1290, including B-cell lymphoma/leukemia (Bcl)2, lysine methyltransferases, tumor protein p63-regulated 1, chronic lymphocytic leukemia upregulated (CLLU) 1 and Bcl tumor suppressor 7A, were identified to be involved in multiple cancers. The Bcl2 gene is a proto-oncogene and inhibits apoptosis. Bcl2 serves an important role in the pathogenesis of breast cancer and increased expression levels of Bcl2 in breast cancer indicates a poor prognosis (24). CLLU1, located on chromosomal locus 12q22, encodes chronic lymphocytic leukemia (CLL)-specific transcripts and patients with increased expression levels of CLLU1 usually exhibit decreased progression-free survival and overall survival times (25). Huang *et al* (26) hypothesized that exosomes may

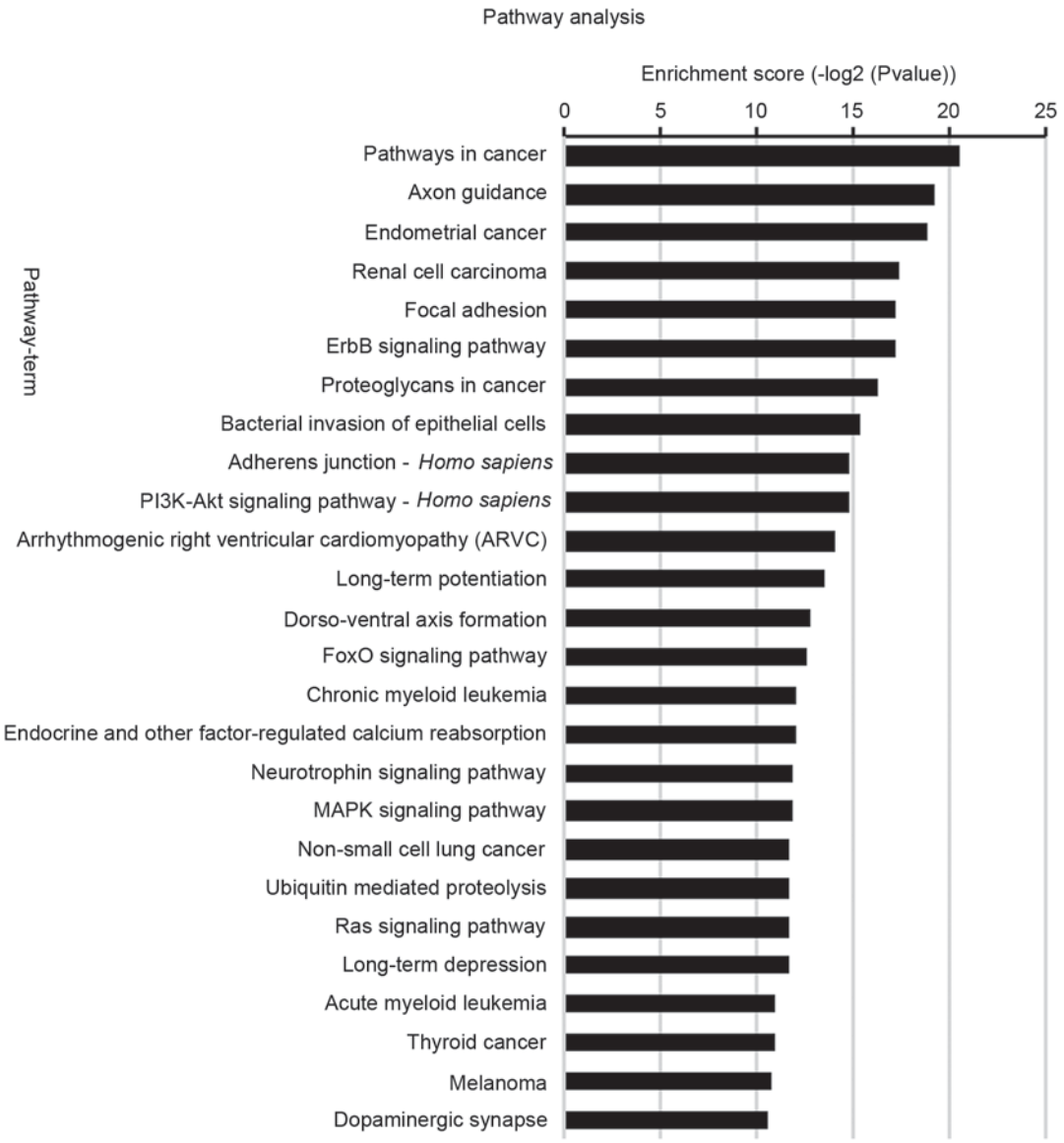

Figure 4. Kyoto Encyclopedia of Genes and Genomes pathway analysis of potential targets of differentially expressed miRNAs. PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; FOXO, forkhead box O; MAPK, mitogen-activated protein kinase.

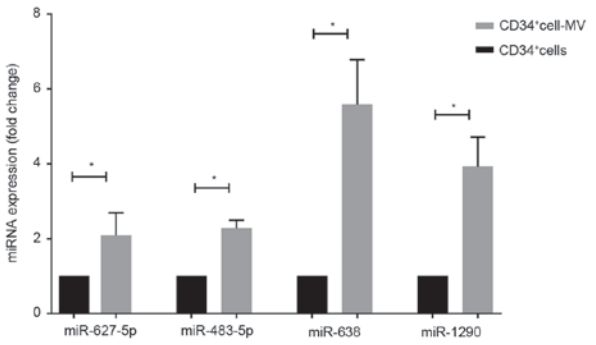

Figure 5. Validation of miRNA expression levels using the reverse transcription-quantitative polymerase chain reaction. Expression levels of miR-627-5p, miR-483-5p, miR-638 and miR-1290 in an independent set of primary CD34+ blasts and blast-derived MVs were determined. miRNA expression in blasts was set as 1; the relative expression of hsa-miR-627-5p, hsa-miR-483-5p, hsa-miR-638 and hsa-miR-1290 in MVs was 2.09±0.35, 2.14±0.01, 5.59±0.69 and 3.92±0.46, respectively. Results are presented as the mean ± standard deviation. *P<0.05. miRNA, microRNA; CD, cluster of differentiation. MV, microvesicle; hsa, human.

be selectively enriched with miR-1290 and miR-375, which allow exosomes to be prognostic markers for advanced prostate cancer. With the exception of hsa-miR-1290, other upregulated miRNAs were also predicted to serve important roles in cancer. For example, miR-638 may be stably detected in human plasma, and the miR-92/miR-638 ratio in plasma may be a useful indicator to distinguish between patients with leukemia and the healthy control group (27). Jaiswal *et al* (28) identified that miR-1246, miR-1308, miR-638 and other human miRNAs may be selectively enriched in microparticles, transferring them to the recipient cells. Similarly, the results of the present study identified that MVs, which cannot be distinguished from microparticles at present, package miR-638. In addition, upregulated miRNAs in MVs were identified to be poor prognostic tumor markers, which is consistent with a previous study (29), which revealed that miR-483-5p and miR-195 were associated with poor prognosis in adrenocortical adenoma. Thus, it can be hypothesized that CD34+ blasts release MVs which contain elevated miRNAs and may be beneficial for the

survival of blasts themselves; however, this requires additional study. Following GO analysis, KEGG was used to analyze the pathways which involved the predicted miRNA target genes. ErbB, PI3K/Akt and FOXO signaling pathways were included. A previous study demonstrated that these pathways serve a critical role in CML (30) and Naka *et al* (31) identified that the transforming growth factor β (TGFβ)/FOXO signaling pathway may be involved in leukemia-initiating cells. Furthermore, in CML FOXO3a$^{-/-}$ rat experiments, TGF-β inhibitor combined with imatinib was demonstrated to be an effective treatment.

To the best of our knowledge, the present study is the first to identify the DE miRNAs between CD34^{+} blasts and blast-derived MVs in CML. Additionally, the present study optimized the flow cytometric method of blast-derived MVs. Using complex pathway analysis, distinct KEGG pathways and GO terms were characterized by altered gene expression and miRNA regulation may be identified. The results of the present study provide evidence that may increase the understanding of physiological functions of stem cell-derived MVs and the potential roles of CD34^{+} blast-derived MVs in CML-associated processes. However, the interactions between miRNAs and their targets in the present study are only bioinformatically predicted, and further study is required to validate them.

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