RESEARCH ARTICLE

Selective miRNA Expression Profile in Chronic Myeloid Leukemia K562 Cell-derived Exosomes

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Abstract

    <u>Background</u>: Chronic myeloid leukemia (CML) is a myeloproliferative disorder of hematopoietic stem cell scarrying the Philadelphia (Ph) chromosome and an oncogenic BCR-ABL1 fusion gene. The tyrosine kinase inhibitor (TKI) of BCR-ABL1 kinase is a treatment of choice for control of CML. <u>Objective</u>: Recent studies have demonstrated that miRNAs within exosomes from cancer cells play crucial roles in initiation and progression. This study was performed to assess miRNAs within exosomes of K562 cells. <u>Methods</u>: miRNA microarray analysis of K562 cells and K562 cell-derived exosomes was conducted with the 6th generation miRCURYTM LNA Array (v.16.0). Gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were also carried out. GO terms and signaling pathways were categorized into 66 classes (including homophilic cell adhesion, negative regulation of apoptotic process, cell adhesion) and 26 signaling pathways (such as Wnt). <u>Results</u>: In exosomes, 49 miRNAs were up regulated as compared to K562 cells, and two of them were further confirmed by quantitative real-time PCR. There are differentially expressed miRNAs between K562 cell derived-exosomes and K562 cells. <u>Conclusion</u>: Selectively expressed miRNAs in exosomes may promote the development of CML via effects on interactions (e.g. adhesion) of CML cells with their microenvironment.

Keywords: MicroRNAs - exosomes - leukemia - myelogenous - chronic - microarray analysis - K562 cells

Introduction

    Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by expansion of a clone of hematopoietic cells that carry the Philadelphia (Ph) chromosome (Druker et al., 2006). The Ph chromosome is a single reciprocal chromosomal translocation between the long arms of chromosome 9 and 22, resulting in the fusion of the Abelson (ABL) oncogene to the breakpoint cluster region (BCR) gene. ABL encodes constitutively activated bcr-abl tyrosine kinase which is involved in pathogenesis of CML (Mineo et al., 2012) . The introduction of tyrosine kinase inhibitors (TKIs) has significantly changed the clinical management of patients with CML (Alikian et al., 2012). However, a minority of CML patients in chronic phase (CP) and a substantial proportion of patients in advanced phase (AP) either are initially refractory to TKIs or eventually develop resistance against TKIs. The resistance and relapse directly correlate with the disease progression (Ernst et al., 2012).

    Exosomes are small vesicles (30-100nm in diameter) that carry proteins, RNAs (including microRNAs) and have been found to mediate the intercellular signal transduction (Henderson and Azorsa, 2012). Exosomes originate from the inward budding of the endosomal membrane and generate the multivesicular bodies (MVBs). They are released into the extracellular space by fusion of the MVBs with the plasma membrane (van Dommelen et al., 2012). Exosomes can be secreted by a variety of cell types such as cancer cells (Yang and Robbins, 2011). Several biological functions have been assigned to exosomes, including immune regulation, intercellular communication, stromal remodeling, signaling pathway activation via growth factor/receptor, intercellular exchange of oncoprotein and genetic materials, induction of angiogenesis and modulation of therapeutic response (Kharaziha et al., 2012). For instance, O'Brien et al. (2013) found that exosomes from triple-negative breast cancer patients' sera significantly increased recipient cells' invasion compared to healthy control sera. It is suggested that exosomes may be involved in cancer cell-to-cell communication.

    About immune regulation function of exosomes, Munich et al. (2012) demonstrated that dendritic cells-derived exosomes could mediate essential innate immune functions. Wahlgren et al. (2012) also found that activated CD3+ T cells could participate in IL-2 mediated immune response signaling via exosomes, to communicate with resting autologous T cells. In addition

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to immune regulation functions, exosomes are involved in the occurrence and development of tumors. Liu et al. (2012) found that exosomes from murine derived GL26 cells promote tumor growth by reducing the number and function of CD8+T cells. Moreover, Roccaro et al. (2013) reported that multiple myeloma BM mesenchymal stromal cells-derived exosomes played a role in multiple myeloma disease progression, and they promoted tumor growth of multiple myeloma. As we know, the release of exosomes from K562 CML cells has been reported, and the exosomes can promote angiogenesis in an src-dependent fashion (Savina et al., 2003; Mineo et al., 2012).

Valadi et al. (2007) reported that exosomes carried both mRNA and miRNA when being released from mast cells, and many of these RNA species could be delivered to other cells. This agrees with several authors working with cancer cells, who have noted that the miRNA content for their originating cancer cells is similar to that found in circulating exosomes (Vlassov et al., 2012). These "bioactive vesicles" shuttle various molecules, including miRNAs, to recipient cells, and affect the metabolism of recipient cells. MiRNAs is small regulatory non-coding RNAs with important roles in a variety of physiological and pathological processes. Aberrantly release of miRNAs from exosomes may cause significant alterations in biological signaling pathways that may affect the disease development, such as cardiovascular disorders, inflammatory diseases, gynecological diseases and cancers (Hu et al., 2012). For instance, aberrantly expressed miRNAs would likely play a role in the leukemic cells. Zimmerman et al. (2010) suggested that miR-181 was associated with myeloid cell leukemia-1 (imatinib-resistant K562 CML cells) expression (a Bcl-2 family member whose expression)and cell survival. Meanwhile, Hu et al. (2010) found that the antisense inhibition of miR-21 in K562 cells suppressed cell migration, promoted cell apoptosis, and inhibited cell growth, and up-regulated the expression of the tumor suppressor gene PDCD4. A recent report by Rokah et al. (2012) showed that miR-31, miR-155 and miR-564 were down-regulated in CML cells, and these down-regulated miRNAs and their putative targets might play pivotal roles in the pathogenesis of CML. The miRNA expression profiles of K562 cells and blood of CML patients have been studied, but the miRNA expression profiles of K562 cell-derived exosomes have not yet been reported. The present study was conducted to investigate whether miRNAs are contained within exosomes of K562 cells, and to determine the differentially expressed miRNAs between K562 cells and K562 cell-derived exosomes.

Materials and Methods

Cell culture

K562 cells (Kangwei, Beijing, China) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin and 100µg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

Isolation of exosomes

FBS used in the isolation of exosomes was centrifuged at 110, 000 g for 1 h to remove exosomes. At the times indicated, the 120 ml culture medium was removed, and centrifugation was performed at 4 degree centigrade once at 300 g for 10 min, once at 2000 g for 20 min, and once at 10 000 g for 30 min. To remove particles larger than 200 nm, the supernatants were filtered through 0.22 µm pore size filters. To harvest exosomes, centrifugation was done at 110 000 g for 60 min at 4 degree centigrade. Exosomes were re-suspended in 20 mL of PBS followed by centrifugation at 110 000 g for 1h at 4degree centigrade. The purified exosomes were re-suspended in PBS and stored at -80°C.

Scanning electron microscopy

For scanning electron microscopy (NOVA NANOSEM 450 instrument; FEI), the exosomes were fixed with 2.5% glutaraldehyde (Sigma-Aldrich GmbH, Taufkirchen, Germany) in PBS for 17h. After washing twice with PBS, the exosomes were dehydrated with an ascending series of ethanol concentrations (15%, 30%, 60%, 80%, 99-100%). After evaporation of ethanol, the samples were left to dry at room temperature for 17 h on a 8 mm × 8 mm aluminum substrate, and then analyzed by SEM after gold–palladium sputtering.

Western blot assay

Proteins were extracted from the purified exosomes and K562 cells using protein extraction reagent (APPLYGEN, Beijing, China) according to the manufacturer's instructions. Protein extracts were mixed in reducing 4% SDS loading buffer followed by incubation for 10 min at 95°C. Then, the mixture was subjected to 10% polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes (Milipore, Bedford, MA). The membranes were blocked with 5% bovine serum albumin (BSA) for 1.5 h at room temperature and subsequently washed thrice with 0.1% Tween 20 in PBS. Membranes were incubated with the Alix antibody (1:500 dilution; Abcam) and then with the horseradish peroxidase-conjugated secondary antibody followed by visualization with enhanced chemiluminescence.

miRNA Expression Profiling

miRNA expression profiling was performed on 4 samples (K562 cells: n=2; K562 cell-derived exosomes: n=2) with the 6th generation of miRCURYTM LNA Array (v.16.0) (Exiqon) containing probes for 1891 miRNAs. Total RNAs were isolated using TRIzol (Invitrogen) and miRNeasy mini kit (QIAGEN) according to manufacturer's instructions, which can efficiently recover all RNA species, including miRNA. The quality and quantity of extracted RNA were determined by using a nanodrop spectrophotometer (ND-1000, Nanodrop Technologies).

After RNA extraction, the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) was used for miRNA labelling according to manufacturer's instructions. After labeling, the Hy3TM-labeled samples were hybridized on the miRCURYTM LNA Array (v.16.0) (Exiqon). Following hybridization, the slides were washed several times using the Wash buffer kit (Exiqon), and

finally dried by centrifugation for 5min at 400rpm. Then, the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Statistical analysis of data from miRNA microarray was performed with GenePix Pro 6.0 software (Axon). Replicated miRNAs were averaged, and miRNAs with intensity of ≥50 in all samples were chosen for calculating the normalization factor. Expressed data were normalized using the Median normalization. After normalization, differentially expressed miRNAs were identified through the Volcano Plot filtering. Hierarchical clustering was performed using the MEV software (v4.6, TIGR).

microRNA quantification

Total RNA was extracted from K562 cells and exosomes using TRIzol (Invitrogen) and miRNeasy mini kit (QIAGEN) according to manufacturer's instructions. The quality and quantity of RNA were measured by using a nanodrop spectrophotometer (ND-1000, Nanodrop Technologies). Two differentially expressed miRNAs identified by miRNA microarray were selected for further validation by quantitative real-time reverse-transcription PCR (qRT-PCR).

Figure 1. Morphology of Exosomes Isolated From K562 Cells Under a Scanning Electron Microscope. Small vesicles of approximately 30-40nm in diameter were noted. a: low magnification of exosomes. b: high magnification of exosomes

SYBR Green MicroRNA Assay was done for detection of hsa-miR-1908 (148687), hsa-miR-298 (46264) and RNU6 (all materials from RIBOBIO, GuangZhou City, China). Total RNA (10ng) was reverse transcribed by PrimeScript RT reagent Kit (TakaRa). qRT-PCR was performed using an Applied Biosystems 7300 Real-Time PCR System and corresponding software (Applied Biosystems, USA) according to the manufacturer's instructions. Detection was done in triplicate. Ct values were averaged and normalized to that of RNU6. Relative expression was determined by the ddCt comparative threshold method.

Gene ontology analysis and pathway analysis

The comprehensive function annotations of the potential targets of differentially expressed miRNAs were done with Gene Ontology analysis and pathway analysis. Gene ontology terms were analyzed with the Gene Ontology database. P-values for all potential targets were calculated in all GO categories, and the threshold of significance was defined as P-value of < 0.01 and FDR of < 0.70. Data of signaling pathways were from Kyoto Encyclopedia of Genes and Genomes (KEGG). All signaling pathways were analyzed, and the threshold of significance was defined as P value of < 0.05 and FDR of < 0.05. The network was built based on up-regulated miRNAs and their potential targets.

Cell to cell adhesion assay

Human umbilical vein endothelial cells (HUVEC) were seeded into a 6-well cell culture plate and were cultured in DMEM with 20% FBS (17 h). We divided the 6-well cell culture plate into two groups, exosome-treated group and control group. Exosome-treated group was treated with 1ml RPMI-1640 contained with exosome (final concentration 16 μg protein/ml). Control group was treated with 1ml RPMI-1640 without exosomes. 9×10^6 K562 cells were labeled with 0.2 μmol/ml fluorescence dye BCECF (Invitrogen, UK) in OptiMEM (37 degree centigrade, 30 min); fluorescence-labeled K562 cells were resuspended in serum free OptiMEM and plated evenly onto above-mentioned two group HUVECs and incubated for another 2 h, non-adherent cells were removed by washing with PBS, PBS was added to each well. These cells were measured using a fluorescence microscope (CX51TR-32FB3-F01, OLYMPUS) at an excitation wavelength of 440 nm and an emission wavelength of 535 nm.

Results

Isolation of exosomes from K562 cells

Exosomes may play a major role in the pathogenesis of cancer, and their functions ranged from regulation of tumor growth, to invasion, angiogenesis and metastasis (Roberson et al., 2010). Exosomes were consisting of an array of molecules derived from the originating cells, including proteins, lipids, RNAs and/or miRNAs. Inappropriate release of miRNAs from exosomes may cause significant alterations in biological pathways that affect disease development (Hu et al., 2012). In the
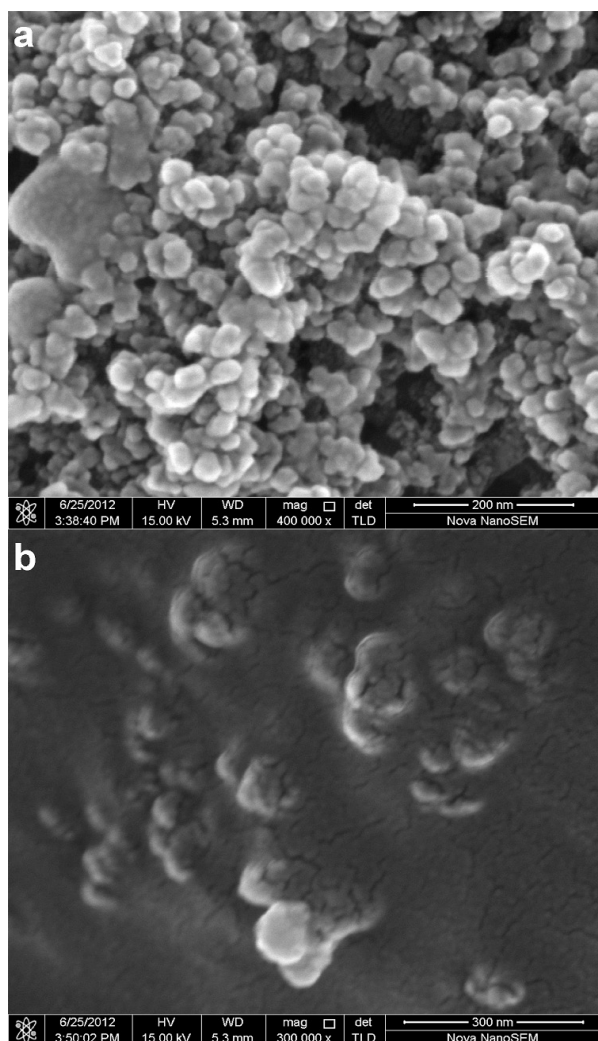
Table 1. Differentially Expressed miRNAs Between Exosomes and K562 Cells

miRNA	Fold change	miRNA	Fold change	miRNA	Fold change
hsa-miR-1908	49.6	hsa-miR-3611	11.4	hsa-miR-629-5p	4.10
Hsa -miR-30c-1-3p	25.6	hsa-miR-519e-5p	11.2	hsa-miR-625-3p	3.96
Hsa-miR-711	24.9	Hsv2-miR-H25	10.0	hsa-miR-107	3.73
Hsa-miR-744-5p	23.6	Ebv-miR-BHRF1-2	9.63	hsa-miR-1827	3.67
Has-miR-4285	21.3	hsa-miR-652-5p	9.39	hsa-miR-483-3p	3.66
Hsa-miR-665	20.6	hsa-miR-585	8.60	Hsv2-miR-H7-3p	3.43
Hsa-miRPlus-C1087	16.9	hsa-miR-4279	7.38	hsa-miR-299-5p	3.31
Hsa-miR-423-5p	16.1	hsa-miR-518b	6.73	hsa-let-7e-p	3.26
Hsa-miR-3686	16.0	Hcmv-miR-US25-1*	6.54	hsa-miR-877-3p	3.25
Hsa-miR-149-3p	15.8	hsa-miR-4268	6.40	hsa-miR-1249	3.22
Kshv-miR-K12-3	15.8	Hsv2-miRH7-5p	6.18	hsa-miR-3915	2.90
Hsv2-miR-H10	15.5	hsa-miR-612	5.72	hsa-miR-3676-3p	2.81
Hsa-miR-516b-5p	14.4	hsa-miR-4290	5.32	hsa-miR-1973	2.72
Sv40-miR-S1-5p	13.7	hsa-miR-498	5.17	hsa-miR-3646	2.53
Hsa-miR-3201	13.5	Hcmv-miR-UL148D	5.00	hsa-miR-298	1.95
Hsa-miR-638	13.4	hsa-miR-943	4.98		
hsa-miR-877-5p	11.8	hsa-miR-525-5p	4.15		

present study, to determine the exosomatic miRNAs profile, exosomes were isolated from K562 cells through microfiltration and centrifugation described in previous studies with slight modification. To confirm that the exosomes were harvested, scanning electron microscopy and Western blot assay were performed. Scanning electron microscopy of the exosomes revealed the particles had spherical shape and were approximately 30-40 nm in diameter (Figure 1 a, b).

miRNA expression in K562 cells and exosomes

To test our hypothesis that miRNAs in the exosomes enabled the communication and influenced the genetic changes within CML patients, miRNA profile of the exosomes extracted from K562 cells was determined. Since we hypothesized that there were differences in the miRNA expression between exosomes and K562 cells, the RNA of K562 cells was also extracted. Thus, 4 samples (K562 cells: n=2, K562 cell-derived exosomes: n=2) were prepared for RNA and hybridized to the miRCURYTM LNA Array (v.16.0). miRNAs expression in K562 cells and exosomes were highly correlated (correlation coefficient: 0.975536 and 0.958076, respectively), indicating that the replicates had a high reproducibility. At the same time, differentially expressed miRNAs were identified between exosomes and K562 cells (correlation coefficient=0.369871). The heat map diagram showed the results of the two-way hierarchical clustering of miRNAs and samples. Each row represented a miRNA and each column represents a sample. The miRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. Cluster analysis arranged samples and miRNAs into groups based on their expression levels, which allowed us to hypothesize the relationships between miRNAs and samples. Interestingly, 49 miRNAs were up regulated in exosomes as compared to K562 cells (Table 1), with both *P*-value of ≤0.05 and more than 1.5-fold changes.

qRT-PCR

In order to validate the findings in microarray, quantitative RT-PCR was done to detect the up-regulated miRNAs. Two miRNAs were selected for further qRT-PCR: hsa-miR-1908 (148687), hsa-miR-298 (46264), and a generally applied small nuclear RNA, RNU6 was tested as a housekeeping gene. Similar results were revealed in the differentially expressed miRNAs as in the miRNA microarray.

Gene Ontology (GO) terms

Lists of predicted genes associated with differentially expressed miRNAs in K562 cell-derived exosomes as compared to K562 cells were generated from Targetscan6.0 (data not shown). Gene Ontology (GO) terms could be assigned to the potential targets. The GO terms associated with the targets were categorized into 66 classes (*P*<0.01). In order to better understand the function of the involved genes, these GO terms were divided into four groups including genetic information processing (8 classes), biological process (33 classes), organismal systems (16 classes) and human cancer associated group (9 classes). In the human cancer associated group, the major functions included homophilic cell adhesion, negative regulation of apoptotic process, and cell adhesion. In the biological process group, axon guidance, proteasomal ubiquitin-dependent protein catabolic process and small GTPase mediated signal transduction ranked in the top three. Chromatin modifications, positive regulation of transcription DNA-dependent and positive regulation of transcription from RNA polymerase II promoter were included in the genetic information processing group. Nervous system development, multicellular organismal development, palate development and many other GO terms were included in the organismal systems. These results suggested that the miRNAs of K562 cell-derived exosomes were associated with cell metabolism, cell cycle, cell adhesion, angiogenesis and tumorigenesis.

KEGG pathway (P-Value)

To better understand the function of potential targets, signaling pathways were analyzed by KEGG (26 signaling pathways, Figure 2). The pathways associated with the up-regulated miRNAs in exosomes (*P*<0.05) included Wnt signaling pathway, pathways in cancer, chronic myeloid leukemia, focal adhesion and adherens junction and many
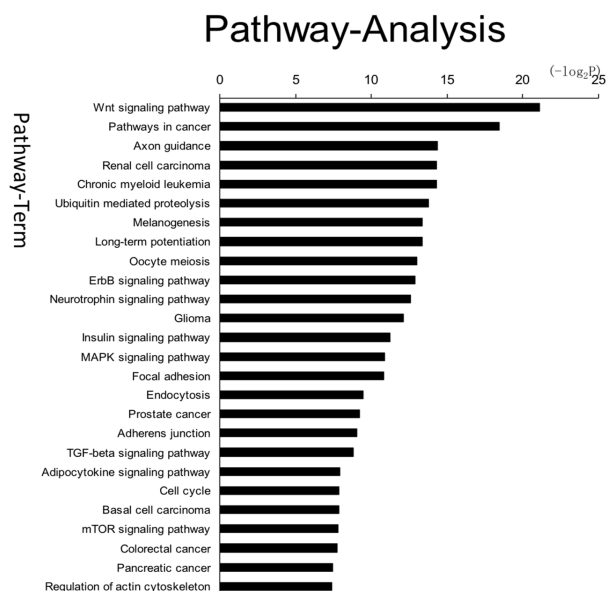
Pathway-Analysis

Figure 2. KEGG Pathway (*P*-Value) of Potential Targets of Differentially Expressed miRNAs
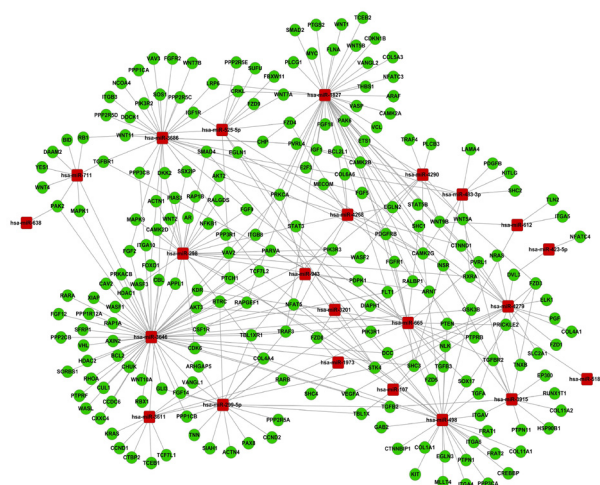

Figure 3. miRNA-target-network of KEGG Pathways. The network shows the relations of 23 up-regulated miRNAs and their potential targets. Red rectangle: up-regulated miRNAs; green ellipses: potential target genes associated with up-regulated miRNAs

other signaling pathways. These pathways play significant roles in cell cycle, cell proliferation, cell adhesion, cell apoptosis, angiogenesis, tumorigenesis, tumor invasion and tumor metastasis.

microRNA-Target-Network

In order to unveil the relationship among miRNAs of interest, network was built base on the up-regulated miRNAs and their potential targets. A total of 344 target genes and 655 target genes associated with five pathways (Wnt signaling pathway, pathways in cancer, chronic myeloid leukemia, focal adhesion and adherens junction) and 9 GO terms (human cancer associated group) were selected from all targets. The 344 target genes associated with five pathways were regulated by 23 up-regulated miRNAs and the 655 target genes associated with 9 GO terms were regulated by 25 up-regulated miRNAs, and the interaction among them is shown in Figure 3.
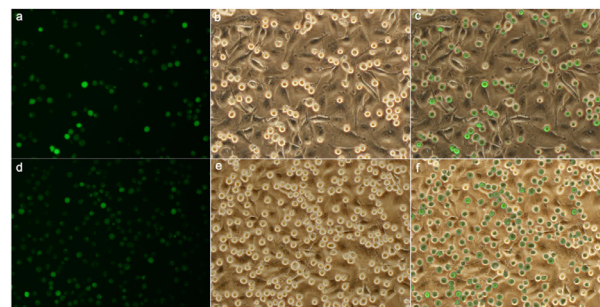

Figure 4. Exosomes Reduced the Adhesion Function of K562 Cells to HUVECs. A, B, C Represents Exosome-Treated Group, D, E, F Represents Control Group. a: photofluorogram of exosome-treated group. b: photogram of exosome-treated group. c: the fusion of a and b. d: photofluorogram of control group. e: photogram of control group. f: the fusion of d and e

Cell to cell adhesion assay

The result of our Gene Ontology and KEGG pathway analysis drop a hint that K562 cell derived-exosomes were associated with cell adhesion. To further investigate the role of exosomes in cell adhesion to endothelial cells, HUVECs seeded in a 6-well cell culture plate were used as cell adhesion substrates, after treated with exosome, K562 cells labeled with BCECF were allowed to adhere. The exosome-treated group was treated with 1ml RPMI-1640 (contains exosomes). The control group was treated with 1ml RPMI-1640 (without exosomes), and cells were cultured in a humidified atmosphere with 5% CO_2 at 37 degree centigrade for 2h. As shown in Figure 4, we found that exosome-treated group had less adherent K562 cells than the control group. It is suggested that exosomes may lead to reduce or defect of adhesion function.

Discussion

In this study, we hypothesized that miRNAs were contained in the exosomes, which enabled the communication and influenced the genetic changes within CML patients. The miRNA patterns were determined in K562 cells and the purified exosomes from K562 cells. Our results demonstrated that 49 miRNAs were up regulated in K562 cell derived-exosomes as compared to K562 cells. Out of these 49 differentially expressed miRNAs, 2 were validated by qRT-PCR and results were similar to those in miRNA array. To better understand the function of miRNAs, bioinformatical analysis was performed, including Gene Ontology and KEGG pathway analysis. Results revealed that 66 GO terms and 26 signaling pathways were associated with the potential targets. Networks of 9 GO terms and 5 pathways of interest were built between up-regulated miRNAs and their target genes.

To better expound the function of these miRNAs, GO terms associated with human cancer were focused in our study. Results demonstrated that a total of 25 up-regulated miRNAs might be involved in the homophilic cell adhesion, negative regulation of apoptotic process, cell adhesion, Wnt receptor signaling pathway, canonical Wnt receptor signaling pathway, positive regulation of mesenchymal cell proliferation, positive regulation of

cell proliferation, negative regulation of canonical Wnt receptor signaling pathway and cell migration. Among these 25 up-regulated miRNAs, miR-3646 was the most important modulator and modulates 107 target genes. However, there is no report about hsa-miR-3646. The targets of miR-3646, such as GATA4, AJAP1, BCL9, ANGPT1, SFRP1 and GSK3B were involved in multiple cancers. Ernst et al. (2009) reported that AJAP1 likely played a role in the pathogenesis of glioblastoma and is significantly associated with overall survival of astrocytic glioma patients. Meanwhile, Willis et al. (1998) suggested that BCL9 might be a target of translocation in some B-cell malignancies with abnormalities in 1q21 and that disordered BCL9 expression might be an important factor in their pathogenesis. Except for hsa-miR-3646, other up-regulated miRNAs were also predicted to play important roles in cancers. For instance, miR-1827 and miR-585 were associated with the initiation and recurrence of lung cancer (Patnaik et al., 2010; Xiong et al., 2011). miR-518b also appeared to play a role in the hepatocellular carcinoma (Wang et al., 2012). In hematological malignances (Pallasch et al., 2009; Tanaka et al., 2009), miRNA-638 was stably present in the human plasma and the ratio of miR-92a to miR-638 in plasma was very useful for distinguishing leukemia patients from healthy ones. Additionally, miR-107, though regulating PLAG1, played an important role in the pathogenesis of chronic lymphocytic leukemia. In summary, our results in GO terms indicated that these 25 up-regulated miRNAs in K562 cell derived-exosomes might promote the proliferation of CML cells.

Following GO analysis, KEGG was employed to analyze the pathways with involvement of predicted miRNA target genes (Figure 2). KEGG analysis showed that these signaling pathways were associated with cell cycle, cell proliferation, cell adhesion, cell apoptosis, angiogenesis, tumorigenesis, tumor invasion and tumor metastasis. In the present study, Wnt signaling pathway was the most associated pathway. MiR-3646, miR-298, miR-299-5p, miR-1827, miR-665, miR-4268, miR-4290, miR-483-3p, miR-498, miR-525-5p, miR-3611, miR-3686, miR-711, miR-423-5p, miR-4279, miR-3915, miR-1973, miR-3201, miR-612, miR-943 and miR-518b might co-modulate the Wnt signaling pathway. It has been suggested that several miRNAs co-participate in the same pathways and play important roles in the proliferation of CML cells. Recent studies showed that Wnt signaling pathway was implicated in the pathogenesis of leukemia. Khan et al. (2007) revealed that the activation of Wnt/beta-catenin pathway led to altering expression of genes involving in cell cycle regulation and apoptosis in leukemia B-cell progenitors, and Wnt-3a pathway promoted proliferation and survival of ALL cells.

As we know, the microenvironment is an important factor of tumorigenesis. Intercellular junctions are essential for normal tissues, cancer formation and communication between neighboring cells. Moreover, cell-matrix adhesions are recruited to the intercellular junctions (Gkretsi et al., 2005). Thus, cell-matrix adhesions are essential for cell migration, tissue organization and differentiation. Rao et al. (2011) showed

that the adhesive capacity to the bone marrow stroma was impaired in leukemia cells. There is evidence showing that a breakdown of adhesive mechanisms governed by an adhesion molecule is present in the leukemia microenvironment. In chronic myelogenous leukemia, the BCR/ABL oncogene itself can lead to adhesion defects and alter the properties of focal adhesion proteins (Weisberg et al., 1997). In recent years, exosomes have become one of the vital components of cancer microenvironment and they can mediate the intercellular communications. Exosomes can carry some proteins, lipids, and nucleic acids and transfer them to recipient cells altering the biochemical composition, signaling pathways, and gene regulation of recipient cells (Ge et al., 2012). Peinado et al. (2012) have shown that bone marrow cell derived-exosomes support the growth and metastasis of metastatic melanoma. MiRNAs (small non-coding RNA sequences) is involved in the deregulated expression of critical genes that play key roles in the tumorigenesis, tumor development, and angiogenesis and have oncogenic or tumor suppressive roles (Fabbri et al., 2012; Fountzilas et al., 2012; Benetatos et al., 2013). Ohshima et al. (2010) showed that the metastatic gastric cancer cell line, AZ-P7a, derived-exosomes could release let-7 miRNAs into the extracellular environment of gastric cancer to maintain the oncogenesis. Out results were in accordance with these works that miRNAs in K562 cell derived-exosomes might promote the proliferation of CML cells via manipulating the microenvironment. In the KEGG analysis, adherens junction and focal adhesion pathways were included. The same as Wnt signaling pathway, adheren junction and focal adhesion pathways were modulated by miR-3646, miR-298, miR-299-5p, miR-1827, miR-665, miR-4268, miR-4290, miR-483-3p, miR-498, miR-525-5p, miR-3611, miR-3686, miR-711, miR-4279, miR-3915, miR-612, miR-943, miR-638 and miR-107. Among them, miR-638 and miR-107 were newly identified miRNAs compared with the Wnt signaling pathway.

Our cell adhesion assay showed that exosome-treated group had less adherent cells than the control group. It is suggested that maybe exosomes lead to adhesion decreased or defected. Chen et al. (2012) found that in colorectal cancer, miR-103/107 through targeting the known metastasis suppressors death-associated protein kinase (DAPK) and Kruppel-like factor 4 (KLF4), decreased cell-cell adhesion. Recently, Umezu et al. (2013) demonstrated that exosomatic miRNAs can be transported from leukemia cells (K562 cells) to endothelial cells and that certain exogenous miRNAs modulate endothelial migration and tube formation. These studies agreed with ours, it suggested that miRNAs in K562 cell derived-exosomes might modulate cell adhesion. Briefly, our findings demonstrated that K562 cell derived-exosomes can selectively encapsulate miRNAs, may promote the proliferation of CML cells via affecting the interaction (e.g. adhesion) of the CML cells and their microenvironment.

In conclusions: To our knowledge, our study for the first time reports the differentially expressed miRNAs between K562 cells and K562 cell-derived exosomes. With complex pathway analysis, different KEGG pathways and

GO terms were characterized by altered gene expression and potentially subjected to miRNA regulation are identified. Our results provided evidence that will not only increase the understanding of physiological functions of cancer derived exosomes, but aid the understanding of potential roles of K562 cell-derived exosomes in CML-related processes. However, it should be emphasized that the interactions between miRNA and their targets reported in the present study are only bioinformatically predicted and further studies are required to validate them.

Acknowledgements

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