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The Potential of Exosomes Derived from Chronic Myelogenous Leukaemia Cells as a Biomarker

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Abstract. *Background/Aim: Exosomes, derived from chronic myelogenous leukaemia (CML) cells, can be used as biomarkers and new targets for the detection of the BCR-ABL transcript. This study aimed to identify these possibilities. Materials and Methods: Human CML cell line-derived exosomes and CML-patients-derived exosomes were isolated with a size-exclusion chromatography column and ExoQuick™ exosome precipitation solution, respectively. Isolated exosomes were analysed by nested PCR to detect the BCR-ABL transcript. Results: Exosomes derived from the two human CML cell lines yielded a 250-bp band. RNA sequence analysis revealed 99% sequence homology with the partial mRNA for the human BCR-ABL chimeric protein. This ~250-bp band was also observed in the exosomes derived from patients with CML. However, only patients at the blast and accelerated phases showed the exosomal BCR-ABL transcript. Conclusion: CML-derived exosomes could act as novel targets for the detection of the BCR-ABL transcript.*

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Chronic myelogenous leukaemia (CML) is a myeloproliferative neoplasm, which can be diagnosed by the clonal expansion of hematopoietic stem cells harbouring the *BCR-ABL* fusion gene. The annual incidence of CML is approximately 0.4-1.75/100,000, and it has not changed for a long time. However, the prevalence of CML continues to increase because of the dramatic improvement in patient survival after treatment with tyrosine kinase inhibitors (TKI) and the increase in life expectancy in the general population (1). Huang *et al*. also estimated that the prevalence of CML in the USA would increase until 2050 (2). Because of the increase in prevalence, appropriate monitoring strategies are gradually emerging.

The *BCR-ABL* fusion gene is a fingerprint of CML and is used as a marker for diagnosis and disease status assessment during TKI therapy. Although the cytogenetic response, determined by metaphase analysis (using more than 20 metaphase cells) of bone marrow (BM) samples, is considered the gold standard for determining the disease status, the most sensitive method is the reverse transcription-quantitative polymerase chain reaction (RT-qPCR), which is used to estimate fusion *BCR-ABL* mRNA levels. In addition to its sensitivity, RT-qPCR has the advantage of being able to analyse peripheral blood (PB), rather than BM (3). However, there are limitations to the use of RT-qPCR in clinical settings. First, control genes or reference materials are essential for reliable and reproducible results. However, the results obtained with current control genes vary with the sample types, local laboratory storage conditions, or protocols (4), and reference materials are limited resources,

that are available to only manufacturers (5). Second, a minimum number of nucleated cells (For example, at least $1\text{-}2\times10^7$) is necessary for the detection of the *BCR-ABL* transcript by RT-qPCR. In other words, the standard RT-qPCR cannot be easily used to analyse body fluids with low numbers of nucleated cells; for example, cerebrospinal fluid (CSF), pleural effusion fluid, and ascitic fluid.

Exosomes are 40-150-nm-wide extracellular vesicles (EVs) derived from various cell types, which likely reflect the phenotypic state of the cell. They may contain all the known molecular constituents of the parent cell, including proteins, RNAs, and DNAs. Exosomes have also been detected in many body fluids, including urine, semen, saliva, amniotic fluid, CSF, bile, ascitic fluid, tears, breast milk, and blood (6). Therefore, exosomes and their components can serve as biomarkers for CML and maybe new targets for the detection of the *BCR-ABL* transcript; however, studies on this property of exosomes are lacking. To verify that CML-derived exosomes could be novel targets for the detection of the *BCR-ABL* transcript, we isolated and characterized exosomes from two human CML cell lines and performed PCR for the *BCR-ABL* transcript. We also isolated exosomes from the BM serum of patients with CML to determine their clinical applicability.

Materials and Methods

Cell culture and its supernatant. All the sera collection procedures were performed per the guidelines of the Internal Review Board of the Korea University Anam Hospital with informed consent from the patients.

Human CML cell lines (K562 and KU812) were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in the Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Carlsbad, CA, USA) with 10% exosome-depleted foetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). Exosome-depleted FBS was prepared by ultracentrifugation. Briefly, normal FBS was ultracentrifuged at 100,000 × *g* for 3 h, and the supernatant was collected after discarding the pellet to deplete the bovine exosomes. After culturing for 36-48 h, the sub-confluent culture medium was harvested and centrifuged at 500 × *g* for 10 min. The supernatant was sequentially centrifuged at 5,000 × *g* for 30 min at 4˚C and at 10,000 × g for 30 min at 4˚C to remove detached cells containing apoptotic bodies and cell debris. The collected supernatant was concentrated using an Amicon® Ultra 100 kDa filter (MWCO 100 kDa) (Merck Millipore, Temecula, CA, USA) at 5,000 × g and at 4˚C until the volume of the concentrated solution was one-hundredth of the original volume (Figure 1).

Exosome isolation from cell culture supernatant. The cell culture supernatant (150 ml) was sequentially centrifuged and concentrated to a volume of 1.5 ml. The concentrated supernatant (0.5 ml) was gradually loaded and distributed, in accordance with the commercial protocol, on the top of a size-exclusion chromatography column packed with 10 ml Sepharose CL-2B (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and has a molecular weight separation range of $70\times10^3\text{-}40\times10^6$ (7). The eluate fractions (6, 7, 8, 9, and 10; 0.5 ml each) were collected. To obtain a more concentrated exosomes solution, the collected eluate fractions (6-10, total 2.5 ml) were concentrated using an Amicon® Ultra centrifugal filter (MWCO 100 kDa) (Merck Millipore, Temecula, CA, USA) at 5,000 × *g* and at 4˚C.

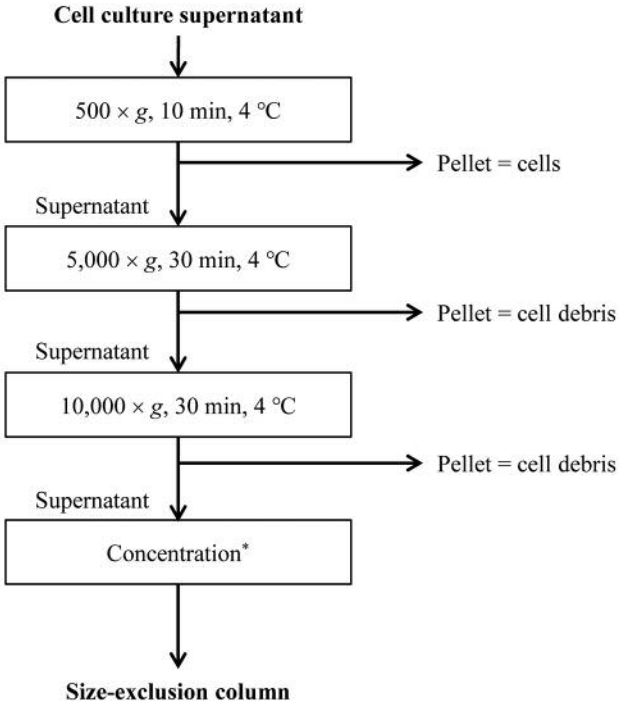


Figure 1. *Overview of the method used for exosome isolation using size-exclusion chromatography. *Samples were concentrated using Amicon® Ultra centrifugal filter (MWCO 100 kDa) (Merck Millipore, Temecula, CA, USA) at 5,000 × g for 30 min at 4˚C.*

Dynamic light scattering and transmission electron microscopy. Dynamic light scattering (DLS) (Zetasizer Nano S90, Malvern, UK) and transmission electron microscopy (TEM) were used to measure the size of the exosomes. For the DLS measurements, the exosomes were suspended in phosphate-buffered saline (PBS), and the size distribution data for each exosome sample from the CML cell lines were collected. Measurements from three different samples were obtained for each exosome population. For the TEM analysis, the exosomes were fixed with 2% paraformaldehyde, loaded on a 300-mesh formvar/carbon-coated electron microscopy grid (Electron Microscopy Sciences, PA, USA), and stained with 2% phosphotungstic acid (PTA). The TEM images were obtained with a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Protein extraction and western blotting. The cells or the exosomes were lysed using the PRO-PREP protein extraction solution (Intron Biotech, Seoul, Korea). The nuclei and membranes of the cells or exosomes were cleared by centrifugation at 10,000 × g for 15 min at 4˚C. Each 30-μg protein sample, as measured by Bradford assay, was separated by 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking with the ProNA™ General-BLOCK solution (Translab, Seoul, Republic of Korea) for 1 h, the membranes were probed overnight at 4˚C with the

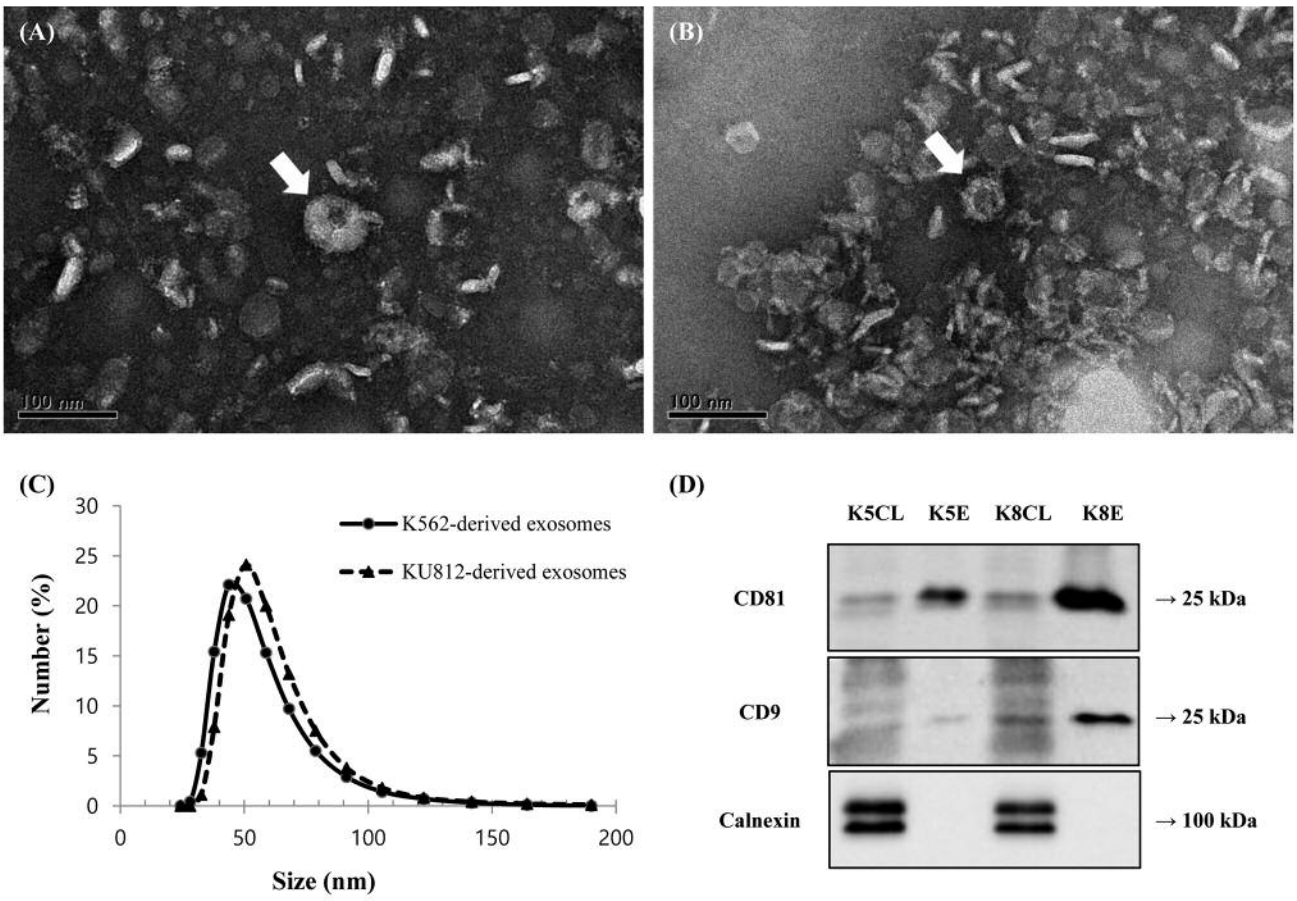


Figure 2. *Characterization of the isolated exosomes derived from the CML cell lines. Transmission electron microscopy was performed to identify the morphology and size distribution of the isolated exosomes. Exosomes derived from (A) K562 and (B) KU812 were cup-shaped vesicles, 20-150 nm in diameter. The size distribution of the isolated exosomes derived from the chronic myelogenous leukemia (CML) cell lines was determined using dynamic lighting scattering; the size ranged between 30 nm and 190 nm in diameter (C). In western blot analysis, exosome marker proteins (CD81 and CD9) were detected; however, the endoplasmic reticulum marker protein (calnexin) was not detected in the exosomes isolated from both CML cell lines (D). K5: K562 cell line; K8: KU812 cell line; CL: cell lysate; E: exosomes.*

following antibodies: CD81 (1:1,000) and CD9 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as exosomal markers; calnexin (1:1,000) (Cell Signaling Technology, Beverly, MA, USA) was used as a control for cell debris contamination; and peroxidase-conjugated anti-mouse (1:2,000) or anti-rabbit (1:2,000) secondary antibody was used for 90 min at room temperature. The antibody-antigen reactions were visualized using the ProNA™ ECL Ottimo (Translab, Seoul, Republic of Korea), and the images were acquired using the ChemiDoc™ Touch imaging system (Bio-Rad, CA, USA).

Total RNA isolation, cDNA synthesis, and nested PCR for the detection of BCR-ABL. Total RNA from cells or exosomes was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Purified RNA (25-100 ng) was reverse transcribed using the Prime Script RT-PCR kit (TaKaRa, Japan). Nested PCR was used to detect the *BCR-ABL* transcripts according to a previously published protocol (8, 9). In brief, 10 µl of cDNA was amplified in 20 µl total volume using the Prime Script RT-PCR kit with the BCR/ABL primer set for 35 cycles (30 sec at 95˚C, 30 sec at 55˚C, and 1 min at 72˚C). Next, 1 µl of the first PCR sample was amplified in 15 µl total volume using the Dr. MAX DNA polymerase (Doctor Protein Inc., Seoul, Republic of Korea) with the bcr/abl primer set for another 35 cycles. The primers used for this study are summarized in Table I.

Exosome isolation from the BM serum of patients with CML. BM serum obtained from one healthy control and three patients with CML were used. The BM serum of the patients with CML was collected consecutively at different time points, and two collections were performed per patient—one sample was collected at the time of diagnosis and the other sample was collected after treatment with TKIs with or without cytotoxic chemotherapy. The ExoQuick™ exosome precipitation solution (SBI, Mountain View, CA, USA) was used according to the manufacturer's instruction to isolate the exosomes from the BM serum. In brief, 2 ml of the human BM serum sample was centrifuged at 3,000 × *g* for 15 min to remove cells and cell debris, and the supernatant was mixed with 504 µl of the ExoQuick™ exosome precipitation solution. The mixture was

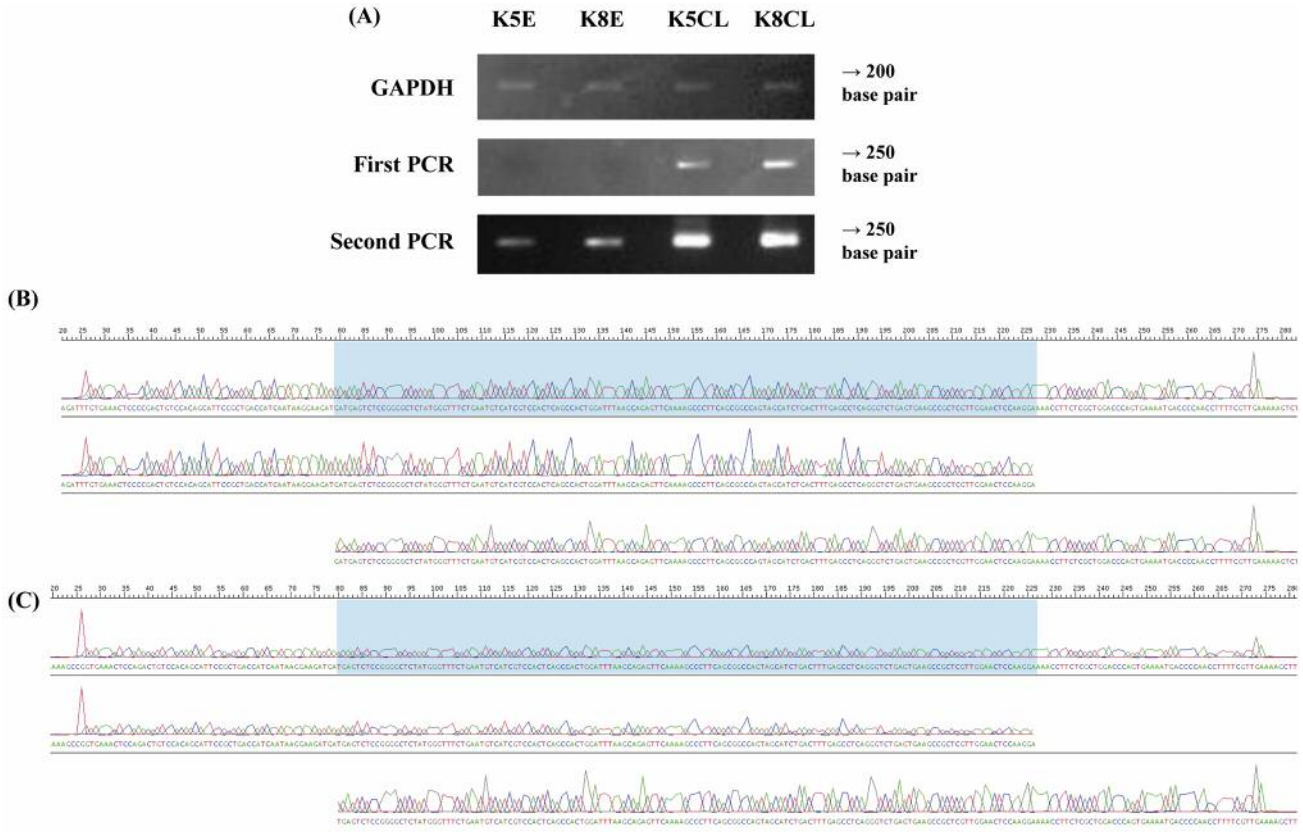


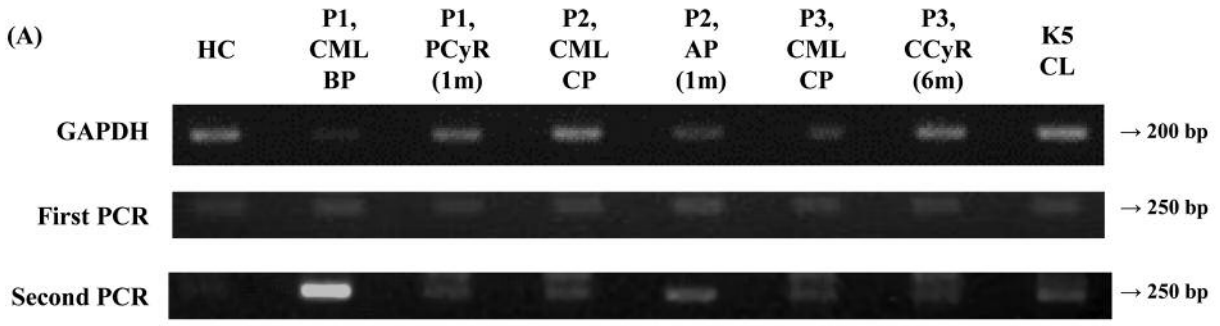
Figure 3. *Nested PCR of exosomes and cell lysate derived from K562 and KU812 CML cell lines. Nested PCR, performed to detect BCR-ABL, showed bands of 250 bp in both K562 and KU12 cell lysate and cell line-derived exosomes (A). NCBI/BLAST of RNA sequence revealed 99-100% BLAST score with the nucleotide sequence of Homo sapiens partial mRNA for BCR-ABL1 chimeric protein in both K562-derived exosomes (B) and KU812-derived exosomes (C). K5: K562 cell line; K8: KU812 cell line; E: exosomes; CL: cell lysate.*

incubated for 30 min at 4˚C and centrifuged at 1,500 × *g* for 30 min at 4˚C. The precipitated exosomes in the pellet were obtained after aspirating the supernatant.

Results

Validation of CML cell line-derived exosomes. The exosomes, shed by the two human CML cell lines (K562 and KU812), were isolated from the debris and the large particles using size-exclusion chromatography; they were subsequently concentrated by ultrafiltration using a 100-kDa molecular weight cut-off filter. First, TEM was performed to determine the morphology and size of the isolated exosomes. K562 and KU812 CML cell line-derived exosomes were visualized as cup-shaped vesicles in the TEM (Figure 2A and B). The size distribution of the isolated CML cell line-derived exosomes, measured by DLS, ranged from 30 nm to 190 nm (Figure 2C), which was in agreement with previously published results (10). Western blotting was performed to characterize the isolated exosomes; the exosomes were positive for the exosomal markers, CD81 and CD9, but were negative for the endoplasmic reticulum marker, calnexin (Figure 2D). These results confirmed that the two human CML cell lines (K562 and KU812) truly released exosomes into their culture media, and that size-exclusion chromatography and subsequent concentration by ultrafiltration could be used to purify exosomes from the culture media.

CML cell line-derived exosomes contain the BCR-ABL transcript. K562 and KU812 cell line-derived exosomal and total cellular RNA were extracted, and nested RT-PCR was performed to detect *BCR-ABL*. The first PCR, performed with the BCR/ABL primer set, showed bands of 250 bp for the K562 and KU812 cell lysates, but no bands were detected in the cell line-derived exosomes; the K562 and KU812 cell lysates were used as positive controls. In the second PCR, performed with the bcr/abl primer set, 250 bp bands were detected in both K562 and KU812 cell lysates and cell line-derived exosomes (Figure 3A). RNA sequence analysis was performed using the NCBI/BLAST (National Center for Biotechnology Information, Bethesda, MD, USA)



(B)

Test	HC	P1, CML BP	P1, PR (1m)	P2, CML CP	P2, AP (1m)	P3, CML CP	P3, CCyR (6m)	K5 CL
BM cellularity (%)	50	93.7	70	100	100	90	50	–
BM cytogenetics (BCR/ABL FISH+, %)	–	91.5	6.6	100	98.7	100	0	–
PB RQ-PCR using IS (%)	–	–	–	21.77	–	26.38	0.106	–

Figure 4. *Nested PCR of exosomes from the bone marrow serum of healthy control and patients with CML. Bands of 250 bp were amplified from the exosomal RNA isolated from patients with CML and the positive control (K562 cell lysate). Only the exosomal RNA of the patients in the blast and accelerated phases yielded RNA sequences, which revealed that the exosomal RNA was the partial mRNA for the human BCR-ABL chimeric protein. Bands with weaker intensities were observed from the exosomal RNA from patients at the chronic phase and cytogenetic response phase; however, these amplicons could not be used for RNA sequence analysis (A). These results were compared with the patient's clinical data (B). HC: Healthy control; P: patient; CML: chronic myelogenous leukemia; BP: blast phase; PCyR: partial cytogenetic response; CP: chronic phase; AP: accelerated phase; CCyR: complete cytogenetic response; K5: K562 cell line; CL: cell lysate; BM: bone marrow; PB: peripheral blood; RT-qPCR: reverse transcription quantitative polymerase chain reaction.*

Table I. *Primers used for the nested PCR for BCR-ABL.*

Primers	Sequence (5' → 3')	Product size (base pairs)	Melting temperature (˚C)
First PCR			
BCR	TTCAGAAGCTTCTCCCTG	252~429	55
ABL	CTCCACTGGCCACAAAAT		
Second PCR			
bcr	GTGAAACTCCAGACTGTC	170~280	55
abl	CAACGAAAAGGTTGGGGT		
Control			
GAPDH5	GTGGATATTGTTGCCATCA	~300	55
GAPDH3	GACTCCACGACTTACTCA		

and the nucleotide sequence of *Homo sapiens* partial mRNA for the BCR-ABL1 chimeric protein, which revealed a 99-100% BLAST score for the PCR product in both K562-(Figure 3B) and KU812-derived exosomes (Figure 3C).

Exosomes from the BM serum of patients with CML also contain the BCR-ABL transcript. Nested PCR was performed for the exosomes isolated from the BM serum (one healthy control and three patients with CML). Bands of approximately 250 bp were detected in the exosomal RNA isolated from the patients with CML and the positive control (K562 cell lysate). RNA sequence analysis revealed that the exosomal RNA, isolated from the patients with CML, at the blast phase (BP) and accelerated phase (AP) showed a 99% sequence homology with the partial mRNA sequence for the human BCR-ABL chimeric protein. Bands with weaker intensities were observed in the exosomal RNA isolated from the patients with CML at the chronic phase (CP) and in that isolated from

the patients who had responded to treatment; however, RNA sequence analysis could not be performed for these amplicons (Figure 4A).

Next, the nested PCR results of the patients with CML were compared with the clinical data of the patients, including those on BM cellularity, cytogenetics, and peripheral blood RT-qPCR (Figure 4B). In patients with CML at CP, the number of BM cells expressing the *BCR-ABL* fusion gene was more than 90%; however, the exosomal *BCR-ABL* mRNA was not expressed sufficiently for RNA sequence analysis. Partial mRNA for the human *BCR-ABL* was detected only in the exosomal mRNA isolated from the patients with CML in the BP or AP by nested PCR. Second, the expression level of the exosomal *BCR-ABL* transcript changed in response to TKI treatment with or without cytotoxic chemotherapy. In patient 1 (P1), the identifiable exosomal *BCR-ABL* transcript, which was expressed before treatment, disappeared when P1 acquired a partial response to treatment. Conversely, in patient 2 (P2), the identifiable transcript appeared with the loss of response to treatment and disease progression.

Discussion

In this study, we isolated and purified exosomes from two human CML cell lines using size-exclusion chromatography, and showed that the exosomes harboured the partial mRNA for the human *BCR-ABL*. The exosomes, which were isolated from the BM serum of the patients with CML in advanced stages of the diseases, such as BP or AP, also expressed the partial mRNA for the human *BCR-ABL*.

Currently, research on EVs is gaining momentum in haemato-oncology because EVs can harbour biomolecules of the parent cell, thereby providing information on tumour microenvironment or immunity; they can also be used as biomarkers or for drug delivery (6, 11). However, EVs are heterogeneous (50~1,500 nm), and their roles can be confounded by the presence of different EV types with different mechanisms of biogenesis, organelle origin, and constituent makeup (12). To overcome these limitations, we purified exosomes that were relatively homogeneous in size and possessed higher sedimentation properties than normal EVs using size-exclusion chromatography. The CML cell-derived exosomes contained partial mRNA for the human *BCR-ABL*. Previous studies also showed that EVs isolated from CML cells contained the genetic information of *BCR-ABL*. Cai *et al*. showed that EVs derived from the K562 CML cell line contained the *BCR-ABL* hybrid gene, which could be transferred into normal neutrophils (13). Krunz *et al*. isolated exosomes from conditioned media of K562 and plasma of paediatric patients with AML and performed deep DNA sequencing to analyse the exosomal DNA; the analysis revealed the presence of genomic double-stranded DNA fragments that were identified as classical *BCR-ABL* translocation (14). On the basis of the results of the present and previous studies, we suggested that the CML cell-derived exosomes could contain the genetic information of the *BCR-ABL* fusion gene, which is the fingerprint of CML, in the form of mRNA or DNA.

Interestingly, only the exosomes isolated from the patients with CML in the advanced phases (BP or AP) of the disease contained the partial mRNA for the human *BCR-ABL*, which called for the need for RNA sequence analysis in this study. Although the number of BM cells expressing *BCR-ABL* transcript in patients in the CP was more than 90%, the exosomal *BCR-ABL* mRNA transcript could not be analysed (Figure 4). These observations suggested that the amount of *BCR-ABL* transcript in exosomes cannot be simply determined by the number of CML cells expressing the BCR-ABL fusion gene in the BM. The biological activity of CML tumour cells appears to be crucial for the incorporation of the *BCR-ABL* mRNA into the exosomes. *BCR-ABL* transcript-induced genetic abnormalities can predispose to transformation and markedly influence the aggressiveness of the progenitor cell clone (15). Moreover, CML cell-derived exosomes participated in angiogenesis (16, 17), promotion of tumour growth (18), and survival of leukemic cells in the BM microenvironment (19, 20). The results of these previous studies support the observations of this study, indicating that there may be an association between exosomal *BCR-ABL* fusion transcript and CML aggressiveness.

Accurate identification of the CML phases is an essential prognostic marker (21). Unlike patients in the CP, who are treated with TKIs, patients in the AP or BP are required to use a higher dose of TKIs or consider allogeneic hematopoietic stem cell transplantation (allo-HSCT) (22). Although it is important to distinguish the different CML phases, the current criteria for defining BP and AP are somewhat unclear. Extramedullary blast proliferation, the proportion of blasts or basophils in PB or BM, persistent thrombocytopenia unrelated to therapy, and clonal chromosomal abnormality upon treatment are usually used to distinguish CP or the advanced phases (BP or AP) (23). However, these markers can only be used subjectively to determine the proportion of blasts or basophils and the platelet count. Different CML phases can be diagnosed based on the definition of the borderline values of the above-mentioned factors by the physician. If the *BCR-ABL* exosomal mRNA could be helpful in differentiating CP from the advanced phases of the disease (BP or AP), it may be a more objective indicator for the accurate identification of the CML phases.

Monitoring of patients treated with TKIs by RT-qPCR for the *BCR-ABL* transcript is an effective milestone in defining patient response. The proportional decrease in *BCR-ABL* transcript levels closely correlates with the level of cytogenetic response (24). On the contrary, increasing levels are strongly predictive of haematological and cytogenetic

relapse after allo-HSCT (25-27). However, a single measurement of *BCR-ABL* transcript levels is not sufficient to determine the need for treatment change. Current guidelines recommend that the results of two consecutive tests in 1-3 months interval or supplementary tests are required to consider a change of treatment (22, 23). In this study, the expression of the exosomal *BCR-ABL* transcript changed in response to TKI. The exosomal *BCR-ABL* transcripts disappeared after acquiring a partial response to treatment and appeared after losing the response. As a result, it is conceivable that the evaluation of exosomal *BCR-ABL* transcript level may be another supplementary test.

The presence of the genetic information of the *BCR-ABL* fusion gene in exosomes has several clinical implications. First, the contents of exosomes derived from CML cells may be used to normalize gene expression in RT-qPCR for the detection of *BCR-ABL*. The use of reference genes is the usual method of choice for normalizing gene expression (28); however, several issues about the comparability of the results of individual samples and accessibility of each laboratory's clinical settings have to be addressed (29). Analysis of exosomes containing partial mRNA for *BCR-ABL* and other biomolecules from the CML cells reduces the complexity of the total cell analysis, thereby aiding in the detection of new reference materials. In addition, since a chip-based device, capable of simultaneously isolating, quantifying, and analysing exosomal mRNAs, is currently being developed (30, 31), the amount or other characteristics of CML-derived exosomes may be used to normalize *BCR-ABL* expression. Second, *BCR-ABL* can be detected even in body fluids with few nucleated cells using exosomal RNA. The ability to distinguish the phases of CML is important for determining the correct therapy for the disease, and one of the criteria for defining the phases is the presence of extramedullary infiltrates of CML cells (22). A biopsy of the involved site is performed clinically if extramedullary infiltration is suspected; however, conventional cell-based examination for the detection of *BCR-ABL* is limited by sample insufficiency and a low number of nucleated cells in body fluids, such as CSF, pleural effusion fluid, and ascitic fluid. Exosomes are released into interstitial or body fluids, and their lipid bilayers enable the storage of their contents for extended periods of time (32, 33). Therefore, the exosome-based detection of *BCR-ABL* may help to overcome the clinical limitations of cell-based examination.

This study has several limitations. First, RNA fluorescence *in situ* hybridization (FISH) was required to determine whether the partial mRNA of *BCR-ABL* is located inside the CML cell-derived exosomes or buried in the periphery. Second, since qualitative PCR was used to detect the *BCR-ABL*, the relative quantitative values were not obtained. Nevertheless, this study suggested the possibility that CML cell-derived exosomes can be used to detect the mRNA of the *BCR-ABL* fusion gene. Nested PCR for the mRNA of the *BCR-ABL* fusion gene can be routinely performed in the clinic using a conventional PCR machine.

In conclusion, exosomes, isolated from CML cells and the BM serum, could contain the *BCR-ABL* transcript. Therefore, CML-derived exosomes could be used as biomarkers and may be new targets for the detection of the *BCR-ABL* transcript.

Conflicts of Interest

The Authors have no competing interests regarding this study.

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