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Plasma-derived exosomes in acute myeloid leukemia for detection of minimal residual disease: are we ready?

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Abstract

The recent emergence of plasma-derived exosomes as biomarkers of leukemic relapse has introduced the potential for more sensitive non-invasive monitoring of leukemia patients based on the molecular and genetic analysis of the exosome cargo. In principle, the protein, lipid, miRNA, mRNA or DNA profiles of exosomes in patients' plasma that associate with leukemic relapse can be identified. The diagnostic/prognostic value of these profiles could then be validated in prospective clinical studies. Here, we consider the potential of exosomes to fulfill the role of future biomarkers of minimal residual disease in AML. The rationale for developing exosome-based methodology for minimal residual disease detection is based on promising early observations. However, standards need to be established for evaluating exosome identity, isolation from body fluids and assessment methods. The rapidly expanding knowledge of the exosome biology suggests that the exosome status as potential biomarkers may become clarified in the near future.

Keywords

exosomes; acute myeloid leukemia; biomarker; minimal residual disease; leukemia relapse

1. Introduction

With currently available intensive chemotherapies and supportive care, a high proportion of patients newly diagnosed with acute myeloid leukemia (AML) achieve morphologic complete remission (CR) [1, 2]. However, if no additional chemotherapy is given to patients in CR, most will eventually relapse. This suggests that current clinico-pathological evaluations are not able to detect microscopic disease remaining after initial chemotherapy. Nevertheless, several studies have demonstrated that using high-sensitivity measurements of

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Declaration of Interest

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the residual disease burden in acute leukemia, it is possible to predict outcomes and to guide therapeutic interventions for minimal residual disease (MRD) in patients who are at a high risk of relapse [3, 4]. The prognostic value of MRD is unequivocal. MRD remains an independent prognostic factor along with other well established factors such as unfavorable cytogenetics and mutational status (i.e., FLT3 positivity) at diagnosis, older age and antecedent hematologic disorders [5]. However, MRD-negative AML patients may relapse. Reasons for leukemic relapse in MRD-negative patients is still under investigation and could be related to instability of the diagnostic leukemic clone, loss of leukemia associated antigens (LAA) expression on blasts or rapid recurrence of disease not previously detected by MRD testing. Testing for MRD in AML is not a trivial task. To date, no MRD assay for AML has been standardized. Among various assays considered as potentially useful, multi-parameter flow cytometry and/or quantitative RT-PCR are most widely used [6, 7]. Both methods have an excellent prognostic value and are used as reliable guidelines for decision making especially in patients with acute lymphoblastic leukemia and acute promyelocytic leukemia. However, despite high sensitivity levels of both methods, MRD monitoring in AML is not widely used [8]. This may be because AML consists of multiple sub-types, each with its own unique genetic/molecular profile. As such, each mutation represents a special challenge in its use as a potential MRD marker. Mutational profiles are patient specific, and while some mutations may be common and may define AML subsets, their detection is not always reliable. Thus, it remains unclear which of genetic/molecular biomarkers can be considered as reliable candidates for the assessment of the MRD status in AML. Recently, the presence of MRD in standard-risk AML, as determined by quantitation of NPM1-mutated transcripts, provided powerful prognostic information independent of other risk factors [9]. Nevertheless, sensitivity levels of RT-PCR might vary, being largely dependent on the specific gene to be amplified, the primers used, quality of nucleic acid extractions, and genes selected as a normal reference. Also, re-emergence or evolution of original clones introduces genetic variability posing additional challenges in RT-PCR detection of MRD in AML.

Immuno-phenotypic detection of MRD by multi-parameter flow cytometry is also challenging. It may be confounded when leukemic cells express an antigenic profile that is different from that at diagnosis or when differences between the MRD antigen profile and that found on normal hematopoietic cells are obscured. In AML, several LAAs have been used to detect MRD, all of which are present, albeit weakly, on normal cells. Thus, the detection of MRD in AML remains a challenge, largely due to the lack of definitive biomarkers, inadequate bone marrow sampling and the stringency of methodological standards which differ between laboratories making comparability of results difficult. Until a method is developed that can be standardized and made commercially available to a global community, currently-available assays for MRD detection remain a research rather than a clinical tool. The question of whether exosomes can circumvent the existing barriers to reliable MRD detection in the near future is, therefore, of considerable interest.

2. Exosome biogenesis, isolation and characteristics

Given the existing challenges in MRD detection in AML, a search for biomarkers that could serve as surrogates of the disease presence has been ongoing. In this context, the emergence

of exosomes and the recognition of their diagnostic and prognostic potentials in cancer have created considerable interest [10]. Exosomes are the smallest (30–150nm in size) type of extracellular vesicles (EVs) that are produced by normal and cancerous cells, including leukemia blasts [11, 12]. Other types of EVs, microvesicles (MVs) formed by “blebbing” of the parent cell membrane and apoptotic bodies (APOs) present in all body fluids along with exosomes can be distinguished from the latter by their larger sizes [13]. The biogenesis of exosomes in the endosomal compartment is well documented [14]. The presence in the exosome cargo of endosomal markers such as ALIX or TSG101 and tetraspanins, CD9, CD63 and CD81, is a characteristic feature of exosomes [15]. They have the vesicular morphology by transmission electron microscopy (TEM) and carry a cargo of proteins/lipids expressed on the plasma membrane of parental cells. In the vesicle lumen, exosomes carry nucleic acids (miRNAs, mRNAs, DNAs) and a variety of molecules present in the cytosol of parental cells [16]. They deliver their cargos to recipient cells by a variety of mechanisms, including the ligand/receptor signaling, phagocytosis and/or the up-take via endocytosis [17]. They operate as message-delivering vehicles and function as an information system which is involved in a broad variety of biological functions [18]. Exosomes carry messages to different systems, tissues, and organs, including those located at a distance from the source of exosomes. By means of the cell-to-cell communication, exosomes regulate immune responses, cell migration, cell differentiation and angiogenesis [reviewed in [19]].

Exosomes have been traditionally isolated from body fluids by high speed ultracentrifugation at $100,00 \times g$ for 2–3 h. More recently, this method of isolation has been replaced by a variety of more convenient procedures, some of which are commercially available [20]. To date, a majority of exosome isolations have used supernatants of cell lines, especially tumor cell lines, where all exosomes are products of one cell type. After undergoing differential centrifugation to remove cell fragments and large extracellular vesicles, these supernatants are ultrafiltrated using 0.22 μ filter, and then exosomes are isolated, e.g., by size exclusion chromatography (SEC) on a Sepharose 2B column [21, 22]. The void-volume fractions containing exosomes are recovered and may be further concentrated either by centrifugation using centrifugal concentrators or by high-speed ultracentrifugation, yielding morphologically intact and biologically active exosomes. Exosome isolation from plasma of normal donors and patients with AML is more complex. As every normal cell and tumor cell can produce exosomes, the exosomal fractions isolated from plasma are mixtures of vesicles in which blast-derived exosomes might represent only a small part, especially in patients with AML in CR after chemotherapy. Further, plasma exosomes are coated with “contaminating” proteins, lipids and glycoproteins likely to cause their aggregation and a potential loss upon centrifugation. Therefore, special methods and additional steps may be required for recovery of plasma exosomes from AML patients, as we have recently described [23]. Once total plasma exosomes are isolated, immunocapture with LAA-specific antibodies (Abs) can be used for separation of blast-derived from normal cell derived exosomes [24]. Studies of exosomes isolated from tumor-cell supernatants indicate that exosomes are enriched in the specific proteins, lipids and nucleic acids present in the parental cell, while lacking others [25]. This suggests that the exosome biogenesis is not a random process. It has been established that exosome biogenesis involves a specialized

sorting mechanism referred to as exosomal sorting complex responsible for transport (ESCRT) [26]. This mechanism provides exosomes with the content that in part resembles that of a parent cell and makes exosomes attractive candidates for biomarkers [27]. Another attractive feature of exosomes is that their vesicular content is protected from enzymatic degradation by the lipid-bilayer membrane. Also, exosomes are present in all body fluids. An increased production of exosomes as well as their molecular content are associated with disease presence and signify disease progression [28, 29]. Exosomes accumulating in plasma of patients with cancer appear to be surrogates for tumor cells. Indeed, our studies of exosomes produced by AML cell lines showed that they carry distinct molecular profiles and carry a unique set of membrane molecules, which mimic those present in the membrane of tumor cells, as illustrated in Figure 1.

3. Exosomes in AML

While exosome secretion occurs under physiologic conditions, and all cells are capable of their release, tumor cells are avid exosome producers. Patients newly diagnosed with AML prior to any therapy have significantly higher plasma levels of exosomes (in μg protein/mL plasma) compared to levels in plasma of normal controls [12]. This by itself does not mean that leukemic blasts are the source of these exosomes. However, the presence in the exosome cargo of LAAs such as CD34 or CD117 suggest that at least a part of the total exosome fraction originates from leukemic cells [12]. Plasma exosome levels change as patients undergo therapy. After a course of induction chemotherapy, a significant reduction in exosomal protein levels occurs, concomitant with the reduction of blasts in the bone marrow [30]. However, protein levels remain elevated in exosome fractions isolated from plasma of many AML patients in CR, at the time when leukemic blasts are undetectable in the bone marrow by conventional hematopathological methods [31]. Furthermore, during consolidation therapy with high-dose cytarabine, exosomal protein levels remained elevated. In our hands, exosomal protein levels in plasma of AML patients who achieved long-term remission were not significantly different from those seen in exosomes in normal control plasma [30]. These data point to the potential significance of exosomal protein levels alone as a sensitive measure of leukemic blast persistence after chemotherapy and as a potential predictor of relapse in AML.

In AML, plasma-derived exosomes contained a different protein cargo compared to normal cell derived exosomes (Figure 1). Blast-derived exosomes contained membrane-associated TGF- β 1, MICA/MICB and myeloid blasts markers, CD34 and CD117 in addition to conventional exosome markers, such as tetraspanins [12, 30]. In addition to changes observed in exosomal protein levels during AML therapy, the exosome cargo was also undergoing qualitative and quantitative changes, as indicated, for example, by changes observed by western blots in TGF- β 1 isoforms in the exosome cargo [30]. Similar to protein levels, the TGF- β 1 levels were elevated and enriched in the mature isoform in exosomes obtained during and after consolidation chemotherapy from plasma of patients who subsequently relapsed. In contrast, TGF- β 1 levels were decreased and the mature TGF- β 1 isotype was not evident in exosomes from plasma of patients in long-term remission. These changes in the exosome cargo had functional consequences, as exosomes of AML patients with high levels of mature TGF- β 1 significantly inhibited natural killer (NK) cell activity in

cytotoxicity assays [30]. Thus, monitoring of exosome molecular cargo at diagnosis and during therapy was also informative about their potential as immune cell-modulating vehicles. Another recent study of exosomes from plasma of AML patients who developed resistance to chemotherapy and whose leukemic blasts are resistant to apoptosis suggests that monitoring of the exosomal protein content could provide clinically-relevant information [32]. Apoptosis-resistant AML blasts produced exosomes highly enriched in survival proteins, including the Bcl-2 family members, and suggesting that changes in a qualitative protein profile of AML exosomes may serve as predictor for the development of drug resistance [32].

The studies described above were performed with total AML plasma-derived exosomes representing a mix of vesicles produced by normal and abnormal cells. To be able to better utilize the molecular and proteomic content of plasma exosomes as a biologic marker in AML, isolation of leukemic blast-derived exosomes and their separation from the bulk of total exosomes is necessary [24]. This is a challenging step that depends on immune capture of tumor-derived exosomes on magnetic beads coated with antibodies specific for LAAs. As a proof-of-principle that blast-derived exosomes can be quantitatively recovered from AML plasma, we used magnetic beads coated with anti-CD34 antibodies [24]. Immuno-affinity-captured CD34+ exosomes were eluted from the beads, recovered and their protein profile analyzed by western blots. Their molecular profile recapitulated that of autologous blasts and was distinct from that of non-captured exosomes [24].

Studies of exosomes produced by primary AML cells and AML cell lines have shown that these exosomes were enriched in coding and non-coding RNAs relevant to the AML pathogenesis. Transcripts relating to AML prognosis (NPM1, FLT3-ITD), response to therapy (CXCR4, IGF-IR) and to the leukemic niche formation (IGF-IR, CXCR4, MMP9) were detected in these AML cell-derived exosomes [33]. Recently, Hornick and colleagues reported in a mouse model that miRNAs in exosomes serve as early biomarker of leukemic relapse [34]. They also reported that both leukemic blasts and bone marrow stroma contributed exosomes enriched in miRNA expression profiles that predicted relapse.

4. AML exosomes as biomarkers of leukemic relapse?

In aggregate, the available results for protein and miRNA contents of exosomes isolated from plasma of AML patients suggest that these exosomes have a potential to serve as biomarkers of leukemic relapse. However, to be classified as a biomarker, the following requirements have to be met: (a) proof of concept; (b) experimental validation; (c) reliable analytical performance (sensitivity, specificity, accuracy, robustness, etc); (d) protocol standardization [35, 36]. At this time, AML plasma-derived exosomes do not meet all of these requirements, as discussed below. Currently, the very definition of exosomes is under extensive investigation [37]. Their distinction from other EVs is not firmly established, and their size (30–150nm) represents an arbitrary measure that may not be sufficient for accurate discrimination. No genetic, molecular or functional markers specific for exosomes exist. The enrichment in tetraspanins (CD9, CD63, CD81) widely used to confirm exosome identity may be useful but is not decisive, because tetraspanins are integral components of all cellular membranes [38]. On the other hand, the biogenesis of exosomes is distinct, as indicated

above, and the presence in their cargo of ALIX or TSG101 testifies to their endosomal origin and differentiates them from vesicles formed by “budding” of cellular membranes.

An optimal method for exosome isolation from plasma has not been agreed upon and the existing methodologies replacing high-speed ultracentrifugation have not been standardized. While these methods are simpler and more efficient, they are being used without attention to the recovery, morphologic integrity and biological functions of isolated exosomes [36]. Commonly, attention is focused on mRNA and miRNA isolation from plasma exosomes with no apparent concerns about the exosome cellular source, their quantitative recovery from plasma or their functional attributes. So far, the criteria for reliable recovery and consistency in biological activity of isolated plasma exosomes have not been established. Given the existing lack of definitive evidence for exosome identity, their cellular origin and validated isolation methods, the entire concept of exosomes as disease biomarkers remains questionable. Various analytical methods have been employed to measure molecular contents of exosomes, but again, the questions of their origin, recovery and purity make these efforts inadequate and even misleading. Proteomic analysis of plasma-derived exosomes has been exceptionally difficult, as these exosomes are coated with “contaminating” immunoglobulins, albumin and other plasma proteins, which mask genuine exosome-associated proteins and interfere with their detection. Removal of “contaminating” proteins with low pH buffers or solvents does not seem to ameliorate the problem and might impair exosome integrity. In place of proteomics, antibody-based protein arrays have been successfully used for molecular and genetic profiling of exosomes [39]. These are especially appealing because of the signal amplification they offer. The disadvantage is that they require substantial quantities of exosomes not always available in plasma of AML patients in CR. It now appears that certain quantitative highly sensitive analytical procedures when applied to plasma-derived exosomes may not detect genuine exosome-associated components which are masked by “contaminating” plasma proteins. Thus, there is a need for the development of novel experimental protocols to measure the molecular content of plasma-derived exosomes. On the other hand, nucleic acids can be readily extracted from these exosomes and successfully analyzed. The two methods widely used for exosomal protein characterization, Western blots and multi-parameter flow cytometry, differ in the type of information they provide: the former informs about the total exosomal content of a component, the latter about its content on the exosome surface. This difference emphasizes distinct mechanisms exosomes use to deliver information to target cells: the up-take followed by a release of the contents vs. surface-mediated signaling [17]. Neither method has been standardized for the use with exosomes, and the experimental protocols differ between laboratories.

It remains unclear whether immunocaptured blast-derived exosomes in AML are better biomarkers of residual disease than flow cytometry or RT-PCR. Comparisons have not been extensively performed. While immunocapture is by definition highly specific, its sensitivity of detection relative to the other two methods will need to be evaluated, especially in AML patients with CR. Antibody-based immunocapture as a method for isolation of blast-derived exosomes is readily applicable to plasma obtained at AML diagnosis, when blast levels in the blood are high [24]. However, in patients who are in CR after initial induction therapies, it might be difficult to recover sufficient blast-derived exosomes for protein or nucleic acid

analysis. The use of CD34 as a blast marker for capture is limited to AML patients who are CD34+, and antibodies to other LAAs will need to be screened for use in immunocapture of blast-derived exosomes. The question of whether blast-derived exosomes might be better markers of leukemic relapse than exosomes derived from normal cells has not been adequately addressed. It is possible that exosomes derived from normal cells which had been altered by the presence of malignancy are equally good or better markers, in which case separation by immune capture of blast-derived exosomes would be superfluous. In this context, the total exosome fraction could potentially serve as an indicator of pre-leukemic vs. leukemic state based either on its total protein content or the qualitative protein profile. In AML, where levels of leukemic blasts are elevated at the time of diagnosis but may be low or not detectable in the plasma of patients who achieve CR after chemotherapy, isolation of total exosome fractions is feasible and may offer a reasonable alternative to the complexity associated with immunocapture of blast-derived vesicles.

Another concern exists in regard to working with frozen and banked plasma specimens in place of freshly, prospectively harvested specimens. Exosome isolations utilize banked plasma or serum specimens without any attention to effects storage and freezing might have on exosome integrity and content. Criteria for biobanking of exosomes have not yet been established. While nucleic acid-based studies seem to be unaffected by banking of plasma, exosome aggregation is enhanced and their integrity, recovery and protein content might be negatively affected by thawing of banked plasma.

5. Expert commentary

The concept that exosomes in plasma of AML patients can serve as biomarkers of leukemic relapse based on the molecular and genetic cargo they carry is intriguing. Qualitative or quantitative changes in the exosomal cargo either by the re-appearance of LAAs on exosomes, increasing levels of immunoinhibitory exosome cargo components or of AML-relevant genetic materials such as miRNAs might serve as markers of disease relapse. However, at this time, plasma exosomes in AML patients are not yet ready for MRD monitoring. First, the proof-of-concept for exosomes as a unique vesicular entity phenotypically, genetically and functionally different from other EVs needs to crystallize. The consensus is needed for establishing a high throughput and inexpensive isolation methods from plasma for exosomes as a whole and for exosomes originating from leukemic blasts in particular. Also, the specific molecular/genetic cargos that are relevant to the AML presence, progression and prognosis need to be identified. These could be thought of as patient-specific “exograms” and could be based on miRNA, mRNA, LAA profiles or panels of immunosuppressive molecules that AML exosomes carry, such as TGF- β 1, for example. Comparisons will be necessary to establish the sensitivity of exosome-based methodology as equal to or superior to the currently used MRD detection methods, RT-PCR and flow cytometry. Only side-by-side assessments will answer the question of whether exosome use for MRD detection offers advantages over RT-PCR and flow cytometry of leukemic blasts. In AML, neither method is full proof [6, 7]. The considerable effort and cost of blast isolation or phenotypic identification necessary for RT-PCR or flow cytometry, respectively, could be offset by the use of easy, inexpensive isolation methods such as, e.g., a miniSEC procedure we recently described [23]. Body fluids can be banked for exosome isolation.

Once isolated, exosomes can be stored frozen indefinitely and serve as an unlimited source of any and all assays for nucleic acids, molecular analysis or functional evaluations. The development and standardization of methods for exosome profiling that meet the stringent criteria required for biomarkers [40] are ongoing but are not yet ready for general consumption. In this context, a collective experience suggests that some of the highly sensitive analytical methods such as proteomics analyses may be less useful than immunoenrichment-based methods for exosome profiling. Further, current studies suggest that changes in qualitative molecular and functional exosome profiles might be more informative, more clinically relevant and simpler to detect than extensive quantitative analyses of the exosome content. Once standardized, assessments of the exosome cargo can be applied to serial patient monitoring. Next, prospective validation studies with large numbers of AML patients will need to be conducted to confirm the role of AML plasma-derived exosomes as reliable biomarkers of MRD.

6. Five-year view

While at this time exosomes are not ready to fulfill the role of biomarkers of MRD, it is expected that within next few years, a rapidly expanding volume of pre-clinical research might usher AML exosomes into the biomarker category. Already studies linking molecular or genetic profiles of plasma-derived exosomes with prognosis and response to therapy are in progress with initially promising results. Accumulating evidence will extend and strengthen the importance of exosomes as opposed to soluble plasma biomarkers, including miRNAs and/or DNA-protein complexes, as more reliable markers of disease. Rapidly emerging scientific insights and technological advances will provide a “proof of concept” for plasma exosomes as biomarkers of AML progression. This will happen gradually and will first of all require that a consensus regarding exosome identity and methods for their isolation and characterization from patients’ plasma be established. The exosome scientific community has been intensively engaged in achieving this goal, and it is expected that it will be realized in the near future. This will allow and encourage the development of high-throughput affordable platforms for exosome monitoring and for the detection of changes in the molecular or genetic profiles in exosomes during therapy. Interestingly, emerging evidence suggests that application of highly-sensitive analytical methods such as MS/MS to exosome profiling may be substituted by qualitative screens for the profiles of components related specifically to AML and MRD. Such qualitative differences in profiles of serially harvested exosomes could inform us better about disease progression or response to therapy. Examples may be alterations in the content of TGF- β 1 isoforms in AML exosomes during chemotherapy reported by us [12] or in the content of survival anti-apoptotic proteins as reported elsewhere [32]. We expect that multi-parameter flow cytometry applied to exosome profiling, which is similar to that used for cell phenotyping and is already in active development, will become a method of choice for exosome profiling. With a vast numbers of mAbs available today for immunophenotyping and the capability to use CyTOF technology with exosomes, this approach will offer unprecedented specificity, reliability and precision to exosome profiling. The capability to measure effects of isolated exosomes on functions of immune cells, either immune suppression of effector cells or immune activation of regulatory cells, will be further optimized adding an additional advantage to gaging the

potential of leukemia to modulate the patients' immune system. Thus, leukemic exosomes will be recognized not only as molecular and genetic biomarkers but also as immunological biomarkers of leukemic progression. Because exosome cargos consist of molecules able to activate various molecular pathways in recipient cells, it may be possible to interrogate leukemic exosomes for the potential to regulate those pathways that are known to be active in leukemic cell differentiation, including, e.g., the Wnt or Notch pathways, as well as in oncogenic transformation. The establishment of exosomes as biomarkers of MRD will require numerous technical innovations and rigorous clinical testing that are likely to consume more than five years. However, it is expected that in five years, a biologically- and clinically-sound approach to using exosomes as surrogate markers of leukemia relapse will begin to be translated to the clinic. The application of exosomes to MRD detection may take even longer but given their potential as biomarkers, it may be worth the effort.

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Key issues

- Exosomes are the smallest type (30–120 nm) of extracellular vesicles present in all human body fluids.
- Exosomes levels in newly diagnosed AML patients are elevated and change with therapy
- Plasma-derived exosomes in patients with AML contained a different protein/ molecular cargo compared to normal cell-derived exosomes
- Criteria for a reliable recovery and consistency in biological activity of exosomes from leukemic plasma have not been established so far
- At this time, AML plasma-derived exosomes are not ready for MRD monitoring
- Prognostic value of AML plasma-derived exosomes needs to be compared to that established with the currently used methods for MRD detection
- The future of exosomes as potential biomarkers for MRD is promising but will require extensive and rigorous technical and clinical efforts

Figure 1.

Exosomes carry a cargo of proteins/lipids which is in part similar to that expressed on the plasma membrane of parental cells. Exosome isolated from AML patients also carry leukemia associated antigens (LAA)