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The role of exosomes in diagnosis of non-haematological malignancies

Dhruv Jain*; Sneha Dhariwal; Honey Sachdeva

Department of Oncopathology, Delhi State Cancer Institute, Delhi, India

***Corresponding Author(s): Dhruv Jain,**

Department of Oncopathology, Delhi State Cancer Institute, Delhi, India

Email: drdhruv_jain@yahoo.co.in

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Abstract

Exosomes are nano-sized particles 'exfoliated' from a variety of cell types. They are known to facilitate exchange of messages between various cells by transporting bio-functional cargo like proteins, nucleic acids and lipids. Exosomes play a pivotal role in cellular signaling under normal physiological conditions, as well as in diseased states like cancer. They are shed in excessive amounts by cancer cells and can be harnessed from a variety of body fluids. Hence, they can serve as a convenient and less invasive biomarker for malignancies. The present work was carried out to decipher the exact status of exosomes as a liquid biopsy tool in non-haematological malignancies. Special emphasis was laid on their isolation and validation techniques. The review of literature revealed that they could serve, both as a diagnostic and prognostic marker in a variety of cancers originating from breast, naso-pharynx, colon, lung, pancreas, prostate and urinary bladder. As of now, the available body of literature on the use of exosomes as a cancer biomarker pointed towards an exciting future ahead. Indeed, exosomes have the potential to bring about a paradigm shift in the practice of personalized medicine for non-hematological malignancies.

Background

Cancer has remained a significant public health problem worldwide. While early diagnosis and increased awareness have improved cancer survival, there remained a need to identify less invasive biomarkers which could prove effective in early diagnosis, aid prognostication and guide therapeutic decision making.

Although a vast body of literature on the role of nano-sized particles like exosomes in various malignancies was available [1], their current status in personalized cancer medicine was still unclear. While the role of exosomes as a diagnostic and therapeutic tool in hematological malignancies has been a subject of intensive research [2], we observed that any work on this research question was a separate review in itself. The present work sought to critically evaluate the existing literature data on the clinical potential of exosomes as a liquid biopsy tool in non-hematological malignancies. Special emphasis was laid on their isolation and detection techniques. An attempt was also made to identify exosomal contents that could eventually be developed as 'tumor signatures' in routine clinical practice.

Exosomes are small, 30–140 nm "exfoliations," shed from normal and neoplastic cells and classically exhibit a 'cup shaped' or 'saucer like' morphology on electron microscopy[3-5]. Of note, the exosomal secretion by cancer cells was almost ten folds greater as compared to normal cells [1]. Formed in the

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endosomal pathway by the inward/reverse budding of multivesicular bodies (MVB), their subsequent release was mediated by fusion of MVB with cell membranes (**Figure 1**). These lipid membrane bound particles had a density varying between 1.15 and 1.19 g/ml in a sucrose gradient [5].

The intercellular transfer of exosomal contents has known to play a key role in the maintenance of normal cell physiology [6-8]. The exosomal cargo included nucleic acids (mRNA, miRNA (miR) and DNA) and proteins (membrane associated proteins: tetraspanin, CD 63, CD 81, CD 82 and CD 9; cytoplasmic proteins: TSG 101, heat shock proteins and protein alex) [9,10]. Additionally, cell-type specific molecules like major histocompatibility complex, Fas L, adhesion molecules, metalloproteinases and tissue specific proteins were other possible contents [1,11-13].

Exosomes exhibit a pro-tumorigenic effect. This resulted from their role in promotion of disease progression, metastasis, angiogenesis, extracellular matrix (ECM) remodeling, immune evasion, chemo-resistance and establishment of a pre-metastatic niche [14,15]. While exosomes could induce angiogenesis by upregulation of angiogenesis regulated genes [16], fibroblasts induction could establish a more favorable tumor microenvironment [17]. Furthermore, exosomes aided epithelial-mesenchymal transition resulted in development of a pre-metastatic niche [18,19]. The exosomes influenced development of drug resistance by mediating intercellular transfer of multi-drug resistance-associated proteins and miRNAs to target cells [20,21]. Also, their effects on drug efflux and drug binding to tumor cells contributed towards multi-drug resistance [22,23].

Secreted by many cell types [24,25], exosomes have been isolated from a multitude of body fluids. The latter includes: urine, amniotic fluid, blood, serum, saliva, ascitic fluid, breast milk, cerebrospinal fluid and nasal secretions [24]. The easy permeability of exosomes through tissue barriers, stability in a myriad of body fluids and resemblance of their contents to the parental cells allowed their use as liquid biopsy tool [6-8].

Isolation of exosomes

Conventionally, ultracentrifugation and size based techniques have been employed for isolation of exosomes [26]. However, these techniques got limited owing to their high costs, being labor intensive and/or having low specificity. These drawbacks were overcome to an extent with newer immuno-affinity capture or micro-fluidics based procedures [27]. Moreover, a recent nano-system based technique like nano-wire-on-micropillar method emerged as a suitable alternative. It utilized the principle of microfabrication to entrap exosomes on porous silicon nanowire and silicon micropillars. The requirement of smaller sample volume reduced chances of clogging with resultant improved performance [28]. Acoustic sorting enabled variously sized exosomes to get separated into different laminar flows by subjecting applied fields across microfluidic channels [29].

Of late, several commercial exosomal isolation kits like Exospin™, ExoQuick™ Total Exosome Isolation Reagent™, PureExo® and miRCURY™ have also come into practice [30]. These required addition of polymeric additives for precipitation of exosomes. Of note, the advantage of doing away with ultracentrifugation provided quick results and improved both, the quality and the quantity of the exosomal yields.

Validation of exosomes

The validation techniques that have been used previously to measure the concentration of exosomes include: nanoparticle tracking analysis (NTA), dynamic light scattering, flow cytometry and transmission electron microscopy [30]. Of these, NTA has emerged as the gold standard [31-33]. It utilized both light microscopy and the data on brownian motion of the particles as they diffused across a field-of-view. The Strokes-Einstein relationship allowed estimation of exosome size in a suspending fluid, whose temperature and viscosity were known [34]. The NTA has allowed measurement of exosomal particles measuring 10 nm–2 µm in size and present in concentrations varying between 10^6 to 10^9 particles per ml [34].

Dynamic light scattering has enabled measurement of exosomes, ranging between 0.3 nm to 10 µm in size. It involved measurement of dynamic alterations in light scatter, when a coherent laser light passed through a suspension of exosomes [35]. Flow-cytometry technique entailed passing individual exosomal particle through a laser spot and measuring their subsequent light scatter and fluorescence. Although flow-cytometry allowed determination of exosomal particles of sizes greater than 300 nm, a newer platform, A-50-Micro-PLUS has further improved this resolution to just around 100 nm [36]. Unlike a conventional light microscope, a transmission electron microscope employed electrons of shorter wavelengths. Besides resolving individual particles, it allowed determination of individual exosomal morphology and presence of any heterogeneity [35].

The recent advancements in exosomal detection techniques utilized: on-chip nanoholographic imaging, nanopore ion occlusion based sensing, diagnostic magnetic resonance and plasmonic exosome detection based methods [30]. Additionally, quantitative RT-PCR, nucleic acid sequencing, western blot and Enzyme Linked Immuno Sorbent Assay (ELISA) have emerged as other alternatives for cancer exosomal proteomics [30].

Role of exosomal proteins in non-haemotological malignancies

Proteomic analyses identified exosomal proteins of use in cancer diagnosis [37]. The non-haematological malignancies wherein exosomes have been investigated as a liquid biopsy tool includes cancers of breast, ovary, prostate, naso-pharynx, bladder, lung, pancreas and colo-rectum (**Table 1**).

In breast cancers, immune-affinity isolation techniques have been employed to characterize CD24 and epithelial cell adhesion molecule (EpCAM) as diagnostic biomarkers [60].

Ovarian cancers, mostly diagnosed in advanced stages have remained as one of the most lethal forms of cancers in women. While the detection of exosomal contents like EpCAM and CD24 offered an opportunity for early diagnosis of ovarian malignancies, CD24 has even proved to be a useful prognostic marker [40,41]. In addition, exosomes like TGF-B1 and MAGE 3/6, isolated in ovarian cancers have served to successfully delineate benign from malignant tumors [42].

In prostate cancer subjects, plasma-derived exosomes like survivin, a member of inhibitor of apoptosis (IAP) was found in significantly higher concentrations [45]. Another group has reported higher levels of urinary exosomes like β–catenin, prostate cancer gene-3 (PCA-3) and transmembrane serine protease 2-ETS transcription factor family member-related gene fusion

(TMPRSS2 –ERG) in prostate cancers [46].

An interesting development in noninvasive diagnosis of bladder cancers occurred, when cancer-associated calcium-signal transducer 2 (TACSTD2) was quantified in raw urine specimens with a commercially available ELISA kit [1]. Significant differences in exosomal CD36 and CD44 between healthy and bladder cancer patients have also been documented [50].

In Nasopharyngeal carcinomas, exosomal LMP1 have been isolated from both blood and saliva [48]. A prior work has reported detection of EBV encoded LMP1 and BARF1 in 62% of their teenaged and 100% of adult subjects with the disease [49].

Role of exosomal nucleic acids in non-haemotological malignancies

Nucleic acids like microRNAs and mRNAs are the exosomal cargo that have generated interest as diagnostic biomarkers [61-62] (**Table 2**). Of these, miRNAs have attracted maximal attention for their supposed stability against RNAse dependent degradation. Hannafon B, et al [63] reported that serum miR-101, miR-372, miR-373 could aid in early diagnosis of breast cancers. Previously, microRNA profiling of circulating tumor exosomes was carried out in ovarian cancer patients [64]. Interestingly, eight miRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214) in this work revealed value as signature tumor biomarkers.

Another study examining lung adenocarcinoma subjects reported marked similarity in the miRNA patterns between the circulating exosomes and those detected in tissue biopsies [62]. Furthermore, these patterns were very different from those observed in healthy controls.

Limitations and unanswered questions

Despite exosomes having emerged as an exciting liquid biopsy tool, there remained many limitations to be overcome and questions to be answered. In the prevailing scenario, the biggest challenge lay in fully decoding their biology and developing more cost effective isolation techniques [69], which could eventually take the research from the 'bench to bedside'. Furthermore, one could also question any significant role of exosomes in clinical practice due to our present inability to definitely establish their cell specific identity. The conundrum regarding tumor heterogeneity and various complex host-tumor interactions on the exosomal yields also needs to be solved [70].

Conclusion

Keeping with the rapid strides made in our understanding of tumor biology, a slow but definite shift in cancer management protocols has taken shape. Of these, liquid biopsy tools like exosomes held promise as a less invasive surrogate biomarker in non-haematological malignancies. Indeed, future research on this subject offers an opportunity to bring about a paradigm shift in the practice of personalized cancer medicine!

Figure

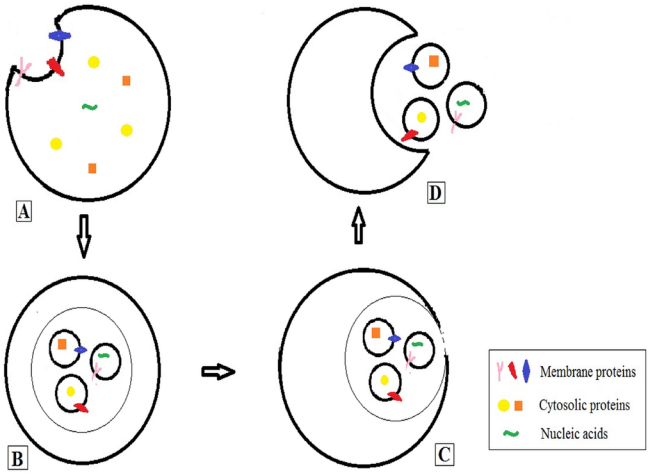

Figure 1: Schematic diagram to illustrate formation and release of exosomes - Inward budding of plasma membrane containing membrane proteins (A), leads to formation of a multivesicular body (MVB), comprised of many endosomes (B). Please note that the cytosolic proteins and nucleic acids are some of the endosomal contents. The subsequent fusion of an intracellular MVB with the cell membrane (C), leads to the release of the exosomes into the extracellular fluid (D).

Tables

Table 1: Use of exosomal proteins in non hematological malignancies

STUDY	SAMPLE TYPE	EXOSOMAL MARKERS	ISOLATION TECHNIQUE
BREAST			
Khan S et al [38]	Serum	Survivin 2B	Exoquick/ELISA
Roberg Larsen H et al [39]	Cell lines	27-hydroxycholesterol	LCMS
OVARY			
Runz S et al [40]	Serum, Ascitic fluid	CD24 & EpCAM	Ultracentrifugation, sucrose gradient /MACS
Liang B et al [41]	Serum, Ascitic fluid	CD24 & EpCAM	Ultracentrifugation, sucrose gradient /MACS
Szajnik M et al [42]	Plasma	TGF B1, MAGE 3/6	Filtratiation and ultracentrifugation
PROSTATE			

Hosseini-Beheshti E et al [43]	Cell lines	Annexin-A2, Calsyntenin 1	Proteomics, molecular lipidomics
Duijvesz D et al [44]	Cell lines	Exportin-1	Proteomics
Khan S et al [45]	Plasma	Survivin	Ultracentrifugation, Exoquick
Nilsson J et al [46]	Urine	PCA-3 and TMPRSS2:ERG	Fitration, ultracentrifugation
NASOPHARNX			
Keryer-Bibens C et al [47]	Cell lines	LMP-1	Differential centrifugation, EM and western blotting
Klibi J et al [48]	Serum & Saliva	LMP-1	ELISA
Houali K et al [49]	Serum , saliva	LMP-1 AND BARF1	Ultracentrifugation and ELISA
BLADDER			
Li W et al [1]	Urine	CACST-2	ELISA
Welton JL et al [50]	Cell lines	CD36 AND CD44	Immunoblotting / flow cytometry
LUNG			
Li Y et al [51]	Urine	Human LRG-1	Ultracentrifugation, EM
Yamashita T et al [52]	Blood	Exosomal EGFR	Targeted ELISA
Jakobsen KR et al [53]	cancer cells	CD317 and EGFR	Targeted ELISA
Sandfeld- Paulsen B et al [54]	Plasma	NY-ESO-1, EGFR, PLAP, EpCAM and Alix	Biotin-conjugated antibodies
PANCREAS			
Kahlert C et al [55]	Serum	Mutated KRAS DNA, p53	Genome sequencing
Melo SA et al [56]	Blood	GPC-1	Mass spectrometry, flow cytometry
Costa silva B et al [57]	Plasma	MIF	Ultracentrifugation, EM
COLORECTAL			
Silva J et al [58]	Plasma	Level of circulating exosomes	Cytometry
Yoshioka Y et al [59]	Serum	CD147, CD9	Exoscreen

Abbreviations: ELISA: Enzyme linked immune sorbent assay; LCMS: Liquid chromatography-mass spectrometry; EpCAM: Epithelial cell surface antigen; MACS: Magnetic activated cell sorting; TGF: Transforming growth factor; PCA-3: Prostate cancer antigen-3; TMPRSS2:ERG: Transmembrane serine protease 2-ETS transcription factor family member-related gene fusion; LMP-1: Latent membrane protein-1; EM: Electron microscopy; CACST-2: Cancer associated calcium signal transducer-2; LRG-1: Leucine rich alpha 2 glycoprotein-1; EGFR, Epidermal growth factor receptor; PLAP: placental alkaline phosphatase; GPC-1:Glypican-1; MIF: Macrophage migration inhibitory factor.

Table 2: Use of exosomal nucleic acids in non hematological malignancies

STUDY	SAMPLE TYPE	EXOSOMAL MARKERS	ISOLATION TECHNIQUE
BREAST			
Hannafon BN et al [63]	Plasma	miR-21, miR-1246	ExoQuick/qRT-PCR
OVARY			
Taylor DD et al [64]	Serum	miR-21, miR-141, miR-200-a,b,c, miR-203, miR-205, miR-214	MACS using anti-EpCAM array
PROSTATE			
Bryant RJ et al [65]	Serum/Plasma	miR141, miR-375	ExoMiR, Qiagenmi R Neasy / qRT-PCR
PANCREAS			
Que R et al [66]	Serum	miR-17-5p, miR-21	qRT-PCR

COLORECTAL			
Ogata- Kawata H et al [67]	Serum	let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, miR-23a	qRT-PCR
Liu C et al [68]	Serum	Serum miR-4772-3p	qRT-PCR

Abbreviations: MiR: micro RNA; qRT-PCR: quantitative real time- polymerase chain reaction; MACS: Magnetic activated cell sorting; EpCAM: Epithelial cell surface antigen.

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