

Diagnostic and therapeutic applications of tumor-associated exosomes

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Abstract

Exosomes are nanometric membrane vesicles released by almost all cell types. Their contents vary, and can include functional miRNA, mRNA, proteins, and lipids wrapped in a double layer of lipid membranes. Tumor-derived exosomes interact with the tumor microenvironment, and are involved in tumor initiation, angiogenesis, invasion, and metastasis. Exosomes have abundant tumor-specific molecules, with the potential to become new biomarkers for tumor diagnosis. Furthermore, an increasing number of studies use exosomes or their modified products as cell-free vaccines and drug delivery carriers in oncotherapy. The present review focuses on the progress of exosomes in tumor diagnosis and therapies.

KEYWORDS

Exosomes, tumor, biomarker, therapy

1 | INTRODUCTION

Exosomes are flattened hemispheres surrounded by lipid bilayers, with a density of 1.13–1.21 g/mL,¹ and a diameter of 30–100 nm; they are generated through the endosomal pathway.² In 1985, Pan *et al.* found by electron microscopy that reticulocytes in mature red blood cells contribute to the development process; the endoplasmic reticulum within the body is a multiple membrane depression with inward bud formation of luminal vesicles, thus into the dynamic subcellular structures – multivesicle bodies (MVB).³ When the MVB are fused to lysosomes, the lumen-like vesicles are degraded. When they are fused to the membrane, the vesicular vesicles within the immature red blood cells allow the formation of small granular vesicles, which are released into the extracellular environment.⁴ Exosomes are generated by most, if not all, cells. The contents of exosomes vary, and can include nucleic acids, proteins, and lipids, which reflect an alternative status and identity of the cells of origin (brain tumor exosomes and microvesicles: pleiotropic effects from tiny cellular surrogates). They can be transferred from host to recipient cells to alter cellular function, with tumor biomarker potential. Additionally, exosomes are ideal candidates for nano-sized drug delivery vehicles, and mediate a variety of therapeutics in oncology, immune therapy, and regenerative medicine.⁵ The present review focuses on the potential of tumor-derived exosomes (TDE) in the diagnosis and treatment of tumors.

Exosomes are secreted by most cell types, including tumor cells, antigen presenting cells (APC), hematopoietic cells, reticulocytes, mast cells, platelets, and intestinal epithelial cells, both *in vivo* and *in vitro*. They function as a mode of intercellular communication and molecular transfer, and facilitate the direct extracellular transfer of specific proteins, and lipids, as well as miRNA, mRNA, and DNA between cells. In cancer patients, most exosomes are secreted by tumor cells,⁶ and are released in virtually all biological fluids, including plasma, urine, saliva, ascites, and the cerebrospinal fluid, playing a major role in tumor microenvironment.^{7,8} However, the specific detection and isolation of tumor cell-derived exosomes in circulation are currently lacking.⁹

Proteins enriched in exosomes include tetraspanin family members (CD9, CD63, CD81, CD82) and endosomal sorting complexes required for transport (Alix and TSG101), as well as members of the GTPase family (Rab27, Rab11, Rab35) and heat-shock proteins (Hsp60, Hsp70, and Hsp90). Exosomes also express specific proteins that could reflect the primary cells; for example, APC-secreted exosomes express the major histocompatibility complex (MHC-I and MHC-II) proteins,¹⁰ costimulatory molecules (CD80 and CD86), and tumor antigens, such as the exosomal protein G250, were obtained from renal cancer, and LMP1 from nasopharynx cancer. Van Giau *et al.*¹¹ showed that exosomes contain genetic material mRNA and microRNA initially, and confirmed that such constituents could play a normal physiological

role when taken up by cells (exosome-mediated transfer of mRNA and microRNAs is a novel mechanism of genetic exchange between cells). Recently, studies have found that exosomes contain double-stranded DNA, whose genetic information has a certain degree of consistency with tumor DNA. Furthermore, genes in exosomes are stable; therefore, exosomes might be used for genetic testing in monitoring tumor development.¹² In addition, exosomes contain specific lipids, such as cholesterol, diacylglycerol, phospholipids, and glycerin acyl phospholipids, which play an important role in improving the stability of exosome membrane.¹³ Lipids also have biological activities,² but studies assessing their physiological and pharmacological effects are scarce.¹⁴

2 | BIOLOGICAL PROCESS OF TDE

TDE, mainly secreted by tumor cells, are important carriers in the tumor microenvironment as they promote angiogenesis, proliferation, and invasion in recipient cells to support tumor growth and a pro-metastatic phenotype. In addition, intercellular communication of TDE is necessary for this progress. Previous studies have shown that TDE can regulate the invasiveness of cancer cells through exosome-mediated delivery of proteins and miRNA.^{15,16}

2.1 | Exosome-mediated tumor proliferation

TDE are known to mediate tumor progression; indeed, not only are they key components of the tumor microenvironment but they could also activate tumor cell proliferation signaling pathways.¹⁷ They are rich in particular miRNAs associated with signaling pathways and overall survival. For example, expression of exosomal miRNA in hepatocellular carcinoma (HCC), including miR-584, miR-517c, and miR-378, could modulate the expression of transforming growth factor- β -activated kinase 1 and associated signaling effectors, resulting in the transformation of recipient cells and induced tumor growth.¹⁸ Similarly, exosomes with growth-promoting oncogenic proteins, including *HER2*, *EGFR*, and *EGFRvIII*, provide sustained proliferative signals in the tumor microenvironment, inducing proliferation.¹⁰

2.2 | Exosome-mediated angiogenesis

Exosomes could also release cargo molecules into the microenvironment, and function with receptors on the surface of endothelial cells to induce angiogenic changes. Studies confirmed that tumor cells could transfer exosomal angiogenic ligands and angio-miR species to endothelial cells in addition to activating hypoxia response during neovascularization.^{19,20} For example, exosomal miR-135 enhances angiogenesis through factor inhibiting HIF-1 PMID: 26784674. Exosomal miR-9 activates the JAK/STAT pathway by reducing cytokine signaling 5 (SOCS5) levels to promote tumor angiogenesis.²¹ Furthermore, cytokines contained in exosomes are responsible for the communication between endothelial and cancer cells,^{22,23} promoting endothelial cell proliferation, migration, sprouting, and progenitor maturation.²⁴ Interestingly, *FGF* and *VEGF* have synergistic effects in these processes.

2.3 | Exosome-mediated tumor invasion and metastasis

TDE are also essential for cancer spread, as Lyden suggested: "Tumor cells are pretty much an innocent bystander. They go to organs where pre-metastatic niches are already established by tumor-secreted factors including exosomes" (tumor cells can send out tiny vesicles that prime organs for cancer to spread). Recent reports confirmed that TDE play a significant role in the pathobiology of tumor metastases, as they provide signals within the tumor microenvironment, including caveolin-1, HIF-1 α , miR-105 and β -catenin to activate the epithelial-mesenchymal transition program. For example, exosomes secreted by breast cancer mediate invasion through the β -catenin pathway.^{10,25} What's more, TDE regulate tumor invasion and metastasis by homing in on future colonization sites and other tumor-associated cells specifically; for example, pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. These findings suggest that targeting such vesicles with a therapeutic agent might inhibit metastatic tumor growth.

2.4 | Exosome-mediated immune response

Many studies have shown that TDE play an especially important role in immunization activities, which are much more complex. They could stimulate antitumor immune response as they carry costimulatory molecules, MHC class I and II molecules, and tumor antigens.²⁶ In addition to their roles in antitumor immune response, TDE might also damage the immune response, as they express Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL) and transforming growth factor- β , which downregulate NKG2D, mediating apoptosis in T lymphocytes PMID: 26784674+PMID:25724562, negatively impacting cytokine secretion and cytotoxic functions of natural killer cells, and decreasing dendritic cells (DCs) activity and APC number, which lead to the tumor escaping immune surveillance.^{4,27} Studies point to impaired natural killer cell function after exposure to murine breast cancer exosomes; this results in defective natural killer cell-mediated tumor clearance *in vivo*.²⁸ TDE cargo molecules regulate the complex immune response in different modes together, although the related molecular basis is not fully defined.

3 | TDE AS CANCER BIOMARKERS

TDE are abundant, stable in blood, changing with tumor physiology, and capable of capturing heterogeneity. Boukouris *et al.* showed that exosomes are highly stable at -80°C .²⁹ In addition, TDE are advantageous in magnetic resonance imaging as they can be detected in the circulation before magnetic resonance imaging-detectable lesions in cancer at lower costs compared with magnetic resonance imaging and computed tomography.⁷ Therefore, exosome diagnostics offers an excellent tool, and allows the capturing of tumor heterogeneity in real time.

3.1 | Exosomal proteins as tumor biomarkers

TDE-derived tumor-associated protein cargoes (e.g. signaling molecules, ligands such as *BRAF*, *EGFR*, *KRAS*, *EpCAM*) constitute a good source for capturing tumor-signaling pathways, and may reveal novel therapeutic targets. It was shown that *EpCAM* and survivin protein levels are higher in body fluids from metastatic breast cancer patients.³⁰ A study showed that GPC1⁺ exosomes contain mutant *KRAS* mRNA, and represent a prognostic marker superior to CA19-9, as they showed sensitivity and specificity of 100%, respectively, at each stage of pancreatic cancer; this suggests that GPC1⁺ exosomes could be a reliable biomarker for monitoring and detecting early pancreatic cancer.⁹

Several human cancers overexpress *EGFR*, which correlates with poor prognosis in a significant number of malignancies; interestingly, exosomes transfer oncogenic *EGFR* from human squamous cell carcinoma to tumor-associated endothelial cells to promote endothelial VEGF expression by activating the mitogen-activated protein kinase (MAPK) and protein kinase B (PKB) pathways.³¹ Meanwhile, Huang *et al.* compared exosome contents between tumor biopsies from nonsmall-cell lung cancer (NSCLC) patients and chronic inflammation lung tissues; they found that 80% of exosomes from NSCLC samples were positive for surface epidermal growth factor receptor (EGFR) by immune staining, for just 2% of those in chronic inflammatory lung tissues.³² Li *et al.* found that high leucine-rich α -2-glycoprotein (LRG1) expression in primary tumors is positively correlated with LRG1 presence in urine samples, suggesting that LRG1 in urinary exosomes might be derived from primary tumor tissues. Furthermore, urinary LRG1 could be a candidate biomarker for non-invasive diagnosis of NSCLC.³³

3.2 | Exosomal RNA as tumor biomarkers

Intact miRNA can be isolated from circulating blood in significant quantities despite high RNase activity levels because of their association with exosomes. The remarkable stability of circulating exosomal miRNA makes them good candidates for disease progression monitoring in a variety of cancers.³⁴

New evidence showed that miR-21 expression is significantly higher in patients with hepatoblastoma compared with the control group in both plasma and exosomes, confirming that exosomal miR-21 could be considered a diagnostic and prognostic biomarker for hepatoblastoma.³⁵ Furthermore, serum exosomal miRNA-21 could serve as a clinical biomarker in advanced human esophageal squamous cell carcinoma because of its higher expression.³⁶ Others found three miRNA (let-7f, miR-20b, miR-30e-3p) with decreased levels in exosomes from NSCLC patients. The levels of let-7f and miR-30e-3p allowed discrimination between two groups of patients with different disease stages, therefore suggesting surgical options. Furthermore, plasma levels of miR-30e-3p and let-7f in NSCLC patients are associated with poor clinical outcome.³⁷ Meanwhile, miRNA differentially expressed in different patient cohorts, especially in individuals with metastatic tumors, could play important roles in tumor progression and metastasis. For example, five miRNA (miR-17, miR-19a, miR-21, miR-126, miR-149) were shown to be expressed at higher levels in patients with sporadic metastatic melanoma compared with values obtained for familial melanoma patients or unaffected con-

trol subjects.³⁴ Serum exosomal miR-141 could distinguish metastatic prostate cancer from primary cancer because of its higher expression in metastatic prostate cancer.³⁸

3.3 | Exosomal DNA as tumor biomarkers

In the present study, the authors found that the exoDNA content remained substantially constant in different treatment conditions, and was stable for 1 week at 4°C, 1 day at room temperature, and after three freeze-thaw cycles, as exosomes can protect it from degradation by the external environment. In contrast, DNA in exosome-free supernatants was unstable; therefore, exoDNA could serve as potential biomarkers for cancer diagnosis and prognosis.¹²

4 | THERAPEUTIC APPLICATIONS OF TDE

The potential of exosomes to serve as biovesicles for nucleic acids, proteins, and lipids, and their roles in intercellular communication, make them a versatile platform for drug delivery and cell-free vaccines, with multiple reports published since 2015.

4.1 | TDE as immunotherapy

In the late 1990s, the notion of exosomes as putative cancer vaccines was making headway in preclinical and, eventually, clinical settings³⁹ because of their ability to stimulate immune responses with comparable effects to parental DC.⁴⁰ The interest grew when tumor-antigen-loaded dendritic cell-derived exosomes (DEX) were shown to induce rejection of established tumors in mice,⁴¹ in addition, DEX could likely be stored, thawed, and utilized more efficiently than DC themselves in theory.³⁹ Furthermore, it was suggested that exosomes can be used to enhance the antitumor immune response, and thus are beneficial to immunotherapy.

Use of DEX in tumor vaccine scenarios has been proposed multiple times, and clinical-grade DEX preparations are increasingly described, leading the way for clinical trials. Interestingly, nine patients with stage III/IV NSCLC and others with stage IIIB/IV metastatic melanoma were given antigen-loaded exosomes derived from dendritic cells obtained from the patient's peripheral blood mononuclear cells. Although both studies showed that exosomes are well tolerated with low toxicity, no tumor-specific CD8⁺ lymphocyte response could be detected.³⁹

Not long after preclinical DEX cancer vaccine reports were published, use of TDE, including the ability of TDE from one tumor to cross-prime antigens leading to the rejection of another tumor type, was reported as a potent source of immunogenic targets in murine tumor models. This strongly suggested that TDE carry tumor antigens, and can trigger efficient antigen presentation of APC. For example, antigens in TDE could be transferred to DC, inducing specific cytotoxic lymphocyte (CTL) activation.⁴²

Several clinical trials have evaluated the therapeutic value of exosomes as cancer vaccines. For example, exosomes isolated from ascites in patients with advanced colorectal cancer were injected subcutaneously to cancer patients at a dose of 100–500 mg exosomes

weekly for 4 weeks, and safety and tolerability were shown to apparently increase.⁴⁰

However, the antitumor immune responses induced by TDE or DEX are relatively weak, and prone to induce immune tolerance. Therefore, several studies evaluated factors that can be altered to increase exosome-induced immune response. For instance, it was shown *in vitro* that both B and T lymphocytes need to be stimulated to elicit an effective response,^{43,44} meanwhile, adjuvants, such as cytidine phosphate guanosine (CpG) or granulocyte-macrophage colony stimulating factor (GM-CSF), can increase the magnitude of immune response *in vivo*.^{45,46}

Subsequently, exosomes collected from the ascites of 40 patients with colorectal cancer were injected with autologous vaccines (with or without GM-CSF) into respective patients. An effect was observed for GM-CSF-containing exosomes at 200 μ g. However, exosomes without GM-CSF required 300 μ g to produce the equivalent effect. These findings showed that GM-CSF significantly enhances the antitumor immune response.⁴⁵

Recently, researchers at Kyoto University in Japan proposed an efficient tumor antigen-adjuvant co-delivery system based on exosomes, using genetically engineered TDE containing endogenous tumor antigens and immunostimulatory CpG DNA. The murine melanoma B16BL6 cells were transfected with vectors encoding proteins that fuse streptavidin (SAV) and *Lactobacillus* adhesion (lactadherin) to produce genetically engineered SAV-lactadherin exosomes (SAV-exo). The latter was combined with biotinylated CpG DNA to prepare CpG DNA-modified exosomes (CpG-SAV-exo). Interestingly, B16BL6-bearing mice immunized with CpG-SAV-exo showed stronger *in vivo* antitumor effects than exosomes or CpG DNA. Thus, the genetically engineered CpG-SAV-exo is an effective exosome based tumor antigen-adjuvant co-delivery system that would likely be useful in immunotherapy of cancer.⁴⁷

4.2 | Therapeutic loading

As the composition of exosomal membrane is similar to that of the parental cell membrane, exosomes can package cellular components selectively. Thus, exosomes could be used as therapeutic delivery vehicles by loading of therapeutic cargo molecules into their lumens, through direct and indirect methods.⁴⁸ Effective loading of therapeutics of interest is a major challenge associated with therapeutic delivery using exosomes.⁴⁹

The direct method of loading cargo molecules into exosomes is much more widespread in the literature.^{41,49} A study showed that exosomes loaded with different chemotherapeutics, doxorubicin (Dox) or paclitaxel, reduce tumor growth in mice without adverse effects compared with the equipotent free drug. Furthermore, the therapeutic effects of Dox-loaded exosomes were shown to be greater than those of commercially available Dox-loaded liposomes, Doxil.⁵⁰

Shantam *et al.* used siRNA against RAD51 and RAD52 to transfect exosomes derived from HeLa cells using lipofectamine. Exosome-delivered siRNA-mediated gene silencing was shown to be associated with massive cancer cell death.⁵¹ Sonia *et al.* used electroporation to transfect exosomes from MDA-MB-231 breast cancer cells, and showed that TDE can process mature microRNA, with ther-

apeutic potential for cancer treatment.⁵² Recently, Kanlikilicer *et al.* suggested that miR-6126 is extensively released from ovarian cancer cells by exosomes, which inhibit ovarian cancer by direct targeting of integrin β 1, a key regulator of cancer cell metastasis. In addition, treatment of cancer cells with miR-6126 analogs resulted in increased miR-6126 levels in exosomes, and reduced integrin β 1 mRNA levels; therefore, exosomes loaded with miR-6126 could serve as a novel therapeutic approach to inhibit ovarian cancer progression.⁵³

However, the indirect method can be more efficiently achieved than the direct one. TDE could be loaded with cargo molecules (antigen/peptide/moiety) of interest by first loading the parent cell to effectively induce antitumor immune response.⁴⁹ A recent study showed that tumor targeting was facilitated by engineering the immature dendritic cells (imDCs) to express a well-characterized exosomal membrane protein (Lamp2b) fused to av integrin-specific iRGD peptide (CRGDKGPDC). Purified exosomes from immature dendritic cells (imDC) were loaded with the chemotherapeutic drug Dox by electroporation, achieving the specific killing of av-integrin-positive cancer cells, with an encapsulation efficiency of up to 20%. These findings suggested that such agents hold great promise for clinical applications if methods can be developed for generating exosomes with high yield and low toxicity.⁵⁴

4.3 | Therapy resistance

Although exosomal depletion might offer treatment benefits for cancer patients, more studies are required to understand the overall effect of exosome depletion on the body, for TDE could enhance cancer chemoresistance by mediating rejection of chemotherapeutics.¹⁷ In addition, TDE might harbor multiple miRNA that transfer a resistance phenotype to sensitive cancer cells by altering cell cycle control and inducing anti-apoptotic programs.⁵⁵ For example, in ovarian carcinoma, exosomes are enriched in other transporter proteins (MDR-2, ATP-7A, ATP-7B), which could induce cisplatin-resistance.⁵⁶

In a recent study of exosomes and DNA-damaging platinum (DDP), A549 cells were found to show DDP sensitivity, which might alleviate resistance of A549 to DDP; meanwhile, exosomes released from A549 cells after DDP decrease the expression of other miRNA in A549 cells. DDP, which might be mediated by the interchange of miRNA and mRNA between cells.⁵⁷ HER-2⁺ exosomes from HER-2-overexpressing breast cancer cells inhibit trastuzumab-induced anti-proliferative activity.⁵⁸ Thus, removal of HER-2⁺ exosomes from the blood of patients might improve patient responses to trastuzumab.⁵⁹ TDE also induce chemotherapy and radiation therapy resistance by activating STAT1-dependent antiviral signaling and Notch3 signaling in cancer cells.⁶⁰

Current studies have focused predominantly on altering stromal cells with exosomes secreted by cancer cells. However, exosomes secreted by many cell types, including supporting cells called fibroblasts, contribute to the microenvironment surrounding cancer cells. A new study showed that exosomes from cancer-associated fibroblasts in pancreatic ductal adenocarcinoma (PDAC) exposed to chemotherapy (gemcitabine) are critical regulators of cancer cell proliferation and survival, with gemcitabine increasing cancer-associated

fibroblast exosome secretion, which could enhance the proliferation and survival of cancer epithelial cells. More importantly, blocking cancer-associated fibroblast exosome secretion with GW4869 (inhibitor of exosome release) resulted in reduced PDAC cell survival. This might constitute a research focus for the future. Collectively, these findings showed that exosome inhibitors could be used as treatment options alongside chemotherapy for overcoming cancer chemoresistance.⁶¹ Thus, a more detailed molecular and genetic profiling will be necessary to identify the mechanisms underlying this process, as exosomes are clearly implicated in transferring drug resistance in cancer.

5 | CONCLUSION

As aforementioned, exosomes have emerged as a new mode of intercellular communication as they contain large amounts of cargo molecules, which can be transferred from tumor cells to recipient cells and participate in multitude biological processes, mediating antitumor immunity, enhancing angiogenesis, invasion, and metastasis. Furthermore, TDE are stable and non-invasive, reflecting tumor heterogeneity. Therefore, there is a wide developing prospect for TDE in personalized diagnosis and therapy. Although TDE content detection is useful in personalized diagnosis, accessibility of sequencing instrumentation, high turnaround time, cost, and low-protein content for arrays and blotting are limiting factors for resource analysis. It is also difficult to isolate and purify cancer-specific mRNAs miRNA or RNA in TDE. Additionally, specific markers that distinguish cancer exosomes from normal ones are unknown as yet, and require further study.

In the past 5 years, studies assessing TDE for therapeutic delivery and cell-free cancer vaccine development have made significant progress. However, various challenges must be overcome before TDE technology is ready for the clinic. As the properties of exosomes depend on their original cells and the microenvironment of their formation, establishing clear characterization practices is essential to ensure reproducibility and safety. In addition, the drug entrapment rate is low, and time for genetic engineering is extended, beside the high cost; meanwhile, not all drugs can be expressed. Thus, in future studies, it might be desirable to identify a drug loaded with the natural exosomes; alternatively, physical means, such as enhancing the hydrophobicity of the exosomal membrane, and intense basic clinical work will be required before TDE can serve as therapeutic targets or cancer biomarkers.

CONFLICT OF INTEREST

The authors declare that they had read the article and there are no competing interests.

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How to cite this article: Wang N, Xie L. Diagnostic and therapeutic applications of tumor-associated exosomes. *Prec Radiat Oncol.* 2017;1:34–39. <https://doi.org/10.1002/pro6.13>