Int J Clin Exp Med 2016;9(8):15040-15049
www.ijcem.com /ISSN:1940-5901/IJCEM0023478

Review Article

Exosomes, microvesicles, and head and neck cancers

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Received January 7, 2016; Accepted July 10, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: The function of exosomes and microvesicles (MVs) in human tumors has been demonstrated to be important. They play important roles in the onset and development of different cancers, and have been shown to be promising objects in studies seeking to discovery and identify tumor markers. However, research on the biological activity and molecular function of MVs in head and neck cancers (HNCs) is uncommon, mainly in nasopharyngeal carcinomas (NPCs). In this review, we studied the isolated methods, the function of exosomes and MVs, and the advance of role in HNCs.

Keywords: Exosomes, microvesicles, head and neck cancer

Introduction

Exosomes and microvesicles (MVs) are membranous vesicles generated by different kinds of cells. These two kinds of vesicles can influence angiopoiesis and the recombination of extracellular matrix, participating in forming the microenvironment that contributes to transforming and transferring tumor cells. However, research on the biological activity and molecular function of MVs in head and neck cancers (HNCs) is uncommon, mainly in nasopharyngeal carcinomas (NPCs). Vesicles derived from HNCs are becoming effective new information sources, not only enriching treatments but also having important effects in promoting anticancer progress. In this review, we summarize current knowledge on exosomes and MVs, and the function of exosomes and MVs in head and neck cancers.

Exosomes and microvesicles

Extracellular vesicles (EVs) are secreted extracellularly through unconventional exocytosis processes by different kinds of cells. In the clinical state, exosomes and MVs exist in multiple fluids, such as in plasma [1], urine [2], cerebrospinal fluid [3], amniotic membrane fluid [4], bronchoalveolar lavage fluid [5], synovial fluid [6], malignant ascites [7], breast milk [8], and saliva [9, 10]. EVs are important mediators of intercellular communication. As information carriers, EVs carry intracellular messengers, such as mRNA, micro-RNA, DNA, protein, and lipids. Additionally, recent studies have shown that exosomes participate in the transfer of virus miRNA, which could influence the tumor microenvironment, growth, and spread of tumors [11]. miRNA carried by exosomes influences multiple signal channels inside IE cells and can induce changes in biological functions, including regulating immunoreactions, promoting intercellular information communications, and promoting antiangiogenesis reactions. Exosomes play an important role in the study of miRNA, and more attention should be paid to the important functions of miRNA carried by exosomes.

Recently, more researchers have turned their attention to studies of EVs. They have described and defined many different kinds of vesicles, including exosomes, deciduous MVs, ectosomes, particles, virus particles, virus-like particles, and oncosomes. However, so far, the properties and functional mechanisms of these vesicles are not fully understood.

The molecular constitution of EVs (diameter 30-1000 nm) [12] has been researched and analyzed widely. They contain many biomolecules, such as membrane lipids, membrane proteins, cytoskeletal proteins, and protein

chaperones. Some elements of MVs are found gathered together around cell surface proteins of parent cells, such as receptors and adhesion molecules [12], and exosomes contain proteins related to their endosome origins and MV formation. Recent research also supports the existence of tumor-derived vesicles (TDVs) that are functional signaling mediums, supporting the theory that exosomes are important components of intracellular signal conduction [13, 14]. It may be expected that improvements in proteomics based on mass spectrometry will further advance the role EVs play part in tumor research.

Exosomes and MVs are two main subspecies of EVs. They have differences in morphology, biophysical features (shape, size, density), and biosynthesis [15]. However, they share a similar molecular constitution, such as cytoskeletal proteins, membrane lipids, and external phosphatidylserine [16, 17].

Exosomes have homogeneous sizes (diameter 40-100 nm) [18] and a cupulate morphology [19]. With the regulation of various signal molecules, multivesicular endosomes (MVEs) bud inward to form exosomes. When MVEs fuse with the cytomembrane, these vesicles are released extracellularly as exosomes. Vesicles contain many characteristic protein substances, such as Alix [20, 21] and TSG101 [22], which are related to endosome/lysosome formation and participate in the endosomal sorting complex required for transport (ESCRT). Although protein components of exosomes differ from cell tissues, to a large extent, they share some common basic components consistent with being mostly derived from cell tissues. Exosomes recognize and fuse with recipient cells specifically, transport autologous substances into recipient cells, and lead to changes in biological function in recipient cells.

Microvesicles are also called deciduous microvesicles, ectosomes, and particulates. They have diameters around 100-1000 nm. The cytomembrane blisters outward to form MVs [23]. This process involves changes in the intracellular calcium ion concentration and cytoskeletal reconstitution [24]. The formation of MVs is related to the activation of cell surface receptors, and intracellular calcium ion concentrations increase after activation. The molecular organizations of MVs are dynamic, depending on cell type and activated state [25]. These vesicles are composed of heterogeneous elements. They contain cell surface markers and lipids, such as phosphatidylserines, integrins, selectins, and CD40 ligands, which distinguish their cell origin [23]. Although there are differences in biogenetic origin and molecular organization between ectosomes and MVs, their definitions require precision. No clear evidence can distinguish their functional roles and no existing marker can distinguish them definitely. Because no specific evidence can distinguish exosomes and MVs, there is some overlap in terminology. Most researchers do not distinguish these two categories and MVs are considered a general term for exosomes and MVs.

Exosomes and MVs have special potential impacts in tumors. They play important roles in the onset and development of different cancers [26, 27], and have been shown to be promising objects in studies seeking to discovery and identify tumor markers.

In the past 10 years, much research about human tumor-derived vesicles (TDVs), such as those from breast cancer, lung cancer, ovarian cancer, prostate cancer, colon cancer, and gastric cancer, has been reported, especially research about cancer diagnosis and characteristic indicators. Vesicles derived extracellularly contain many proteins and lipids, which are the same as the structural components of cytomembranes and intracellular cysts. The compositions of vesicles derived extracellularly reflect the types of source cells and help to confirm the way in which these vesicles were produced. Molecules in these vesicles are transported to target cells and can continue to have effects there: for example, DNA and RNA can induce epigenetic changes in target cells.

Currently, various methods are used to isolate EVs of different origins from bodily fluids and cell culture supernatants [28]. The most common process is based on a series of differential centrifugation steps. First, cell debris and contaminants are removed; then, high-density vesicles are pelleted by high-speed centrifugation. MVs are usually obtained at 10000× *g*, and exosomes at 100000× *g* from the supernatant pellet [29]. These methods are simple and useful, and have been used widely. Sample preparations can be highly polluted by other apoptotic vesicles, cell debris, and protein aggregates, so that the methods mentioned may be combined with additional filtering steps or the

use of density gradient centrifugation [30]. There are now different kinds of density gradient methods: linear or discontinuous gradients can be used, such as cane sugar [31], Optiprep [32], and Percoll [33]. Exosomes can be separated from other types of vesicles or cellular constituents based on density (of 1.12-1.20 μg/mL, in sucrose solutions [34]). Recently, System Biosciences (SBI) Company developed a precipitation solution named 'TC ExoQuick', which can separate human exosomes from serum, ascites, urine, and cell culture fluid [30]. In comparison with other methods (e.g., magnetic beads and gel filtration chromatography), this reagent can produce peak RNA and protein amounts, but this product also uses density gradient ultracentrifugation to get high-quality and integrated exosomes. ExoQuick precipitation solution may be effective in quick separations and in facilitating the recovery of exosomes, but the purity and the quality of samples remains to be confirmed. In addition, ExoQuick TC precipitates other proteins [30], so it does not separate tumor-originated exosomes preferentially.

Recently, with new techniques, separation methods have improved, such as nano-membrane-filter procedures [35] and immunomagnetic beads extraction methods [32]. These technologies have good prospects, and have been applied successfully in different kinds of cells and bodily fluid, such as antigen-presenting cells [36], breast cancer cells [37], colon cancer cells [32], ascites fluid [37], and serum [38]. However, these technologies had not yet been widely used in vesicles, because their application needs definite knowledge of positive protein markers for different vesicles, which is lacking for most samples presently.

After vesicles are separated, many analytical methods, such as transmission electron microscopy (TEM), flow cytometry (FC), Western blot analysis [39], and LC-MS/MS^{10}, can be used to assess sample quality and sample size (vesicle completeness, size, density and expression of known positive markers). The reproducibility of these separation methods has been shown to be appropriate for protein biology research on cell culture and biofluids. Exosomes and MVs can be isolated from many clinical samples, such as plasma, urine, cerebrospinal fluid, amniotic fluid, bronchoalveolar lavage fluid, synovial fluid, ascites, breast milk, and saliva, by cane sugar differential gradient centrifugation.

For optimizing and normalizing the extraction process of exosomes, although cell culture supernatant fluid is an ideal model, there have been many technological problems in getting highly purified vesicles from clinical specimens. Body fluids have different properties, like complexity, dynamic range, and viscosity, and they contain exosomes and MVs secreted by different kinds of cells, including malignant cells, normal cells, and immune cells.

To solve some of the limitations of these techniques, extraction techniques need to be optimized further, such as removing albumin from urine and serum samples, and diluting specimens (plasma and ascites). Urine is the most widely studied biological agent, because it can be gathered non-invasively and easily [40]. The isolation techniques for saliva exosomes should also be improved [41].

Microvesicles and head and neck cancers

Head and neck cancers (HNC) are the fifth most common cancers globally: there are about 650,000 newly diagnosed cases every year [42]. More than 50% of patients with HNCs have local advanced cancers at diagnosis, and overall survival has not improved over the past several years. One of the challenges of HNCs is that this disease refers to many anatomical sites, such as the nasal cavity, paranasal sinus, oral cavity, nasopharynx, oropharynx, laryngopharynx, and larynx. Oral cavity squamous cell carcinoma (OSCC) and oropharyngeal carcinoma (OPC) are two common kinds of HNCs. OSCCs are closely related to the environment and life style, such as smoking and drinking. Viral infection is the main pathology behind at least two kinds of HNCs: oropharyngeal and nasopharyngeal carcinomas.

Human papilloma virus (HPV) has been shown to be a risk factor for cancer, and to have a relationship with most newly diagnosed OPC cases [43]. However, there has been no reported research about exosomes in HPV-related cancers (including OPCs) yet. Increasing evidence indicates that exosomes play an important role in virus infection and intercellular communication, so studying exosome components in HPV-related cancers is important.

Many studies have reported exosomes derived from virus-related nasopharynx cancers. Nasopharynx cancer is a unique kind of head and neck epithelium-derived cancer, characterized by relatively young patients (average age of about 50 years old) and specific geographic areas, such as Southeast Asia, where the annual incidence rate reaches 1/4000 [44]. Almost all cases of nasopharynx cancer are related to Epstein-Barr virus (EBV) [45, 46]. Epstein-Barr virus belongs to the γ-herpes viruses, a cluster of large enveloped viruses with long, straight double-stranded DNA genomes. These viruses are divided into α-, β-, and γ-herpes virus subfamilies, and can cause life-long latent infections [47]. EBV infections are asymptomatic in many instances, but sometimes the virus can cause infectious mononucleosis and related human malignancies, like Burkitt's lymphoma, NK/T cell lymphoma, and gastric cancer [48]. EBV in nasopharynx cancers typically exists in the form of the type II incubation period.

Compositions of exosomes derived from EBV-positive nasopharynx cancer have been reported commonly but, so far, research on the biological activity and molecular function of MVs in HNCs is rare [49, 50]. Some scholars had reported that MVs exist in serum of patients with HNCs [49, 50]. Although the origins of these MVs are controversial, the hypotheses that TDVs exist in bodily fluids and that parent cancer cells express tumor-specific antigen has been supported by many reports [51]. Within tumor-specific antigens detected on vesicles, soluble FasL and its receptor Fas, two members of the tumor necrosis factor (TNF) family, are regarded as having a relationship with tumor progression and apoptosis resistance in multiple human malignancies, such as gastric cancer [52], prostate cancer [53], glioblastoma [54], and OSCC [55]. That is, a link with progression of head and neck cancers could be created by regulation of cell apoptosis and immunological surveillance.

$FasL^+$ microvesicles were detected from serum in patients with oral squamous cell carcinomas by Kim et al. [49]. This study linked the biological activity of MVs and tumor progression in patients with HNCs, and suggested that the existence of carcinogens could promote escape mechanisms of tumors. $FasL^+$ MVs sheltered immunosuppressive and apoptotic effects of immunocompetent cells, indicating their prognostic value in OSCCs.

To define the molecular distribution in HNCs of MVs and deepen our understanding of their correlations with the development of HNCs, the functional activity of MVs and well-known tumor growth markers were analyzed, such as disease activity, lymph node involvement, and tumor stage. Some research indicated that HNC MVs could induce cell apoptosis in $CD8^+$ effector cells. Moreover, MVs from the sera of patients with head and neck squamous cell carcinomas (HNSCCs), could induce the production of high-level pan-caspase active species in $CD8^+$ Jurkat cells. If MVs were extracted from advanced tumors, these effects could be further enhanced, compared with those from early stage tumors [50].

Exosomes and nasopharynx cancers

Exosomes have been regarded as a new mechanism that could be used by cancer cells and virus-infected cells to regulate the microenvironment [56]. Additionally, viruses may use exosomes to spread infection and escape acquired immunity. Genetic materials, including viral DNA and micro-RNAs, exosomes, and cytokines, have been detected in the peripheral blood of patients with nasopharynx cancers [57, 58]. The relationship between exosomes and nasopharynx cancers was first shown by exosomes from tumor cells detected in the saliva, serum, and plasma of mice and patients with nasopharyneal carcinomas [59]. Further research showed that exosomes released by latent EBV-infected nasopharynx cancer cells contained LMP1, signal transduction molecules, and EBV-encoded micro-RNAs [59].

Galectin 9

Many studies have found that malignant epithelial cells derived from nasopharynx cancer xenografts can release $HLA\text{-}II^+$ exosomes that contain Galectin 9 or LMP1 [60]. Using anti-HLA-II-coated beads for magnetic capture, nasopharynx cancer-specific exosomes have been separated from the plasma of patients with nasopharynx cancers. Galectin 9 has been detected from exosomes of patients with nasopharynx cancers, whereas no Galectin 9 was found in exosomes from control groups [57]. Galectin 9 is a β-galactose-combined agglutinin, and was first characterized in cell analyses of nasopharynx cancers [61]. This protein can interact with membrane receptor TIM1 and induce T cell apoptosis. Exosomes from circu-

lating tumors contained MHC-II molecules and Galectin 9 can promote T-reg expansion and induce apoptosis of mature $CD4^+$ Th1 cells, so exosomes play important roles in immunosuppression [61].

LMP1

LMP1 has often been used to describe EBV-related cancers. LMP1 is a major EBV oncogene and has transformational characteristics in cultured cell lines [62, 63]. LMP1 is like a constitutive active element of the tumor necrosis factor receptor family. It can activate multiple signaling pathways, including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), phosphatidyl inositol 3-kinase (PI3K)/Akt, and NF-κB [63]. LMP1 can also induce certain behaviors of specific genes, such as cell apoptosis, cell cycle progression, and the expression of cell proliferation-specific genes [63].

In EVs from nasopharynx cancer cell strains, LMP1 protein in cells infected by EBV was the most common cancer-causing viral protein [60]. Exosomes derived from malignant epithelial cell-conditioned medium from a nasopharynx cancer xenograft were also found to contain LMP1 [60]. LMP1 was also found in serum exosomes from patients with nasopharynx cancers [59]. These EBV-infected cells released exosomes, which contained LMP1 and signal transduction molecules, and could suppress *in vitro* effects of T cells [64, 65]. Additionally, Meckes et al. [11] showed that exosomes released by nasopharynx cancer cells contained EGFR, PI3K, and high levels of LMP1. It has been shown that LMP1 can promote EGFR releasing nasopharyngeal carcinoma cell-derived exosomes. Exposure to these exosomes could activate ERK and PI3K/AKT signal transduction pathways in recipient cells. This indicated that this virus might regulate the tumor microenvironment and promote the growth of adjacent tumor cells.

LMP1 has been shown to be a target of BART miRNAs and many miRNAs distributed in the tissues of patients with nasopharynx cancers [11]. BART miRNA downgraded LMP1 to adjust LMP1-induced NF-κB signals [11]. Additionally, LMP1 could influence the miRNAs of host cells. LMP1 could upregulate miR-146a, affecting IFN-responsive genes and suppressing antiviral defenses of the host, reducing immune-mediated monitoring [66].

Micro-RNAs

miRNA is the most common genetic material in exosomes, and is regarded as an important regulator of mRNA and protein expression. miRNAs have been associated with almost all kinds of cancers, hematological and malignant tumors, and primary malignant solid tumors [67]. According to the target transcription fragments of miRNAs, it is known that miRNAs have effects in tumor suppression and on oncogenes [68]. For example, miR-21 is one of the most common miRNAs in human cancers; it can suppress PTEN [69] and apoptosis genes [70, 71], thus increasing the survival rates of cells. Considering their ubiquity, much research has described potential biomarker roles of miRNAs. Recently, miRNAs have been shown to enter into the extracellular environment via exosomes [38].

EBV-encoded miRNAs

EBV was found to express its own miRNAs. EBV-encoded miRNA were of two forms, a) colony BHRF1, related to a transcript encoding protein BHRF1 [72] and b) colony BART [73]. An *in vitro* study indicated that their patterns were related to the latency stage of the virus [74, 75]. miRNA of type BHRF1 has been found in an EBV-transformational B lymphoblastoid cell line (LCL) [76]. Most exosomes from nasopharynx cancer cells contain BART miRNA [74, 75]. In total, 22 miRNA precursors encoded by BARTs have been described from high-throughput sequencing of EBV-infected cell lines. Each miRNA hairpin structure can code for two mature miRNAs, so the number of BART miRNAs has reached a potential 44 [77, 78]. These EBV-encoded miRNAs could be secreted via exosomes by tumors, and have enough stability to ensure distribution via the circulating blood. Various kinds of BART miRNAs can coexist in one cell and only a few miRNAs may be expressed [75]. Meckes et al. also pointed out that exosomes released by EBV-positive nasopharynx cancer cells showed different abundances of BART miRNAs, indicating that miRNAs of some viruses might be packed or transported into exosomes selectively [11]. A recent study confirmed that BART miRNAs could be detected in exosomes of nasopharynx cancer

cells *in vitro*, heterogeneous xenograft tumors from mice, and plasma samples from nasopharynx cancer patients [79]. Additionally, these BART miRNAs were functional after transfer to recipient cells and could downregulate target mRNAs or proteins [11].

EBV-encoded miRNAs function in nasopharynx cancer exosomes

Although there have been many studies about nasopharynx cancer exosomes, their exact effects and mechanisms remain to be determined. Nasopharynx cancer exosomes have been thought to take part in intercellular communication between cancer and recipient cells; they may control virus infection, immune escape, and the tumor microenvironment by changing gene expression in adjacent tumor cells and matrix cells [80]. Intercellular communication mediated by exosomes has often been described in terms of the transfer of hereditary materials from infected nasopharynx cancer cells to adjacent cells [81], such as LMP1, Galectin 9, signaling molecules, and viral miRNA, thus regulating gene expression of virus and host cells [11, 81, 82].

EBV-encoded miRNAs could regulate genes in host cells, interrupt regulatory pathways of host cells, and escape immunoreactions, which could promote tumor progression [83]. For example, miR-BART7 and miR-BART19-3p could regulate the WIF1 and APC genes in the Wnt signaling pathway [84], while miR-BART5 could reduce cell apoptosis via PUMA [85, 86]. MIR-BART2-5p could regulate the MICB gene and help to avoid immunodetection. EBV has also been shown to suppress the secretion of exosomes by T cells, affecting the immune system of the host [65, 87].

Beyond the regulation of cellular genes, EBV-encoded miRNAs can also affect viral genes. Methods of increasing the persistence of viral infection in nasopharynx cancer cells include using a targeted virus gene to maintain the incubation period, or to escape immunodetection by maintaining a low level of latent gene expression [80]. BART miRNA can be fully expressed in nasopharynx cancer tissues, targeting the 3'-UTR of LMP1, downregulating its expression [79]. miR-BART22 can also downregulate latent membrane protein 2A (LMP2A), which is an immunogenic viral antigen recognized by cytotoxic T cells [88]. The downregulation of expression levels of latent genes LMP1 and LMP2A could lead to EBV-infected cells escaping immunological surveillance by the host [80]. Additionally, this BART miRNA may also regulate LMP1-induced NF-κB signals and sensitivity to cis-platinum [79]. LMP1 can upregulate miR-146a in host cells in return [66].

Exosomes containing BART-miRNA can be detected in nasopharynx cancer transplants and the plasma of nasopharynx cancer patients, indicating that they may serve as biomarkers or auxiliary detection tools, and detecting plasma DNA and BART could provide more specific information about tumor phenotypes [79]. However, as yet, little is known about dynamic changes and transfer mechanisms of miRNAs in exosomes.

Exosomes and laryngeal carcinomas

A few studies have been reported on exosomes in laryngeal carcinoma. Wang et al. isolated exosomes from serum samples of 52 laryngeal squamous cell carcinomas. CD63, a specific exosomal marker protein, was detected. miR-21 and HOTAIR were also found in the purified exosomes. Moreover, serum miR-21 and HOTAIR were associated with clinical stage and lymph node metastasis [89].

Exosomes and esophageal carcinoma

Exosomes have also been found to be non-invasive diagnostic and prognostic markers in esophageal carcinoma. Warnecke-Eberz et al. found that some exosomal onco-miRs, miR-223-5p and miR-483-5p, were upregulated in esophageal adenocarcinoma. However, miR-224-5p, miR-452-5p, miR-23b-5p, miR-203-5p, miR-1201-5p, miR-149-5p, miR-671-3p, miR-944-5p, miR-27b-3p, and miR-22-3p were significantly downregulated. These exosomal onco-miRs may be useful for the non-invasive diagnosis and treatment monitoring of esophageal adenocarcinoma [90]. Takeshita et al. also found that serum exosomal miR-1246 may be a diagnostic and prognostic biomarker for esophageal carcinoma [91]. Chiam et al. found that serum exosomal multi-miRs may detect esophageal adenocarcinoma [92].

Acknowledgements

This research was supported by the National Natural Science Foundation of China (grant nos. 81372903).

Disclosure of conflict of interest

None.

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