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TOPICAL REVIEW

Exosome Analysis: A Promising Biomarker System with Special Attention to Saliva

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Abstract Today, exosome-related studies have become a focus in science and technology. Recently, three scientists won the Nobel Prize for determining the mechanisms of exosomal transport, making exosomes a promising biomarker system for disease diagnosis and treatment. This review provides a general introduction of exosomes and explores the recent progress on the function, application, isolation, and identification of exosomes as biomarkers in blood and other body fluids, especially in saliva. Detailed information of exosomal proteins and RNAs is discussed in the paper because of their ability to determine the function of exosomes. Due to their noninvasive assessment for quick and convenient diagnosis of diseases, salivary exosomes may well be promising biomarkers.

Keywords Exosome · Saliva · Biomarkers · Function · Isolation · Identification

Introduction

Over recent decades, studies on exosomes have become a hot area and have played an important role in several areas of science. James E. Rothman, Randy W. Schekman, and Thomas C. Südhof shared the 2013 Nobel Prize in Physiology and Medicine for solving the puzzle of intracellular transport mechanisms. Previously, although scientists regarded exosomes as messengers that transmitted information between cells, the specific mechanisms of the transfer were not understood. Now that the mystery has been solved, we believe that in the future, exosomes will be valuable for use in the diagnosis and treatment of various diseases, in the production of immunosuppressive drugs, and in many other applications.

What is an exosome? Each cell of an organism is involved in the production and export of molecular products. For example, insulin is manufactured and released into the bloodstream, and neurotransmitters pass from one nerve cell to another. These molecules are transmitted in the form of small vesicles from the cells, called "cell exosomes." Cells continue to discharge "small vesicles" and other macromolecular complexes into intercellular spaces, all of which are referred to as subcellular structures. There are now known to be many kinds of subcellular structures, distinguished in terms of their density, size, and mechanism of formation. Among these, exosomes have diameters of 30–120 nm, and they are produced in the endosomes, forming multivesicular bodies (MVBs) in the cytoplasm, and are discharged through the plasma membrane. In the intercellular space, MVBs fuse, rupture, and discharge their contents (Vlassov et al. 2012).

Exosomes are essentially spherical. Their surface consists of a lipid bilayer, which contains polysaccharides, protein receptors, and other structures (Tan et al. 2013). The interior contains lipids, RNAs, and proteins. The structure of exosomes can be considered as a miniature version of the original donor cell approximately (Raimondo et al. 2011). Exosomes exist in almost all cell types

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around the body and in most bodily fluids. The cells include hematopoietic cells, B cells, T cells, dendritic cells, mast cells, platelets, intestinal epithelial cells, Schwann cells, adipocytes, neuronal cells, fibroblasts (NIH3T3), numerous tumor cell lines, and antigen-presenting cells. Body fluids containing exosomes include blood, urine, saliva, breast milk, epididymal fluid, amniotic fluid, semen, and malignant effusions (Yamada et al. 2012).

The contents of the exosomes are variable. Exosomes may contain multiple active enzymes and proteins, including glycoproteins, membrane-trafficking and fusion proteins (GTPases, annexins, flotillin), CD antigen proteins (CD9, CD63, CD81, CD82), heat-shock proteins (Hsc70, Hsp90), proteins involved in multivesicular body biogenesis (Alix, TSG101), as well as lipid-related proteins and phospholipases. Although most of these proteins are derived from the original cells, they may change after the exosomes leave the cells. Exosome membranes contain lipids, such as cholesterol, ceramide, sphingolipid, and phosphoglyceride. Furthermore, exosomes have been reported to contain significant amounts of RNA. Indeed, the concentration of miRNAs in the exosomes is very high (Zhang and Grizzle 2014). Although numerous proteome and transcriptome studies of these particles exist, little is known about their glycobiology. While exosomes actually contain large amount of carbohydrate including mannose, polylactosamine, glycoprotein, N-glycan, etc. (Batista et al. 2011). In turn, polysaccharide can also help with the formation of exosomes (Baiett et al. 2012). Nowadays, with the focus of exosomal carbohydrate becomes hotter, we believe the study of it will play an important role in exosomal areas.

Exosomes have many functions; they are involved in immune regulation, communicating substances between cells, and spreading prion proteins and retroviruses in infections. They may also be useful for drug delivery, using mesenchymal stem cells as a cargo carrier, to release exosomes (Lai et al. 2013). Because we can regard exosomes as similar to their cells of origin, analysis of the biological functions of exosomes may also be useful in the interpretation of the functions of the original cells. Studies have shown that exosomes not only play an important role in RNA processing, but also in RNA degradation. To degrade pre-RNA, unspliced mRNA is spliced first. In this respect, exosomes operate as an autoantigenic complex of exoribonucleases (Reis et al. 2013). Furthermore, exosomes can work as a cargo carrier for transport. The use of exosomes in protein transport was confirmed long ago, but a role in RNA transport was not validated until 2007.

It was once thought that the function of exosomes was to allow cells to discharge waste proteins. Now they are seen as an intercellular messenger system, transferring substances and forming communications. Based on this characterization, the hope that exosomes can be used in clinical diagnoses and the treatment of diseases is very promising.

How are vesicles transported? This problem has now been answered. Randy. W Schekman found a series of genes involved in the exosomal transport mechanism (Jin et al. 2012). James Rothman discovered the mechanism by which exosomes fuse with their targets, allowing the transfer of their "cargo" (Sollner et al. 1993). Thomas Südhof revealed how signals control exosomes, allowing them to achieve the precise distribution of their cargo (Kaeser et al. 2011). The three Nobel Prize-winning scientists characterized the molecular mechanisms by which exosomes are transported at the right time to the right place. First, receptors and ligands bind inside the cell body to form the early exosome. The early exosome undergoes two kinds of transformation. In one, the proteins of the early exosome combine with the plasma membrane. In the other, the exosome transforms into the internal luminal vesicle (ILVs) of MVBs. ILVs and MVBs bud from the limiting membrane and enter the lumen of endosomes. Degradative MVBs can be generated by the endosomal sorting complex for transport and ubiquitination. Exocytic MVBs fuse with the plasma membrane and release exosomes into the microenvironment. The cytoplasmic contents of the exosomes can be internalized by neighboring cells, thereafter releasing their exosome contents. Exosomes that are internalized by a neighboring cell can also change into MVBs (Simpson et al. 2009; Chlebowski et al. 2013).

Functions of Exosomes

First, exosomes were thought to function primarily in the removal of unwanted proteins and RNAs from cells. However, more recent studies on exosomes have shown that they are involved in many other functions.

RNA Processing and Degradation

Exosomes can act as a tool for processing and degrading RNAs. As early as 2000, Brouwer et al. (2001) described PM/Scl-100 and PM/Scl-75 autoantigens found in human exosomes. The composition of human exosomes, was largely the same as that of yeast and contained autoantigens. The antigens PM/Scl-100, PM/Scl-75, hRrp4p, hRrp42p, and other autoantigens are homologous to those in the exosomes of yeast. The study validated that the PM/Scl-100 complex was a protein complex and an autoantigen complex in myositis and scleroderma. Those exosomes are involved in RNA processing and degradation. Comparing the exosomes with those of yeast, the study described the

structure of the PM/Scl complex and also suggested that exosomes were linked to ribosome biogenesis. The antibody epitope on PM/Scl may also be involved in mediating apoptosis as exosomes of Staphylococcal entorotoxin B anchoring breast cancer cells (Mahmoodzadeh Hosseini et al. 2014).

Pathogen Spread

Exosomes have the ability to exchange pathogens between cells. Ritchie et al. (2013) found that prion proteins pRPSC and prpc were related to transmissible spongiform encephalopathies. Studies showed that both prion proteins were linked to exosomes in animal blood, indicating that exosomes could be an important medium for the spread of transmissible spongiform encephalopathies. Further studies showed that prpc was closely associated with exosomes, confirming that they were exosomes, mainly from plasma, that transmit variant Creutzfeldt-Jakob Disease (vCJD).

Tumor Promotion

Exosomes are associated with apoptosis, coagulation, inflammation, and they can control the polarity of cell development and differentiation. They may also be involved in the growth, survival, differentiation,transmission and stress reactions of cells (Beninson and Fleshner 2014).

Tumor cells can discharge small exosomes, and oncogenes can be spread in this manner. Glioma cells and lung cancer cells can secrete exosomes containing the epidermal growth factor receptor, EGFRvIII (Yamashita et al. 2013). After exosomes enter the blood, they can integrate with tumor cells that lack EGFRvIII, by which they activate transforming signaling pathways (MAPK and Akt), change the expression of EGFRvIII-regulated genes, and cause morphological transformations. Thus, in tumor secretions, exosomes secreted by tumor cells can promote the expression of tumor growth factors and even change the phenotype of some tumor cells (Al-Nedawi et al. 2008).

Immune Function

Exosomes can carry large amounts of proteins. These proteins may be related to cell movement, gathering, shaping, and combination. They may also be involved in immune functions. Research has shown that miRNAs of exosomes in the breast milk vary with different stages of lactation. For example, miR-181a and miR-155 are present in large numbers of exosomes from the different stages of lactation and play a significant role in immune responses. Thus, studies suggest that miRNA from exosomes in the breast milk may modulate the immune response in infants. Moreover, Zhou et al. (2012) used deep sequencing technology to examine the expression and distribution profiles of immune-related miRNAs of the exosomes in breast milk and reached the interesting conclusion that these miRNAs had high stability and resistance to harsh conditions, such as extreme temperatures and RNase digestion.

Exosomes can also produce an immune reaction in tumor cells. Exosome immunotherapy is similar with cell therapy but is more convenient because there is no need to culture exosomes. Exosomes are more stable and easier to be preserved (O'Loughlin et al. 2012). It is more convenient to extract exosomes, and they are resistant to deterioration. The application of exosomes may be helpful for vaccines and drug combinations. The only drawback is that the use of a tumor exosome vaccine should be individualized, so that the patient uses autogenous vaccines from him/herself. Perhaps in the future, large-scale use of synthetic vaccines may occur.

Hartman et al. (2011) discussed the potential of tumor vaccines that involve fusing the carcinoembryonic antigen (CEA) and HER2 antigens (two non-mutant tumor-associated antigens, "TAA") into the C1C2 domain of lactoferrin proteins. Locating the TAA on the target exosome not only increased the immune response to the native protein, but it also enhanced the immune tolerance to TAA. There is also the possibility that TAA-targeted exosomes can be used in viral diseases. Rountree et al. (2013) studied MVA-BN-PRO, which is an immunotherapy product for prostate cancer derived from a modified vaccinia Ankara (MVA) virus, known as "MVA-BN." By attaching the antigen targets to the exosomes, their immunogenic properties can be altered. To obtain exosome targets, we need to fuse the antigen with the C1C2 domain of lactoferrin proteins. Experiments used two different MVA-BN-PRO, targeting prostate-specific antigen (PSA, MVA-BN-PSA-C1C2) and prostatic acid phosphatase (PAP, MVA-BN-PAP-C1C2) exosomes. The treatment of mice with MVA-BN-PAP-C1C2 resulted in a substantial increase in the immune response; antibody titers were increased 10- to 100-fold compared with MVA-BN-PRO. The MVA-BN-PSA-C1C2 construct also increased the immunogenicity of the PSA. This research suggests that locating a target antigen on exosomes can greatly improve the immunogenicity of vaccines, improving the immune therapeutic potential of the disease caused by the virus.

Exosomes in Saliva

Exosomes contain RNAs, proteins, carbohydrates and lipids, and can act as messengers intercellularly. They are present in various bodily fluids. By extracting and analyzing exosomes, the early diagnosis of diseases may be

possible. This may mean that previous invasive methods will be used less, and new methods that are more economical, convenient, and efficient will be developed.

Studies have shown that most exosomes are present in plasma, while the number of studies on salivary exosomes is relatively small. Human saliva is a liquid medium containing proteins and minerals. It maintains oral integrity through lubrication, antibacterial and buffering properties, and promotes chewing and swallowing.

Most of the contents of exosomes in saliva resemble those in plasma. As early as 2005, exosomal secretion of RNPs from non-neoplastic salivary gland epithelial cells (SGECs) were detected (Kapsogeorgou et al. 2005). RNPs are immune antigens involved in systematic rheumatic diseases. Using electron microscopy, immunoblotting and immunoprecipitation, saliva has been shown to contain large amounts of exosomes, similar to other bodily fluids.

Salivary exosomes can be obtained relatively readily, which will be of great value if they can be used for the diagnosis of diseases. Salivary exosomes may not only be used for the diagnosis of oral and other diseases, but they also act as a way of observing the health status of the body. Lau et al. (2013) confirmed that biomarkers of tumor-derived exosomes provided a degree of contact between pancreatic cancer and the oral cavity. This provided an example of using a noninvasive method to diagnose a systemic disease. In that study, experimental mice suffering from pancreatic cancer were developed by implanting the pancreatic cancer cell line Panc02 into the pancreas. The inhibition of biogenesis and secretion of tumor-derived exosomes resulted in inhibited development of the discriminating disease-specific salivary biomarkers and showed that tumor-derived exosomes play a role in salivary tumor-specific biomarkers (Principe et al. 2013). Previous studies have confirmed that many salivary biomarkers are related to tumors and cancers. However, previous studies did not clearly explain why a cancer located far away from the oral cavity could affect salivary biomarkers. However, this study set up a model and examined the question. The interaction between tumor-derived exosomes changed the transcriptome of the salivary gland, thus changing the contents of the discriminating salivary biomarkers. The study also considered that salivary diagnosis was superior to blood diagnosis, because the concentration of macromolecules in blood is high and specific biomarkers may be hidden and difficult to find.

The use of salivary exosomes for diagnosis depends mostly on two macromolecular substances: proteins and RNAs. Human saliva contains many proteins, such as amylases, mucins, and blood group proteins (Al-Tarawneh et al. 2011). Proteomic studies of saliva exosomes began some time ago. Multiple studies have now shown that many proteins of exosomes show higher expression in disease states. For example, CD24 peptides are highly expressed in various diseases, such as SLE and hepatitis B, as well as in plasma, urine, and saliva. An SNP in which alanine (A) is substituted for valine (V) has been detected in an exosome RNA template for CD24.

Human whole saliva (WS) may contribute to the catabolism of biologically active proteins, thus playing an important role in oral local immune defenses. Exosomes in human saliva can help to heal the wounds in oral cavity by activating proteins (Brand et al. 2013). Ogawa et al. (2011) discovered that there are two kinds of exosomes in saliva, exosomes I and II. They differ in size and protein composition. Proteomic analyses indicated that both exosomes contained exosomal biomarkers, such as Alix, Tsg101, and Hsp7, and they both contained immunoglobulin A and polymeric immunoglobulin receptor. Dipeptidyl peptidase IV (DPP IV), also called CD26, was mostly found in exosome II and was metabolically active in cleaving chemokines (CXCL11 and CXCL12) (Ogawa et al. 2008). Thus, exosome II plays a role in degrading polypeptides. The researchers found that the average diameter of exosome I was 83.5 nm and that of exosome II was 40.5 nm, using electron microscopy, Western blot analysis, and two-dimensional polyacrylamide gel electrophoresis. Proteins expressed at higher levels in exosome II included DPP IV, carbonic anhydrase 6, cystatin family proteins, IgG Fc-binding protein, and galectin-3 binding protein. In contrast, ezrin, moesin, radixin, Rab GDP dissociation inhibitor beta, alpha enolase, guanine nucleotide-binding protein Gi/Gs/Gt subunit beta-1, and annexins were expressed only in exosome I. Alpha-amylase and proline-rich proteins were both abundant in WS, but none or only low amounts could be detected in exosome I or II.

The heterogeneous structure of salivary exosomes may indicate that exosomes derive from different parts of the salivary glands. For example, pIGR localizes mostly in acinar and ductal epithelial cells. However galectin-3 binding protein, which is detected in both I and II, is only located on the surface of ductal epithelial cells. The difference in the distributions of the salivary proteins may indicate different origins for exosomes I and II. Furthermore, both exosomes I and II contain large quantities of IgA, showing that they are likely both involved in immune responses. However, when implanting exosomes I and II into different mice, researchers observed that the mice implanted with exosome II generated antibodies to DPP IV more successfully, demonstrating that exosome II had better immunogenicity. This research was the first reported proteomics study in human WS that showed exosomes I and II had different proteomic properties.

Similar to proteins, exosomal salivary RNAs show high potential in disease diagnosis. Most RNAs in exosomes are small RNAs, comprising mostly miRNAs. RNAs are more

Table 1 Isolation and identification of exosomal biomarkers

Year	Samples	Primary isolation and validation methods	Biomarkers	References
2013	Serum of healthy people	1. Differential ultracentrifugation 2. ExoQuick precipitation methods	375 miRNAs	Rekker et al. (2014)
2013	Urine of healthy people	1. Nanoparticle tracking analysis (NTA) 2. Ultracentrifugation	CD24 and aquaporin 2(AQP2)	Oosthuyzen et al. (2013)
2013	Urine of people suffering from kidney disease	1. Differential centrifugation 2.Immunoelectron microscopy	CD2AP mRNA	Lv et al. (2014)
2013	Cultured human proximal tubular cells and human urine	1.Serial centrifugation 2. Transmission electron microscopy 3. Western blot 4. Flow cytometry	Osteoprotegerin (OPG)	Benito-Martin et al. (2013)
2013	HeLa cell culture media and human serum	1.Total exosome isolation(from serum) reagent (Invitrogen) 2. Total exosome isolation(from cell cultire media) reagent (Invitrogen) 3. Nanoparticle tracking analysis 4. Western blot	miRNAs, mRNAs, rRNAs, tRNAs, and other RNA types	Schageman et al. (2013)
2013	Hela and HT180 human fibosarcoma cells	1. Differential centifugation 2. Micro-filtration 3. Atomic force microscopy(AFM)	siRNAs	Shtam et al. (2013)
2012	Human metastatic mammary gland epithelial adenocarcinoma cell line MDA-MB-231 cells(231) and human submandibular gland (HSG) cells	1. Ultracentrifugation and micro-filtration 2. Electron microscopy 3. SDS-PAGE and protein staining 4. Western blot	Proteins like amylase proteins and mRNAs	Lau and Wong (2012)
2012	Serum of the healthy people	1. Repeated centrifugation and filtration steps 2. Electron microscopy 3. Flow cytometry 4. Western blot	CD63, 18 s rRNA and 28 s rRNA	Lässer et al. (2012)
2011	Normal and oral cancer patient's saliva	1. Ultracentrifugation 2. High-resolution AFM 3. Single molecule force spectroscopy	CD63	Sharma et al. (2011)
2009	Human parotid saliva	1. Serial centrifugation 2. Immunoelectron microscopy 3. Gel electrophoresis 4. Western blot	491 proteins	Gonzalez-Begne et al. (2009)

prone to decompose in saliva and serum, versus miRNAs, which have specificity in many cancers and other diseases and even in some non-disease conditions, such as pregnancy. One reason why miRNAs may be useful biomarkers is that they remain in exosomes and are protected by them. Specific exosomal miRNAs are present in the saliva of patients with ovarian and lung cancer. Thus, tumor-associated exosome miRNA may be a powerful biomarker (Gallo et al. 2012). What's more, through the detection of the male-specific zinc finger gene contained in exosomes, we can even identify the sex of a fetus (Keller et al. 2011). On the other hand, because of the ready decomposition of small interfering RNAs and recombinant proteins in biological medicines, further studies should focus on the transport systems by which drugs are precisely targeted to the cell. The use of extracellular exosomes to transport biological drugs may be promising and effective. However, the target of the system could be easily missed. Due to the low utilization rate of the contents in exosomes, their substitution, using synthetic vesicles, has been proposed. Functional synthetic exosomes will result from assembly of the key contents of exosomes and the phospholipid membrane. Such a system is not complex and has desirable characteristics, which may improve the acceptance of drugs (Kooijmans et al. 2012).

In 2013, Ogawa et al. (2013) studied the small RNA transcriptomes of the two kinds of exosomes found in human saliva using next-generation sequencing technology. Because of rapid developments in next-generation sequencing, we can analyze large numbers of RNAs to determine gene annotations, including those of protein-coding genes. This was the first study of salivary exosome RNAs conducted using next-generation sequencing technology. Through such technology, we can incorporate the conclusions of previous studies. Exosomes I, exosome II, and WS express different miRNAs and most of the miRNAs expressed in WS are not expressed in exosomes. In the exosome, GW182, a component of the RNA-induced silencing complex, shows great significance in the stability of exosomal RNAs. Furthermore, hsa-mir-378 in exosome I, exosome II, and WS is associated with cell survival, tumor growth, and angiopoiesis. Salivary exosomes also contain other kinds of small RNAs, such as piRNAs and snoRNAs, the functions of which need further study.

Isolation and Identification of Exosomes

The isolation and identification of exosomes from biological materials has been difficult, because their isolations are easily contaminated with non-exosomal proteins. The cytomembrane can discharge many kinds of subcellular bodies, not all of which are exosomes. The use of transmission electron microscopy can identify specific exosome. Researchers further examined exosomal structures, chemistry, and mechanics using the new ultrasensitive technique of low-force atomic force microscopy (Raimondo et al. 2011). When conducting analysis of exosomal proteomes, the identification of specific exosomal proteins is very important. Furthermore, as studies of exosomes continue, there is a need for new isolation methods to be developed. Wei et al. (2013) at UCLA established a new way to isolate exosomes, detecting exosomal biomarkers using "electric field-induced release and measurement" (EFIRM). Researchers have also used new ways to disrupt exosomes to secrete their contents while detecting exosomal RNA and proteins. When exposed to an irregular electric field, the rapid release of exosomal RNAs and proteins results in rapid detection of those molecules. The technique is fast and simple and can quantify molecules found in exosomes. Researchers used a lung cancer cell line to detect H460 and hDC63-gfp. Thus, hDC63-gfp appears to be expressed in saliva and serum. This was the first time that exosomes shed from a tumor in another organ were discovered in saliva, and it introduced the new idea of detecting salivary biomarkers. However, existing methods of isolating exosomes need to be further optimized and the stabilization of exosomes needs to be improved. Kalra et al. (2013) compared three methods (differential centrifugation coupled with ultracentrifugation, epithelial cell adhesion molecule immunoaffinity pull-down, and OptiPrep density gradient separation) for extracting exosomes from plasma. Through validation using MS, microscopy, and Western blots, the study concluded that the OptiPrep density gradient separation was better than the two other methods due to less plasma protein contamination. Extracted exosomes can be stabilized for 90 days. However, scientists still need to develop and use other techniques for studying exosomes, allowing the possibility of discovering many more exosomal biomarkers and using them to diagnose other diseases (Table 1).

In summary, exosome researches may play important roles in many fields. Thanks to the three scientists who provided our understanding of the mechanisms of exosomal transport, the prospect of developing more exosomal biomarkers is quite promising. What's more, challenges still exists in the isolation and validation of exosome markers. Despite this, it seems certain that the ongoing rapid development of technologies and research will inevitably result in the accumulation of further useful biomarker data.

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Conflict of interest The authors declared that they have no competing interests.

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