

The Role and Application of Salivary Exosomes in Malignant Neoplasms

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Abstract: The study of salivary exosomes in malignant neoplasms has attracted widespread attention in the clinical setting. Although a variety of diagnostic and treatment approaches have been proposed, there are some limitations to their application. In recent years, the role of salivary exosomes in cancer has been increasingly studied. Salivary exosomes not only renew and regulate the biological behavior of tumor cells, such as malignant proliferation, migration, and invasion, but they also serve as ideal markers for early diagnosis of diseases and may represent an effective therapeutic target. This article reviews the current research on salivary exosomes in malignant neoplasms.

Keywords: liquid biopsy, exosome, malignant neoplasm, diagnosis, treatment

Introduction

An epidemiological survey of malignancies estimated that about 14 million new cancer cases worldwide were diagnosed in 2012 and this number will rise to 24 million by 2035.¹ Of these, the number of new cancer cases in the elderly is expected to double, which may increase the global economic burden of cancer in terms of the demand for clinical services. At present, the early diagnosis of malignant tumors is difficult, and common treatment mainly includes surgery, chemotherapy, radiotherapy, and targeted therapy, however, the impact of treatment on outcomes is still not ideal. Further, treatment may also damage normal tissues and lead to serious complications. Therefore, finding new and effective approaches for diagnosis and treatment and exploring the mechanisms that affect the occurrence and development of tumors have always been the focus of ongoing research.

Saliva is a mixture of water, electrolytes, enzymes, and other elements.² Molecules in the saliva can be exchanged with blood through capillaries in the glands, hence, they share similar bioactive substances.³ In addition, as a sampling material, saliva has a widely accessible and is easy to collect. Compared with clinical diagnoses using other body fluids, such as cerebrospinal fluid, thoracic effusion, and blood, sampling of saliva has the advantages of being safe, non-invasive, of low cost, and convenient.

Exosomes are nanosomes with a diameter of about 50 to 140 nm that are actively secreted by a variety of cells (macrophages, tumor cells, and mast cells) in the body, with an outer shell composed of a lipid bilayer structure.⁴ Exosomes were first identified by Johnstone et al⁵ in the reticulocytes of mammals, and were initially only regarded as “waste” at that time; hence, they were not given much attention. Recent studies have found that exosomes are spherical, their cores are

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composed of hydrophilic structures, and vesicles are rich in proteins, lipids, and genetic material.⁶ Exosomes are widely present in bodily fluids such as saliva, blood, cerebrospinal fluid, and urine, and play a role in cell communication or as mediators of cell communication through the release of bioactive substances contained in their vesicles.^{7–9}

Studies have confirmed that bioactive substances in salivary exosomes are involved in physiological or pathological processes of the body,³ and are closely related to head and neck malignancies such as laryngeal, hypopharyngeal, thyroid, oral, nasopharyngeal cancers, and many other systemic tumors. It is relatively easy to isolate salivary exosomes. Compared with other body fluids, salivary exosomes have unique advantages, high stability, and strong tissue targeting capabilities. A close relationship between exosomes and malignancies has been demonstrated, allowing them to regulate tumor growth, invasion, and metastasis, and to monitor the prognosis of malignancies. Due to their structural characteristics, salivary exosomes present their own structural characteristics, which are not easily degraded by enzymes in the body. When transmitting messages, the integrity and biological function of vesicles are maintained, which is conducive to their extraction as experimental samples and detection of diseases.¹⁰ Studies have shown that the majority of RNA in salivary exosomes are microRNA (miRNA), whose content and structure vary in tumor patients. Therefore, salivary exosomes can also be used as a tool for early diagnosis of tumors.¹¹ In addition, salivary exosomes can be used as carriers of anti-tumor drugs, and may play a role in the communication between tumor cells to reshape the tumor microenvironment, thus becoming an effective target for tumor therapy. Fu et al¹² extracted human T cells and evaluated their activity on tumors *in vitro* and *in vivo* in nude mice. The authors found that T cells expressing chimeric antigen receptor (CAR-T) secreted surface exosomes carrying CAR that could bind tumor cells, inhibit tumor growth, and kill tumors. Exosomes with reversible fusion and fission capabilities have been developed to control the delivery of drug-carrying exosomes during Fe²⁺ and Fe³⁺ interredox processes.¹³ Engineered exosomes can be re-programmed for the release of exosomal-targeted drugs under photothermal induction, suggesting that exosomes can be used for precision oncology therapy.¹⁴ The study of salivary exosomes and their association with malignant tumors have recently attracted

much attention, and the findings provide broad prospects for noninvasive detection of tumors.

Overview of Salivary Exosomes

Composition of Salivary Exosomes

Salivary exosomes can be secreted by salivary glands or blood. They are round vesicles in shape and contain shared proteins, such as heat shock protein 70 (HSP70) and tetraaminohydrin. These proteins, which can also be found in exosomes from other sources, are closely related to calcium transport, signal transduction, protein processing, and transportation, and also contain specific proteins, such as AQP5 or aquaporin 5, involved in salivary water regulation.^{10,15} Some proteins co-exist in salivary exosomes and saliva, and are highly expressed in the former, indicating that these proteins may originate from salivary exosomes.³ In addition to proteins, salivary exosomes also contain bioactive molecules such as mRNA, miRNA and cytokines. Studies have shown that mouse proteins can be expressed in human mast cells by adding mouse mast cell exosomes to human mast cells, and mast cell-derived exosomes can exchange RNA through exosomes. Exosome RNAs that can travel between cells are called “esRNAs”, and may involve miRNAs, a class of non-coding mRNAs with sizes that vary from 19 to 25 nucleotides in length and that mediate cell-to-cell communication in the microenvironment, as well as play an important role in tumor diagnosis.^{16–18}

Separation of Salivary Exosomes

Based on chemical and physical properties, a variety of separation methods can be used to isolate salivary exosomes, including membrane affinity ultracentrifugation, tangential flow filtration, field flow fractionation, sucrose gradient centrifugation, ExoQuick extraction, and size exclusion chromatography. To separate components of saliva secreted by the body there are two types of separation methods. The first is a form of ultracentrifugation and the other involves chemical precipitation.¹⁹ Both methods present some limitations: the ultracentrifugation method is simple to perform, but is time consuming and produces exosomes of low purity. Sedimentation can obtain complete exosomes and increases their recovery, but it is only suitable for specific scenarios.^{20,21} Due to the fact that exosomes are easily damaged and contaminated, and viscous substances are obtained following ultracentrifugation, the separation of salivary exosomes is still difficult to

achieve at present. A newly developed ultra-fast separation and purification system (EXODUS) presents the advantages of low cost, high stability, and reproducibility, and improves the purity, efficiency, and yield of exosomes. This improved exosome separation technology will allow to discover more exosomal diagnostic disease markers, to be exploited for early disease screening.²²

Function of Salivary Exosomes

Studies have shown that salivary exosomes contain tissue factor (TF) and participate in the initial stages of coagulation in the body, shorten the coagulation time, and play a role in hemostasis.²³ Minor variations in salivary exosome content are diagnostic for a variety of diseases, such as autoimmune disease,²⁴ periodontitis,²⁵ and oral lichen planus.²⁶ In tumors, molecules in saliva can be exchanged with blood through capillaries in glands, and about 80% of proteins coexist with exosomes in saliva and serum.

Studies have shown that exosomes can be used as diagnostic markers in multiple tumors.^{27,28} Salivary exosomes are rich in angiogenic proteins that deliver growth factors and stimulate endothelial cells, thereby promoting angiogenesis in tumor tissues.²⁹ In addition, salivary exosomes can adapt to and participate in host humoral immunity, enhance immune resistance of tumor cells, evade the immune surveillance of NK cells, and promote tumor immune escape.^{21,22} Salivary exosomes also have the ability to deliver P-glycoprotein (P-gp), which mediates mechanisms of drug-resistant metastasis, and enhances tumor resistance.³⁰ Thus, salivary exosomes create favorable conditions for tumor migration and invasion.^{31,32} At present, salivary exosomes can also assist in the delivery of anti-tumor drugs or interfere with tumor growth to exert their an anti-tumor role.³³

Role of Salivary Exosomes in Diagnosis and Prognosis Evaluation of Malignant Tumors

Salivary exosomes are suitable for tumor fluid biopsies, as they are rich in a variety of active substances and can be used for early diagnosis and prognosis evaluation of tumors.³⁴ Salivary exosomes containing miRNA and circulating tumor genes (ctDNA) can be used as biomarkers for cancer screening.³⁵ A head and neck squamous cell carcinoma (HNSCC) study found that using ctDNA as a marker extracted from oral saliva, the early tumor positive diagnostic rate was 100%, which was significantly higher

than blood tests, and also presents higher sensitivity for diagnosis of cancers of the distal oral cavity (oropharynx, larynx, and hypopharynx).³⁶ Studies including 5 groups of HNSCC and healthy controls showed that miR-486-5p or miR-486-3p and miR-10b-5p were significantly upregulated in patients with HNSCC, and miR-486-5p was also significantly altered in early lesions (stage I) and thus could be applied as a biomarker for early diagnosis of HNSCC.³⁷

Liu et al compared salivary exosomes of oral cancer patients and healthy controls by quantitative real-time polymerase chain reaction (qRT-PCR), and found that the increased expression of miR-31 could promote higher expression of miR-29a-3p mediated by exosomes by regulating the SOCS1/STAT6 signaling pathway in macrophages. Further, the expression of miR-125a and miR-200a was decreased, and a reduction of miRNA was observed after tumor resection, which can be used to monitor tumor prognosis.^{38,39}

Lin et al identified higher levels of GOLM1-NAA35 RNA, a chimeric RNA (seG-NchiRNA) secreted by salivary exosomes in mice and humans in early- and advanced-stage esophageal squamous cell carcinomas (ESCC) compared with the normal control group.⁴⁰ Nano-particle tracking analysis showed that the level of seG-NchiRNA in ESCC patients was 2.1 times higher than that in the normal control group, and the changes in the level of seG-NchiRNA were correlated with changes before and after ESCC surgery, which provide a scientific and effective rationale for monitoring the prognosis of ESCC patients.

Zhang et al⁴¹ found that the changes in the expression of various mRNAs and carbonic anhydrase 6 (CA6) in salivary exosomes of breast cancer patients were more marked than those in healthy controls, which could be used for the early diagnosis of breast cancer. Lau et al established an in vitro model of breast cancer and found through microarray analysis that exosomes derived from breast cancer cells could process the transcription of salivary exosomes, thereby changing the content of the salivary exosome.⁴²

In a mouse model, exosomes secreted by pancreatic cancer cells were able to regulate the transcription of genes in the saliva, and further, pancreatic cancer-specific transcriptional genes were identified as having increased expression in salivary exosomes, indicating the presence of markers of pancreatic cancer cells in salivary exosomes.⁴³

Sun et al used quantitative methods to compare the salivary exosome proteomics profile of normal subjects and patients with lung cancer, and confirmed that four proteins (BPIFA1, CRNN, MUC5B, and IQGAP) derived from lung tissue cells and could be used for non-invasive detection of lung cancer, and further, approximately 80% of the proteins in salivary exosomes and serum exosomes were shared. These findings indicated bi-directional communication of information and correlation of composition between salivary exosomes and distal tumors.^{44,45} To further confirm this relationship, a tumor transplantation model of nude lung cancer mice was established. H460 lung cancer cells were engineered to stably express HCD63-GFP, and were then injected into the thoracic cavity of nude mice. Human GAPDH mRNA was detected in the HCD63+ saliva exosomes of experimental nude mice. These results indicated that specific mRNAs in salivary exosomes could be used to identify tumors and have the potential to be biomarkers for tumor diagnosis.¹⁵

Role of Salivary Exosomes in the Treatment of Malignant Tumors

Due to its own structural characteristics, exosomes become “cargo” nanocarriers with unique advantages, including the inclusion and protection of its specific contents, low rejection rates, and drug resistance, permeability of the blood-brain barrier, high efficiency of drug delivery, and maintenance of drug activity. Therefore, exosomes have the potential for wide application in disease treatment.⁴⁶ Paclitaxel (PTX) presents unique anti-tumor activity by inhibiting cell microtubule mitosis. Exosome inclusion of PTX can improve drug stability and blood drug concentration, and enhances the inhibitory effects on cancer cell proliferation.^{47,48} Ma et al found that exosomes secreted by breast ductal cancer cells carrying antitumor drugs such as methotrexate can help inhibit reverse the proliferative activity of CD133+ tumor stem cells, indicating that exosomes can enhance the efficacy of antitumor drugs and kill tumor cells.⁴⁹ Exosomes carrying antioxidant drugs can reach neuron cells through intranasal administration and provide a protective effect on neuron activity.⁵⁰ Exosomes carrying IFN- γ can increase the secretion of IFN- γ by NK cells, which has a potential role in the treatment of tumor cells.⁵¹

Salivary exosomes can also be used as targets for tumor therapy. Baroni et al found that salivary exosomes carrying miR-9 are significantly increased in oral squamous cell carcinoma (OSCC) and could promote tumor

growth by inducing fibroblast-like characteristics of breast cancer cells, while the introduction of LNA-9, an inhibitor of miR-9, could reduce tumor metastasis and invasion.^{52,53}

Li et al demonstrated that $\gamma\delta$ T cell extracellular vesicles ($\gamma\delta$ T DES) rich in miR-138 can increase the number of G1 stage tumor cells in nude mice, inhibit the biological activity of CAL-27 cells, slow down the growth rate, and inhibit tumor formation.⁵⁴ In addition, knockdown of miR-133b-3p in saliva exosomes reduces the expansion of epithelial progenitor cells, providing a new rationale for tumor therapy.⁵⁵

Studies have shown that exosomes released by cancer cells can inhibit the activation and proliferation of CD8+T cells, promote the regulation of T cells, and play an immunosuppressive role. Immunotherapy of exosomes plays an important role in human tumors, and lays the foundation for the research and development of tumor immunotherapy vaccines.^{56,57}

Mechanism of Action of Salivary Exosomes in Malignant Tumors Promotion of Tumor Progression

Tumorigenesis is a dynamic and complex process.⁵⁸ Salivary exosomes can influence the progression of tumor cells through signaling molecules, and are closely associated with multiple biological processes of malignant tumors, thereby mediating the malignant progression of tumor cells.⁵⁹

Salivary Exosomes and Tumor Proliferation

About 90% of head and neck malignancies are HNSCC, which are common systemic malignancies. Oral cancer is a common subtype of head and neck malignancies and is increasingly affecting younger individuals in recent years, likely due to an association with viral infections and personal lifestyle habits.⁶⁰ Distant metastasis may occur early in the disease course and the prognosis is poor. When most patients are diagnosed clinically, they have already developed to the advanced stage and have missed the optimal treatment time. Therefore, it is extremely important to explore early and efficient detection methods. Gai et al⁶¹ showed that salivary extracellular vesicle-associated miRNA can affect OSCC, mainly through miRNA ErbB signaling pathways, while targeted regulation of CD44 can promote tumor cell proliferation and invasion. In addition, miR-512-3p and miR-412-3p can target multiple genes in the TGF β signaling pathway (for example,

TGF β R2) to decrease the regulation ability of suppressor genes. The specific upregulation of miR-302b-3p and miR-517b-3p expression was confirmed by qRT-PCR in OSCC patients. Hypoxia-induced OSCC cell-derived exosomes stimulate miR-21 expression by inducing HIF-1 α and HIF-2 α , thereby promoting tumor growth.⁶² He et al⁶³ up-regulated levels of salivary exosome miR-24-3p, which then directly acted on the transcription initiation of target genes in cancer cells, and thereby inhibited the expression of PER1 and improved the growth rate and colony-formation efficiency of OSCC cells. Wang et al⁶⁴ extracted exosomes from salivary glands derived from adenoid cystic cancer cells using a commercial kit. These extracted exosomes were found to enhance tumor cell invasion and metastasis by regulating kinase ERK1/2 phosphorylation, the MAPK signaling pathway, and promoted fibroblast morphogenesis through the NGF-NTRK1 signaling pathway, thus promoting tumorigenicity. Zhang et al⁶⁵ found that the salivary exosome miR-512-3p in patients with TSCC promoted the proliferation of tumor cells by down-regulating GAS2. Salivary exosomes could also reduce the cytotoxicity of NK cells by down-regulating the expression of CD69 and NKG2D, which resulted in an immune escape mechanism, which promoted tumor proliferation.⁶⁶

Salivary Exosomes and Tumor Metastasis

Chen et al⁶⁷ identified in Epstein-Barr virus-positive nasopharyngeal carcinoma that exosomes could induce lymphatic ducts and tube formation, thereby promoting nasopharyngeal lymphatic metastasis. Latent membrane protein 1 (LMP1) was identified in the saliva of exosomes.⁶⁸ LMP1 protein can up-regulate the expression of NTRK2 through NF- κ B to activate Akt and ERK signal transduction and enhance the hypoxic tolerance and metastasis of tumor cells.⁶⁹ EB-LMP1 can also activate the IRF-7 signaling pathway, increase the expression of A3S, cause mtDNA hypermutation, and alter the expression of miRNA, and ultimately promotes tumor cell invasion and distant metastasis.⁷⁰

Tumor Inhibition

Cancer-derived inhibitory salivary exosomes exert an anti-tumor role by influencing angiogenesis and regulating stem cell properties. Gonzalez-Begne et al identified angiopoietin-like protein 1 (ANGPTL1) in salivary exosomes, which is widely present in human

tissues, and found that it regulates miR-630 promoter activity and inhibits zinc finger protein (SLUG) expression by down-regulating the ERK/SP1 signaling pathway. SLUG is overexpressed in a variety of malignant tumors and is related to the stem cell properties and drug resistance of tumor cells, and thus has an inhibitory effect on the metastasis, proliferation, and progression of cancer.⁷¹⁻⁷³ Studies have shown that miRNA16, an carried by exosome secreted by mesenchymal stem cells from mice, reduced the expression of vascular endothelial growth factor (VEGF) in T1 cells in a dose-dependent manner, reduced tumor proliferation and migration, and altered tumor progression.⁷⁴ VPS4A is tumor suppressor that mediates miRNAs, such as miR-122-5p, miR-29b-3p secretion, via exosomes derived from liver cancer cells. Through the PI3K/Akt signaling pathway in liver cancer cells, VPS4A regulates the uptake of tumor exosome miRNAs, thereby inhibiting tumor progression.⁷⁵

Bioactive substances of salivary exosomes play different roles in different malignant tumors. The bioactive substances and the mechanism of salivary exosomes in different types of malignant tumors are shown in [Table 1](#).

Summary and Prospects

As a non-invasive material, salivary exosomes have the advantages of relatively stable sampling and convenient storage, which are helpful for the diagnosis and prognosis of a variety of tumors, and thus, represent a new direction in the diagnosis and treatment of malignant tumors. Several studies have examined the role of exosomes in diagnosis, treatment and mechanisms underlying of salivary exosome-mediated metastasis in malignant tumors. However, the mechanisms involved in the regulation of salivary exocrine exosomes by the body are currently not clear; thus, exploring separation technologies, improving precision medical treatment by understanding the properties of saliva secreted by body and regulatory mechanism controlling malignant tumors should be further clarified. Salivary exosomal carriers of antitumor drugs can be engineered for clinical use, as their immunosurveillance capabilities and potential application for immunotherapy treatment show great potential. Extracting salivary exosomes and designing engineering exosomes for clinical application in precise tumor treatment will represent the research direction in the future.

Table I Mechanism of Action of Salivary Exosomes in Malignant Tumors

Disease	Bioactive Substance	Pathways	Effect	Reference
OSCC	miR-512-3p	ErbB and CD44	Proliferation, differentiation	[61]
	miR-21	TGFβ	Proliferation, differentiation	[61]
	miR-24-3P	HIF-1α and HIF-2α	Proliferation, differentiation	[62]
		PER1	Proliferation, differentiation	[63]
HNSCC	miR-486-3p, miR-10b-5p	KEGG	Proliferation, differentiation	[37]
The oral cavity	miRNA31	SOCS1/STAT6	Proliferation, differentiation	[38]
Salivary glands adenoid cystic cancer	mRNA	ERK1/2, MAPK and NGF-NTRK1	Proliferation, differentiation	[64]
TSCC	miR-512-3p	Gas2	Proliferation, differentiation	[65]
Pancreatic cancer	mRNA	NK cells	Transfer, metastasis	[66]
NPC	LMPI	NTRK, 2Akt and ERK	Transfer, metastasis	[69]
		IRF-7	Transfer, metastasis	[70]
Breast cancer	miR-1246	CAVI/PDGFRβ	Transfer, metastasis	[76]
Colorectal cancer	ANGPTL 1	ERK/SPI	Inhibition	[73]
Stomach cancer	miR-122-5p	DUSP4 MMP2 and MMP9	Inhibition	[77]
Melanoma	miR-33a-5p	Snai2	Inhibition	[78]

Abbreviations: OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; TSCC, tongue squamous cell carcinoma; NPC, nasopharyngeal carcinoma.

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Disclosure

The authors report no conflicts of interest in this work.

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