Review

Secretory MicroRNAs by Exosomes as a Versatile Communication Tool

Takahiro OCHIYA

Division of Molecular and Cellular Medicine, National Cancer Center Research Institute
5–1–1 Tsukiji, Chuo-ku, Tokyo, 104–0045 Japan

Abstract: In the past several years, the importance of microRNA (miRNA) in cancer cells has been recognized. Proper control of miRNA expression is essential for maintaining a steady state of the cellular machinery. Dysregulation of miRNAs leads to the cancer development, meaning that expression profile of miRNAs can be used as cancer biomarker, and recovery of down-regulated miRNAs or inhibition of up-regulated miRNAs will be a novel approach for cancer therapy. Recently, it was discovered that extracellular miRNAs circulate in the blood of both healthy and diseased patients. Most of the circulating miRNAs are included in protein, lipid or lipoprotein complexes, such as RNA-binding proteins, apoptotic bodies, microvesicles, or exosomes, and are, therefore, highly stable. The existence of circulating miRNAs in the blood of cancer patients has raised the possibility that miRNAs may serve as a novel diagnostic marker. However, the secretory mechanism and biological function, as well as the meaning of the existence of extracellular miRNAs, remain largely unclear. Our recent study revealed the secretory mechanism of miRNAs and showed their cell-to-cell transfer. Here we summarize current approaches to modulate the intercellular and interindividual network via silencing signals exported by secretory miRNAs and discuss about the usage of circulating miRNAs as a novel communication tool.

Key words: microRNA, exosome, diagnosis, micro-environment, communication.

Finding of secreting microRNAs in body fluids

Growing evidence suggests that extracellular microRNAs (miRNAs) stably exist in human body fluids, including plasma, saliva, and urine, although ribonucleases (RNases) also circulate throughout the body.[1)] This finding indicates that miRNAs are excreted after they are contained in RNase-resistant lipid vesicles, such as exosomes and apoptotic bodies. Recent studies have revealed the novel genetic exchange between cells using miRNA either in microvesicles (up to 1 μm) or in small membrane vesicles of endocytic origin called exosomes (50～100 nm).[2～11)] One of the first reports showing the existence of miRNA in exosomes was studied by Valadi et al., who reported that exosomes released from human and murine mast cell lines contain mRNAs and miRNAs.[11)] Hunter et al. demonstrated that miRNAs contained in the microvesicles from blood were known to regulate the cellular differentiation of blood cells and metabolic pathways and to modulate immune function.[12)] Apart from microvesicles and exosomes, more recent evidence has suggested that circulating microRNAs found in sera of the human are coupled with some specific proteins and lipoproteins. However, very little is known about the secretory machinery of miRNAs.

Oral cancer miRNAs in saliva

Considering that exosomes and microvesicles are evident in several types of body fluid from cancer patients, miRNA surely be able to be found not only in serum/plasma but also in other body fluid. Indeed, Michael et al. showed the presence of miRNAs within exosomes isolated from human saliva.[13)] Furthermore, analyzing patient saliva with a polymerase chain reaction (PCR) technique, Park et al. found that miR-125a and miR-200a were present in significantly lower levels

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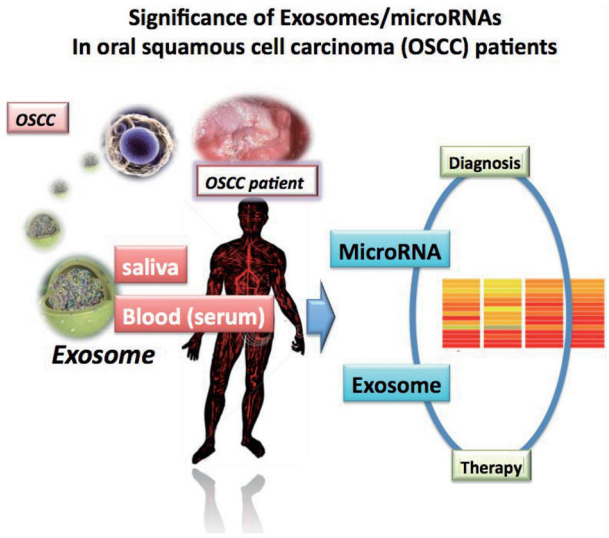

Fig. 1 Significance of Exosomes/microRNAs in oral squamous cell carcinoma (OSCC) patients. Circulating microRNAs in OSCC patients-exosomes (saliva and serum) are useful for a novel diagnosis and therapeutics against OSCC.

in the saliva of oral squamous cell carcinoma (OSCC) patients than in control subjects.[14] These findings suggest that the detection of miRNAs in saliva can be used as a noninvasive and rapid diagnostic tool for the diagnosis of oral cancer (Fig. 1). OSCC is the sixth most common cancer in the U.S., accounting for 90% of oral cancers and leading to 8,000 deaths per year. The average five-year survival rate for OSCC is about 50%, and this number has not changed in last three decades. Therefore, an early detection method for OSCC is needed to increase long-term patient survival. In this connection, finding of OSCC specific miRNAs highlighted a novel means of early cancer detection, and that the presence of salivary miRNA adds a third type of molecule, in addition to proteome and transcriptome, that can be measured in human saliva.

Exosomes in saliva

As human plasma, saliva contain exosomal particles. Saliva from healthy human donors was collected in tubes. For the RNA isolation, 100 μl of the protease inhibitor and RNase inhibitor were added per 20 ml of saliva. The saliva was diluted 1:1 with phosphate buffered saline (PBS) and centrifuged at 20,000×g for 20 min to remove cells and cell debris. The supernatant was filtered through a 0.2 μm filtration system and then ultracentrifugation at 100,000×g for 70 min to pellet the exosomes. By electron microscopic analysis, salivary exosomes look like round shape with 120～150 nm in a diameter. It is reported that exosomes from saliva can be taken up by human macrophages, as shown by the uptake of fluorescently stained exosomes.[15] It has been shown that other cells can take up exosomes in a similar way to macrophages, which means that this is a common feature of exosomes. It has not been determined the cellular origin of saliva exosomes, but it has been shown that primary cultures of salivary glands can release exosomes-like particles suggesting that exosomes in saliva are partly derived from salivary gland epithelial cells.

Molecular mechanisms of secreting miRNAs and exosomes

Our group demonstrated that the secretion of miRNAs depends on the cellular amount of ceramide, a bioactive sphingolipid, whose synthesis is tightly regulated by neutral sphingomyelinase 2 (nSMase2).[16] Treatment of a chemical compound, GW4869, which can inhibit the enzymatic activity of nSMase2, markedly blocked the secretion of miRNAs and exosomes. These data suggest that miRNAs are secreted by an exosome-dependent pathway that involves ceramide biosynthesis. The molecular dissection of miRNA secretory mechanisms is likely to assist in a better understanding why the expression of secretory miRNAs is perturbed during the development of various diseases, including cancer, diabetes, and immune disorders.[17~19] This finding may be beneficial to confer reliability and credibility to the diagnostic use of secretory miRNAs.

Intracellular communication by exosomes

As evidenced by many reports,[20~24] small RNAs are currently regarded as a category of intercellular signal entities, which are called, for instance, mobile small RNAs, systemic silencing signals, or just secreted RNAs. To answer the question as to whether

the secretory exosomal miRNAs that we observed can function biologically in a similar manner to other members, we set up *in vitro* and *in vivo* experiments pertaining to intercellular transfer. Purified exosomes labeled with a green fluorescent reagent PKH67 were successfully incorporated into recipient PC-3 cells. We also detected the migration of secretory nucleic acids into PC-3 cells by using SYTO dye, a specific probe for vital DNAs and RNAs including mRNAs and miRNAs. Furthermore, we reported that secretory miR-146 inducing a phenotypic change in the incorporated cells. It is generally acknowlkedged that normal epithelial cells regulate the secretion of autocrine and paracrine factors that prevent aberrant growth of neighboring cells, leading to healthy development and normal metabolism. One reason for tumor initiation is considered to be a failure of this homeostatic cell competitive system. Kosaka et al. identify tumor-suppressive miRNAs secreted by normal cells as anti-proliferative signal entities.[25] Among these miRNAs, secretory miR-143 could induce growth inhibition exclusively in cancer cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs can act as a death signal in a cell competitive process. Taken together, the findings indicate that exosomal secretory miRNAs can spread translation-inhibitory signals, leading to the elicitation of a wide array of biological events.

Secretory miRNAs could be an interindividual communication tool

The relevance of miRNA transfer cannot be limited within an individual organism. Kosaka et al. found that the expression of immune-related miRNAs in human breast milk culminates in the first 6 months of lactation, in agreement with the significance of colostrum in the context of passive immunity.[26] This paper appears to suggest the two important concepts such as dietary intake of miRNAs and vertical transfer of miRNAs. If the miRNAs intake by daily food maintain their biological activity after digestion, they will be highly valued as a crucial ingredient that can modulate gene expression of our own cells. In fact, current reports suggested that plant (rice origin) miRNAs could found in the circulating blood.[27] The microRNAs seem to come from ingested rice. Presumably the micoRNAs are taken up in the intestine and secreted into the blood in small vesicles, since microRNAs packaged into exosomes are highly stable in low pH such as stomach acid. Based on the second concept, genetic exchanges between mother and child would be mediated by milk-exosomes as well as amniotic fluids. The excretions from the body, such as tears, saliva, semen, and vaginal discharge, also include secretory miRNAs, suggesting that these fluids may act as a mediator of horizontal genetic materials transfer. It is within the bounds of probability that secretory miRNAs could be an interindividual communication tool among humans.

Perspective

The existence of circulating miRNAs and microvesicles in the blood of cancer patients has raised the possibility that disease-specific miRNAs and exosomes may serve as a novel diagnostic marker. Moreover, exosomes secreted from tumor cells contribute maicro-managing tumor microenvironment. Intracellular communications via microRNAs and microvesicles including exosomes are important novel therapeutic targets for inhibiting tumor development and metastasis including oral cancer.

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