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Exosomal long non-coding RNAs as biomarkers in human diseases

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ABSTRACT

The intensive study of extracellular vesicles was started about a decade ago revealing alterations of their amount and content to several cellular stimuli, highly depending on the releasing cell type. Exosomes, a type of extracellular vesicles, are released by every cell type and are present in most body fluids, what makes them attractive targets of biomarker research. Several studies have indicated that their content – including proteins and coding, as well as non-coding nucleic acids – could represent the disease state and serves as specific disease biomarkers. Out of these molecules, a special interest was gained by long non-coding RNAs (lncRNAs). Just as exosomes, lncRNAs are specific to their cell of origin and often specific to diseases, also found extracellularly, mainly contained in extracellular vesicles. Thus, recent efforts in biomarker research has turned to circulating exosomal lncRNAs, which might lead to the development of highly specific disease markers.

Here we summarize the current knowledge on disease-associated exosomal long non-coding RNAs. The

intensive studies in this area have revealed numerous potential targets for biomarkers, and highlighted the potential of their combination with other exosomal markers to represent a highly sensitive and specific diagnostic tool. However, we believe that additional functional data on both exosomes and lncRNAs are necessary for understanding their deregulation in diseases and developing their use as diagnostic approaches.

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INTRODUCTION

The detection of soluble biomarkers from biological fluids — referred to as liquid biopsy — is a method that is more and more frequently applied for the early diagnosis of several diseases. It is believed that altered levels or the appearance of several circulating molecules potentially are indications of disease and can be detected at early stages for which other — often invasive — diagnostic tools are ineffective. Since extracellular vesicles are present in body fluids and their content depends on the cell of origin, circulating extracellular vesicles could potentially serve as early diagnostic markers for malignant diseases (1).

Extracellular vesicles are omnipresent in human tissues and many types of biological fluids, including blood, breast milk, urine, sperm, amniotic fluid, saliva, bronchoalveolar lavage, cerebrospinal fluid, synovial fluid, pleura effusions and ascites (2). Initially, extracellular vesicles were considered a mechanism by which cells rid themselves of cytoplasm and membrane proteins (3); however, not long after their discovery, they were found to be truly functional and actively released from cells for a variety of reasons. Extracellular vesicles are generally classified by their size, morphology and biochemical composition as well as their biogenesis as apoptotic bodies, microvesicles and extracellular vesicles (2). Apoptotic bodies are the largest — with diameters of up to 5000 nm — and are released during apoptosis by direct budding of the membrane. These vesicles contain nuclear content, cell organelles, DNA, ribosomal RNA and messenger RNA (mRNA). Microvesicles or microparticles are in the mid-range of extracellular vesicles with diameters of 100–1000 nm and are formed by direct shedding of the plasma membrane through outward invaginations. Microvesicles contain plasma membrane and cytosolic proteins as well as nucleic acids. Exosomes are small membrane nanovesicles released from various cells (B and T cells, dendritic cells, mast cells, mesenchymal stem cells, epithelial cells, astrocytes, endothelial cells and cancer cells) into the extracellular environment. Exosomes are the smallest extracellular vesicles, with diameters of 30–150 nm, and have a lipid bilayer containing various proteins, coding and non-coding RNAs and bioactive lipids, depending on their cell of origin. Exosomes are formed by inward budding of endosomal membranes that produce multivesicular bodies in which intraluminal vesicles develop. Intraluminal vesicles are subsequently secreted into the extracellular space as exosomes when multivesicular bodies fuse with plasma membrane (2). Exosomes have a plethora of biological functions. In addition to mediating cell-to-cell communication, signal transduction and transport of genetic material, exosomes play important roles in immune modulation and are involved in the pathogenesis of various human diseases, such as cancer and autoimmune inflammatory diseases (4-6).

During infection, exosomes carry pathogen-derived proteins, nucleic acids, lipids and carbohydrates and, thus, serve as antigen presenters, activating innate immune receptors to induce host defense (2). Exosomes shuttle both coding and non-coding RNAs, which maintain their function when transferred to recipient cells. This epigenetic signaling has an important role in cell-to-cell communication (7).

Long non-coding RNAs (lncRNAs) are longer than 200 nucleotide and lack protein-coding potential. These transcripts are involved in many cellular processes, including the regulation of chromatin modification, gene transcription, mRNA translation and protein function. The expression of lncRNAs is generally tissue- or cell-type specific and malignant cells have been shown to have a specific lncRNA signature (8). Since these molecules have a role in the pathogenesis of many diseases, they might be used as biomarkers for detection of disease at early stages (6,9); however, the biological functions of circulating lncRNAs and the mechanisms regulating the levels of circulating lncRNAs still need to be evaluated. Numerous studies have confirmed that lncRNAs play crucial roles in various multifactorial human diseases, such as cancer and neurological diseases, and that they also have an impact in the differentiation and activation of immune cells. These results together suggest that lncRNAs contribute to the pathogenesis of human inflammatory diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and psoriasis (10).

LncRNAs have been found to be enriched in exosomes compared to the cell of origin (11, 12). Of the circulating extracelluar vesicles, exosomes are the richest reservoirs for almost all lncRNAs (13). When using lncRNAs as biomarkers, the circulating exosome fraction is more useful than whole body fluid, as exosomal lncRNAs are protected against RNases, they are enriched in the exosome fraction compared to the whole-body fluid and their exosomal expression levels is dependent on the cells of origin (14). Several hypotheses have been proposed for the mechanism by which molecules are loaded into exosomes. Most probably, structural motifs in the lncRNAs interact with the proteins responsible for RNA localization and exosomal loading. As certain RNAs are enriched in exosomes, it is likely that these RNAs are actively loaded into extracellular vesicles (2,12).

Most studies that investigate exosomes as biomarkers have focused on cancerous diseases (15), and the study of exosomal lncRNAs as biomarkers is more advanced for cancer than any other disease. Numerous studies indicate that the expression pattern of circulating lncRNAs probably carries information about the size of the tumor or malignancy or other important characteristic of the disease, and, therefore, the presence of circulating and exosomal lncRNAs may reflect disease progression in particular cases. The use of circulating lncRNAs as biomarkers is also emerging for chronic inflammatory diseases, as many lncRNAs associated with these diseases are found in circulating exosomes. In this review, we summarize our knowledge about exosomal lncRNAs and their potential use as biomarkers for several human diseases, including chronic inflammatory and cancerous diseases.

EXOSOMAL lncRNAs IN CHRONIC INFLAMMATORY DISEASES

Rheumatoid arthritis

RA is a common chronic inflammatory autoimmune disease that is characterized by the infiltration of lymphocytes and macrophages into the synovial fluid, hyperplasia of the synovial membrane, degradation of cartilage and bone erosion (6,9). LncRNAs seem to be implicated in the development of the disease, as altered expression is associated with the severity and activity of the disease (for an extensive review see reference (16)). The first lncRNA that was associated with RA was the 2.3 kb H19 RNA, which exhibited significantly higher expression in the synovial tissue of RA patients compared to the tissue of healthy individuals (16,17). Since this discovery, numerous high-throughput analyses were completed to describe the lncRNA profile

of RA from cells as well as from serum. Xu et al. investigated the expression of lncRNAs in serum samples from RA patients and healthy donors and identified 5 lncRNAs that were expressed at significantly higher levels in RA samples than controls: RNA143598, RNA143596, HIX0032090, IGHCgamma1 and XLOC_002730 (18). Comparing the lncRNA expression in serum exosomes, Song et al. found significantly higher expression of HOTAIR, LUST, anti-NOS2A, MEG9, SHNG4, TUG1 and NEAT1 and significantly lower expression of mascRNA, PR antisense transcripts, PRINS, and HOXA3 expression in RA patients than in healthy individuals (19). These molecules are likely to serve as biomarkers for RA, and combined analysis of these molecules may be a robust method to determine disease prognosis. However, to date there is a lack of information about the sensitivity and specificity of these molecules in RA, therefore, until further studies elucidate these characteristics, the diagnostic application of exosomal lncRNAs is greatly limited.

Systemic lupus erythematosus

SLE is a chronic autoimmune disease, characterized by the production of multiple autoantibodies against nuclear auto-antigens and double-stranded DNA. Abnormal interaction between the innate and adaptive immune system and activation of the complement system leads to tissue or organ damage (10,16,20). Lupus nephritis (LN) is one of the most serious manifestations of SLE, and 10–30% of LN patients progress to end-stage renal disease. Renal biopsy is still the “gold standard” to predict renal outcome, and a non-invasive method to assess glomerular damage would be major improvement. Recent results suggest that exosome-derived markers, especially from urine samples, might be appropriate for such an assay (9,21,22). Circulating exosomes in the plasma of SLE patients are derived from platelets, endothelial cells and leukocytes and have clinical and serological correlations (22). Circulating exosomes are also involved in the pathogenesis of SLE, since serum exosomes isolated from SLE patients were able to induce cytokine production in peripheral blood mononuclear cells of healthy donors (23).

LncRNAs are strongly associated with susceptibility to SLE. The gene for the GAS5 lncRNA is located in the SLE-susceptibility locus of chromosome 1q25 and is closely linked to SLE susceptibility (10). The GAS5 lncRNA is also implicated in RA pathogenesis (24), and GAS5 levels are also downregulated in the serum of SLE patients (25). Moreover, serum GAS5 level could be a highly specific but not sensitive biomarker for SLE, and in combination with linc0597 lncRNA could be used as a highly sensitive (83.44%) and specific (93.75%) biomarker (25). Expression of another lncRNA, NEAT1, is increased in the peripheral blood cells of SLE patients, as it is in RA patients (19), and is positively correlated with disease activity (26). NEAT1 often colocalizes with MALAT1, which is also an abnormally upregulated lncRNA in both RA and SLE. MALAT-1 is a key factor in the pathogenesis of SLE, as it regulates the expression of IL-21 and SIRT1 in monocytes from SLE patients (16,27). These studies on lncRNAs in SLE focused on either circulating lncRNAs (25) or cellular lncRNA expression. The fact that the circulating and cellular lncRNAs in SLE are also present in the serum exosomes of RA patients (19) suggests that these lncRNAs might also be present in serum exosomes of SLE patients. Future studies on exosomal lncRNAs in SLE could provide the necessary information to include circulating exosomal lncRNAs as disease specific markers in SLE.

Psoriasis

Psoriasis is a chronic inflammatory skin disease, characterized by the abnormal proliferation and

differentiation of basal keratinocytes and their deregulated interplay with professional immune cells (28). Many non-coding RNAs, including microRNAs (miRNAs) (29) and lncRNAs (30), have been found to be deregulated in this disease and implicated in disease pathogenesis.

One of the first lncRNAs to be associated with psoriasis was described by our research group in 2005: psoriasis associated non-protein coding RNA induced by stress (PRINS). The PRINS lncRNA is upregulated in non-lesional skin samples of psoriatic patients (31) and functions as a regulator of cellular apoptotic functions by interacting with nucleophosmin (32) and miR-491-5p (33), and effecting G1P3 gene expression (34). PRINS also regulates inflammatory cytokine expression through interactions with their mRNA (35).

Another regulatory lncRNA expressed in the epidermis, TINCR, is localized to the cytoplasm of differentiated human keratinocytes and is able to stabilize mRNAs linked to differentiation (36), many of which are implicated in psoriasis pathogenesis (30).

Approximately 10–30% of psoriatic patients are also affected by psoriatic arthritis (PsA), which causes skin symptoms as well as joint erosion and new bone formation. The analysis of lncRNAs in blood samples from PsA patients indicate that lncRNAs are involved in disease pathogenesis (37) and that these molecules could be used as new biomarkers and possibly therapeutic targets for PsA.

Although lncRNA expression in psoriatic skin and immune cells was described by numerous research groups, circulating lncRNA levels and exosomal lncRNAs have not received much attention to date. Importantly, it has been suggested that circulating exosomes could serve as a tool for prognosis and for monitoring therapy efficiency (5).

EXOSOMAL lncRNAs AS BIOMARKERS IN CANCER

Early diagnosis of malignant neoplasms is important for successful treatment and survival of patients. Cancers have a high mortality rate due to the lack of suitable, specific and early detection of diagnostic tumor biomarkers. Tumor cells release exosomes, which facilitate communication within the local environment and primary tumor cells, supporting tumor-cell growth, tumor-associated angiogenesis and tissue inflammation in both autocrine and paracrine manners. Numerous studies show that cancer-derived exosomes activate signal-transduction pathways involved in cancer cell proliferation and survival (38,39). Exosomes regulate immune modulation, including immunosuppression that supports the growth of the tumor (4). Moreover, as exosomes are known to alter cellular functions, they have been intensively studied for their potential in metastasis formation, especially through the mechanism of epithelial-to-mesenchymal transition (40).

The peripheral blood of cancer patients contains significantly more exosomes than blood samples from healthy individuals (41), as tumorous cells release higher amounts of exosomes. Circulating exosomes support the dissemination of the tumor, are involved in the initial events of metastasis, and carry a unique molecular fingerprint from their cell of origin. The high number of circulating exosomes and their molecular content makes them ideal candidates for tumor biomarkers and for predicting the metastatic potential of a tumor in liquid biopsies (38,42,43). However, these biomarkers have not been integrated into clinical routines, as their isolation is expensive and time consuming (44).

Downregulation of tumor-suppressive miRNAs and upregulation of oncogenic miRNAs have been described for various human cancers.

Cancer-associated miRNAs regulate tumorigenesis, survival, angiogenesis, migration and invasion of tumors (45); moreover, clinical studies have correlated dysregulated expression of particular miRNAs with tumor responsiveness to chemotherapies (46).

Similarly to microRNAs, lncRNAs have been shown to play a fundamental role in cancer cell growth, proliferation, cell death, invasion and formation of metastasis (47); however, the majority of these genes have not yet been characterized functionally. Currently, many attempts focus on the use of lncRNAs as prognostic markers for cancer patients, as tumor cells can be characterized by their distinct lncRNA profile (48). These lncRNAs can be packaged within exosomes and, as such, offer great potential for use as markers for specific tumors (Table 1), especially as exosomal release rate is increased in cancer cells compared to healthy cells (41).

The use of the exosomal lncRNAs as cancer biomarkers is also supported by the high sensitivity (70% to 94%) (49–60) and specificity (72% to 94%) (49–60) of these markers. Moreover, the combination of either the lncRNA markers (58–60) or lncRNAs with miRNAs (61) or already used protein diagnostic markers (56,60) can increase both the sensitivity and specificity of these markers.

This possibility was tested in prostate cancer, where the level of prostate specific antigen (PSA) is used for screening for prostate cancer with high specificity (~93%) but low sensitivity (~20–25%) at a cutoff value of 4 ng/ml (62), however it can be also modestly elevated in benign prostatic hyperplasia. Wang *et al*. suggest, that exosomal lncRNA expression could help differentiation between prostate cancer and benign prostatic hyperplasia in cases, where PSA levels (4–10 ng/ml) alone have little diagnostic value (60).

EXOSOMAL lncRNAs IN OTHER HUMAN DISEASES

Bacterial and viral interaction with host cells

Extracellular vesicles are produced by both Gram-negative and Gram-positive bacteria (73) and have several functions, including molecular transport, mediation of stress response, biofilm formation and influence on hosts cells (2). Both normal human flora and pathogenic bacteria communicate with the host cells through extracellular vesicles, and infected host cells respond by releasing exosomes to alert surrounding cells (74). The extracellular vesicles released by infected cells contain both pathogen- and host-derived factors and play key roles in pathogen-host interactions, including pathogen uptake and replication and regulation of the host immune response (75). Viruses also modify the number and content of exosomes released by infected cells, which often contain virus-associated miRNAs, as in the case of Epstein-Barr and human immunodeficiency viruses, or parts of the viral genome (2).

Monoclonal gammopathies

Multiple myeloma is a heterogeneous disease with focal lesions in the bone marrow, and analysis of a biopsy specimen obtained from a single site in the bone marrow is not sufficient for the prediction of disease outcome. However, circulating molecules (DNA, miRNAs and lncRNAs) in peripheral blood could serve as potential diagnostic, prognostic and predictive markers. PRINS, for which exosomal expression correlates with characteristic chromosomal aberrations in the disease, is one such candidate molecule (76).

Neurodegenerative diseases

LncRNAs are expressed in the central nervous system, which allows the possibility that they play roles in normal neurological development

and growth and, possibly, in tumorigenesis. Circulating and cerebrospinal-fluid-derived exosomes could be used to detect biomarkers for neurodegenerative diseases and for tumors of the central nervous system; however the lncRNA content of such exosomes has not yet been thoroughly investigated (77).

Osteoarthritis

By analyzing the lncRNA profile of exosomes derived from plasma and synovial fluid, Zhao and Xu found that, whereas plasma-derived exosomal lncRNAs had no diagnostic value in the disease, PCGEM-1 was significantly upregulated in the synovial fluid of late-stage osteoarthritis patients.

They hypothesize that PCGEM-1 expression can be used to distinguish early osteoarthritis from the late-stage disease (78).

Chronic kidney disease

In urine-derived exosomes of patients suffering from chronic kidney disease 30 differentially expressed non-coding RNAs were identified as suitable biomarkers for early diagnosis, of which the most powerful disease marker is miRNA-181a, while lncRNAs were found to be less than 1% of all deregulated RNA molecules in the disease (79).

DISCUSSION

Liquid biopsies represent a non-invasive and painless method for monitoring health and disease states of individuals. Protein and RNA content of extracellular vesicles, which are present in all body fluids, is receiving increasing levels of attention for their potential as biomarkers. Although it seems that expression levels of many lncRNAs within exosomes are sufficient to serve as disease markers that can potentially be used in diagnosis or prognostic tools for human diseases, most research studying lncRNAs in body fluids has not determined whether the transcripts are freely circulating in the body fluid or are contained in exosomes. Moreover, most reports about exosomal lncRNAs show that their expression is deregulated in several diseases (Table 1), indicating that these molecules might not be sufficiently disease specific to be used as biomarkers (19,24–26).

To overcome this issue, some studies used a combined analysis of exosomal lncRNAs, miRNAs and proteins as biomarkers increasing the sensitivity and specificity of the diagnostic test (56,58–61). Studies using such combined analysis are also helpful for building interaction networks and databases of exosome-derived molecules and support the functional study of these molecules necessary to understand their role in

Table 1 Exosomal lncRNAs as potential biomarkers in cancer

Exosomal lncRNA	Cancer type	Reported findings
CRNDE-h	Colorectal cancer	High levels correlate with poor prognosis (49)
ENSG00000258332.1	Hepatocellular carcinoma	Higher levels in serum exosomes compared to liver cirrhosis and chronic hepatitis B (50)
H19	Bladder cancer	High levels in serum exosomes associated with poor disease prognosis (51)

HOTAIR	Bladder cancer	High levels in urine-derived exosomes (63)
	Cervical cancer	High levels (64)
	Glioblastoma multiforme	High levels in serum and exosomes (52)
	Laryngeal squamous cell carcinoma	High levels in exosomes (61)
HOTTIP	Gastric cancer	Expression in exosomes is an independent prognostic factor (54)
HOX-AS-2	Bladder cancer	High levels in urine-derived exosomes (63)
LINC00161	Hepatocellular carcinoma	High levels in serum-derived exosomes (55)
LINC00635	Hepatocellular carcinoma	Higher levels in serum exosomes than observed for liver cirrhosis and chronic hepatitis B (50)
lncRNA 91H	Colorectal cancer	Early biomarker for colorectal recurrence or metastasis (65)
lncRNA-ATB	Hepatocellular carcinoma	Independent predictor of mortality and disease progression in combination with miRNA-21 expression (66)
lncRNA-HEIH	Hepatocellular carcinoma	High levels in serum and serum exosomes (67)
lncRNA-p21	Prostate cancer	Different levels in benign prostate hyperplasia and prostate cancer (56)
lncRNASNHG14	Breast cancer	High levels in patients resistant to trastuzumab (57)
lncUEGC1/2	Gastric cancer	Highly sensitive and stable biomarker (68)
MALAT1	Bladder cancer	High levels in urine-derived exosomes (63) associated with poor prognosis (58)
	Cervical cancer	High levels (64)
	Epithelial ovarian cancer	Correlated with an advanced and metastatic phenotype and independent predictive factor for overall survival (69)
	Non-small cell lung cancer	High levels (70)

MEG-3	Cervical cancer	Low levels (64)
PCAT-1	Bladder cancer	Expression in urine-derived exosomes associated with poor disease prognosis (58); serum exosomal expression used as biomarker (59)
SAP30L-AS1	Prostate cancer	Used in combination with SChLAP1 to differentiate between benign prostatic hyperplasia and prostate cancer (60)
SChLAP1	Prostate cancer	Used in combination with SAP30L-AS1 to differentiate between benign prostatic hyperplasia and prostate cancer (60)
SNHG16	Bladder cancer	High level in serum exosomes is a diagnostic marker (59)
SPINT1-AS1	Colorectal cancer	High level associated with poor prognosis (71)
SPRY4-IT1	Bladder cancer	Presence in urine-derived exosomes associated with poor disease prognosis (58)
UBC1	Bladder cancer	High level in serum exosomes is a diagnostic marker (59)
ZFAS1	Gastric cancer	High serum-exosomal level (72)

disease pathogenesis and promote their use as biomarkers.

The intensive study of both lncRNAs and exosomes only started a decade ago, thus there are plenty of open questions. Most studies on both fields are still descriptive, comparing expression levels of lncRNAs or number and contents of exosomes in diseased tissues, cells or liquid biopsies to healthy samples. But there is still a lack of knowledge on the function of both exosomes and lncRNAs in both healthy and diseased states. It is also debatable whether the identified differences are specific to one disease or general features of related diseases. The expression of the same lncRNAs in several inflammatory diseases (19,24–26) suggest that their expression is rather specific to chronic inflammation than the disease itself. There are plenty of open questions in this topic: What are the target cells of the circulating exosomes and how their cargo – especially their lncRNA content – alters the function of target cells? Are the targeted cells specific to the disease and have a function in the disease course? Although there are some evidences that the amount of exosomes and their content changes with therapy (46,57), whether their characteristics return to healthy state is unknown. Nevertheless, the lack of consensus on exosome isolation is one of the biggest issue to overcome (44,80–84). Most methods used so far were shown to have high laboratory-to-laboratory and method-to-method differences in the amount and quality of the isolated extracellular RNA (80), which is the biggest barrier before their implementation as routine biomarkers.

Taken these limitations into account we believe that the already described disease specific expression of one or more lncRNAs in exosomes should be the starting point to their functional

study, to provide the necessary information for future implications in detecting, monitoring and treating disease.

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