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Non-coding RNAs in cancer brain metastasis

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1. ABSTRACT

More than 90% of cancer death is attributed to metastatic disease, and the brain is one of the major metastatic sites of melanoma, colon, renal, lung and breast cancers. Despite the recent advancement of targeted therapy for cancer, the incidence of brain metastasis is increasing. One reason is that most therapeutic drugs can't penetrate blood-brain-barrier and tumor cells find the brain as sanctuary site. In this review, we describe the pathophysiology of brain metastases to introduce the latest understandings of metastatic brain malignancies. This review also particularly focuses on non-coding RNAs and their roles in cancer brain metastasis. Furthermore, we discuss the roles of the extracellular vesicles as they are known to transport information between cells to initiate cancer cell-microenvironment communication. The potential clinical translation of non-coding RNAs as a tool for diagnosis and for treatment is also discussed in this review. At the end, the computational aspects of non-coding RNA detection, the sequence and structure calculation and epigenetic regulation of non-coding RNA in brain metastasis are discussed.

2. INTRODUCTION

Cancer cells that are originated from primary tumors in the body often metastasize to other organs. In the United States, approximately 150,000 cases of brain metastases occur each year (1). It is also reported that 9%-17% cancer patients develop brain metastases (1,2), and accounts for 20% of total cancer deaths annually. As the technologies have advanced for early diagnosis and more effective treatments, is the prevalence of brain metastases is ironically increasing as patients are living longer. A variety of primary tumors from various organ sites can develop brain metastasis through complex mechanisms that involve various genetic factors during the multiple-step metastatic process (Figure 1). Breast, colorectal, renal, lung and melanoma are the most common cancers that metastasize to brain, and patients with these primary tumors are susceptible to brain metastasis. Table 1 summarizes the recent trend of incidence proportions (3) and metastatic rates (4-9) of these five types of primary tumors. Patients with brain metastasis may present with central nervous system symptoms such as seizure, headache, nausea and vomiting. Brain metastasis is responsible for high morbidity and mortality. Despite that the evolution and development of targeted systemic agents and more sensitive diagnostic methods, the prognosis of patients with brain metastasis remains poor. The median survival of patients with brain metastasis is less than 10 months (10), and this is in part due to the problem of permeability of drugs through the blood-brain barrier as well as resistant mechanisms to drugs. Therefore, the brain is a unique sanctuary for tumor cells.

3. PATHOPHYSIOLOGY OF BRAIN METASTASIS

The conventional theory of cancer metastasis involves six distinct steps (11). First, cancer cells need

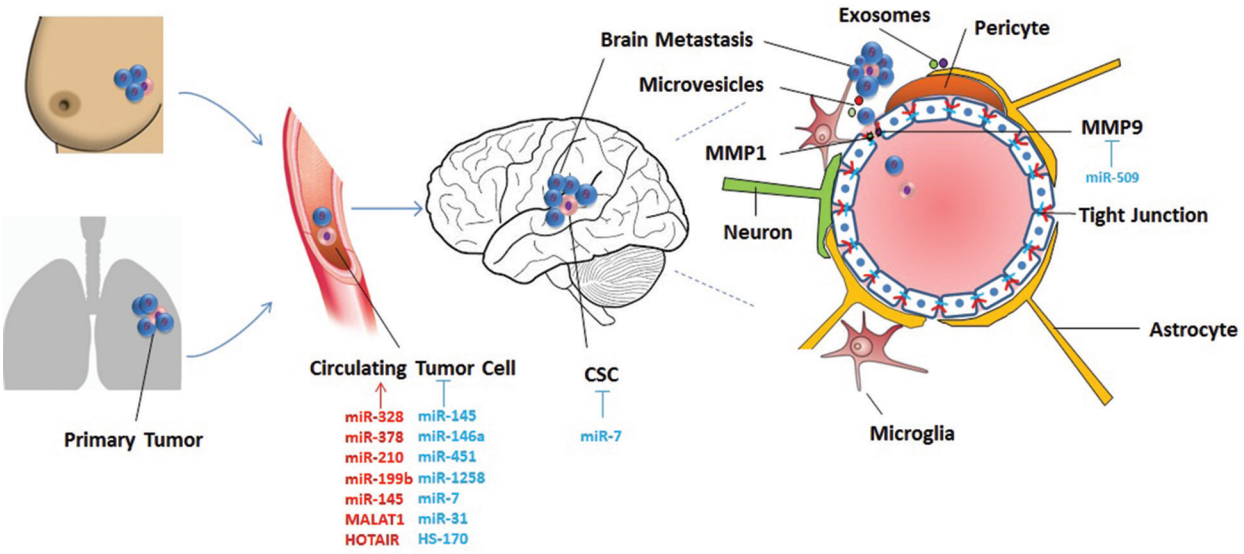

Figure 1. The metastatic process of cancer cells to the brain. Cancer cells invtravasate from its primary tumor into lymphatic and blood vessels, circulate through the lymphatic system or the bloodstream, and finally extravasate into the brain by disrupting the tightness of blood-brain barrier. Secretory proteases such as MMP1 degrade tight junction proteins to open the way for cancer cell entering brain, where it interact with environmental cells to support the colonization. Red color indicates up-regulated microRNAs; blue color indicates down-regulated microRNAs. Abbreviations: CSC, cancer stem cell; MMP, matrix metalloproteinase.

Table 1. Statics of brain metastasis

Primary tumor	Lung	Melanoma	Renal	Breast	Colorectal
Incidence proportions[1]	19.9	6.9	6.5	5.1	1.8
Metastasis rates[2]	15-30	10-40	5-10	15-30	2-12
[1]The percentage of total brain metastasis events contributed by one type of primary tumor, [2]The chance of brain metastasis for one type of primary tumor					

to acquire the invasive property within the primary tumor before metastasis. The major changes during the first step include promotion of cell motility, induction of epithelial–mesenchymal transition (EMT) and secretion of molecules modulating the environment to carve out the metastasis trail (12-14). Following these changes, cancer cells invade into local stroma where they later intravasate into surrounding blood vessels. These cells entering the circulation system are called circulating tumor cells (CTCs). Understanding the roles of CTCs in tumor progression is an area of active investigation and one where there is a need for intensive study to identify the biological properties of metastasis in addition to increasing the technical detection sensitivities. However, it is believed that CTCs are the intermediate stage between primary tumors and metastatic colonization when cancer cells go through differentiated changes and gain heterogeneity (15). These CTCs will finally sojourn in the capillary of a foreign tissue where extravasation occurs. Cancer cells take advantages of several different mechanisms to extravasate into the new neighborhood. They can secrete cytokines and chemokines to mimic leukocytes so that the endothelial cells will increase the permeability for them (16). In addition, some cancer cells also secrete enzymes to disrupt endothelial cells. It is reported that breast cancer brain metastatic cells secrete MMP1 to degrade tight junction proteins which seals the adjacent endothelia cells (17). Thus, cancer cells slide into brain microenvironment through the paracellular gap (17). Alternatively, the cancer cells grow intraluminally within the microvessel and finally break through the wall as the volume increases (18).

Despite the fact that many cancer cells translocate from primary tumor to distant sites, survival in the new microenvironment is still challenging for these cells. The majority of them die while only those cells inheriting the heterogeneity fitting the destined new environment or the cells capable of adaption will finally be selected to survive (19-22). During this process, interaction with the new microenvironment plays a key role to the final fate of the cancer cells (Figure 2). In the context of the ‘seed and soil’ theory (22), different cancer cells are different seeds containing distinct genetic bases while different organs are different soils with distinct inner environments. Only those seeds with the appropriate properties will survive and grow in the “soil” of distant tissue. This also explains the preference for metastasis destinations of different tumors. It is known that prostate cancer often prefers bone (23) while breast cancer metastasizes to lung, liver, bone, and brain (24). On the other hand, muscle is the barren soil for seeds from

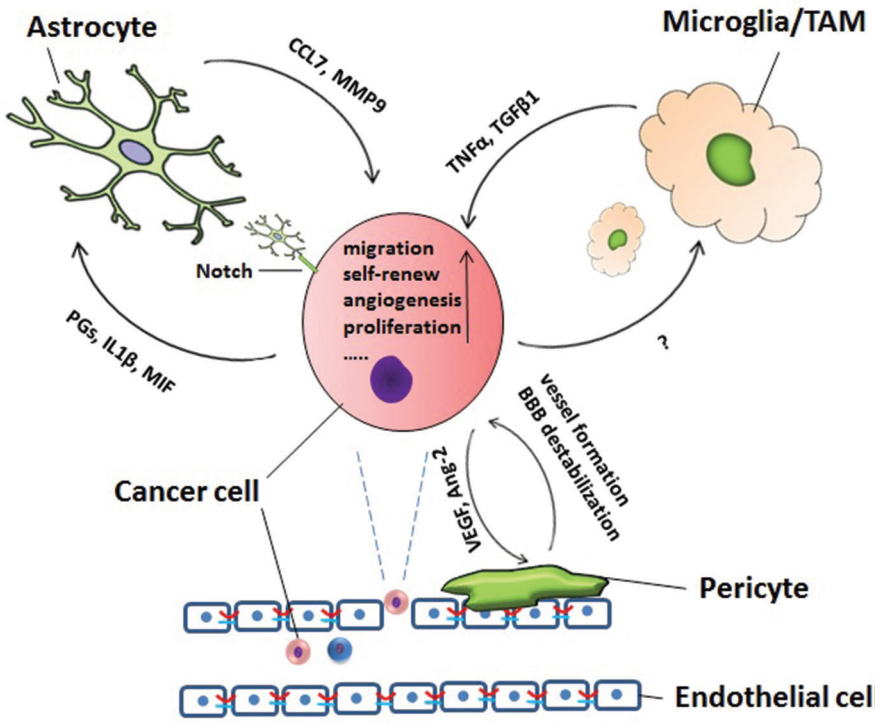

Figure 2. Interaction between cancer cell and brain microenvironment cells. Schematic of some regulatory factors mediate the crosstalk between cancer cell and surrounding cells, including astrocyte, microglia/TAM, pericyte, and endothelial cells. Abbreviations: PGs, prostaglandins; IL1β, Interleukin-1 beta; MIF, macrophage migration inhibitory factor; CCL7, Chemokine (C-C motif) ligand 7; MMP9, matrix metalloproteinase9; TNFα, tumor necrosis factor alpha; TGFβ1, transforming growth factor beta 1; TAM, tumor-associated macrophage; VEGF, vascular endothelial growth factor; Ang-2, angiopoietin-2; BBB, blood-brain barrier.

tumors since tumors rarely metastasize to muscle (25). These observations indicate the importance of the microenvironment for the final step of metastatic cancer cells to establish colonization at the distant organs.

4. SPECIFIC HOST FACTORS INVOLVED IN BRAIN METASTASIS

In the case of brain metastasis, while cancer cells share the universal patterns for distant colonization (Figure 1), there are also special factors that are involved in the metastatic process (Figure 2). The microvasculature of the brain parenchyma is composed of continuous endothelial cells with tight junctions to seal the gap between cells (26). This structure, called blood–brain barrier (BBB), differs from blood vessels in other sites of body and the endothelium within it is non-fenestrated (27). This structure of BBB greatly limits the entrance of circulating tumor cells into the brain. However, cancer cells often modulate the endothelial cells to increase the permeability for extravasation across BBB (28-30), and the tight junction proteins can be liable as the targets of cancer cells (31). It is reported that various proteases are employed by cancer cells to blaze a way into brain. For example, melanoma releases serine proteases to cause the disintegration of the interendothelial junctional complexes (32). Breast cancer cells secrete matrix metalloproteinase 1 to degrade tight junction components Occludin and Claudin 5 (17). Intact tight junctions between endothelial cells enable the paracellular traversing of cancer cells across BBB to the brain microenvironment.

As described above, after extravasation, lodging into the brain microenvironment is still challenging to cancer cells. However, crosstalk between cancer cells and brain cells contributes to the cancer cells survival. Glial cells constitute up to 90 percent of the brain cells, while astrocytes are the most abundant type of glial cells in the CNS (32). It is known that astrocytes maintain the homeostasis of the brain microenvironment (33). It modulates the tightness of blood-brain barrier (34) as well as the neural signal transduction (35) and it also supports nutrition to neurons (36). Astrocytes also buffer the microenvironment by taking up excess ions and neurotransmitters, and then releasing them when needed (37-39). Cancer cells appear to take advantage of the nourishing and protective properties of astrocytes to fertilize themselves (40). It was reported that cancer cells activate astrocyte at the metastatic site to trigger positive feedback effect so they can adapt to the new environment and initiate colonization in the

Table 2. Non-coding RNAs in brain metastasis

	Name of Non-coding RNA	Role in brian metastasis	Primary tumor	Function	Reference
Micro-RNA	miR-145	Suppress	Lung	Increase proliferation	(106)
	miR-328	Promote	Lung	Increase migration through PRKCA	(55)
	miR-378	Promote	Lung	Promote cell migration, invasion and tumor angiogenesis	(56)
	miR-146a	Suppress	Melanoma	Upregulation of β-catenin and downregulation of hnRNPC	(107)
	miR-210	Promote	Melanoma	Unknown	(69)
	miR-451, miR-126, miR-30c, etc	Suppress	Renal	Unknown	(108)
	miR-199b	Promote	Renal	Unknown	(108)
	miR-1258	Suppress	Breast	Target HPSE	(109)
	miR-7	Suppress	Breast	Target KLF4	(58)
	miR-509	Suppress	Breast	Target RhoC	(59)
	miR-145, miR-1, miR-146a, etc	Promote	Colorectal	Unknown	(110)
	miR-31, HS-170	Suppress	Colorectal	Unknown	(110)
Lnc-RNA	MALAT1	Promote	Lung	Induce EMT	(67)
	HOTAIR	Promote	Lung	Induce cell migration and anchorage-independent cell growth	(68)

brain (41-45). Xing *et al* reported that reactive astrocytes promote breast cancer brain metastasis by activating Notch signaling in brain (46). Other microenvironment factors also contribute to metastasis by transporting or up-regulating pro-survival signaling. For example, hypoxia is reported to activate Notch signaling which supports breast cancer metastasis and self-renewal of cancer stem cells (CSCs) at initial stage (47). However, nutrition from blood is necessary for the expansion of cancer cell number. Cancer cells interact with pericyte and endothelial cells through cytokines such as VEGF, Ang2 to promote blood vessel destabilization and accelerate aberrant angiogenesis (48,49). In addition, microglia/TAM (tumor-associated macrophages) is also found to interact with cancer cells. Both of patients' tissue samples and cell culture experiments showed differential activation of microglia/TAM around cancer cells (50-52). Aberrant activation of microglia/TAM leads to secretion of cytokines supporting cancer cell growth, such as TNF-α, TGF-β1 (50,52). Therefore, a variety of host factors and tumor microenvironment contribute to the process of metastatic colonization and these factors are also considered to be potential therapeutic targets in the future.

5. ROLE OF MICRORNA IN CANCER BRAIN METASTASIS

Metastatic cancer cells harbor aberrant signaling proteins and express dysregulated non-coding RNAs (ncRNAs) to promote mobility and survival of tumor cells. One major component of the dysregulated factors in metastatic cancer cells is microRNA (miRNAs). MicroRNAs are a class of small and non-coding RNAs. They are important regulatory molecules in animals and plants. The first microRNA was discovered in 1993 by Lee, Rosalind C, *et al* (53); however, it wasn't until 21st century when researchers began to explore the relationship between microRNAs and cancers (54). MicroRNA regulates gene expression in multiple ways including translational repression, mRNA cleavage, and mRNA decay initiated by microRNA-guided rapid deadenylation. Recent studies describe how some microRNAs are important for cancer brain metastasis through the regulation of cell proliferation and mobility in the brain. Diverse brain metastatic tumors are universally reported to harbor dysregulated endogenous expression of metastasis-related microRNAs (Table 2). For example, several microRNAs were found to be associated with lung cancer brain metastasis. MiR-328 was found to promote brain metastasis in non-small cell lung cancer (NSCLC) patients (55). Even though the direct target of miR-328 in NSCLC is not clearly defined, protein kinase C alpha (PRKCA) was up-regulated upon overexpression of miR-328. High level of PRKCA is also correlated with increased migrating ability of cancer cells, which was significantly reduced when miR-328 was suppressed (55). Another microRNA that was also found to promote brain metastasis in the lung cancer is miR-378 (56), which was also shown to be up-regulated in brain metastasis patients with NSCLC. MiR-378 appears to increase the risk of brain metastasis by promoting

cell migration, invasion and tumor angiogenesis. While up-regulation of these miRNAs promotes metastasis, lung cancer brain metastatic cells also down-regulate the expression of other metastasis suppressive microRNAs. The expression of MiR-145 was found to be low in lung cancer brain metastasis (57). MiR-145 was shown to directly target MUC1, a gene associated with metastatic ability of cancer cell. Suppression of MUC1 leads to decreased level of β-catenin and cadherin 11, which correlates with decreased cell invasive ability. Breast cancer also takes advantage of microRNAs to promote brain metastasis. MiR-7 was recently found to be down-regulated in breast cancer brain metastasis (58). Profiling analysis revealed that cancer stem cells that are highly metastatic to brain express significantly lower level of miR-7, which was found to modulate the stem-like capacity of cancer cell through KLF4 (58). Another metastasis suppressive microRNA found to be down-regulated in brain metastasis of breast cancer is miR-509 (59). The miR-509 targets RhoC and TNF-α. Low expression of miR-509 leads to high MMP9 secretion induced by RhoC and TNF-α, while MMP9 is a well-known proteinase involved in cancer cell migration and extravasation (60).

6. ROLE OF LNCRNA IN CANCER BRAIN METASTASIS

Another type of non-coding RNA regulating metastatic ability of cancer cells is called long non-coding RNAs (lncRNA). LncRNAs are a large family of diverse RNA molecules with the length of more than 200 nucleotides. LncRNAs do not encode proteins but they alter gene expressions (61). The regulatory effect of lncRNA is a consequence of intervention of gene transcription during different steps. Chromatin modification is one of the ways lncRNA induces chromosome silencing. For example, it is known that the lncRNA Xist recruits Polycomb Repressive Complex 2 to X chromosome where repressive H3K27me3 modification is widely established (62). The Xist induced heterochromatin to inactivate X chromosome is essential during early embryonic development (62). Transcription initiation is also regulated by lncRNA through positive and negative regulation. Evf2 (Embryonic ventral forebrain-2) lncRNA is reported to form a complex with the transcription factor Dlx2 (Distal-Less homeobox 2) to initiate the transcription of genes with Dlx2 binding sites (63), while Alu (Arthrobacter luteus) lncRNA binds and deactivates RNAPII to inhibit the expression of heat shock genes (64). LncRNA can also regulate gene expression after transcription (post-transcriptional regulation). For example, NAT lncRNA protects Zeb2 mRNA from splicing to increase the translation rate (65). Uchl1-AS increases polysome loading onto Uchl1 mRNA to promote translation (66). In addition, lncRNA affects mRNA splicing, transportation and translation. Protein translocation is also known to be associated with lncRNA expression. The dysregulation of lncRNA in cancer cells greatly affect their metastatic ability and destination preference. Brain metastasis has been reported to be associated with up-regulation of certain metastasis-promoting lncRNAs (Table 2), though more comprehensive study is needed to address the remaining biological issues related to the specific mechanisms involved. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) has been shown to be over-expressed in variety of tumors including non-small cell lung cancer (NSCLC) primary tumors that metastasizes to brain (67). The mechanism for MALAT1 to support cancer cell migration to brain is associated with its ability to induce EMT, which can be suppressed by silencing MALAT1. Despite the change in migration ability induced by MALAT1, the mechanism of this driving force is still unknown. Nonetheless, the target of MALAT1 is still unrevealed, and the detailed mechanism by which the MALAT1 silences its target needs further exploration. There is another lncRNA that also performs highly similar activity. It was reported that brain metastases patients of NSCLC have high expression of lncRNA HOTAIR (68). *In vitro* studies have shown that HOTAIR indeed enhances cell migration and anchorage-independent cell growth. Similar to MALAT1, the target and the exact role of HOTAIR is still unclear. The relationship between MALAT1 and HOTAIR in NSCLC brain metastasis is still unknown. It is unclear if the up-regulation of these two lncRNAs are a coincidence or part of the synergism within an uncharted brain metastatic pathway. Deciphering the roles of MALAT1 and HOTAIR may lead to further understanding of the pathology of NSCLC brain metastasis. Due to the relatively short history of studies on lncRNA, only a handful of lncRNAs are found to be associated with brain metastasis. A more systematic approach is necessary for screening of lncRNAs involved in cancer brain metastasis. LncRNAs which promote/inhibit cell migration and proliferation are a significant event in cancer biology, and likely the number of lncRNAs to be discovered playing a major role in the biology of cancer metastasis to the brain will grow before long.

7. TUMOR MICROENVIRONMENT AND EXTRACELLULAR VESICLES

The microRNAs and lncRNAs described above are generated by and influence cancer cells. However, brain metastasis is a multi-step event involving other cells in their tumor microenvironments. For example, cancer cells need to overcome the obstruction posed by the extracellular matrix and require disruption of endothelial cells for extravasation. It's also challenging for cancer cells to survive in the circulation and establish colonies in the distant organs. In order to achieve colonization in distant metastasis, cancer cells communicate with the environmental cells so that they influence changes within cells or bring modulation to the microenvironment,

aiming to establish a niche for tumor growth. These communications include physical interaction, ions, cytokines, and chemokines. More recently, non-coding RNAs, such as microRNA and lncRNA are also found to serve as the messenger between cancer cells and environmental cells. It is known that cells secrete extracellular vesicles including microvesicles and exosomes to transport microRNA and lncRNA to surrounding cells (69). Tumor associated macrophages are reported to deliver miR-223 to cancer cells through microvesicles (70). Incorporation of TAM-derived miR-223 through microvesicles was found to increase the invasiveness of breast cancer cells (71). On the other hand, cancer cells also modulate environmental cells through delivery of non-coding RNAs by exosomes. Rat adenocarcinoma cells transfer exosomal miR-494 and miR-542-3p to target cadherin-17 in lymph node stroma cells (72). Down-regulation of cadherin-17 increases matrix metalloproteinase transcription, for a preparation of premetastatic niche in lymph nodes (72). LncRNA was also found to embroil cancer cells through extracellular vesicles (73). Despite the fact that more exosomal non-coding RNAs are found to promote the malignancy of cancer cells, the argument about the physiological role of extracellular vesicles remains controversial. However, some recent clues shed insight on the potential answer for this question. It was found that certain lncRNAs are enriched in exosomes while their endogenous expression is low (74). Interestingly, microRNAs were found to perform cell-independent biogenesis within cancer cell exosomes (74). The processed mature microRNAs are able to get into surrounding cells and promote tumorigenesis; however, there appears to be unknown mechanism by which microRNAs choose between staying in the cells and being excluded into exosomes. These findings all indicate the potential value of studying extracellular vesicles involved in the interaction of cancer cell with surrounding cells.

8. NON-CODING RNAS AS POTENTIAL BIOMARKERS AND THERAPEUTIC TARGETS

MicroRNAs and lncRNAs are now known to be associated with the progression and classification of various types of malignancy. It is well established that patients with distinct cancer types have up-regulation of certain non-coding RNAs in circulation (75). Some of them have been proven to serve as biomarkers for metastatic destination. For example, miR-10b and miR-373 were shown to be the blood makers in detecting lymph node metastasis of breast cancer (76). Serum miR-29a is a promising novel marker for early detection of colorectal liver metastasis (77). The discovery of these non-coding RNAs that are up-regulated in metastatic cancers makes an early diagnosis of metastasis event possible. At present, no non-coding RNAs have been found to be specific for brain metastasis. The study of secretory non-coding RNAs is still at its nascent stage; however, active investigations are ongoing in this field because of the potential usage of these molecules as a powerful non-invasive diagnostic tool for the metastatic disease.

The implication of non-coding RNAs in therapeutic purpose is also under active research. The basic strategies of taking advantage of non-coding RNAs in therapeutics include increasing suppressive non-coding RNA expression and decreasing oncogenic non-coding RNA level. To increase the suppressive non-coding RNAs, microRNA mimics can be used in cancer patients. MicroRNA mimics are small, chemically modified double-stranded RNAs that mimic endogenous microRNAs. The suppressive microRNA mimics are supposed to maintain the same biological function as endogenous microRNAs. This was proved both *in vitro* and *in vivo* as numerous researchers successfully restrained tumor progression with microRNA mimics (78,79). Currently a microRNA mimic based drug MRX34 is under phase 1 clinical trial (NCT01829971). An alternative choice for delivering microRNAs other than miRNA mimics is the adenovirus-associated vector (AAV). The advantage of AAV is transducing target cells without integrating into the genome. Systemic delivery of AAVs carrying miR-26a suppressed hepatocellular carcinoma cell proliferation and induced cancer cell apoptosis (80). Compared to increasing suppressive non-coding RNA expression, more choices exist for inhibiting oncogenic microRNAs. MicroRNA biogenesis needs the processing of several machinery proteins such as DICER complex (74). Drugs targeting these proteins can theoretically suppress the miRNA production. However, the side effect coming from the off target effects of these drugs is a great concern. Molecules that can directly modulate non-coding RNAs are of investigational interest, and the one example of such molecules are called antagonistic microRNAs (antagomiRs). These are chemically modified, cholesterol-conjugated single-stranded RNA analogues complementary to microRNAs (81). However, due to poor pharmacokinetic properties, their clinical translation may be limited. Development on this strategy led to another antisense oligonucleotide agent called locked nucleic acid (LNA) (82). LNA has evaluated binding affinity with improved stability supported by six modified nucleotide bases. Currently, LNA is under phase 2 clinical trials and showed promising effect on the Hepatitis C patients. In the field of cancer brain metastasis, the oligonucleotide is also applicable to target oncogenic molecules while modification in drug delivery system may be needed to improve the pharmacokinetic properties of RNA drugs due to its poor stability and the presence of blood-brain barrier. Except for the chemical modifications mentioned in antagomiRs and LNA drugs, nanoparticles are also used for delivery of oligonucleotide drugs. Nanorod was proven to be capable of transporting DARPP-32 siRNA across blood-brain barrier to dopaminergic neurons (83).

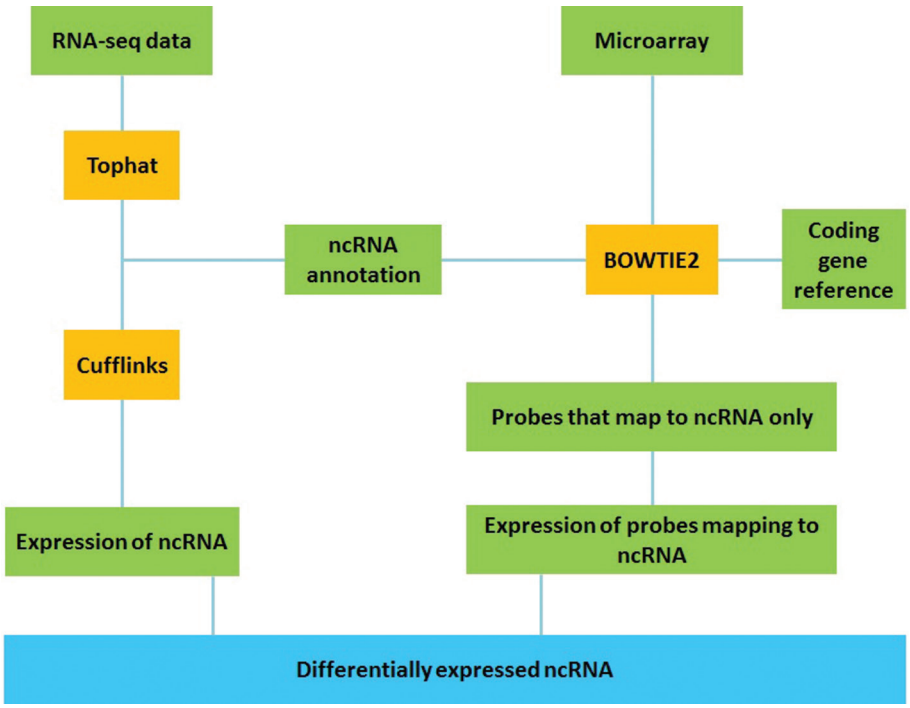

Figure 3. Flowchart for lncRNA detection from microarray and RNA-Seq data.

In addition, endogenous exosomes have also been proven to be a choice of carrier for RNA drugs targeting brain environment (84). Therefore, these nanocarriers appear to be suitable for delivery of oligonucleotide-based drugs for brain metastatic cancer therapy.

9. COMPUTATIONAL DETECTION OF NON-CODING RNA IN BRAIN METASTASIS

Since the transcriptome produces complete genome sequences for different organisms in rapid manner, the computational distinguishing non-coding RNAs from whole genome sequence in specific tissue/cancer cell types became challenging (85, 86). Various computational methods were proposed to identify the non-coding RNAs in different tissues from specific cancer types (87). In general, the detection of non-coding RNAs that are differentially expressed in brain metastasis patients and healthy donors can be done by using either microarray or RNA-seq. The general work-flow of non-coding RNA detection for brain metastasis is given in Figure 3. To identify the brain metastasis associated non-coding RNAs, the non-coding RNA reference library must be created with reference sequence from the NCBI and non-coding RNA annotation files from the GENCODE. Then the raw sequencing reads are mapped to non-coding RNA reference library using Tophat (88), and are assembled into non-coding RNA transcripts by using Cufflinks (89). Expression levels of these transcripts are quantified as FPKM (reads per kilobase of transcript per million mapped reads). Then the entire sample's transcription data is merged together by Cuffmerge. Finally, CummeRbund package is applied to detect non-coding RNAs that are differentially expressed in patients and healthy donors. Du *et al*. (2013) summarized more detailed non-coding RNA detection process in various cancer types (87).

10. SEQUENCE AND STRUCTURE BASED NON-CODING RNA FUNCTIONAL ANNOTATION

Though, high throughput techniques produce enough sequence and expression information, their molecular functional mechanism can only be understood by deciphering their sequence and structural information. There is a general theory that the sequence encodes the structures and such structures determine the functions. For novel non-coding RNA sequence/structure, simple BLAST (90, 91) sequence alignment as well as complex machine learning approaches are employed to annotate their functions by finding the homologous functional class/domain/binding-sites/binding-partner. The homology and machine learning approaches are useful in detecting the functional regions for non-coding RNAs, however they have limitation on resolving non-coding RNA sequence length and dearth of ability to categorize their interacting partners (proteins). In the recent years, methods were developed to find the binding partners for novel non-coding RNAs (92-97). Suresh *et al*., (2015) summarized

non-coding RNA -protein interaction prediction methods that use sequence and/or structure information and he also proposed RPI-Pred (RNA-protein interaction predictor) method (97) to find correct interacting transcription factor/protein. The RPI-Pred (97) was tested with the all non-coding RNAs from six model organisms and was further extended to non-coding RNAs -protein interaction network construction. One step advance, the algorithm, 'fast predictions of RNA and protein interactions and domains at the Center for Genomic Regulation, Barcelona, Catalonia' (catRAPID) (94), was developed to predict the possible binding sites for given protein and non-coding RNA sequences. However, the result of catRAPID (94) was not tested with any experimental approaches, thus in some cases the result was not reliable. Nevertheless, the methods still need improvement to predict the exact binding site/ domain for given non-coding RNA sequence. Once the binding site or functional domain is identified with specific transcription factor, it will be mapped with cancer specific pathway to understand cellular mechanism of such non-coding -protein interactions.

11. EPIGENETIC UNDERSTANDING OF NON-CODING RNA FOR POTENTIAL BIOMARKERS

On other hand, the correlation between epigenetic alterations (DNA methylation and histone modification) and non-coding RNAs in various cancer types revealed epigenetic related non-coding RNA biomarkers. Our recent studies have also proved that the DNA methylation has significant importance for understanding various cancer types (98-100). Li *et al*., (2015) performed genome-wide DNA methylation analysis and discovered epigenetically dysregulated non-coding RNAs in human breast cancer (101). Zack *et al*., (2013) performed an analysis on somatic copy number alterations (SCNAs) promoted cancers and the results improve current understanding of the development and functional consequences of cancer-related SCNA (102). Recently, White *et al*., (2014) concluded that transcriptome data provides the foundation to understand the role of cancer associated non-coding RNAs and helps to improve knowledge of transcriptome-cancer associations as well as to develop novel biomarkers (103). These studies on other cancer types are helpful to understand the regulatory mechanisms of brain cancer associated non-coding RNAs.

12. FUTURE DIRECTIONS

Brain metastasis is a multi-step process involving various components such as extracellular matrix, collateral-vascular system, blood-brain barrier and brain microenvironment. The roles of non-coding RNAs in these steps of brain metastasis still remain unclear. For example, cancer stem cells are considered as an essential factor for metastasis and ample evidence leads to the notion that the brain microenvironment supports stemness of cancer cells (104). However, it's not clear which non-coding RNAs are involved in this step. It's also unknown whether non-coding RNAs are involved in cancer cell-microenvironment interactions involved in the disruption of the blood-brain barrier and its role in inducing cancer cell dormancy. Moreover, even though exchange of non-coding RNAs between cancer cells and surrounding cells through extracellular vesicle have been observed in several types of cancers (72, 97, 105), it is still unclear whether a similar process is involved in brain metastasis. There are also several technical issues limiting the study of non-coding RNAs in brain metastasis that need to be resolved. One of them is the need to develop a proper animal model which can be used to monitor cancer cell-brain microenvironment interactions. The brain microenvironment is enriched with different types of parenchymal cells such as vascular cells, glial cells, pericytes, fibroblasts and neural cells. The major limitation of *in vitro* models is the inability to properly track the roles of non-coding RNAs in a complicated multi-interaction network. At the same time, while extracellular vesicles may take major role in cancer progression and treatment, a lack of specific markers of distinct extracellular vesicles makes the study of non-coding RNAs in different types of extracellular vesicles ambiguous. Recent evidence suggests that there is heterogeneous regulation of secretory non-coding RNAs and endogenous non-coding RNAs (74). Isolating extracellular vesicles for secretory RNA profiling independent of cells is, therefore, needed. Another challenge is the assessment of complete small non-coding RNA transcriptomes involved in the initiation and progression of different types of cancer. More comprehensive profiling and bioinformatics are needed for revealing novel non-coding RNAs and elucidating their roles in brain metastasis. Profiling extracellular vesicles that are independent of cells will help address the significance of heterogeneous regulation of secretory non-coding RNAs. Novel techniques such as RPI-Pred (97), which predicts non-coding RNA-protein interaction, may increase the resolution of screening and reveal the underlying mechanism, in particular for lncRNAs, as their roles remain largely unclear.

Translational use of non-coding RNAs is still at an infant stage, leaving many unsolved and poorly understood concepts. However, there is great hope to develop novel non-invasive biomarkers as more attention has been drawn into microenvironment, extracellular vesicles and secreted non-coding RNAs from cancer cell and stromal cells. At the same time, many promising studies have been undertaken to target non-coding RNAs for therapeutic purposes utilizing cutting edge technologies. Further systematic studies on non-coding RNAs with a new *in vivo* model system for brain metastasis and development of innovative target

screening technology may lead to a discovery of novel therapeutic drugs for patients with brain metastasis.

13. CONCLUSIONS

Brain metastasis is a complex, multi-step process where non-coding RNAs play a significant role. Although discoveries have been made on the roles of various non-coding RNAs including microRNAs and lncRNAs, their detailed characteristics remain unclear. The microenvironment is known to be tightly associated with the metastatic colonization. Crosstalk between cancer cells and environmental cells greatly dictate the fate of the metastasis event. However, the exact biology of the non-coding RNAs and extracellular vesicles, which transport content between cells, is not well studied in brain metastasis. At the same time, non-coding RNAs are also shown to have potential as a tool for early diagnosis and treatment for brain metastasis. Brain metastases continue to pose a significant clinical challenge and progressive understanding of these basic concepts are required in order to translate potential biomarkers and existing targets to improve outcome of patients with brain metastasis.

Further, the bioinformatics and systems biology approaches are helpful to investigate the functional roles of non-coding RNAs in brain cancer cell types, by integrating genetic data to build and characterize non-coding RNA regulatory networks, and by using non-coding RNA primary sequences and secondary structures to discover its associations with functional proteins, such as transcription factors and chromatin remodeling enzymes, and consequently to reveal its regulatory mechanism. This integrative network analysis will lead to a comprehensive understanding of non-coding RNA functions in biological and cellular process, and their specific regulatory roles in brain cancer.

14. ACKNOWLEDGEMENT

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