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The potential diagnostic and prognostic role of extracellular vesicles in glioma: current status and future perspectives

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Abstract

Lack of appropriate diagnostic/prognostic tools for Glioblastoma (GB) is considered one of the major setbacks in the early diagnosis and treatment of this deadly brain tumor. The current gold standard for its diagnosis and staging still relies on invasive biopsy followed by histological examination as well as molecular profiling. Nevertheless, non-invasive approaches are being explored and one example is through the investigation of extracellular vesicles (EVs) in the biofluids of GB patients. EVs are known to carry molecular cargoes such as DNA, mRNA, miRNA, proteins and lipids in almost every type of body fluids. Thus, molecular signature of GB may be present in the EVs derived from these patients. This review focuses on the diagnostic/prognostic potential of EVs in GB, through presenting recent studies on (i) molecular components of EVs, (ii) links between EVs and GB tumor microenvironment, and (iii) clinical potential of EV biomarkers, together with the technical shortcomings researchers need to consider for future studies.

Introduction

Glioblastoma (GB), the deadliest and most prevalent form of brain tumor, arises from glial cells with an incidence rate of 3.2 per 100,000 populations (1) and the numbers are increasing every year (2). GB can develop as primary tumor (*de novo*) without known clinical/histological evidence, or secondarily from low-grade tumors by transformation. Most GB are primary which occur predominantly in older patients and have poorer prognosis (3). Genetic changes vary in GB, for example, IDH1 mutation is evident in secondary GB only (4) and p53 mutations are more common in secondary GB than primary (5). Other common mutations in GB include, epidermal growth factor receptor (EGFR) (40-57%) (6), platelet-derived growth factor receptor (60%) (7), mouse double minute homolog 2 (10-15%) (5), the phosphatase and tensin homolog (PTEN) gene (20-34%) (8), NF1 (13.7%) (9), and PIK3CA (12%) (9). Loss of heterozygosity on the chromosome arm 10q accounts for 60-90% in all GB cases (10). Based on these genetic variances and their gene expression, GB can be further divided into four subtypes, namely, proneural, neural, classical and mesenchymal (11, 12). Distinct genetic and epigenetic variations are associated with each subtype that may be a result from intra-tumor heterogeneity (13).

GB patients have poor prognosis and short median survival time (only 14.6 months). Current treatment modalities include maximal surgical resection (the first line treatment), followed by radiotherapy with complementary chemotherapy using temozolomide (14).

However, these aggressive measures failed to suppress its recurrence in almost every case. This is because of the highly heterogeneous nature of GB tumors (12) and their associated diverse cellular and invasive phenotypes (15-18). In addition, within the same tumor displaying common gene profile there exists a variety of gene copy number variations and subtype-specific signatures (19). These patient-specific variations may be useful as biomarkers for prognosis during therapy.

Like other cancer cells, GB cells are capable of communicating with neighboring cells through shedding a range of molecules. Numerous recent studies have shown that molecules secreted by tumor cells are often encapsulated in lipid layer-based structures, known as extracellular vesicles (EVs). These tumor-derived EVs are involved in immune regulation (20-22), angiogenesis (23), tumor progression (24) and intercellular communication by exchanging proteins and RNA (25, 26). Based on recent literature, this review explores the potential roles of extracellular vesicle in GB pathogenesis and discusses their possibility as a source of diagnostic, prognostic and predictive biomarkers for GB.

Extracellular vesicles: definition, biogenesis, release and uptake

According to their origin and/or size, EVs can be classified into three subclasses, which are apoptotic bodies, microvesicles and exosomes (27). During the programmed cell death process, cells release apoptotic bodies as blebs. Their size ranges between 1000 and 5000 nm (28). Microvesicles, also known as ectosomes, are produced in the plasma membrane by a process of external budding with size ranging from 100 to 1000 nm (29). The smallest of EVs are the exosomes (30 - 100 nm), which are produced inside the cell through internal budding of vesicles in the lumen of early endosome (30). EVs were originally described as a means of removing biological waste from cells (31). It is now evident that they play a very significant role in intercellular communication, not only for normal body functions but also in disease states such as cancer (32, 33). Furthermore, EVs are also known to determine tissue organization (34), promote sperm egg formation (35), and affects the mating behavior (36). In addition, EVs are suggested to be involved in structural remodeling of central nervous system (37, 38).

Microvesicle and exosome biogenesis are different. Microvesicles are produced through the outward budding of the plasma membrane while exosomes are generated by fusing the multivesicular bodies to the plasma membrane. We will focus our discussion on the biogenesis, release and uptake mechanism of exosomes. The biogenesis of exosome begins at endosome formation through invagination of the plasma membrane. The endosomes are divided into three different compartment inside cell during the endocytic process and they are early, recycling and late endosomes (39). Early endosomes are formed after plasma membrane invagination and are able to fuse with endocytic vesicles leading to different cellular fates e.g., recycling, secretion or degradation (40). After sorting the recycled amount into recycling endosomes, the remaining early endosomes transform into late endosomes (32). They accumulate to form intraluminal vesicles through three different pathways: (i) endosomal sorting complexes required for transport (ESCRT) dependent pathway (41, 42), (ii) alternative ESCRT pathway (43), and (iii) ESCRT-independent pathway (44). Cytosolic molecular cargoes including proteins, lipids and nucleic acids are inserted into exosome during intraluminal vesicles formation and the contents differ according to their different

biogenesis pathways. Intraluminal vesicles are stored within multivesicular bodies before being degraded by lysosome or released as exosome through fusion with the plasma membrane (39).

Like its biogenesis, exosome is released through different mechanisms. One mechanism is through Rab GTPases and it is evident that RAB11, RAB27A, RAB27B and RAB35 are involved. EVs release via RAB11 and RAB35 are enriched in flotillin, Wnt, transferrin receptor and PLP (45). But, RAB27A/B-dependent release involves late endosomal proteins e.g., TSG 101, CD63 and ALIX (46, 47). RAB7 is only involved in exosome release by breast tumor cells (43, 47). Rab GTPases independent mechanisms are also evident in some cases (48-51).

Endocytosis, ligand/receptor interaction and fusion with the plasma membrane are mechanisms described so far for cellular uptake of exosome (52). Different endocytosis processes such as clathrin-mediated endocytosis, phagocytosis, macropinocytosis are also responsible for exosome internalization. Cell types, physiological states, surface ligands and receptors may affect the uptake mode. For example, in neurons exosome uptake involves phagocytosis or clathrin-dependent endocytosis (53). Ligands/receptors such as heparin sulfate proteoglycans and scavenger receptor type B-1 are important in exosome uptake because blocking them decreased exosome uptake by cells *in vitro* (54, 55). Hydrogen ion concentration can affect the plasma membrane fusion process as exosome fusion and the release of its contents into the cytosol can be promoted by pH-sensitive fusogenic peptides (56, 57).

The molecular cargoes of extracellular vesicles

EVs contain a variety of proteins, receptors, lipids and nucleic acids in its lipid bilayer or within its aqueous compartment (58). However, the contents can vary according to their biogenesis and cell of origin. Proteins related to membrane function (e.g., ICAM1, integrins), EV biogenesis (TSG101, ALIX), uptake, and release (Annexins, Rab proteins) are commonly found in EVs (59). In addition, a large array of tetraspanins e.g., CD9, CD37, CD53, CD63, CD81 and CD82 (59); proteins related to antigen presentation e.g., HLA-G, MHC; cytokines e.g., VEGF-A (60), semaphorin 3A (Sema3A) (61), TGF-beta (62) and EGFRVIII (63) could also be present. The lipid composition of EVs has not been fully investigated but it has been shown that their lipid bilayer is enriched with sphingomyelin, cholesterol, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, prostaglandin and ganglioside GM3 (64, 65). EV nucleic acid contents are diverse in nature, and they are usually fragmented (66). Previous research showed that, most of them are small RNAs, especially rRNAs and tRNAs (67), but other small RNAs are also present e.g., short and long non-coding RNAs, mRNAs, and miRNAs (68-70). Data on mitochondrial and genomic DNA content in EVs are scarce (71, 72).

Extracellular vesicles and the GB tumor microenvironment

It has been postulated that one of the major roles of EVs in the central nervous system is to exchange molecules between different types of cells and help to maintain their normal functions (73). In pathological conditions such as GB, tumor cells use EVs for their own benefits to promote angiogenesis, clonogenicity, heighten cell proliferation and invasion (74,

75). By transferring non-coding RNAs (76), oncogenic EGFRvIII (77), histones (78), PTEN (79), and pro-migratory factors (80), EVs themselves can influence tumor microenvironment and transform normal cells into malignant cells.

Like other cancers, hypoxia in GB upregulates the production of angiogenic factors (81). Both *in vitro* and *in vivo* experiments confirmed that the level of hypoxia relates to mRNA and protein increase in EVs (82). A recent study has demonstrated that EVs could transfer hypoxia-dependent miRNAs between CD133⁺ U87 glioblastoma cells and vascular cells (83). Invadopodia, a structure formed in highly metastatic cancer cells, can proteolytically degrade extracellular matrix by protruding its actin rich structure into the cell membrane (84). Functional invadopodia are present in glioma cell lines (85) and increased levels of Tks5, an adaptor protein critical for invadopodia formation, is correlated with poor survival (86). EV biogenesis can also affect invadopodia formation, stabilization and protein secretion (87). Recently, it has been reported that five genes, namely actin-related protein 3, insulin-like growth factor 2 receptor, integrin- β 1, annexin A1 and programmed cell death 6-interacting protein, related to invadopodia formation are highly expressed in GB tumor-derived EVs (88). Furthermore, EVs may affect angiogenesis through its action on endothelial cells (89) and through transferring miR-1 (90) and Sema3A (61). A recent study confirmed that, glioma stem cell derived EVs could alter endothelial cell angiogenesis through miR-21/VEGF/VEGFR2 signaling pathway (91). In fact, immune activity of cells of monocytic lineage may be altered by up taking GB-derived EVs and shift their protein expression and cytokine secretion toward a pro-tumoral phenotype (92). *In vivo* experiments also showed change in phenotype of brain immune cells that had taken up GB-derived EVs (93). These observations suggest that, EVs can alter tumor microenvironment by exchanging signals between brain cells, which ultimately provide suitable environment for tumor growth.

Diagnostic/prognostic potential of EV biomarkers

The diagnosis of GB is heavily dependent on neuroimaging (e.g., MRI) and tissue biopsies. However, both approaches for predicting response to therapy are somewhat error prone. MRI can only detect established tumors with sufficient mass (94). It is also difficult to assess the therapeutic response by MRI, because imaging properties of the tumor are often interfered by intervention. Increased or decreased contrast enhancement may have happened due to the interaction of chemo radiation and anti-angiogenic agents with the tumor microenvironment, respectively (95, 96). On the other hand, tumor biopsy is invasive in nature and causes brain swelling and hemorrhage resulting in de-regulation of brain function (97). It is also impossible to get longitudinal and/or repeated sampling, and to determine the real picture of intra-tumoral heterogeneity (98). This limits our ability to predict chemotherapeutic resistance, monitor treatment response, clear differentiation between pseudo progression and real progression (99).

Recently, EVs emerge as a promising source of biomarkers for diagnostic and prognostic purposes. They can be non-invasively collected for longitudinal sampling (97); their large array of molecules allows characterization of the global tumor genome and transcriptome (100); their short half-life enables detection of rapid changes in the tumor milieu (101). Besides, its inherent stability and capability to maintain the integrity of its contents allow researchers to analyze DNA, RNA and proteins from solid tumors. It also provides a potential

link between tumor drug-resistance and metastasis. Skog et al. first suggested the potential diagnostic role of EVs in brain cancer in 2008 (102). His group concluded that, EVs isolated from serum could be used to detect the evolving genetic changes relative to tumor progression at any given time. In line with this idea, Noerholm et al. showed that distinct RNA expression pattern is present in serum EVs of GB patients compared with controls (103). The miR-301a level in EVs is significantly increased and correlated with overall survival (104). In fact, nucleic acid variations could be detected in EVs collected from GB patients e.g., IDH1 (105), EGFRvIII (63), miR-21 (106), miR-1587 (107) and EPHA2 (108). EV nucleic acids can also serve as a source of biomarkers that depicts chemotherapeutic resistance in GB patients. For example, O⁶-methylguanine-DNA-methyltransferase mRNA expression level was found to be increased in EVs collected from GB patients resistant to temozolomide (109). Other nucleic acid biomarkers like miR-181d and miR-603 (110), alkylpurine-DNA-N-glycosylase (108), and miR-221 (111) may serve similar purposes. EV protein content may also serve as biomarkers. Pinet et al. highlight that TrkB expression is increased in EVs isolated from plasma of GB patients (112). Another study showed that Sema3A is expressed on the surface of EVs released by patient-derived glioblastoma cells (61).

Temozolomide (TMZ) is an alkylating agent commonly used in GB treatment together with radiation therapy after surgical resection of the tumor (113). It is now proven that TMZ affects EVs secretion (114) and could confer drug resistance to recipient cells by transferring molecular cargos through EVs (115). Mass spectrometry based analysis reveals that protein levels related to cell adhesion, e.g. β 1-integrin, are increased in EVs after TMZ treatment (114). Whereas TMZ resistant cell derived EVs containing miR-151a are able to generate drug resistance in recipient cells (115). It is therefore possible to monitor TMZ failure by analyzing the molecular components of GB tumor-derived EVs (116). Other EV surface proteins such as CD44 and CD133 (117) may serve as biomarkers for chemoresistant GB patients. It is because glioblastoma stem-like cells responsible for chemoresistance and tumor recurrence also express the same cell surface markers (118). Selected EV-derived molecules and their roles in glioblastoma are summarized in Table 1.

Although EVs have the potential for GB diagnosis, most research conducted to date are proof-of-principle studies highlighting their roles in tumor biology. Nevertheless, several EV components such as EGFR mutation, mutant IDH1 and miR-21 have gained more attention recently. Molecular methods such as qRT-PCR for the detection of EGFRvIII in EVs derived from serum and cerebrospinal fluid (CSF) have shown high specificity (>95%). Unfortunately, the sensitivity is rather low (~60%) (102, 119). Similarly, IDH1 mutant detection using the more sensitive BEAMing ddPCR technique was positive only in CSF EVs but not those from serum (105). Comparable findings are also reported in EV associated miRNAs detection (120). In contrast, more promising results are obtained in solid tumors including those of prostate, lung, esophagus and ovary (121-125). In particular, using EVs for early detection of pancreatic cancer have been studied extensively (126-129).

Challenges and future perspectives

The current techniques used for EV isolation have their own advantages and disadvantages, as described in Table 2. The most challenging task regarding utilization of EVs and their components in clinical settings is to isolate pure EV populations without contamination with similar sized particles (130). Some unwanted biomolecules may non-specifically attach to

the surface of EVs and act as confounding variables. There is also a lack of knowledge on whether compositional difference in EVs from different origins reflect their functional aspects or not. Other challenges include the standardization of the quantitation and characterization methods, as well as downstream analysis of EV contents. Electron microscopy is currently used for characterizing EVs along with flow cytometry, but both techniques require expensive equipment and may not be suitable for routine use (131, 132).

EVs are present in a wide array of biofluids but it is still uncertain which source is the most suitable in terms of their isolation and compositional representation (133). The concentration of DNA and RNA in EVs differs significantly (134). Their nucleic acid content varies greatly from patient to patient, and different isolation methods can give very different results (135). EVs purified from serum, plasma, CSF, cell culture media and other sources vary greatly, in terms of yield, and the amounts of nucleic acid recovered (136, 137). For example, a recent study showed that miRNA species and their abundance are highly variable among EVs isolated from plasma and cell culture media (120).

For glioma diagnosis, EVs from CSF is preferred since their source is confined to the central nervous system. Whereas EVs from blood is contaminated by those derived other cell types in the circulation and thus reduce the abundance of glioma-specific EVs (138). Physical characteristics of blood (e.g., viscosity and density) and high abundance of lipoproteins also limit the availability of target EVs. High and low density lipoproteins can transport miRNA that may be co-precipitated with those derived from EVs (139). Furthermore, small lipoproteins such as chylomicrons that have similar sizes as EVs may interfere with their isolation (140). Apart from relevance to brain tumor, CSF-derived EVs exhibit higher reproducibility and accuracy in determining miRNA compared with blood (141, 142). In spite of this advantage, CSF is a poor source for detecting circulating tumor cells (143).

Other than the source of EVs, their collection, isolation and storage could also pose problems for downstream analyses (144). Time of collection and physical activities can influence the level of circulating EVs (145). The latter is suggested to help release more EVs in the circulation (146). Regarding isolation methods (summarized in Table 2), none of them could easily be translated into routine clinical use in terms of cost, time, user friendliness and reproducibility. The tube quality and preservatives used may cause artifactual vesicles formed and some cells are capable of producing EVs on storage (147).

There are a number of existing techniques available for analyzing the molecules present in EVs, including RNAseq, droplet digital PCR, microarrays, and real time PCR. Each technique has its own sensitivity and specificity. Their properties are summarized in Table 3. In summary, the major bottleneck for EVs to be useful in clinical diagnosis is the standardization of their sources for different disease, and the collection and isolation methods to be used.

Conclusion

EVs hold great promises as a non-invasive source of biomarkers for clinical applications. Their ease of collection and the stability of their contents provides a window into the pathological status of the patient. They could even be used for early diagnosis of disease like

GB in which the clinical symptoms are non-specific! A major hurdle though is in the purification of EVs without contamination by particles of similar sizes. Furthermore, the profile of biomarkers in the EVs must be clearly defined and correlated to the disease state.

Molecules	Biological source	Biological significance	Method of detection	References
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With more interdisciplinary efforts, these hurdles will be overcome leading to the concrete use of EVs for diagnosis and prognosis in GB, and many other types of diseases.

Table 1: Selected EV-derived molecules and their roles in glioma.

miR-301a	Serum	up regulated in GB patients, promote proliferation and invasion of glioma-derived H4 cells, levels downregulated after tumor removal and independently associated with overall survival	qRT-PCR	(104)
miR-124a	Mesenchymal stem cells	viability and clonogenicity in glioma stem cells reduced in vitro, in vivo transfer of miR-124a showed that 50% of animals lived long	qRT-PCR	(148)
miR-373	U87 cell culture medium	elicit proangiogenic response, cell proliferation	Affymetrix microarrays	(83)
miR-1587	Mesenchymal stem cells	increase proliferation and clonogenicity	Illumina next-generation sequencing	(107)
VEGF-A	Patient-derived glioma stem cells	promote angiogenesis, increase endothelial permeability	qRT-PCR , ELISA	(61)
EGFRvIII	1) Serum 2) Cerebrospinal fluid	1) EGFRvIII expressed both in serum exosome and tumor tissue samples (the sensitivity and specificity in serum were 79.31% and 51.58% respectively), expression level inversely correlated with survival. 2) EGFRvIII mutation present in CSF Extracellular vesicles but not in normal tissue (the sensitivity and specificity are 61% and 98% respectively), amplification of wild-type EGFR present in both CSF derived EVs and normal tissue.	qRT-PCR	(63, 119)
miR-221	Cell culture	positively correlated with proliferation, migration and temozolomide resistance	qRT-PCR	(111)
Annexin A1	Cell culture	GB tumors have higher level of annexin A1 compared with normal brain in silico	quantitative high-resolution mass spectrometry	(88)
RBM11	Cell culture	apoptotic EVs transfer of RBM11 to recipient cells transform Cyclin D1 and MDM4 to pro-oncogenic Cyclin D1a and MDM4s isoforms, which indicate worse survival of GB patients.	qRT-PCR	(149)

CRYAB	Cell culture	upon stimulation by cytokine, U373 cells produce and secrete more CRYAB and increase the cancer progression related proteins	ELISA	(150)
miR-151a	CSF	miR-151a overexpression correlates with improved prognosis, whereas reduced expression indicates poor prognosis	FISH	(115)
RNU6-1	serum	small noncoding RNA (RNU6-1) at higher levels in GB patients	qRT-PCR	(151)
miR-148a	serum	miR-148a levels elevated in GB patients, affects cell proliferation and metastasis	RT-PCR	(152)
PTRF	serum	PTRF/CD63 ratio significantly higher in Grade IV glioma	Western blot	(153)
miR-21	CSF	EV miR-21 levels higher than control group	qRT-PCR	(154)
miR-10b	CSF	miR-10b levels significantly higher in GB patients compared with non-neoplastic control group	qRT-PCR	(141)
IDH1	CSF	mutant IDH1 mRNA detected in GB CSF samples	ddPCR, BEAMing	(105)
miR-24, miR-103, miR-125	CSF	levels of miR-24, miR-103, miR-125 significantly higher in experimental group	qRT-PCR	(120)
cric-TTBK2	Tissue, cell culture	expression level of cric-TTBK2 higher in GB tissue and cell culture; promotes cell proliferation, migration, and invasion, while inhibits apoptosis	qRT-PCR, in situ hybridization	(155)

Table 2: Overview of extracellular vesicles isolation.

Technique name	Isolation procedure	Benefits	Demerits
Ultracentrifugation	EVs separated based on their size, shape and density	Cost effective in terms of reagents, high-throughput	Expensive in terms of instrumentation, extensive manual labor, long procedure, may interfere downstream analysis
Ultrafiltration	EV isolation is exclusively based on their size or molecular weight	Rapid, simple instrumentation, easy to operate, direct RNA extraction possible	Purity not so high, clogging and vesicle trapping may reduce the yield
Precipitation	Precipitation done through altering their solubility by tying up water molecules	Easy to handle, requires simple instrumentation, sizable sample capacity	Frequent contamination, time-consuming, requires pre and post cleanup
Immuno-affinity capture-based techniques	Interaction between surface antigens of EVs and immobilized antibodies	Able to isolate specific EVs, purity of EVs are extremely high, able to sub-categorize.	Costly, need to design immobilized antibody every time, low quantity and low output, binding capacity hampered by tumor microenvironment
Microfluidics-based approaches	Micro/Nano scale isolation based on physical and biochemical properties of EVs	Quick, inexpensive, handy, easy to operate, high portability	Need standardization on large cohort of clinical samples, low input and low yield may affect downstream analysis
Size exclusion chromatography	EVs are separated on the basis of size on a single column	High recovery rate, fast, no EVs-aggregation, high purity and integrity	Concentration of isolated EVs are too low, similar sized particles may co-isolate
Density gradient centrifugation	EVs are isolated in a sucrose and iodixanol density gradient according to their size and mass density	Separation efficiency and purity are higher than ultracentrifugation, samples remains in intact form	Laborious, time-consuming, low throughput, isolated samples may be contaminated by same size particles, complex instrumentation process.

Table 3: Techniques currently used for extracellular vesicles research with their test indexes.

Experiment Name	Test Indexes	Advantages	Disadvantages
Flow cytometry	Marker proteins, concentration	Able to determine the cellular origin, high-throughput, it's possible to analyze thousands exosomes in one sample.	Low sensitivity, limited resolution, a significant number of particles remain undetected, sometimes EVs are counted as a single event signal.
Nanoparticle tracking analysis	concentration	Can precisely measure small particles, samples remain intact during experimental period, sample preparation is very fast and easy, fast measurement, recovery of samples possible after measurements.	It's very tough to get proper dilution, larger particles may mask the smaller ones.
Transmission electron microscopy	concentration	High resolution, it's possible to detect heterogeneous population	Extensive and multistep sample preparation required, the electron beam may also cause damages to biological samples.
Western blot	Marker proteins	Able to detect as little as 0.1 ng, selectively detect a target protein.	Time consuming, High cost and high technical demand, need to optimize the process, prone to false or subjective result.
ELISA	Concentration, marker protein	Highly sensitive and specific, quick and convenient, it is possible to measure a particular surface marker protein both qualitatively and quantitatively.	Requires large amount of EVs, low detection limit.
Quantitative real time PCR	Mutation, RNA	Can be performed easily in most labs, Inexpensive, well established method, no post PCR processing required.	Less sensitive in compare to ddPCR, complexity arises due to simultaneous thermal cycling and fluorescence detection, it's quite challenging to quantify EV miRNAs.
Digital droplet PCR	Mutation, DNA methylation, copy number variation	Superior in sensitivity and feasibility in compare qRT-PCR, highly tolerant to inhibitor, no need to rely on references and standards, able to detect copy number variation.	It requires highly allele-specific probes, overestimation may be caused due to DNA denaturation during partitioning, Molecular dropout and sample inhomogeneity causes underestimation, the initial cost of equipment are quite high, expensive in terms of consumables.
Next generation sequencing	RNA, Mutation	It can distinguish miRNAs at resolution of single base, able to analyze hundreds of miRNAs individually or in panel in a single experiment, it's possible to detect abnormalities	Library preparation for RNA-Seq could be a challenging job, sophisticated bioinformatics systems required, highly expensive and time

		across the entire genome.	consuming, the sensitivity of identification of rare mutations are quite low.
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