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REVIEW PAPER

MicroRNAs as precise diagnostic biomarkers: A review

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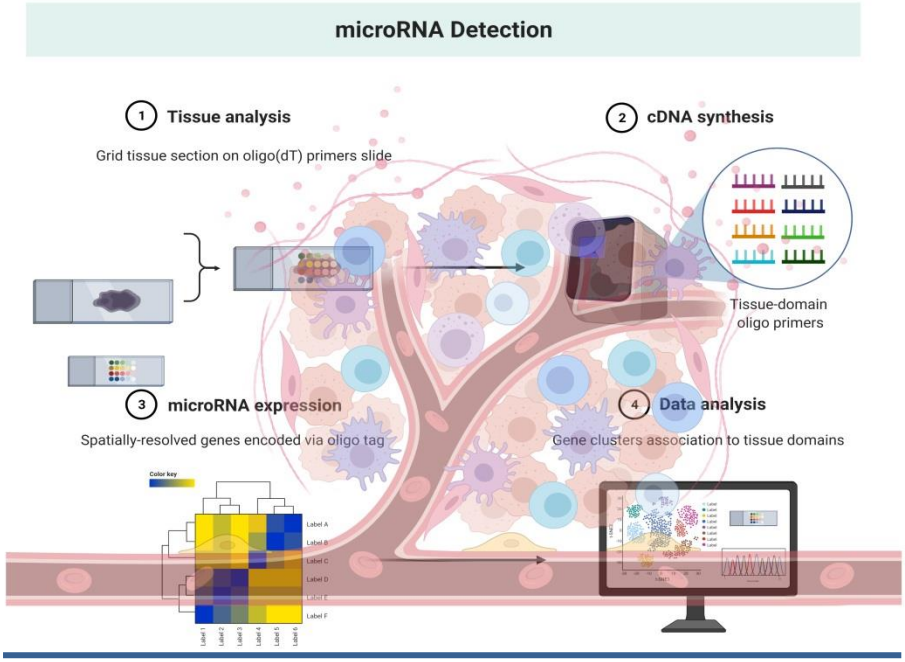
Highlights

- Intracellular miRNA levels affect the expression of many genes, and are connected to cellular signaling and cellular metabolisms such as Cell division, regeneration, and apoptosis.
- Real-time qPCR is widely used for miRNA identification because of its large dynamic range, excellent accuracy, and sequence uniqueness.
- NGS is capable of collecting a variety of materials, including body fluids, and generating sequence information for almost 10 million randomly selected nucleic acid molecules.
- NGS is a great technique for doing research and identifying genetic variants that need multi-gene analysis due to a large number of readings.

Graphical Abstract



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Abstract

miRNAs (also known as endogenous noncoding RNAs, or "ncRNAs") are a family of endogenous ncRNAs that are small (about 22 nucleotides in length). Too far, about 2,500 distinct microRNAs have been reported in various publications and new interest in miRNA biological function has arisen due to the recent advancement in molecular biology, As a result, it's unsurprising that abnormal miRNA expression plays a role in the pathogenesis of a variety of illnesses, including cardiovascular disease, neurodegenerative disease, and cancer. Current needs are not being satisfied by conventional miRNA detection techniques. In contrast, immunoassay techniques, and next-generation sequencing (NGS) have been extensively used to detect miRNA with great sensitivity. These novel methods have advanced the functional study and clinical diagnostics of miRNAs. In this paper, we highlight the latest advancements in miRNA detection methods and their possible future uses. It will guide follow-up methods that are very sensitive and specific as well as pertinent to disease diagnosis and treatment.

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Introduction

MicroRNAs (miRNAs) are tiny noncoding RNAs (21-24 nucleotides in length) that act as negative posttranscriptional regulators of gene expression. The organism's regulatory network includes a wide range of miRNAs and messenger RNAs, with a single miRNA regulating hundreds of mRNA molecules. Intracellular miRNA levels affect the expression of many genes, the effects of which are connected to cellular signaling and cellular metabolism (Figure 1). Cell division, regeneration, and apoptosis are all under their supervision (1). As a result, it's unsurprising that abnormal miRNA expression plays a role in the pathogenesis of a variety of illnesses, including cardiovascular disease, neurodegenerative disease, and cancer. So far, about 2,500 distinct microRNAs have been reported in various publications (2-4). MicroRNAs have also been discovered in the extracellular fluid - blood plasma, serum, and urine. microRNAs are non-random and hence have been demonstrated to reflect the occurrence of certain events within the body (5, 6). While circulating miRNA's collection of data, along with the fact that circulating miRNA is known to be rather stable, bolsters the case for the use of circulating miRNA as a clinically meaningful biomarker, further research is necessary to determine its therapeutic relevance. At present, there is a large amount of research ongoing that is investigating the identification of individual miRNAs and groupings (profiles) of several miRNAs as a diagnostic or prognostic technique (7, 8).

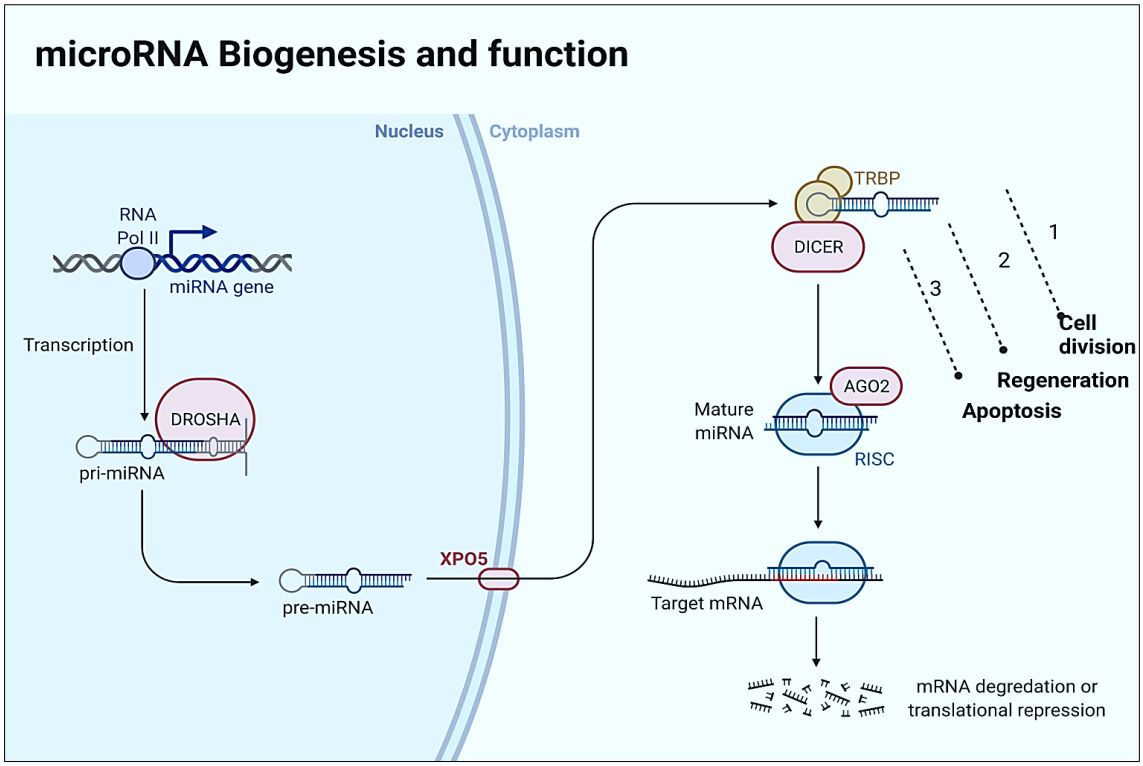

Figure 1. Intracellular miRNA levels affect the expression of many genes, the effects of which are connected to cellular signaling and cellular metabolisms such as Cell division, regeneration, and apoptosis.

microRNAs detection methods

Traditional methods

Northern blotting

The much more widely utilized approach for miRNA detection is northern blotting. Both mature and precursor miRNAs can be identified. It is not reliant on specialized equipment. The general idea is that when a restriction enzyme digests the RNA sample, it is separated on an agarose gel, then denatured, and transferred to a nitrocellulose film or nylon membrane, which is followed by a step in which the isotope or other probes are added before the RNA hybridizes with it (9, 10). Autoradiography or other appropriate methods may be used to identify miRNAs after washing the free probe. Despite having several advantages, there are downsides to

Northern blotting such as being semi-quantitative, limited throughput, laborious, and prone to RNA degradation. As a result, a very precise experimental design is required. Northern blots are insensitive, which means they cannot detect small RNAs. While specific radioisotope-labeled probes assist in improving sensitivity, they also increase the likelihood of eliciting a response (11, 12). Numerous improvements have happened in recent years in light of the aforementioned restrictions. Locked nucleic acid (LNA) is a special type of oligonucleotide molecule with a rigid structure made by linking the carbons at the 20th and 40th positions on ribose with a methylene group. The possibility exists to enhance the primer or probe's melting point through the use of this type of nucleic acid (Tm value). LAN is beneficial for hybridization processes by improving their heat stability, hybridization effectiveness, and miRNA detection sensitivity. By replacing DNA oligonodonucleotide probes with LNA oligonucleotide probes, the researchers improved the thermal stability of the complex formed between the target miRNA and the oligonucleotide probes, which in turn improved the experimental sensitivity and shortened the experiment time (13, 14). To reduce the risk, used probes that were not radiolabeled. They sped up the experiment by using a reagent that linked RNA to the proper membrane, such as EDC. The sensitivity and capability of northern blotting still fall short, however, in determining the quantity of RNA in specific cells (15).

Real Time PCR

Real-time qPCR is widely used for miRNA identification because of its large dynamic range, excellent accuracy, and sequence uniqueness. The gold standard for miRNA detection is real-time qPCR. Such methods begin with reverse transcription of the target miRNA into cDNA. Following that, real-time fluorescence detection was enabled via polymerase chain reaction (PCR) amplification (16, 17). It is possible to synthesize cDNA by reverse transcription using stem-loop primers (which have around 15, 41, and 42 sequence spots), poly(T) adapters (which have 16 sequence spots), or a gene-specific primer (which has a tail sequence) with Taq-Man, but in this case, Taq-Man and SYBR Green fluorescent dye are frequently used to monitor qPCR. SYBR Green is a fluorescent dye that serves as an intercalating agent between dsDNAs, amplifying the fluorescence signal by an order of 800-1000 times. The reliability of qPCR assessments of particular amplification products is reduced using SYBR Green due to the inability to distinguish nonspecific products such as primer dimers. The homogeneity of qPCR results is evaluated using the dissociation curve analysis (18, 19). dsDNA melts when its length and base composition coincide with the degree of polymerization, whereas the number of inflection points on the melting curve reveals the total number of PCR products, including primer dimers. As a result, an appropriate dissociation curve should have a single point of inflection, while numerous inflection points indicate the existence of nonspecific products (20).

Taq-Man probes are oligonucleotide probes tagged with a reporter group at the 50th position and a fluorescence quenching group at the 30th position. Between the two primers are their binding sites. The procedure is more specific and repeatable than the SYBR Green method because it does not utilize primers that generate nonspecific signals or primer dimers that provide a fluorescent signal. While real-time qPCR is highly sensitive, it has limitations in terms of false positives and the added complexity of building primers to guarantee correct findings (21). Quantification is accomplished via the use of real-time qPCR, which entails the integration of several steps. Each of these stages must be changed independently prior to testing. Numerous variables must be taken into account in order to get repeatable results. RNA extraction, RNA integrity check, cDNA synthesis, primer design, amplicon detection, and data normalization are just a few of the procedures available. Also, was established a new quantitative two-step real-time polymerase chain reaction (RT-PCR) technique for miRNA identification (22). They are used real-time stem-loop primers to reverse-transcribe miRNAs. The reverse transcriptase amplification was completed, and the resultant cDNA samples were analyzed using TaqMan real-time quantitative PCR equipment. This stem-loop structure may be used to discover miRNAs that have been missed by microarrays, as well as other non-coding RNAs such as those generated from endogenous genes (including RNA transcripts) and other non-coding RNAs such as pirRNA

and siRNAs. This is the first time that computer prediction and bioassay screening have been combined, resulting in a large-scale method capable of screening thousands of miRNA species. For miRNA identification, was compared northern blotting to real-time qPCR. The northern blot's use of gel electrophoresis enables the quantification of RNA on a membrane via RNA electrophoresis (23). Additionally, research indicates that it surpasses other widely utilized transcription factors in degrading RNA and conducting transcription experiments. Unlike Northern blotting, which requires a certain length of cDNA probe and a specific quantity of RNA, Southern blotting requires a specific amount of RNA and a specific length of cDNA probe. As a result, it is ineffective for identifying defective genes or screening the whole population (24). Real-time qPCR is more sensitive and specific than conventional PCR, but has a wider quantification range. It confronts many challenges, including variability in RNA templates, poor experimental design, uneven data processing, and insufficient data standards (25).

Microarrays

Complementary DNA and RNA sequencing on microarrays (DNA/RNA chips) is accomplished via the use of natural parallelization. Covalently attaching a synthetic DNA probe to a particular location on a solid surface complements the DNA sequence existing on that surface. Numerous kinds of probes may be covalently bonded to a tiny surface, and hybrids can be detected using fluorescent probes in a range of hues (26). Micro multiplexing's main benefit is that it enables the development of many channels. Today's commercial miRNA detection methods (e.g., Thermo Fisher Scientific's GeneChip miRNA 4.0 arrays or EXICON's miRCURY LNA microRNA array) are capable of efficiently assessing hundreds of miRNAs in a single sample, and customers may choose miRNA targets based on their particular needs (27). It should be possible to get very precise information on the amounts of nucleic acids using microarrays. However, there is a quantitative bias among the many genes and transcripts. Rather than performing absolute measurements, microarrays are frequently used to quantify microRNA (miRNA). Microarray detection has limitations owing to the fluorescence detector's sensitivity, which requires the use of costly equipment. In contrast to routine/standard concentration measurements, the primary use of microstructures in today's market is the differential expression of miRNA (28).

Newly developed methods

NGS

NGS is a term that refers to a collection of techniques for massively multiplexed sequence analysis of DNA and RNA. Unlike conventional Sanger sequencing, which needs a homogeneous DNA template as an input, NGS is capable of collecting a variety of materials, including body fluids, and generating sequence information for almost 10 million randomly selected nucleic acid molecules (29). NGS is a great technique for doing research and identifying genetic variants that need multi-gene analysis due to a large number of readings. A comprehensive selection of NGS methods and associated equipment is available for purchase (30). The Illumina technique is the most commonly utilized since it progressively uses fluorophore-labeled nucleotides and fluorescence imaging. The most sensitive method for identifying and measuring miRNAs is a next-generation sequencing (NGS). The benefit of this technique is that it enables the discovery of hitherto uncharacterized short RNAs. However, the whole NGS procedure is lengthy and needs a large number of samples. Additionally, it is very costly-it necessitates the purchase of chemicals, instruments/equipment, and extremely complex assessment software (31-36).

Immunoassay techniques

Numerous molecules, including microRNAs, have been identified as disease indicators for a variety of human diseases. Only a tiny percentage will be converted into regular clinical usage. A significant issue is determining the reliability of new markers on platforms widely used in clinical labs (37-39). In order to detect

miRNA biomarkers such as qRT-PCR and next-generation sequencing, which are available in a restricted number of clinical laboratories, current molecular methods such as qRT-PCR and next-generation sequencing are very expensive. Additionally, interoperability with high-throughput clinical processes is a challenge (40). MiRNA assays may be more widely used if they are adapted to platforms and technologies that address these problems. Immunoassays include many features that set them apart from current test formats. They have a reduced time to result, are faster than NGS, and use immunoassay technology. Additionally, the test is inexpensive (41). The most important factor in immunoassay is that a substantial number of central labs and hospitals have deployed the required equipment. Immunoassay platforms are already widely used in clinical labs around the globe, and a variety of immunological assays are performed on these commercial systems. Additionally, immunoassays may be adapted for use with immunoassay analyzers. In essence, immunoassays look for or detect the analyte (microRNA) in a sample by using an antibody that interacts with it and generating an immunological reaction between it and the analyte (42-46).

Discusion

Currently, there are several professional immunoassay platforms in use throughout the world, and these platforms include a broad range of immunological tests. Another feature of immunoassays is that they may be used with analyzers that perform immunoassays. Immunoassay uses antibodies to collect samples that have an immunological reaction that can then be detected using the method. As we stated before, a new ELISA-based technique for measuring miRNA was developed. These simple methods are usually seen in non-laboratory settings.

Conclusion

Numerous investigations have indicated that miRNAs are differently expressed in sick tissues and highly abundant in plasma, serum, and other body fluids, suggesting that they may be relevant for routine clinical diagnosis. Researchers have primarily sought to identify miRNA signatures that are representative of diseases, such as cancer, viral infections, nervous system disorders, cardiovascular disorders, muscular disorders, and diabetes, and several experiments have been conducted particularly to verify miRNAs as biomarkers and to further understand their function in regulating physiological and pathological processes. To make the most of miRNAs as biomarkers, we need to learn more about their molecular features in these diseases. The major hurdles to miRNA patterns being employed in in vitro diagnostics are collecting unique miRNAs that can be consistently used as distinct and dependable "ideal" biomarkers for diseases across a broad patient population and creating simple and inexpensive detection tools.

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