

DETECTION TECHNIQUES OF CIRCULAR RNAs AS NEW BIOMARKERS IN SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT

Systemic lupus erythematosus (SLE) is severe, chronic autoimmune disease affecting mainly young active individuals, leading to disability and premature death. Recent studies have reported long non-coding RNAs (lncRNAs) to participate in the pathogenesis of the disease. Among lncRNAs, the circular RNAs (circRNAs) gain growing scientific attention due to their stability in body fluids. This makes them suitable for new non-biomarkers for evaluation of SLE activity and promising therapeutic targets. Methods for detecting of circRNAs are evolving rapidly. The aim of this review is to present these techniques and their advantages and disadvantages.

Keywords: *circRNAs, long non-coding RNA, SLE*

INTRODUCTION

Systemic lupus erythematosus (SLE) is the prototype multisystemic autoimmune disease with a chronic course of remissions and exacerbations, leading in many cases to severe end-organ damage and substantial disability (1,2). Its etiology is complex and includes environmental, hormonal, genetic and epigenetic factors (3). Typical for the disease

are vigorous autoantibody production, generation of immune complexes and immune-mediated tissue injury (2,4). The clinical presentation can vary among patients from mild mucocutaneous or joint manifestations to life-threatening renal or central nervous system involvement.

Given this heterogeneity, SLE is a challenge not only to treat, but also to diagnose and monitor. That is why in recent years there has been an intensive search of biomarkers for early diagnosis and for more accurate evaluation of disease activity. In the wide spectrum of biomarkers, non-coding RNAs (ncRNAs) appear to have the potential of being the ideal biomarker - specific, sensitive and noninvasive. ncRNAs are the dominant product of eukaryotic transcription and are associated with key biological processes and diseases. Generally, there are: 1) small non-coding RNAs (sncRNAs) with a length equal to

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or less than 200 nucleotides and 2) long non-coding RNAs (lncRNAs) - with a length of over 200 nucleotides (5). lncRNAs are involved in various important biological processes such as chromatin remodelling, gene transcription, RNA splicing and others. The functional consequences of a change in the expression of lncRNAs are large and pathogenetically significant. Recent studies have shown that lncRNAs are involved in the pathogenesis of immune-mediated inflammatory diseases such as rheumatoid arthritis, autoimmune thyroiditis and SLE (6-8). This makes them suitable targets for therapy and allows them to be studied as potential biomarkers for early diagnosis and assessment of activity in SLE (9-12). Recently, a newly discovered type of lncRNAs – circular RNAs (circRNAs) have received great scientific attention. They are resistant to exonucleases and have a stable structure, which prolongs their half-life; they also have a tissue-specific expression and a relation to developmental stage or age abundance (13). Since a growing number of studies have revealed circRNAs as a potential clinical biomarker for SLE (14-17) and for lupus nephritis (LN) (11, 18-20), their detection is of great importance. Along with the increasing knowledge about these RNAs, the methods

for their detection are also being developed. The aim of this article is to review these techniques and present their advantages and disadvantages.

METHODS FOR CIRC RNAS DETECTION

In the early studies, the detection of circRNAs has been achieved with electron microscopy (21). Nowadays, various techniques are used to detect circRNA expression and to investigate their functions. For example, by using RNA-Seq and microarray researchers can identify circRNAs (22). Validation methods include polymerase chain reaction (PCR) (23), northern blot (24), in situ hybridization (25). Usage of enzymatic methods like RNase R and RNase H degradation assay is optional and helps researchers to achieve correct identification of circRNAs (26,27). Distinguishing between circRNAs and linear transcripts is possible with 2D gel electrophoresis (28) and gel trap electrophoresis (29). In circRNA analysis, several methods are combined for detection and validation of this type of lncRNAs. In this context, the order of the used detection methods often is as it follows: circRNA selection, RT PCR, Sanger sequencing (Fig. 1).

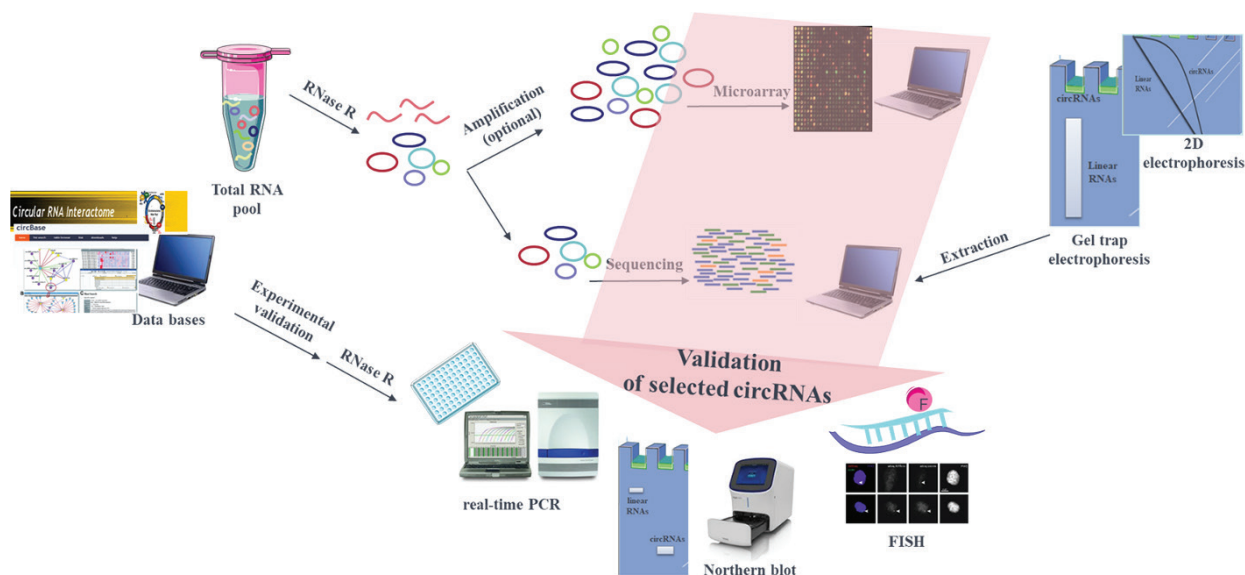


Fig. 1. Workflows in circRNA analysis where different methods for detection and validation are combined.

Total pool of RNA can be treated with RNase R and enriched with circRNAs for microarray or sequencing and subsequently data analysis. The amplification step is recommended when there is a low-input material. Next, for validation of circRNAs of interest real time PCR, northern blot or fluorescent in situ hybridization (FISH) can be used. Another strategy begins with electrophoresis - the separated circRNAs are then extracted, sequenced and validated. A third possible workflow starts with circRNA selection from available databases. They give an information about the circRNA sequence, network interactions, expression and disease-related data.

PCR technique is the most frequently used one for validation of in silico-selected circRNAs.

CircRNA Selection

CircRNAs can be selected from databases, microarray or RNA sequencing. High-throughput analyses like microarray and RNA sequencing include prior treatment with RNase R for linear RNA reduction and enhancement of circRNA detection (30). If RNA sequencing is performed at high depth (100-150 million reads), enrichment of circRNAs is not necessary (31). Microarrays profile only known circRNAs through hybridization. While microarrays recognize circRNAs based only on the junction sequences, they do give information on the internal sequence of a given circRNA. Moreover, microarray cannot distinguish among circRNAs with common junction sequences (31). However, microarray offers good annotation and analysis related to circRNA biology like miRNA binding sites (32). On the other hand, RNA Seq has limited annotation information and generates large data (32). At the same time, RNA sequencing has a higher discovery power and is recommended for the detection of novel circRNA transcripts (30) (Table 1). For circRNA detection from RNA-Seq data different bioinformatics tools are used. They can be divided into two groups: 1) with “pseudoreference-based” or “candidate-based” strategy, when they require circRNA sequence with the gene annotation and 2) with “segmented-based” strategy, when they relay backsplicing junction identification from the mapping information generated by alignment of the reads to the reference genome or transcriptome (33). All computational approaches have their own advantages and some authors find dramatic differences in their sensitivity (34). As a conclusion, computational circRNA analyses are still being developed – still the choice of algorithm for the genome-wide detec-

tion of circRNA significantly impacts false positive and false negative rates of detection, and they need further development.

Many databases have been developed to facilitate circRNA studies. Some of them provide the sequences of given circRNAs or clarify circRNA position in the genome and expression pattern in tissues and cells (circBase); and propose a design of divergent primers and prediction of miRNA or protein targets (CircInteractome) (33).

Enzymatic Methods

Enzymatic methods are optional and often performed in combination with other techniques since they provide circRNAs enrichment due to the enzyme-based depletion of linear RNA fragments (35,36,37). Limitations in exoribonuclease RNase R usage are related to incomplete efficiency of the enzyme and the possibility to introduce technical noise (37,38). It is possible that stable structures in linear RNAs block the enzyme action too.

Other less frequent enzymatic methods, which leave circRNAs but not most of the linear RNAs intact, include RNase H (26), tobacco acid phosphatase, and terminator exonuclease treatment (35). When two short DNA probes are annealed to the RNA of interest, RNase H cleaves that RNA. Run on a gel and blotted for the fragments, the circular RNA of interest appears as two bands. If RNA is cleaved in the presence of each DNA probe separately, circular species are shown by one band (38).

Usually, the enzymatic methods go before RNA-seq, microarray or RT-PCR analyses in order for true circRNAs to be detected and quantified. However, some linear RNAs such as snRNA can overcome the enrichment step and affect downstream analyses

Table 1. Comparison between microarray and RNA-seq in circRNAs detection

Microarray	RNA Seq
Detect only known circRNAs	Detect novel circRNAs
Principle of hybridization	Principle of cloning & sequencing
High input of total RNA	Low input of total RNA
Limited to the probes on the array	May include linear RNAs resistant to RNase R
Relies on junction sequences and does not inform on mature sequences	Does not inform on variants
Good annotation	Still evolving annotation
Desktop computational resources	Large data and high demand of computing power

(37). To resolve this problem, Pandey et al. developed the so-called RPAD method, based on initial RNase R treatment followed by poly(A) tailing and poly(A) depletion (39).

PCR

The PCR technique is the speediest and most effortless detection method, which is usually used for validation of the selected by microarray or NGS circRNAs. Divergent and not convergent primer pairs are required for expression validation of the selected circRNAs (40). In comparison with convergent primers, divergent ones can amplify the circular form and are resistant to RNase R (41). There are already web tools for the design of divergent primers (42). Linear RNAs, such as GAPDH and β -actin, are usually used as internal control genes (43,44). There are several studies that have reported that circRNAs are better choice reference genes for the detection of other circRNAs (45). Zhong et al. suggest hsa_circ_0000284 and hsa_circ_0000471 may be used as reference genes for circRNAs and for other RNAs, too (46).

Sanger Sequencing

When a not characterized or not yet validated circRNA is being analysed, Sanger sequencing of the obtained PCR product is recommended for final validation (40). Sanger sequencing requires purification of PCR products using phenol/chloroform/isoamyl alcohol precipitation, and then the existence of the back-splice site is validated.

Northern Blotting

In general, the Northern blot starts with denaturation and separation of RNA samples by gel electrophoresis. Agarose gels are recommended for circRNAs from 0.2 up to a few kb. However, if circular and linear RNAs have the same size, they cannot be distinguished by their running behavior. In polyacrylamide gels circRNAs have mobility relative to linear markers, therefore these gels are suitable for circRNAs only up to 1 kb (24). The separated RNA samples with different size are transferred and immobilized onto a membrane. Then a labelled probe against specific RNA sequences is hybridized with the membrane. After membrane washing and depending on the probe labelling, the hybrid signals are detected through different methods. The specific circRNA detection is determined: 1) by the choice of probe regions and the detection principle; 2) by

the choice of a gel matrix (24). So, the design of the probe is one of the important steps in this analysis. The probe usually has 100-200 nt in length and could be selected against continuous exon region or against exon-exon junction. The exonic probe allows simultaneous detection of a linear transcript, thus their usage requires further validation of the circularity by RNase R, for example.

FISH

Visualization of different RNA species within the cells is allowed by fluorescence *in situ* hybridization (FISH) (47). When FISH is coupled with high-resolution microscopy it becomes a powerful method for analyzing the subcellular localization of RNA. However, the detection of circular RNAs (circRNAs) using microscopy is challenging since circRNA junction is the only target for the FISH probe in order to avoid linear RNA recognition (48).

2D Electrophoresis and Gel Trap Electrophoresis

For distinguishing circRNAs from linear RNAs two-dimensional denaturing polyacrylamide gel electrophoresis and gel trap electrophoresis can also be used. When total RNA is run on a 2D gel, circular and linear RNAs show different migratory patterns. Linear RNA migration is along the diagonal, whereas circRNA migration is off of the diagonal and resembles an arc (28). circRNAs can be excised from the gel and used in next generation sequencing (NGS) as an enrichment step (49). In gel trap electrophoresis only linear RNAs migrate in the gel. CircRNAs are held in the well, thus forming the circRNA pool. It is possible for circRNAs to be extracted from the gel and sequenced via NGS (49).

CONCLUSION

The functions of the majority of circRNAs are still unknown. However, circRNA detection and validation are an initial step before biogenesis and functional analysis. That is why the developing of these identification methods will give a precise way to explore the functional relevance of circRNAs that are involved in SLE pathogenesis as well as the opportunity for these circRNAs to be used as biomarkers of a new generation.

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