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Methods to Quantify microRNAs in the Myc Gene Network for Posttranscriptional Gene Repression

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Abstract

As a global transcription factor, Myc regulates both protein-coding genes and noncoding microRNA genes. Myc-activated or repressed miRNAs are involved in various pathways to affect tumorigenesis, mediate apoptosis, proliferation, angiogenesis, metastasis, and metabolism downstream of Myc. Functional characterization of miRNAs in the Myc network requires the accurate detection and quantification of miRNA expression levels. Here, we describe two widely used methodologies to determine miRNA expression, including miRNA real-time PCR and miRNA northern analysis.

Keywords

Myc; miRNA expression; Real-time PCR; Northern analysis

1 Introduction

Myc encodes a basic helix-loop-helix transcription factor that acts as global gene regulator [1]. The aberrant activation of the *Myc* gene has been frequently observed in many human tumor types. *Myc* activation constitutes the molecular basis of many aberrant cellular processes during malignant transformation, including uncontrolled cell proliferation and cell growth, evasion of programmed cell death, enhanced angiogenesis, increased motility, cancer metabolism, and metastasis [2, 3].

As a potent transcription factor, Myc acts as a global gene regulator that activates or represses numerous target genes in the large network of major oncogenes and tumor suppressors. To date, majority of studies on Myc have focused on the Myc-regulated protein-coding genes and their diverse biological functions downstream of Myc. Yet recent studies have revealed the functional importance of noncoding RNAs, particularly microRNAs (miRNAs), in the gene network regulated by Myc. miRNAs are a class of small, noncoding RNAs that mediate posttranscriptional gene silencing of many mRNAs [4, 5]. In addition to its direct transcriptional regulation of protein-coding genes, the ability of Myc to regulate many miRNAs—thus to indirectly regulate gene expression posttranscriptionally—further expands its capacity as a global gene regulator. Hence, miRNAs regulated by Myc

constitute an important molecular mechanism underlying its pleiotropic functions during diverse developmental and pathological processes.

The polycistronic *miR-17-92* is the first miRNA gene identified as the target of Myc [6]. Myc can directly bind to the E-box sequence located at the *miR-17-92* promoter, increasing the level of both pri-miR-17-92 and, subsequently, its mature miRNA components [6]. Enforced *miR-17-92* expression accelerates *c-Myc-induced* B-lymphomagenesis due to impairment of *c-Myc-induced* apoptosis [7]. As a miRNA oncogene downstream of Myc, *miR-17-92* mediates pleiotropic biological functions, promoting cell survival, cell proliferation, and angiogenesis in a cell type- and context-dependent manner [8–11] (Fig. 1). Following the initial discovery of *miR-17-92* as a key mediator of Myc function, a number of miRNAs have been identified as major Myc targets that mediate its diverse developmental and pathological functions. In response to the activation of the EGFR signaling, Myc activates the transcription of *miR-7*, which subsequently enhances cell proliferation and tumor formation [12] (Fig. 1). Both Myc and N-Myc upregulate *miR-9* in human mammary epithelial cells, which targets the epithelial cell adhesion molecule E-cadherin to promote cell motility and tumor invasion [13] (Fig. 1).

In addition to transcriptional activation, Myc can also regulate the transcriptional repression of miRNA genes. miRNA profiling studies using both human and mouse B-cell lymphoma cells identified the widespread repression of miRNA expression upon Myc activation, including *miR-15a/16-1*, *let-7*, *miR-34a*, *miR-26a*, and *miR-23a/b* [14]. Myc-repressed *miR-15a/16-1*, *let-7*, *miR-34a*, and *miR-26a*, when overexpressed, all promote apoptosis and decrease proliferation by repressing antiapoptotic genes and cell cycle genes in a cell type-dependent manner [15–22] (Fig. 1). Myc-repressed miRNAs, *miR-23a*, and *miR-23b* also mediate cancer metabolism. For instance, glutaminase (GLS) is a direct target of *miR-23*. The transcriptional repression of *miR-23a/b* by Myc leads to increased expression of GLS, which ultimately upregulates glutamine metabolism in cancer [23] (Fig. 1).

Studies of miRNAs in the Myc network require sensitive and reliable methods to detect and quantify the expression of miRNAs. Here, we describe two methods to fulfill this purpose: miRNA real-time PCR and miRNA northern blot. Both methods are optimized based on previous reports [24, 25], providing a powerful and quantitative methodology for determining miRNA expression levels in the complex Myc signaling network.

2 Materials

2.1 miRNA Real- Time PCR

1. RNA isolation: TRIzol Reagent (Life Technologies), chloroform, isopropanol, 75 % ethanol, and nuclease-free water.
2. Poly(A) tailing of miRNAs: Poly(A) Polymerase Tailing Kit (Epicentre), RNaseOUT (Life Technologies), and nuclease-free water.
3. cDNA synthesis of poly(A) tailed miRNAs: small RNA reverse transcription primer
(CGAATTCTAGAGCTCGAGGCAGGCGACATGGCTGGCTAGTTAAGCTTG

GTACCGAGCTCGGATCCACTAGTCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
N), 10 mM dNTP, nuclease-free water, RNaseOUT (Life Technologies), and SuperScript III reverse transcriptase (Life Technologies).

4. Real-time PCR: miRNA-specific forward primer, universal reverse primer (CGAATTCTAGAGCTCGAGGC), TaqMan probe (6FAM-CTCGGATCCACTAGTC-MGBNFQ, Life Technologies), Taq Man Universal Master Mix without UNG AmpErase (Life Technologies), nuclease-free water. miRNA-specific forward primer is designed based on the sequences of target miRNAs, and this design should obey the general guidelines of primer design. The primer sequences can be the exact sequences of target miRNAs, or few nucleotides shorter at 5' or 3' end to make qualified primers. U6 snRNA (GCAAATTCGTGAAGCGTTCC) is used as the endogenous control for real-time PCR.
5. NanoDrop 1000 Spectrophotometer (Thermo Scientific).
6. 96 well clear low-profile PCR microplate, ultra clear pressure-sensitive sealing film (Axygen), 7. 7900 HT Fast real-time PCR system (Applied Biosystems).

2.2 miRNA Northern Blot

1. Sequa Gel, 7.5 M urea gel system (National Diagnostics).
2. Dye loading buffer: 95 % formamide, 0.025 % bromophenol blue, 0.025 % xylene cyanol FF, 5 mM EDTA, stored in aliquots at −20 °C.
3. 10× TBE: 1 M Tris base, 1 M boric acid, 0.02 M EDTA, stored for up to 6 months at room temperature.
4. Decade marker system (Life Technologies): Mix 1 µl decade marker RNA (100 ng), 6 µl nuclease-free water, 1 µl 10× Kinase Reaction Buffer, 1 µl [gamma-³²P] ATP >3,000 Ci/mmol, and 1 µl T4 Polynucleotide Kinase (New England Biolabs). Incubate at 37 °C for 1 h. Add 8 µl nuclease-free water and 2 µl 10× cleavage reagent. Incubate at room temperature for 5 min. Add 20 µl dye loading buffer. Heat the mixture at 95 °C for 5 min. Store the labeled marker at −20 °C.
5. Running buffer and transfer buffer: 0.5× TBE in nuclease-free water.
6. Hybond N + nylon membrane (GE Healthcare).
7. Whatman 3 MM chromatography paper (Whatman).
8. 100× Denhardt's solution: 2 % Polyvinylpyrrolidone, 2 % Bovine serum albumin, 2 % Ficoll 400, 2.5 mM EDTA; store at −20 °C for long-term storage.
9. 20× SSC: 3 M NaCl, 0.3 M Trisodium citrate; adjust the pH to 7.0 before autoclave, and store at room temperature.
10. Pre-hybridization/hybridization mix: 50 % formamide, 5× SSC buffer, 3× Denhardt's solution, 200 µg/ml Salmon Sperm DNA, 0.1 % SDS; prepare fresh before each use.

11. Washing buffer: 2× SSC, 0.1 % SDS in nuclease-free water.
12. HL-2000 HybriLinker including HB-1000 hybridization oven and UV cross-linker (UVP).
13. Typhoon Trio variable mode imager (Amersham Biosciences) and storage phosphor screen (Molecular Dynamics).
14. Microspin G-50 columns (GE Healthcare).
15. T4 Polynucleotide Kinase (New England Biolabs).
16. [γ - 32 P] ATP, 6,000 Ci/mmol, 10 mCi/ml (Perkin Elmer).

3 Methods

3.1 miRNA Real-Time PCR

3.1.1 RNA Isolation

1. Add 1 ml TRIzol Reagent to ~50 mg tissue or $\sim 5 \times 10^6$ harvested cells.
2. Homogenize tissue sample using tissue grinder or lyse cell sample by pipetting the cells up and down several times (*see* Note 1).
3. Incubate the homogenized sample for 5 min at room temperature.
4. Add 0.2 ml of chloroform. Shake tube vigorously by hand for 15 s.
5. Incubate for 2 min at room temperature.
6. Centrifuge the sample at $12,000 \times g$ for 15 min at 4 °C (*see* Note 2).
7. Transfer the aqueous phase into a fresh Eppendorf tube. Avoid drawing any of the interphase or organic layer into the pipette.
8. Add 0.5 ml of 100 % isopropanol to the aqueous phase and mix well.
9. Incubate for 10 min at room temperature.
10. Centrifuge at $12,000 \times g$ for 10 min at 4 °C (*see* Note 3).
11. Discard the supernatant from the tube, leaving the RNA pellet in the tube.
12. Wash the RNA pellet with 1 ml of 75 % ethanol.
13. Centrifuge at $7,500 \times g$ for 5 min at 4 °C. Discard the supernatant.
14. Air dry the RNA pellet for 5–10 min (*see* Note 4).
15. Resuspend the RNA pellet in nuclease-free water by pipetting up and down several times.
16. Measure the concentration of the dissolved RNA using a NanoDrop.

¹It is critical to homogenize the samples as quickly as possible in TRIzol. Make sure that the solution is clear and uniformly viscous.

²The mixture separates into an upper aqueous phase, an inter-phase, and a lower phenol-chloroform phase. RNA remains only in the aqueous phase.

³RNA will form a white pellet on the bottom of the tube after centrifugation.

⁴Do not overdry the RNA pellet because the pellet can lose its solubility.

3.1.2 Poly(A) Tailing of miRNAs

1. Combine the following components: 2 µg RNA, 1 µl Poly(A) Polymerase 10× Reaction Buffer, 1 µl 10 mM ATP, 0.5 µl RNaseOUT (40 U/µl), 0.25 µl Poly(A) Polymerase (4 U/µl). Add nuclease-free water to total 10 µl.
2. Incubate at 37 °C for 30 min.
3. Stop the reaction by immediate freezing at −20 °C (*see* Note 5).

3.1.3 cDNA Synthesis of Poly(A) Tailed miRNAs

1. Add 1 µl small RNA reverse transcription primer (1 µg/µl), 2 µl 10 mM dNTP, and 10 µl nuclease-free water to 10 µl of poly(A) tailed small RNAs (*see* Note 6).
2. Heat the mixture to 65 °C for 5 min, followed by incubation on ice for at least 1 min.
3. Collect the content of the tube by brief centrifugation, and add 7 µl 5× First-Strand Buffer, 2 µl 0.1 M DTT, 2 µl RNaseOUT (40 U/µl), and 1 µl SuperScript III reverse transcriptase (200 U/µl).
4. Mix by pipetting gently. Incubate the mixture at 50 °C for 60 min.
5. Inactivate the reaction by 15 min of heating at 70 °C.
6. Add 65 µl nuclease-free water to bring final volume to 100 µl. Store the cDNA at −20 °C.

3.1.4 Real-Time PCR

1. Dilute the stock cDNA 20 times to the working concentration using nuclease-free water (*see* Note 7).
2. Combine the following components into one reaction: 1 µl cDNA at the working concentration, 0.5 µl miRNA-specific forward primer (10 µM), 0.5 µl universal reverse primer (10 µM), 0.4 µl TaqMan probe (10 µM), 7.6 µl nuclease-free water, and 10 µl TaqMan Universal Master Mix without UNG AmpErase (*see* Note 8).
3. Transfer the reaction mixture to each well of an optical 96 well PCR microplate. Seal the plate with an ultra clear sealing film and centrifuge briefly to position reaction mix at the bottom of the well.
4. Run the following program on a real-time PCR system:

95 °C, 10 min (enzyme activation);

95 °C, 15 s (denaturation) and 60 °C, 1 min (annealing/extension) for 40 cycles (*see* Note 9).

⁵This reaction can be stopped by phenol/chloroform extraction and salt/alcohol precipitation.

⁶It is recommended to use the primer purified by RNase free HPLC.

⁷The dilution factor depends on the abundance of the target small RNAs in the tissue or cell samples.

⁸TaqMan probe is light sensitive. Therefore, the tubes containing TaqMan probe must be wrapped with aluminum foil.

⁹3-step cycling can be used alternatively, anneal at optimal temperature for 30 s followed by extension at 60 °C for 30 s. The T_m of primers determines the optimal annealing temperature.

5. Analyze the results according to instrument user guide.

3.2 miRNA Northern Blot

1. Resuspend RNA samples (~10–15 µg of total RNA) in 10 µl of dye loading buffer, and heat it at 95 °C for 5 min.
2. Load the RNA samples and the radioactively labeled decade marker onto a high-percentage (10–15 %) denaturing poly-acrylamide-urea gel. Run the gel ~ 25 W until the bromophenol blue is near the bottom of the gel (*see* Note 10).
3. Disassemble the gel and place it in transfer buffer for 5 min. Cut six sheets of Whatman 3 MM paper and Hybond N + nylon membrane into the same size of the gel, and wet them with transfer buffer.
4. Place three Whatman 3 MM papers on anode plate of Semi-Dry Transfer Cell (Bio-Rad), and then put pre-wet Hybond N + nylon membrane on top of the papers. Lay the gel on top of membrane and add another three Whatman 3 MM papers. Remove any air bubbles by rolling a pipette on the gel sandwich. Finally, add a few drops of transfer buffer before putting the safety lid on to keep the sandwich wet.
5. Transfer at constant 800 mA for 1 h with the maximum voltage of 25 V.
6. Disassemble the sandwich and put the membrane on a Whatman 3 MM paper.
7. Expose the membrane, with the RNA surface facing up, to UV light cross-link for 1 min (254 nm, 1,200 mJ auto-cross-linking setting). Dry the membrane overnight between two Whatman 3 MM papers at 4 °C (*see* Note 11).
8. Put the membrane in glass hybridization bottle with 5 ml pre-hybridization mix. Pre-hybridize in a pre-hybridization mix with rotation for at least 2 h at 42 °C in a hybridization oven.
9. End label a radioactive northern probe using the complementary DNA oligo to the mature miRNA sequence. To do so, 100 ng DNA oligo is diluted in 3.25 µl nuclease-free water. Add 1 µl 10× Reaction Buffer, 0.75 µl T4 Polynucleotide Kinase, and 5 µl 6,000 Ci/mmol [gamma-³²P] ATP to the reaction mix, and incubate at 37 °C for 1 h. Add 60 µl nuclease-free water to the reaction, and clean the probe with G-50 spin column. Denature the probe at 95 °C for 5 min.
10. Add the radiolabeled probe in fresh hybridization mix and hybridize at 42 °C overnight (*see* Note 12).
11. Dispose the hybridization mix, and wash the membrane at 42 °C for 20 min in washing buffer. Repeat this washing step one more time (*see* Note 13).

¹⁰It is important to thoroughly rinse the wells before loading the samples in order to have sharp and clear bands. Remaining urea will result in fuzzy bands on the blot.

¹¹Do not UV cross-link the membrane more than once. Multiple cross-linkings will reduce the hybridization efficiency of the probe. EDC chemical cross-linking can be used as an alternative method [24].

¹²Hybridization temperature may vary. If cross hybridization happened, higher temperatures should be used instead of 42 °C. It is also recommended to increase the hybridization time for low abundant miRNAs.

¹³Higher stringent washing buffer should be used in case of high background. On the other hand, lower stringent washing buffer must be used if the signal is weak.

12. Cover the membrane with plastic wrap and expose it to a phosphor imager screen for phosphorimaging. Scan the screen at 50 μ m high sensitivity on Typhoon scanner after exposure for overnight or longer (*see* Note 14).

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¹⁴Membrane can be stripped with 0.2 % SDS in nuclease-free water. Strip at 80 °C for 1 h. Do not strip one membrane for more than 3 times. When stripping, keep the membrane wet, otherwise probe will be permanently bound to the membrane.

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Fig. 1.
Representatives Myc-activated or repressed miRNAs in various cellular processes during tumor development, including angiogenesis, cell proliferation, metastasis, apoptosis and metabolism