厚生労働科学研究費補助金

創薬基盤推進研究事業

高速シークエンサーを用いたnon-coding RNAまで包括されたトランスクリプトーム解析による新規安全性バイオマーカーの同定

平成２０年度　総括研究報告書

研究代表者　土屋　創健

平成２１（２００９）年４月

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研究代表者　土屋　創健　京都大学大学院薬学研究科助教

研究要旨

本研究の目的は、ギガシークエンサーを用いて、non-coding RNA (ncRNA) まで包括されたデジタルトランスクリプトーム解析を世界に先駆けてトキシコゲノミクスに適用し、従来よりも高感度・高精度な薬物毒性予測を実現する肝・心毒性の新規安全性バイオマーカーを同定することである。

近年、生理作用や病態発現に重要な役割を果たす機能性 ncRNA の存在が明らかとなり、ncRNA に着目した新たな生命科学研究に注目が集まっているが、現在までに ncRNA に着目したトキシコゲノミクスの研究報告は皆無である。そこで、ncRNA まで包括された網羅的なデジタル発現情報を用いることにより、従来までの限局された解析では発見できなかった従来よりも高感度・高精度な薬物毒性予測を実現する肝・心毒性の新規安全性バイオマーカーの同定を試みるとともに、ncRNA の関与する薬物毒性の発症・分子メカニズムの解明、さらには肝細胞や心筋細胞の細胞障害・細胞死を抑制するための治療標的分子の発見を目指す。

初年度である平成２０年度はトログリタゾンによる肝毒性モデルを評価・構築し、ncRNA まで包括されたデジタルトランスクリプトームデータを取得した。現在、これらのデータのバイオインフォマティクス解析を行っており、トキシコゲノミクスにおけるギガシークエンスデータ解析の方法論を確立しつつある。

A. 研究目的

近年、transfer RNA や ribosomal RNA 以外のタンパク質をコードしない RNA、non-coding RNA （ncRNA）が messenger RNA (mRNA) と同等数の２万種類以上転写されていることが明らかとなり、とりわけ、短鎖の ncRNA、microRNA が生理作用や病態発現に重

要な役割を果たすことが見いだされ、ncRNA に着目した新たな生命科学研究が注目・展開されている。しかしながら、現在までに ncRNA に着目したトキシコゲノミクスの研究報告は皆無である。そこで本課題では、次世代型高速シークエンサーであるギガシークエンサーを用いて ncRNA まで包括したデジタルトランスクリプトーム解析を世界に先駆けて行い、従来よりも高感度・高精度な薬物毒性予測を実現する肝・心毒性の新規安全性バイオマーカーを同定することを目的とする。本研究課題の特色としてギガシークエンサーによるデジタルトランスクリプトームデータ解析をトキシコゲノミクスに初めて適用する点であり、それにより mRNA と ncRNA の発現情報をデジタル情報として同時に取得でき、マイクロアレイを用いた解析では困難であった未同定の RNA の検出と各 RNA 内・間に渡る高精度なバイオインフォマティクス解析が可能となる。その結果、従来の解析では見いだせなかった新規安全性バイオマーカーの同定、薬物毒性の新たな発症・分子メカニズムの解明、さらには肝細胞や心筋細胞の細胞障害・細胞死を抑制するための治療標的分子の発見が可能となり、早期毒性予測による医薬品開発期間・コストの軽減化と、国民における医薬品使用の安全性の向上に繋がるものと期待される。具体的には、当該平成２０年度にトログリタゾンによる肝毒性モデルの ncRNA まで包括されたデジタルトランスクリプトームデータの取得を行った。平成２１年度には、このデータを用いて ncRNA を包括したトキシコゲノミクス解析の有用性を評価し、さらに四塩化炭素及びクマリンによる肝毒性データを重層し、個々の薬物に特異的または共通な肝毒性発現メカニズムと新規安全性バイオマーカーの同定を目指し、平成２２年度にはドキソルビシン・シクロフォスファミドによる心筋細胞毒性について解析を行い、心毒性発現メカニズムと新規安全性バイオマーカーの同定を行い、さらに肝細胞と心筋細胞という多種の組織・細胞で共通の安全性バイオマーカーの存在を検証するする予定である。

B. 研究方法

細胞培養：非動化（５６℃、３０分）された 10% (v/v) fetal bovine serum 含有の Dulbecco’s Modified Eagle Medium (Sigma) を用い、コラーゲンコートディッシュにて 37℃、5% CO_2 の条件で HepG2 細胞を培養した。

WST-1 細胞増殖測定：WST-1 を基質とした Nicotinamide adenine dinucleotide (NADH) 還元酵素の活性を指標に、細胞増殖を測定した。HepG2

細胞をトログリタゾン(vehicle (0.1% dimethylsulfoxide; DMSO), 1μM、3μM、100 μM)で刺激し、各０，６，１２，２４、４８時間後に回収した。1x10^4 cells の HepG2 細胞に飽和 WST-1 溶液 (Roche) を加え、37℃、5 % CO_2 の条件下で 1 時間反応させた後、還元型 WST-1 (Formazan) の 460nm とバックグランド補正用の650nmの吸光度をマイクロプレートリーダーで測定した。ギガシークエンスデータの取得：各濃度のトログリタゾン (vehicle, 1, 3, 100 μM) で刺激した HepG2 細胞をそれぞれ０，６，２４時間後に回収し、acid guanidiniumthiocyanate-phenol-chloroform 法を用いて、total RNA を抽出した。Ribominus (Invitrogen)を用いて total RNA から ribosomal RNA を除去した後に RNA の断片化を行い、さらにランダムプライマーをアニールさせて逆転写反応により 1st strand DNA を合成し、引き続いて RNase H と DNA polymerase I を用いて 2nd strand DNA を合成した。この二本鎖 DNA の両末端にシークエンス用のアダプターを結合させ、in situ PCR 及びシークエンスに最適なサイズのもののみをゲル電気泳動により分画した。PCR 反応により in situ で個々のクラスターを形成させ、蛍光標識塩基を用いた１塩基伸長反応を用いることにより、それらの塩基配列情報を取得した。

（倫理面への配慮）

本課題の包括的デジタルトランスクリプトーム解析は商業的に販売されているヒト培養細胞を対象としていることから、倫理面での問題は特にないものと判断される。また、本課題を遂行するにあたり、組換えＤＮＡ実験を行うが、文部科学省並びに京都大学大学院薬学研究科施設の取り扱い規定に準拠し、組換えＤＮＡ実験安全委員会の承認を得るとともに、研究機関等における組換えＤＮＡ実験等の実施に関する基本指針に従う。

C. 研究結果 及び D. 考察

初年度である平成２０年度において、ヒト肝癌由来培養細胞株におけるトログリタゾンの毒性発現条件の最適化とそのncRNAまで包括されたデジタルトランスクリプトームデータの取得を計画・実行した。本研究事業による前年度までの研究の進捗は計画通りである。

ncRNA を包括したデジタルトランスクリプトームデータ解析の意義・有用性を評価するため、*in vitro* での肝毒性評価系として広範囲の薬物において高頻度に使用されているヒト肝癌由来細胞株HepG2と肝毒性を有する薬物としてトログリタゾンを用いて解析を行った。まず、肝細胞毒性の分子

メカニズムを解明し、新規安全性バイオマーカーを同定するために最適な薬物処理時間・用量をWST-1法による細胞生存活性を指標に探索した。vehicle と比較してトログリタゾン100 μM刺激群において刺激後6時間から有意な細胞生存活性の低下が検出され、時間経過に伴い刺激後４８時間において最も顕著な低下効果が検出された。トログリタゾン 1 μM 及び3 μM 刺激群ではそのような低下は検出されなかった。また、薬物活性ではなく薬物毒性に特異的な変動を捉えるため、vehicle とともに、トログリタゾン肝細胞毒性のネガティブコントロールとして、トログリタゾンと同じく peroxisome proliferators activated receptor gamma (PPARγ)のリガンドでチアゾリジン系抗糖尿病薬でありながら肝毒性の少ないピオグリタゾンを使用したところ、ピオグリタゾン刺激（1μM、 3 μM、100 μM）群では有意な細胞生存活性の低下は検出されなかった。以上のことと PPARγ に対するトログリタゾンとピオグリタゾンの EC50(ともに約 1 μM)から、HepG2 細胞に対する TGZ の毒性用量として 100 μM、薬効(非毒性)用量として 1 μM と定義し、高速シークエンサーを用いてこれらの条件における薬物処理後２４時間と未処理の ncRNA 包括デジタルトランスクリプトームデータを取得した。２年目の本年度において、このデータを用いて ncRNA 包括デジタルトランスクリプトームデータの解析アルゴリズムの開発・評価を行い、トキシコゲノミクスにおける ncRNA 包括デジタルトランスクリプトームデータのバイオインフォマティクス解析手法を確立し、ncRNA を包括したトキシコゲノミクス解析の有用性を評価する予定である。

E. 結論

トログリタゾンを用いた肝毒性モデルにおける ncRNA まで包括されたデジタルトランスクリプトームデータを取得した。

F. 健康危険情報

特記事項無し

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得
   無し
2. 実用新案登録
   無し
3. その他
   無し

研究成果の刊行に関する一覧表

書籍

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雑誌

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Takano H., Nakazawa S., Shirata N., Tamba S., Furuta K., **Tsuchiya S**., Morimoto K., Itano N., Irie A., Ichikawa A., Kimata K., Nakayama K., Sugimoto Y. and Tanaka S.	Involvement of CD44 in mast cell proliferation during terminal differentiation.	***Lab. Invest.***	89	446-455	2009

Biogenesis and Function Mechanisms of Micro-RNAs and Their Role as Oncogenes and Tumor Suppressors

Soken Tsuchiya, Kazuya Terasawa, Ryo Kunimoto, Yasushi Okuno, Fumiaki Sato, Kazuharu Shimizu, and Gozoh Tsujimoto

Introduction

Micro-RNAs (miRNAs) are evolutionarily conserved small noncoding RNAs (20–23 nucleotides). MiRNAs regulate various physiological pathways such as differentiation, proliferation, and apoptosis by negative regulation of the gene expressions at the posttranscriptional level [1–3]. Currently, more than 800 human miRNAs have been identified and registered in the miRNA database miRBase [4]. Strikingly, 30% of protein-coding transcripts in humans is predicted to be regulated by miRNAs [5,6]. Recently, miRNAs have been reported to work as oncogenes or tumor suppressor genes and be directly involved in the initiation, progression, and metastasis of various cancers [7–9]. Therefore, we focus on the role that miRNAs play in cancer and the use of miRNAs in drug discovery. Collection of evidence suggests that miRNAs can be potentially useful for understanding tumorigenesis and finding novel strategies for cancer diagnosis and therapy.

Biogenesis of miRNAs

MiRNAs are generated in multiple steps (Fig. 1). Initially, miRNAs are transcribed by RNA polymerase II as long RNA precursors (pri-miRNAs) [10–12]. Pri-miRNAs are usually several kilobases in length, and contain a 7-methyl

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S. Nakanishi et al. (eds.), *Systems Biology*,
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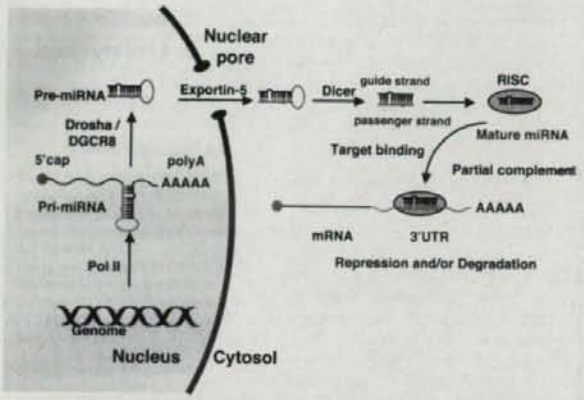

Fig. 1 Schematic diagram of biogenesis and function in micro-RNAs (miRNAs). MiRNAs are transcribed by RNA polymerase II and sequentially processed by Drosha/DGCR8 and Dicer. MiRNA-loaded RNA-induced silencing complex (*RISC*) causes the cleavage or translational silencing of target mRNAs

guanosine cap structure and a poly(A) tail similar to protein-coding mRNAs. The pri-miRNAs are processed into precursors of approximately 70 nucleotides (pre-miRNAs) with a stem-loop structure and a two-nucleotide 3′-overhang by the RNase III enzyme, Drosha, and a double-stranded RNA-binding protein, DGCR8/Pasha [13–15]. Then, pre-miRNAs are transported to the cytoplasm by a member of the Ran transport receptor family, exportin-5 [16,17]. Pre-miRNAs exported in the cytoplasm are further processed by another RNase III enzyme, Dicer, and unwound by a helicase [18]. Finally, only one mature miRNA strand (guide strand) is incorporated into a RNA-induced silencing complex (RISC) that mediates cleavage or translational inhibition of target mRNAs, while the other strand (passenger strand) is quickly degraded [19–21]. The stability of the base pairs at the 5′-end of the duplex determines which strand is incorporated in RISC [22,23]. RISC is composed of Dicer, Argonaute2 (Ago2), and the double-strand RNA-binding protein, TRBP [21,24], and cleaves target mRNAs more efficiently by using pre-miRNAs rather than the duplex RNAs that do not have the stem-loop structure, suggesting that processing by Dicer may be coupled with assembly of the mature miRNA into RISC [21]. The incorporated miRNA guides the RISC to the complementary sequence in the 3′-untranslated region (UTR) of target mRNAs. MiRNAs base-pair to the 3′-UTR of target mRNA with perfect or near-perfect complementarity, leading to the target mRNA degradation by Ago2,

a component of RISC [25]. In contrast, partial base-pairing between a miRNA and a target mRNA leads to translational silencing of a target mRNA without RNA degradation [26]. In partial base-pairing, the binding of some nucleotides in the 5′-region of miRNAs has been indicated to be functionally important by systematic mutation experiments [27,28].

The Role of miRNAs in Cancer

Recently, it has been reported that the expression of several miRNAs is altered in a variety of human cancers, suggesting potential roles of miRNAs in tumorigenesis [29]. Calin et al. [30] showed that more than 50% of miRNAs were located in cancer-associated genomic regions or in fragile sites. In fact, miR-15a and miR-16 genes exist as a cistronic cluster at 13q14, which is deleted or downregulated in most cases (~68%) of B-cell chronic lymphocytic leukemias [31]. Cimmino et al. [32] found that both these miRNAs negatively regulate the expression of B cell lymphoma 2 (BCL2), which inhibits apoptosis and is present in many types of cancer including leukemias. In fact, overexpression of miR-15 and miR-16 in the MEG-01 cell line induces apoptotic cell death.

Alterations in gene copy number of miRNAs are detected in a variety of human cancers [33–35]. Zhang et al. [33] showed that miRNAs exhibited high-frequency genomic alterations in human ovarian cancer, breast cancer, and melanoma using high-resolution array-based comparative genomic hybridization. Hayashita et al. [34] found that the expression and gene copy number of the miR-17–92 cluster composed of seven miRNAs is increased in lung cancer cell lines, especially with small-cell lung cancer histology. Enforced expression of miRNAs included in this polycistronic cluster enhances cell proliferation in a lung cancer cell line. The increase in expression and gene copy number of miR-17–92 cluster was also found in B-cell lymphomas [35]. The expression of miRNAs in this cluster is upregulated by c-Myc, whose expression and/or function is one of the most common abnormalities in human cancers, and miR-17-5p and miR-20a in this miR-17–92 cluster negatively regulate the expression of transcriptional factor E2F1 [36]. Furthermore, it was indicated that the miR-17–19b cluster included in miR-17–92 cluster inhibited apoptotic cell death and accelerated c-Myc-induced lymphomagenesis in mice reconstituted with miR-17–19b cluster-overexpressed hematopoietic stem cells [35]. Furthermore, the miR-17–92 cluster has been reported to augment angiogenesis in vivo by downregulation of antiangiogenic thrombospondin-1 and connective tissue growth factor in Ras-transformed colonocytes [37].

Global expression profiling analysis of protein-coding genes is known to be useful for cancer diagnoses and prognosis predictions [38]. Recently, Lu et al. [29] indicated that miRNA expression profiles can successfully classify poorly differentiated cancers that cannot be classified by mRNA expression profiles. Accordingly, miRNA expression profiles are more accurately correlated with clinical severity of cancer malignancy than protein-coding gene expression profiles. This result

indicates the potential of miRNA expression profiles in cancer classification and prognosis prediction [29].

Because miRNAs act as oncogenes or tumor suppressor genes, miRNAs are potential targets of therapeutic strategies. Recently, Krutzfeldt et al. [39] indicated that chemically engineered oligonucleotides, termed antagomirs, efficiently inhibited miRNAs in vivo. Additionally, it is reported that introduction of 2′-*O*-methoxyethyl phosphorothioate antisense oligonucleotide of miR-122 (abundant in the liver; regulates cholesterol and fatty acid metabolism) decreases plasma cholesterol levels and improves liver steatosis in mice with diet-induced obesity [40]. These findings indicate that antisense oligonucleotides are also potential targets for drug discovery, suggesting the possibility that intractable cancers may become curable by overexpression and/or inhibition of miRNAs. However, for miRNAs to be used in gene therapy, further improvements to make miRNAs more effective and less toxic than other cancer therapy are required.

Prediction of Target mRNAs

Identification of the miRNA-targeted genes provides deep biological insights to understand the new mechanisms of the physiopathological phenomena. Therefore, development of a comprehensive assay to rapidly identify target mRNAs would greatly assist understanding of miRNAs and lead to novel therapeutic approaches against cancer. Although there are many bioinformatics approaches for target prediction, which have predicted that a single miRNA may have hundreds of target genes [5,6,41–45], they have room for many improvements in accuracy and completeness of prediction, respectively. Thus, we have developed a new computational approach to extract regulatory networks between miRNAs and their target mRNAs (Fig. 2). We assumed that the expression pattern of miRNA would present inverse correlation with that of its corresponding target mRNA. Hence, selecting the inverse correlative pairs among the miRNA–target pairs predicted by the sequence-based algorism (ex. miRanda) from expression profiles of mRNAs and miRNAs, we have successfully reduced the false-positive pairs of target prediction results. Moreover, overlapping target information of the predicted pairs onto the coexpressed gene networks, we have also generated the gene networks coregulated by the common miRNA. We hope the inferred networks can be helpful to understand the biological systems of the miRNAs world.

It has been established that the abnormalities of specific miRNA expression contribute to tumorigenesis. Therefore, miRNAs are expected to be powerful tools for cancer classification, diagnosis, and prognosis prediction as well as potential targets of cancer therapy. Furthermore, identification of target mRNAs regulated by miRNAs, elucidation of the oncogenic or tumor suppressive molecular mechanisms by miRNAs and identification of genetic variation in miRNAs and miRNA–target sites of mRNAs may lead to the discovery of new molecular targets related to oncogenesis.

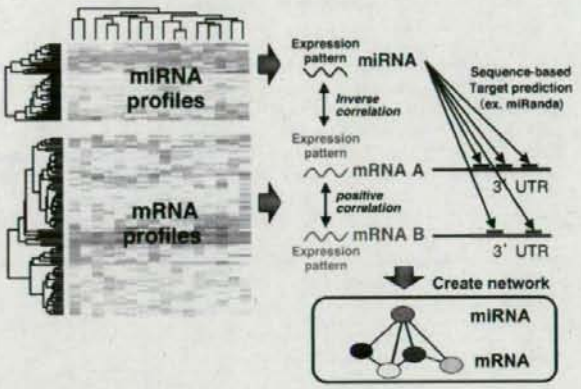

Fig. 2 Scheme of formulation of networks between miRNAs and their target mRNAs

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4 MicroRNAs and Discovery of New Targets

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CONTENTS

SUMMARY

MicroRNAs are endogenous short non-coding RNAs that regulate gene expression mainly at the post-transcriptional level by base pairing to the 3′ untranslated region of target messenger RNAs. At present, hundreds of microRNAs have been identified in humans, and some of them have been revealed to play a critical role especially in the initiation, progression, and malignant potential of various cancers. In this chapter, we discuss the role of microRNAs in cancer and its potential application for cancer therapy.

Key Words: MicroRNA; non-coding RNA; translational suppression; cancer; oncogene; tumor suppressor gene; diagnosis; antisense oligonucleotide; drug discovery

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1. INTRODUCTION

MicroRNAs (miRNAs) are evolutionarily conserved "non-coding RNA" molecules (~22 nucleotides). miRNAs regulate various physiological pathways such as differentiation, proliferation, and apoptosis by cleavage or translational suppression of target messenger RNAs (mRNAs) *(1, 2, 3)*. Currently, over 400 human miRNAs have been identified and registered in the miRNA database miRBase *(4)*, and they are predicted to regulate 30% of protein-encoding transcripts *(5,6)*. Computational analysis estimates the presence of up to 1,000 miRNAs *(7)*. Recently, miRNAs have been reported to work as oncogenes or tumor suppressor genes and be directly involved in the initiation, progression, and metastasis of various cancers *(8,9,10)*. Therefore, this chapter focuses on the role that miRNAs play in cancer, and the use of miRNAs in drug discovery. Collection of evidence suggests that miRNAs can be potentially useful for understanding tumorigenesis and discovering novel strategies for cancer diagnosis and therapy.

2. BIOGENESIS OF MIRNAS

The majority of miRNA genes are located in the introns of protein-coding genes or outside genes *(11)*. Unlike Drosophila, most human miRNA genes exist sporadically, although some miRNAs are found as clusters *(12,13,14)*.

miRNAs are generated in multiple steps (Fig. 1). Initially, miRNAs are transcribed by RNA polymerase II as long RNA precursors (pri-miRNAs) *(15,16,17)*. Pri-miRNAs are usually several kilobases in length, and contain a 7-metyl guanosine cap structure and a poly(A) tail similar to protein-coding mRNAs. The transcribed pri-miRNAs are processed into precursors of approximately 70 nucleotides (pre-miRNAs) with a hairpin-shaped stem-loop secondary structure, a 5′ phosphate and a two-nucleotide 3′ overhang by the RNase III enzyme, Drosha, and a double-stranded-RNA-binding protein, DGCR8/Pasha *(18, 19, 20)*. The pre-miRNAs are then transported to the cytoplasm by a member of the Ran transport receptor family, Exportin-5, in a Ran guanosine triphosphate-dependent manner *(21, 22)*. Pre-miRNAs exported in the cytoplasm are further processed by another RNase III enzyme, Dicer, and unwound by a helicase *(23)*.

Finally, only one mature miRNA strand (guide strand) is incorporated into an RNA-induced silencing complex (RISC) that mediates cleavage or translational inhibition of target mRNAs, while the other strand (passenger strand) is quickly degraded *(24,25,26)*. The stability of the base pairs at the 5′ end of the duplex determines which strand is incorporated in RISC *(27,28)*. RISC is composed of Dicer, Argonaute2 (Ago2), and the double-strand RNA binding protein, TRBP *(26, 29)*, and cleaves target mRNAs more efficiently by using pre-miRNAs rather than the duplex RNAs that do not have the stem-loop structure, suggesting that processing by Dicer may be coupled with assembly of the mature miRNA into RISC *(26)*.

The incorporated miRNA guides the RISC to the complementary sequence in the 3′ untranslated region (UTR) of target mRNAs. miRNAs base-pair to the 3′UTR of the target mRNA with perfect or near perfect complementarity, leading to the target mRNA degradation by Ago2, a component of RISC *(30)*. On the contrary, partial base pairing between a miRNA and a target mRNA leads to translational silencing of a target mRNA

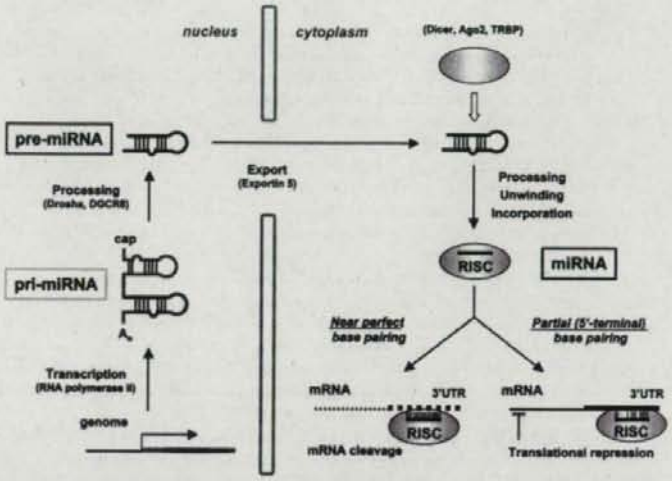

Fig. 1. Mechanism of biogenesis and function in miRNAs. A schematic diagram of miRNA biogenesis is shown. miRNAs are transcribed by RNA polymerase II and sequentially processed by drosha/DGCR8 and dicer. miRNA-loaded RISC causes the cleavage or translational silencing of target mRNAs.

without RNA degradation *(31)*. In partial base pairing, the binding of some nucleotides in the 5′ region of miRNAs has been indicated to be functionally important by systematic mutation experiments *(32,33)*.

3. THE ROLE OF MIRNAS IN CANCER: DIAGNOSIS AND DRUG DISCOVERY

miRNAs have distinct expression patterns among tissues and cells in different differentiation stages *(34,35)*. Lim et al. *(36)* showed that over-expression of miR-124, a brain-specific miRNA, shifted the gene expression profile of HeLa cells toward that of the brain. Similarly, over-expression of muscle-specific miR-1 shifted the expression profile toward that of muscle. These results indicate that miRNAs play important roles in cell differentiation and characterization. Therefore, miRNAs are considered to have a significant influence on various disorders.

Recently, it has been reported that the expression of several miRNAs are altered in a variety of human cancers, suggesting potential roles of miRNAs in tumorigenesis *(37)*. Calin et al. *(38)* showed that more than 50% of miRNAs were located in cancer-associated genomic regions or in fragile sites. In fact, miR-15a and miR-16 genes exist