of a malignant cholangiocyte cell line. The up-regulated expression of miR-21 is also detected in human colon, lung, pancreas, prostate, and stomach cancer *(49,55)*, suggesting the possibility that miR-21 inhibits apoptotic cell death in these cancers.

Interestingly, it has been reported that the expression level of let-7 is reduced in human lung cancers *(56)*. This result suggests that let-7 might act as a tumor suppressor gene in lung cancer. In fact, regardless of disease stage, lung cancer patients with down-regulation of let-7 had shortened post-operative survival *(56)*. Furthermore, Johnson et al. *(57)* found that let-7 negatively regulated the expression of human RAS family members, which possess potent oncogenic activity. Actually, RAS protein levels are inversely correlated with let-7 expression levels in human lung cancers, suggesting a possible mechanism for let-7 in lung cancer.

To identify novel miRNAs involved in cellular transformation, Voorhoeve et al. *(58)* performed functional genetic screens using a library of vectors expressing human miRNAs and in vitro neoplastic transformation assays. They showed that miR-372 and miR-373 accelerate proliferation and tumorigenic development in primary human cells that express oncogenic RAS and tumor suppressor p53, possibly through suppression of p53-mediated CDK inhibition by down-regulation of large tumor suppressor homolog 2 (LATS2) *(58,59)*. Furthermore, miR-372 was found to be exclusively over-expressed in most human testicular germ cell tumors that rarely exhibit loss of p53 function, suggesting contribution of miR-372 to the development of human testicular germ cell tumors by inhibition of the p53 pathway *(58)*.

Recent evidence indicates that polymorphisms and genetic variation in germ line as well as somatic cells have a critical role in cancer predisposition and malignancy *(60,61)*. However, in spite of comprehensive scanning of protein coding genes, the molecular basis of familial cancers remains largely unknown. Recently, a germ line mutation of the miR-16-1–miR-15a primary precursor, which impaired mature miRNA expressions, was identified in B-cell chronic lymphocytic leukemia patients *(62)*. Furthermore, germ line or somatic mutations of miRNAs were found in 11 of 75 patients with B-cell chronic lymphocytic leukemia, but none of these mutations were found in 160 persons without cancer *(62)*. These results suggest that genetic variation of miRNAs in a germ line may play important roles in cancer predisposition and malignancy. In addition, germ line mutation in miRNA-target sites of mRNA 3′ UTR were found in KIT and slit and trk-like family member 1 (SLITRK1), suggesting genetic variation of miRNA-target sites in a germ line may also play significant roles in disease predisposition *(50,63)*.

Human cytochrome P450 (CYP) 1B1, which is abundantly expressed in malignant tumor tissues, is a member of drug-metabolizing enzymes and catalyzes the metabolic activation of various procarcinogens. Recently, it was found that CYP1B1 expression was post-transcriptionally inhibited by miR-27b *(64)*. Furthermore, decrease of miR-27b expression and increase of CYP1B1 expression in most breast cancer tissues was detected *(64)*. These results indicate that miRNAs may play important roles in not only physiologic events but also drug metabolism and production of carcinogens.

Global expression profiling analysis of protein coding genes is known to be useful for cancer diagnoses and prognosis predictions *(65)*. Recently, Lu et al. *(37)* 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

Table 1
Canser-Associated miRNAs

miRNA	*Cancer types*	*Targets*[a]	*References*
Oncogene			
miR-17–92	BCL, lung	CTGF, E2F1,Tsp1	42–45
miR-21	breast, cholangiocyte, colon, glioblastoma, lung, pancreas, prostate, stomach	PTEN	49, 52–55
miR-155	BCL, breast, colon, lung, thyroid	AT1R	46, 49–51
miR-372/373	testicular germ cell	LATS2	58
Tumor suppressor gene			
let-7a	breast, lung	RAS	55–57
miR-15a/16	B-CLL	BCL2	39, 40

[a] Target genes identified by the biological experiments are listed.
Abbreviations: AT1R, angiotensin II type I receptor; B-CLL, B-cell chronic lymphocytic leukemia; BCL, B-cell lymphoma; BCL2, B-cell lymphoma 2; CTGF, connective tissue growth factor; LATS2, large tumor suppressor homolog 2; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Tsp1, thrombospondin-1.

protein-coding gene expression profiles. This result indicates the potential of miRNA expression profiles in cancer classification and prognosis prediction *(37)*.

Because miRNAs act as oncogenes or tumor suppressor genes (Table 1), miRNAs are potential targets of therapeutic strategies. Recently, Krutzfeldt et al. *(66)* indicated that chemically engineered oligonucleotides, called 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 and regulates cholesterol and fatty-acid metabolism) decreases plasma cholesterol levels and improves liver steatosis in mice with diet-induced obesity *(67)*. These findings indicate that antisense oligonucleotides are also potential targets for drug discovery, suggesting the possibility that intractable cancers may become curable by over-expression and/or inhibition of miRNAs. However, for miRNAs to be used in gene therapy, further improvement is required to make miRNAs more effective and less toxic than other cancer therapy.

4. PERSPECTIVE

It has been established that miRNAs play critical roles in cell differentiation, proliferation, and apoptosis, and the abnormalities of specific miRNA expression contribute to tumorigenesis. Additionally, recent studies show that polymorphisms or genetic variation of miRNAs and miRNA-target sites of mRNAs in a germ line may play important roles in cancer predisposition and malignancy *(50,62)*. Therefore, miRNAs are expected to be powerful tools for cancer classification, diagnosis, and prognosis prediction, as well as to be 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. Bioinformatics approaches have predicted that a single miRNA may have hundreds of target genes *(5, 6, 68, 69, 70, 71, 72, 73, 74)*, although detailed experimental validation has yet to be done. Development of a comprehensive assay to rapidly identify target mRNAs would greatly assist our understanding of miRNAs and lead to novel therapeutic approaches against cancer.

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2．最新技術/システム
2）高感度マイクロアレイ

土屋創健・清水一治・辻本豪三

DNAマイクロアレイのスポット部位に柱状構造を有する黒色樹脂の基板とその柱状構造を利用したビーズによる撹拌を用いることで，従来のガラス基板のマイクロアレイと比べて，約100倍高感度なマイクロアレイが開発された。この高感度マイクロアレイは，わずか0.01μgのtotal RNAから遺伝子増幅を行うことなく正確な遺伝子プロファイルを取得することが可能であった。今後，高感度マイクロアレイを用いることにより，臨床の場において治療前生検標本から迅速かつ簡便に正確な遺伝子プロファイルを取得できると期待される。

はじめに

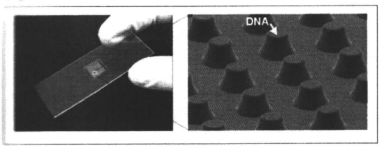
一度に大量の遺伝子の量的変動を分析するDNAマイクロアレイは極めて強力な研究ツールとしてこれまで主に基礎研究分野において使用され，ゲノム機能科学研究の発展に多大な貢献を果たしてきた。このDNAマイクロアレイを医療へ応用しようする試みは当然盛んであり，創薬ターゲット分子の同定や病態・予後診断のための疾患特異的なバイオマーカーの探索のためにDNAマイクロアレイが使用され，実際，病態の発現や予後に関連する遺伝子が数多く同定された。また，DNAマイクロアレイを用いた解析から薬物の治療効果や副作用に関与する遺伝子も同定され，DNAマイクロアレイは患者個人の体質，病態，薬剤応答性に応じて薬の種類や量を決定するなどの患者個人に最適化した医療，個別化医療を実現するためのツールとしても期待されている。

このように病態の診断や個別化医療の実現に向けてDNAマイクロアレイに大きな期待がかけられているが，個々の遺伝子の検出感度やその信頼性に関しては定量PCR法などの他の検出方法と比較しても劣っており，とりわけ低発現の遺伝子の検出においてはいまだ十分な性能が得られていなかった。そのため，低発現ながら生理作用や病態発現に重要な役割を担っていることの多い転写因子や受容体をもれなく検出できるような高感度のDNAマイクロアレイが渇望されていた。また従来，治療前生検標本などの微量なサンプルをDNAマイクロアレイで解析するためには，RNAポリメラーゼによる転写反応やPCR法などによる遺伝子増幅が必要不可欠であったが，複数の遺伝子間で増幅効率が異なることや，これらの操作は複雑かつ煩雑で時間も要し，専門的な技術の習得が必要であること，消耗品および設備の面で非常にコストがかかることから，臨床の場においてDNAマイクロアレイが使用されるには，生検標本のような微量の検体からでも遺伝子増幅を行うことなく，迅速・簡便に正確な解析を行うことができる高感度なDNAマイクロアレイが必要であった[1]。

われわれは東レより開発された立体構造を有するマイクロアレイの性能評価を共同研究で行った

key words
高感度，柱状構造，黒色樹脂，ビーズ撹拌，診断，個人化医療，臨床，生検標本，microRNA

図❶ 高感度マイクロアレイの柱状構造（文献2より改変）



結果，0.01 μgという少量のtotal RNAから遺伝子増幅を行うことなく遺伝子プロファイルを取得できることが明らかとなったので，以下にこの高感度マイクロアレイの特徴と性能評価について概説する。

Ⅰ．柱状構造によるスポット形状の安定化とバックグラウンドノイズの低下

DNAマイクロアレイの高感度化を実現するため，スポット形状の安定化とバックグラウンドノイズの軽減を目的にして，検出部に直径0.15 mmおよび高さ0.2 mm柱状構造を有する黒色樹脂のDNAマイクロアレイ基板が考案された（**図❶**）。その結果，柱の上部にプローブを固定化することでスポットの形状が安定化し，より高密度にプローブを固定化することが可能となった。また，従来のDNAマイクロアレイのバックグラウンドノイズは主に周囲のガラスへの非特異的な標識核酸の吸着に起因していたが，この柱状構造を有する高感度マイクロアレイはスキャン時に読み取り面を柱の上端に設定することでスポット周囲に空気のみの空間を作り出し，バックグラウンドノイズを大幅に軽減させた（**表❶**）[2]。さらに，バックグラウンドノイズの低下には黒色樹脂自体の自家蛍光がガラスよりも低いことも寄与していることが，底面でのバックグラウンドノイズの測定結果から明らかとなった（**表❶**）。

Ⅱ．柱状構造を利用したビーズ撹拌による反応性の向上

一般にハイブリダイゼーション溶液中の核酸の拡散は遅く（～200 μm/hr），特に希薄な標識核酸濃度のハイブリダイゼーション溶液中では溶液中の標識核酸とプローブの十分な反応効率が期待できなかった[2]。そこで，柱状構造を利用して柱間にビーズを封入し，ハイブリダイゼーション時にマイクロアレイを振盪させてビーズを動かすことで物理的に溶液の撹拌を加速させたところ，感度が約3倍亢進することが明らかとなった（**表❷**）。この際，ビーズの直径をカバーのガラス板と柱上部の間隙より大きくすることで，ビーズは柱間のみを移動し，スポット部位を傷つけることがないように工夫を施している。

Ⅲ．高感度マイクロアレイの性能評価

上記の改良を行った高感度マイクロアレイの性能を評価するため，Cy3標識したヒト脳由来のcDNAとCy5標識したヒト肝臓由来のcDNAをハイブリダイゼーションさせた際の蛍光画像を従来のガラス基板のDNAマイクロアレイと比較した結果，高感度マイクロアレイではバックグラウンドノイズの軽減とシグナル値の上昇が観察された（**図❷**）。また，ハイブリダイゼーションさせる標識核酸の量を1，0.1，0.01 μgと段階的に減らしたところ，従来のガラス製チップでは0.1 μgにおいて著しくシグナルが減弱し，0.01 μgではほとんどシグナルが検出されなかったのに対し，高感度マイクロアレイでは0.1 μgにおいてシグナルの減弱

表❶ 基板底面からの焦点距離とノイズシグナル値の関連（文献2より改変）

基板底面からの焦点距離（μm）	0	50	100	200
高感度マイクロアレイ基板	330	250	190	180
従来のガラス基板	460	−	−	−

表❷ シグナル強度に対するビーズ撹拌の効果
（文献2より改変）

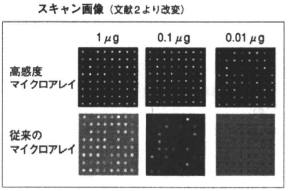
標識核酸濃度（ng/μl）	0.15	0.30	0.75
ビーズ撹拌あり	2,020	2,470	4,510
ビーズ撹拌なし	660	770	1,400
シグナル強度比（ビーズ撹拌あり/なし）	3.1	3.2	3.2

ほとんど検出されず，0.01 μgにおいても大部分のスポットにおいて同等のシグナルが検出された（図❷，図❸）。また，0.01 μgにおいても高感度マイクロアレイではほぼ同等のダイナミックレンジが維持されていたのに対し，従来のガラス基板のDNAマイクロアレイでは0.1 μgにおいてすでにダイナミックレンジが顕著に狭まっていた（図❸）。さらに，異なるインプット量間におけるCy3とCy5のシグナル比の相関係数を従来のガラス基板のDNAマイクロアレイと比較したところ，高感度マイクロアレイにおいてより高い正の相関が得られることが明らかとなり，高感度マイクロアレイの高感度性と信頼性の高さが確認された（表❸）。

高感度マイクロアレイから得られた遺伝子の発現差の精度を調べるため，高感度マイクロアレイより得られる発現差が定量PCR法から得られる発現差と一致するかどうかを，9つの遺伝子（*HLA-F*, *PARVB*, *NR2C1*, *MDH1*, *MTI1E*, *RPL23AP7*, *BRDT*, *CD84* および *SERPINA1*）に関して検証を行った（図❹）。その結果，いずれの遺伝子においても高感度マイクロアレイと定量PCRから得られた発現差はよく一致し，両者の相関係数は0.94であった。これは，他の市販されているDNAマイクロアレイを用いて検討された報告結果と比べても，高感度マイクロアレイの結果が定量PCRの結果とよりよく相関していることを示している[4]。

以上の結果から，この高感度マイクロアレイが遺伝子増幅を行わずに0.01 μgのtotal RNAから高精度の遺伝子発現プロファイルを取得できることが確認され，この高感度マイクロアレイは，臨床の場において使用されるに十分な性能を有していることが明らかとなった。

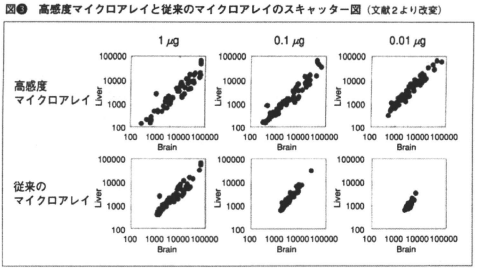
図❷　高感度マイクロアレイと従来のマイクロアレイのスキャン画像（文献2より改変）



表❸　異なるインプット量におけるCy3/Cy5比の相関係数（文献2より改変）

インプット量（μg）	1 and 0.1	1 and 0.01
高感度マイクロアレイ	0.87	0.80
従来のマイクロアレイ	0.49	0.10

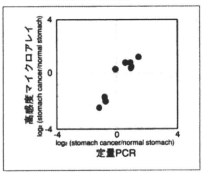
図❸　高感度マイクロアレイと従来のマイクロアレイのスキャッター図（文献2より改変）



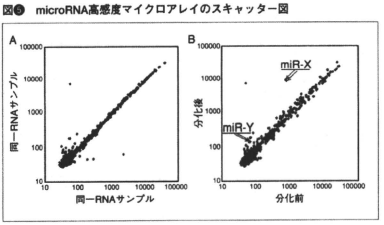
Ⅳ．microRNA高感度マイクロアレイ

近年，短鎖（約20塩基）のnon-coding RNAであるmicroRNAが細胞の発生・分化や癌の発症・悪性化などに重要な役割を果たすことが明らかとなり，これまで単なる伝達役に過ぎないと思われてきたRNAの機能性に着目した新たな生命科学の展開に大きな期待が寄せられている。しかしながら，microRNAは非常に短鎖であるがゆえに，DNAマイクロアレイでのプローブ部位の選択ができないなどの問題があり，従

図❹ 高感度マイクロアレイより得られた発現差の定量PCRによる検証（文献2より改変）

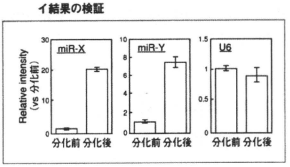


図❺ microRNA高感度マイクロアレイのスキャッター図



来のガラス基板のDNAマイクロアレイでは精度の高いマイクロアレイ解析を行うのは困難であった。そこで，この高感度マイクロアレイにヒトmicroRNAのプローブ471種類（miRBase：Release 9.1）をduplicateに固定化し，マイクロアレイ間における再現性の確認を行ったところ，相関係数が0.99という非常に高い再現性が得られること，3桁を超える幅広いダイナミックレンジが得られることが明らかとなった（図❺A）。さらに，あるヒト由来細胞株に分化を誘導し，その前後におけるmicroRNAの変動をこの高感度マイクロアレイで解析したところ，スポットしたduplicateがそろって2倍以上変動したmicroRNAが2種類（ともに仮名：miR-X，miR-Y）検出された（図❺B）。これらのmicroRNAの発現変動を定量PCR法で検証したところ，高感度マイクロアレイから得られた結果が確認され，コントロールとして用いたU6の発現には差はみられなかった（図❻）。以上のことから，この高感度マイクロアレイがmicroRNAのような短鎖のRNAの検出においても優れた性能を有していることが確認された。

図❻ 定量PCRによる高感度microRNAマイクロアレイ結果の検証



おわりに

2006年9月，米国食品医薬品局（FDA）によって進められていたMicroArray Quality Control（MAQC）プロジェクトのフェーズIが終了し，マイクロアレイ解析による遺伝子発現プロファイルの互換性・再現性が確認され，遺伝子発現プロファイル診断薬の実用化への展望が開けた。さらに2007年2月には，最初のマイクロアレイ体外診断法として，FDAにより遺伝子発現プロファイルから乳癌の再発リスクを評価するDNAマイクロアレイMammaPrint®が認可され，個々の患者の体質・病状を加味した個人化医療がいよいよ現実のものになろうしている。

高感度マイクロアレイは，微量の検体からでも遺伝子増幅なしに正確な遺伝子プロファイルをすることができる臨床の場でのニーズに応えたマイクロアレイであり，今後，病態の診断や個別化医療に活用されることが期待される。

謝辞
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Technical Tips

DNAマイクロアレイはこれまで主に医療および基礎研究分野においてその威力を発揮してきたが，最近では土壌汚染浄化などの環境や食品といった分野においても脚光を浴びてきている。

安価で安全な土壌や地下水の浄化方法として，微生物を用いて汚染物質の分解を行う方法があるが，これには汚染物質を分解するために有効な微生物を事前に特定する必要がある。この微生物を用いた浄化方法の有効性を事前検証するための方法として，マイクロアレイが注目されている。従来までの方法と比較して，短時間かつ低コストで検証を行うことができ，さらに多種類の微生物を同時に検出できることから，汚染物質の異なる分解過程に携わる微生物を組み合わせた浄化方法の検討が可能となる。

また，マイクロアレイは食品や飼料中にどのような生物種由来のものが含まれているのかを迅速かつ網羅的に同定することが可能であり，昨今の食品の品質・安全性に対する関心の高まりを受け，食品の品質鑑定におけるマイクロアレイの使用増大が見込まれている。

今後，さらに様々な分野においてマイクロアレイの適用が拡大することが期待される。

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

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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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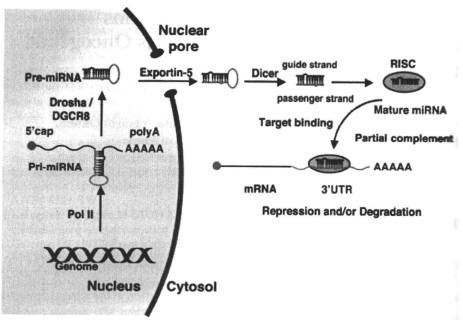

Fig. 1 Schematic diagram of biogenesis and function in micro-RNAs (miRNAs). MiRNAs are transcribed by RNA polymerase II and sequentially processed by Drosha/DGCR8 and Dicer. MiRNA-loaded RNA-induced silencing complex (*RISC*) causes the cleavage or translational silencing of target mRNAs

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.

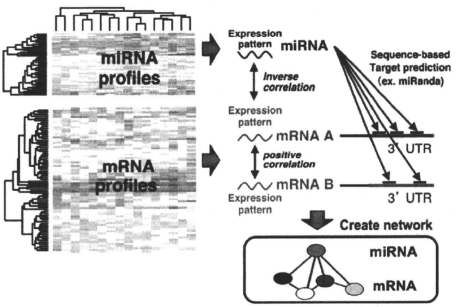

Fig. 2 Scheme of formulation of networks between miRNAs and their target mRNAs

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Characterization of gene expression profiles for different types of mast cells pooled from mouse stomach subregions by an RNA amplification method

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Abstract

Background: Mast cells (MCs) play pivotal roles in allergy and innate immunity and consist of heterogenous subclasses. However, the molecular basis determining the different characteristics of these multiple MC subclasses remains unclear.

Results: To approach this, we developed a method of RNA extraction/amplification for intact *in vivo* MCs pooled from frozen tissue sections, which enabled us to obtain the global gene expression pattern of pooled MCs belonging to the same subclass. MCs were isolated from the submucosa (sMCs) and mucosa (mMCs) of mouse stomach sections, respectively. 15 cells were pooled, and their RNA was extracted, amplified and subjected to microarray analysis. Known marker genes specific for mMCs and sMCs showed expected expression trends, indicating accuracy of the analysis.

We identified 1,272 genes showing significantly different expression levels between sMCs and mMCs, and classified them into clusters on the basis of similarity of their expression profiles compared with bone marrow-derived MCs, which are the cultured MCs with so-called 'immature' properties. Among them, we found that several key genes such as *Notch4* had sMC-biased expression and *Ptgr1* had mMC-biased expression. Furthermore, there is a difference in the expression of several genes including extracellular matrix protein components, adhesion molecules, and cytoskeletal proteins between the two MC subclasses, which may reflect functional adaptation of each MC to the mucosal or submucosal environment in the stomach.

Conclusion: By using the method of RNA amplification from pooled intact MCs, we characterized the distinct gene expression profiles of sMCs and mMCs in the mouse stomach. Our findings offer insight into possible unidentified properties specific for each MC subclass.

Background

Mast cells (MCs) are derived from hematopoietic stem cells and play important roles in allergic responses, innate immunity and defense against parasite infection. Unlike other blood cells, MCs migrate into peripheral tissues as immature progenitors and differentiate into mature mast cells. One of the unique features of MCs is that they show a variety of phenotypes depending on the different tissue microenvironment of their maturation [1]. In MCs, various MC-specific serine proteases are stored in the secretory granules, and their gene and protein expressions are dramatically altered when their cell environment is altered. For example, Reynolds *et al.* have shown that at least six distinct members of mouse MC-specific serine proteases are expressed in different combinations in different mast cell populations [2]. In addition, recent studies have shown that mature MCs vary in terms of what surface receptors and lipid mediators they express [3,4]. Because each mast cell population *in vivo* must play a specific role in the body, it is important to determine the character of each population of MCs.

Comprehensive gene expression analysis is a powerful approach to understand the characterization of various MC subpopulations. To date, several studies on microarray analysis of MCs have been conducted [5-7], but most of them dealt with MCs cultured *in vitro*. Alternatively, gene expression profiles of MCs isolated from skin and lung have been analyzed [3,8-10]. However, the numbers of MCs analyzed as one sample were relatively high and they were exposed to physical forces, enzymes and the anti-Kit antibody for purification, during which the original properties of the MCs may have been affected.

In the gastrointestinal tract, there are MCs that are mainly classified into two subclasses; mucosal MCs (mMCs) and submucosal MCs (sMCs) on the basis of their location, morphology (size and shape) and granule contents [11,12]. mMCs are mainly found in the mucosa of the gastrointestinal system, having chondroitin sulfate-containing granules, which are stained with toluidine blue but not safranin, and their activation occurs during parasite infection [13], while sMCs are localized in the submucosa of the gastrointestinal tract and their granules are rich in heparin and stained with both toluidine blue and safranin [1,11]. However, the molecular basis determining the differences in biochemical properties of these two MC subclasses remains uncertain, partially due to the difficulty of their isolation.

To overcome these problems, here we established a method of RNA amplification from intact MCs isolated from frozen tissue sections, which enables us to conveniently obtain the global gene expression pattern of MCs in various tissues. To validate this method, we first determined the minimum cell number required to achieve reproducible RNA amplification. We then compared the gene expression profiles obtained from small numbers of mMCs and sMCs in the mouse stomach, and found several key genes to be specifically expressed in one subclass of MCs, which may reflect some aspects of the distinct properties between the two MC subclasses in the gastrointestinal tract.

Results and discussion

Development of an RNA amplification protocol to obtain gene expression profiles from a small amount of RNA

To gain insight into the functional differences between the different subclasses of MCs, we employed three rounds of the T7-based RNA amplification method. Based on the preliminary experiments using peritoneal MCs and bone marrow-derived MCs (BMMCs), we estimated that a single MC yields 2 pg of RNA. Before we performed comparative analysis of MCs from different tissues, we first evaluated the accuracy and reproducibility of three rounds of the T7-based RNA amplification method, starting with the amount of RNA that can be obtained from a single MC. To assess this, we first compared the microarray results obtained from 5 μg of BMMC RNA prepared by the standard protocol with those obtained from the same RNA diluted 10^5- or 10^6-fold (30 pg, 10 pg and 2 pg) and subjected to three rounds of T7-based amplification (Figure 1a–c). Although three rounds of amplification yielded enough quantity of RNA for microarray analysis (>20 μg) even in the case of 2 pg RNA, scatter plot analysis revealed that the qualities of the obtained results were quite different between the samples from 5 μg and 2 pg RNA. The genes judged as 'Presence' in both 30 pg and 5 μg of RNA were 8,149 genes, which corresponded to 72% of genes judged as 'Presence' in the 5 μg of RNA (11,344 genes; Figure 1a), while only 4,116 genes were judged as 'Presence' in both 2 pg and 5 μg of RNA, which corresponded to only 36% of genes judged as 'Presence' in the 5 μg RNA (Figure 1c). The decrease in the number of genes judged as 'Presence' in the diluted samples (30 pg, 10 pg and 2 pg) may be due to the loss of low copy number RNA species during amplification.

We next examined the reproducibility of the microarray results obtained from two sets of 30 pg BMMC RNA samples (30 pg-1 and 30 pg-2) or two sets of 2 pg samples (2 pg-1 and 2 pg-2) (Figure 1d and 1e). In the 30 pg RNA samples, 7,537 (30 pg-1) and 8,777 (30 pg-2) genes were judged as 'Presence'. However, only 4,324 (2 pg-1) and 4,460 (2 pg-2) genes were judged as 'Presence' in each 2 pg RNA sample, again suggesting the loss of low copy number RNAs during amplification from a small amount of RNA. As to the reproducibility, 86% of the 'Presence' genes in the 30 pg-1 and 74% of 'Presence' genes in the 30 pg-2 sample were judged as 'Presence' in both 30 pg RNA