ORIGINAL RESEARCH

Identification of a new microRNA expression profile as a potential cancer screening tool

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Abstract

Purpose: Small non-coding microRNAs (miRNAs) are key components of cancer development and are considered as potential biomarkers for cancer diagnosis and treatment monitoring. This study investigated miRNA expression profiles of human cancer cells in order to develop a screening method for lung cancer.

Methods: A series of lung cancer related miRNAs (miR-21, miR-145, miR-155, miR-205, miR-210, miR-92, miR-17-5p, miR-143, miR-182, miR-372, let-7a) were selected as candidates for miRNA expression profiles of human lung cancer cell lines (A549, SK-mes-1). MicroRNA u6 was the endogenous control. Cancer cell lines for positive controls; breast MCF-7, prostate Du-145, and glioblastoma U118. The negative control was normal lung fibroblast cell line MRC-5. RT-PCR was performed on StepOnePlus (Applied Biosystem, USA). MiRNA expressions of malignant cells were compared with normal fibroblast cells as well as endogenous control (u6) using the thermal cycle at threshold. Assessment of miRNA expression profiles were then performed using agglomerative hierarchical cluster analysis software (SPSS13, USA).

Results: We demonstrated that miR-21, miR-182 and let-7a were over-expressed, and miR-145 and miR-155 were under-expressed in all cancer cell lines. Combined with the cluster analysis we were able to clearly distinguish cell lines for normal fibroblasts, breast cancer, prostate cancer, glioblastoma, and lung cancer.

Conclusion: There is potential utility of screening for lung cancer with miRNA expression profiles. Future work will focus on the sensitivity of such miRNA expression profiles in screening sputum for lung cancer, which can be performed in real time.

Lung cancer remains the leading cause of cancer-related deaths in Canada, the United States and Europe. In fact, more people die from lung cancer each year than from breast, colon, and prostate cancers combined.^{1,2,3} Conventionally-treated early stage lung cancer patients have a five year survival of approximately 60 to 70%.⁴ One reason that lung cancer mortality is so high is the lack of effective standardized screening. As a result, most patients present with advanced stage disease and subsequent poor overall survival. One recent review states: "Overall, no single cheap and reliable test has yet been identified for the effective screening of lung cancer in large population studies, but work is in progress."⁵ Effective and financially feasible screening for patients at a high risk of developing lung cancer is, therefore, a high research priority.

Non-small-cell lung cancers (NSCLC), mainly comprised of adenocarcinomas and squamous cell carcinomas of the lung, originate from the respiratory pluripotent stem cells and therefore exhibit varied histology and molecular markers.⁶ DNA and miRNA analyses show clear potential to improve or refine diagnosis as an improvement over conventional cytology examination. Molecular genetic analysis can identify cells bearing tumor-related molecular genetic aberrations, and may be more sensitive than cytology in identifying neoplastic cells.⁷⁻⁹ Assessment of deletions of tumor suppressor genes, *HYAL2* and *FHIT*, can be used to detect abnormal cells not only in cytological positive sputum, but also in 55% of cytological negative sputum from stage I NSCLC patients.^{10,11}

miRNAs are a class of small non-protein-coding RNAs that can post-transcriptionally regulate the expression of hundreds of their target genes through inhibition of messenger RNAs, thereby controlling a wide range of biological functions such as cellular proliferation, differentiation, and apoptosis.¹²⁻¹⁸ miRNAs have been verified as highly tissue-specific, and have been proposed as promising candidates for biomarkers for cancer and other diseases.^{12-14,17} If researchers can determine which of these miRNAs are indicative of cancer, future screening techniques could focus on a simple miRNA analysis.^{17,18} Furthermore, miRNAs may function as tumour suppressors, or oncogenes, and deregulated miRNA expression has been shown to participate in cancer development and progression.^{14,19-21} Over- and under-expression of miRNAs in surgically resected lung tumour tissues have been used for diagnosis and prognosis of lung cancer patients.^{12-14,20,21} Therefore, miRNAs could potentially be useful in the diagnosis and classification of human malignancies.¹²⁻¹⁴ One study has investigated the feasibility of examining aberrant miRNA expression in non-small-cell lung cancer. Xie, *et al.* report that miR-21 over-expression can be used as a marker in sputum for the diagnosis of non-small cell lung cancer (NSCLC).¹⁹ Using a single miRNA expression as a biomarker of cancer may be easier but

less accurate than using multiple miRNAs, as multiple miRNAs are over- or under-expressed in a variety of cancers and may have diagnostic or prognostic significance.^{12-14,22-24} The result is that a specific miRNA may be variably over-expressed in several different tumor types and would, therefore, not be able to reliably distinguish a specific tumor cell origin.

Researchers recently identified miRNA “profiles”, combinations of specific miRNA expressions that indicate the presence of lung cancer in patients. Yu used a five miRNA panel to predict survival and relapse in lung cancer patients.²³ The use of a miRNA panel overcomes the lack of specificity in single miRNA determination and can potentially distinguish between lung cancer and other conditions.¹²⁻¹⁴ Analysis of miRNA may form the basis of an accessible, reliable screening method. However, the feasibility of analyzing the signatures of miRNA combinations in different cancer cell lines, including lung cancer cell lines, has not been investigated. The objective of the present study is to develop a reliable and clinically practical method of generating miRNA profiles from a range of cancer cells, including lung cancer cell lines. In this study, 12 miRNAs were selected as candidates for profiling based on previous published work.¹²⁻³⁰ miRNA profiles were measured with quantitative RT-PCR (qRT-PCR).

Cluster analysis is a methodology for classifying objects based on data collected to characterize those objects. In medicine, the objects can be patients or cells, and the data can be various signs, symptoms, or test results.^{31,32} Cluster analysis can lead to diagnosis when a distinct grouping based on the data is observed. This methodology can be very rigorous and, as described by Riffenburgh: “Clustering is obtained mathematically by statistics that compare numerical similarities or distances in multivariate space.”³³ In this paper hierarchical cluster analysis of the miRNA profiles was used to distinguish different cancer cell lines from each other and from normal cells.

Methods

Cell lines

The following human cancer cell lines were used in the experiments: A549 (lung adenocarcinoma); SK-mes-1 (lung squamous cancer); MCF-7 (breast cancer); Du-145 (prostate cancer), and U118 (glioblastoma). Human normal lung fibroblast (MRC-5) was also used as a non-cancerous control. Cells were obtained from ATCC (American Type Culture Collection).

Isolation of RNA

TaqMan microRNA Cell-to-CT kit (Cat: 4391848, Applied Biosystem, USA) was used to extract total RNA from cell lines. Briefly, The 5×10^4 cells were plated into 96-well plate and incubated at 37 °C with 5% CO₂ for 6 hours. After cells were completely attached, they were washed with 4 °C PBS. The mixture of 49 µl lyses solution and 1 µl RNase I was added into wells containing cells and incubated at room temperature for 8 min. Then 5 µl stop solution was added and incubated again at room temperature for 2 min. The as-received total RNA solution was stored at -70 °C for subsequent reverse transcription.

Reverse Transcription of RNA

The 12 miRNAs included: miR21, miR145, miR155, miR205, miR210, u6 (endogenous control), miR92, miR-17-5p, miR143, miR182, miR372, let-7a. TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem, USA) was used for miRNA reverse transcription (RT). Briefly, the mixture of RT reaction included 50 nM stem-loop miRNA-specific primer, 1X RT buffer, 0.25 mM of dNTPs, 3.33 U/µl MultiScribe reverse transcriptase (all from Applied Biosystem), and 5 µl extracted RNA to total 15 µl. RT reaction was conducted at 16 °C for 30 minutes and 42 °C for 30 minutes, 85 °C for 5 minutes with StepOnePlus instrument (Applied Biosystem, USA). 15 µl RT

products were stored at -70 °C for subsequent RT-PCR.

Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

TaqMan Universal PCR Master Mix, and Taqman microRNA Assay (miRNA-specific primer and probe) were from Applied Biosystem (USA). The qRT-PCR was conducted with StepOnePlus instrument (Applied Biosystem, USA).

The PCR reactions include 1.33 µl RT products, and 10 µl 1X TaqMan Universal PCR Master Mix and 1 µl stem-loop miRNA-specific primer and probe.²⁵ 20 µl PCR reactions were incubated in a 96-well plate at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The threshold cycle (C_T) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. $\Delta\Delta C_T$ method was applied using qRT-PCR for miRNA expression; that is, 11 miRNA expression folds were calculated based on reference sample MRC-5 and endogenous control u6.³⁰ Relative Expression of target 11 miRNAs were normalized to expression of endogenous control U6, a commonly used internal control for miRNA quantification assay,³¹ and to a reference sample of normal lung fibroblast, MRC-5.

Sensitivity and dynamic range of miRNA quantification

To determine specificity of miRNA quantification in human cell lines by qRT-PCR assay, the RNA isolated from lung cancer cell line (A549) and breast cancer (MCF-7) was diluted by five orders of magnitude: from 1~2000 (cell number) for sensitivity (lower detection limit) and dynamic range (from 3 to 20000). The sensitivity and dynamic range of miRNA (miR-21) was determined using the standard curve that was obtained with qRT-PCR.

FIGURE 1. Sensitivity and dynamic range of miR-21 quantification in cancer cell lines (all experiments performed in triplicate).

(A) standard curve of miR-21 extracted from A549: ($R^2 = 0.995$, slope = -3.37). Demonstrates number of cycles needed to reach lower detection limit for varying quantity of cells at start of qRT-PCR.

(B) standard curve of miR-21 extracted from MCF-7: ($R^2 = 0.997$, slope = -3.44) Demonstrates number of cycles needed to reach lower detection limit for varying quantity of cells at start of qRT-PCR.

(C) Amplification curve of miR-21 extracted from A549 cell line: Extracted RNA from range of 3 to 20,000 cells. Used standard curve of quantification method of qRT-PCR to generate amplification curves.

Data Analysis

The series-ordered miRNA expression folds for each cell line were arranged into miRNA profiles as bar graphs of series-ordered miRNA expressions in each cell line. In these graphs, the increase and decrease of each miRNA expression fold create the unique pattern of miRNA expression for each cell line. The experimental-normalized miRNA expression profiles were used to evaluate the cell lines by cluster analysis using average linkage and correlation similarity. ANOVA was used for data analysis. Pearson's-correlation method for cluster analysis was performed using hierarchical cluster analysis software (SPSS13,

USA). This is a pre-clinical validation study for our analytical methodology.

Results

Sensitivity and dynamic range of miRNA quantification

The miRNA-21 in A549 and MCF-7 cell lines were specifically determined using qRT-PCR. The serially diluted cell solution served in experimental samples for measuring expression of miR-21, miR-92, and U6. The results showed excellent linear correlation ($R^2 = 0.995-0.997 \gg 0.99$) between the miRNA input and

FIGURE 2. : Profiles for 5 human cancer cell lines and human normal lung fibroblast (MRC-5): For each cell line the 11 miRNAs are repeated left to right in the order: m21, m145, m155, m205, m210, m92, m17-5p, m143, m182, m372, let7a. Cell line MRC-5 miRNA expressions normalized to 1.

the C_T values for the qRT-PCR assay. Standard deviations of C_T value (maximum 0.7% to minimum 0.2%) for triplicate experiments indicated that accuracy and reproducibility of qRT-PCR experiments were excellent and meet the qRT-PCR quantification requirements. The miRNA qRT-PCR assay had a dynamic range of at least four orders of magnitude (from 3 to 2×10^4 cells), and was capable of detecting a minimum of three A549 cells for miR-21 in (Fig. 1A) and 16 MCF-7 cells (Fig. 1B). According to the TaqMan microRNA Cell-to- C_T kit, the upper limit was 10^5 cells, so the highest number of cells used in the dynamic range experiments was 2×10^4 . Figure 1C demon-

strated the typical amplification curves of miR-21 for A549 in standard curve qRT-PCR experiments.

miRNA expression for different cell lines:

With the exception of miR-205 in Du-145 cells, the 11 miRNAs plus endogenous control u6 were expressed in all cell lines, however, the magnitude of their expression varied greatly between cell lines. For five of the human cancer cell lines, miR-21, miR-182 and let7-5a were over-expressed in comparison with human normal fibroblast MRC-5, but the magnitude of over-expression was different depending on cell line. In contrast, miR-145 and miR-155 were under-expressed for the five human cancer cell lines. Table 1 and Figure 2 demonstrate the 11 miRNA expression profiles, except for endogenous control U6, for all five human cancer cell lines and human normal lung fibroblast cell line (MRC-5).

Cluster Analysis of miRNA profiling

In addition to differences in tissue origin, the cell lines were also developed from different cell lineages: epithelial (lung (A549, Sk-mes-1, MRC-5), prostate (Du-145), breast (MCF-7), and endothelial (glioblastoma (u118)). Agglomerative hierarchical cluster analyses identified major features of the miRNA expression profiles in these cell lines. First, we observed that miRNA profiles from normal and malignant cell lines were distinctly different. Cluster analysis separated the normal lung fibroblast cell line

TABLE 1. 11 miRNA expression based on endogenous control u6 and reference sample MRC-5

	m21	m145	m155	m205	M210	m92	m17-5p	m143	m182	m372	let7a
A549	300.7059	1.4019	0.7780	534.5244	3.6011	23.8085	17.1541	3.2603	2641.0099	92.5450	67.2648
Mes-1	93.8476	0.0522	1.1418	20.1156	11.9864	14.5939	16.0697	1.4266	1579.0979	215.8976	32.8895
Du145	4.3550	0.0214	0.1498	0.0000	0.9818	1.8638	3.7348	0.0327	182.9222	1.8458	2.4395
MCF-7	17.5396	0.0242	0.0057	5425.5569	1.6735	3.2948	0.8853	0.3891	877.1706	6.8601	3.2161
U118	4.5523	0.2589	0.1115	7498.3400	63.4418	19.4408	5.8712	378.7598	3267.0274	31762.2872	1900.4043
MRC-5	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

In the first column are the cell lines: A549: adenocarcinoma from lung, Mes-1: squamous cell carcinoma from lung, Du145: prostate cancer, MCF-7: breast cancer, U118: glioblastoma multiforme, MRC-5: normal lung fibroblasts. In the first row are the different miRNAs that are expressed as multiples of the quantity found in the MRC-5 cells.

TABLE 2. Similarity Matrix of miRNA Profiling Cluster Analysis

Cell Type	Correlation between Vectors of Values					
	A549	Mes-1	Du145	MCF-7	U118	MRC-5
A549	1.000	.973	.977	.243	.009	.000
Mes-1		1.000	.992	.046	.093	.000
Du145			1.000	.052	-.029	.000
MCF-7				1.000	.116	.000
U118					1.000	.000
MRC-5						1.000

The MRC-5 cell line is normal lung fibroblasts, which has no similarity to the cancer cell lines. Among the cancer cell lines the higher the number, the more similar the cell lines and the closer they will cluster in the Dendrogram (Figure 3).

MRC-5 from the other five human cancer cell lines. Second, cluster analysis produced complete separation of endothelial cell (U118) and the other epithelial cells (MCF-7, Du145, A549, Sk-mes-1). Third, the sub-cluster of epithelial cancer cell lines can be further divided into three groups: the first group of breast cancer, the second of glioblastoma, and third of lung and prostate cancer.

Table 2 lists the similarity matrix of cluster analyses for six cell lines' miRNA profiles, based on the 11 miRNA relative expressions. Figure 3 demonstrates the results of cluster analyses of cell lines based on the 11 miRNA expression profiles. From the analyses, the 11 miRNA expression profiles for the two lung cancer cell lines and prostate cancer have a high correlation between vectors of values. Despite their relatively close clustering, in Figure 3, the profiles of the cell lines are distinct from one another.

Discussion

It is widely accepted that over- or under-expression of a single miRNA expression is not a reliable biomarker for cancer.^{12,14,22,28} It has been suggested that simultaneous assessment of a panel of tumour-specific miRNA biomarkers will lead to higher sensitivity and specificity for diagnosis of lung cancer.¹⁹ Recently, Yu reported changes in a five miRNA panel correlated with treatment outcome of lung cancer patients.²³ In contrast, Yanaihara found that 43 different miRNAs

FIGURE 3. Dendrogram: Average linkage clustering was performed with uncentered correlation metric between groups. Length of lines from branching represents relatedness of clusters.

were over- or under-expressed in lung cancer cell lines.¹⁴ Therefore, the optimum number of miRNAs to be included in a profile is not clear at this time. We selected our 11 miRNAs, which were reported to be closely related to various cancers in the literature. We hypothesed that these 11 miRNAs would give a profile that could distinguish cancer cells from normal, and differentiate between cell lines, without requiring a larger number of miRNAs. We chose the specific miRNAs for our profiling based on previously reported changes in expression in a wide range of cancerous tissues. miR-21 is expressed in most solid cancer cells but not in non-cancerous tissue.^{14,18-21} miR-17-5p is expressed in breast, colon, lung, pancreas and prostate cancer. The let-7a cluster, miR-372, and miR-182 expression profiles have been reported to predict clinical outcome of NSCLC patients.^{22,23} Markou reported that miR-205 is a highly specific marker for squamous cell lung carcinoma.²¹ miR-155 is a important cancerous regulator and expressed in both NSCLC and breast cancer.^{14,30} miR143 is down-regulated in NSCLC and colon cancer.²⁹ miR-210 is over-expressed in lung cancers.¹⁸ Finally, the other miRNAs were selected to help refine identification of cancer cell's tissue of origin. In this study we have shown that this combination of miRNA expressions in a profile can identify and differentiate cancer cell lines.

In our study, for miRNA testing, as few as three to 16 cells was found to be sufficient for detection. The qRT-PCR assay showed high sensitivity and broad dynamic range. Owing to variations in amount of different miRNAs in each type of cell, the lowest detection limits for different miRNAs were different. Therefore, future work must take this into account when selecting which miRNAs to include in any profiling panel. With the qRT-PCR method, our precise quantification of the 11 microRNAs expression profiles provides another dimension to the molecular phenotype of malignancy and potentially provides a powerful data set for genomic analysis of tumorigenesis.

In this study, miR-21 relative expression in human lung cancer cell line A549 and SK-mes-1 is 300.71 and 93.85, respectively, which is significantly greater than in the other human cancer cell lines. Specifically, miR-21 expression in Du145 was 4.36 and in MCF-7 was 17.54, and in U118 it was 4.55. Deng reported that miR-21 is located in chromosome 17q23.1,⁹ a site of translocation breakpoints or amplification in various cancers, which leads to miR-21 over-expression. Xie and Markou both used miR-21 over-expression as a biomarker to distinguish lung cancer cells from normal lung tissue.^{19,21}

Additional analysis showed that miR-205 was over-expressed in human lung cancer cell lines A549 (534.52) and Sk-mes-1 (20.12), the breast cancer line MCF-7(5425.55), and glioblastoma U118 (7898.34), not expressed in the prostate cancer cell line Du-145(0). This large variation in expression of miR-205 can potentially be used within the miRNA expression profile as a unique biomarker for squamous cell lung carcinoma (Sk-mes-1) and prostate cancer (Du-145).

These results indicate that let-7a is over-expressed in our human cancer cell lines, including both lung cancer cell lines (A549 and Sk-mes-1) and the breast cancer cell line MCF-7. This is in contrast to previous reports that indicate that let-7a is under-expressed in both lung cancer and breast cancer.^{14,30} This discrepancy

may indicate that expression of let-7a may vary even within tumour types and warrants further study.

In this study, we identify an optimal miRNA profiling methodology that combines a panel of 11 tumour-specific miRNAs. This panel was able to distinguish cancer cells from normal cells, and to differentiate different cancer cell lines. Quantitative real-time PCR array technology was used to determine a profile based on a small number of miRNAs. The qRT-PCR assay was highly reproducible and shows clear potential to improve diagnosis in comparison with conventional methodologies. With the qRT-PCR array, we determined the miRNA molecular markers in as few as three to 16 cancer cells. Utilization of the mathematical methodology brings consistency and reproducibility to the miRNA expression analysis. In addition, by applying cluster analysis, we were able to clearly distinguish the origin of all six cell lines and to accurately sub-type the different lung cancer cell lines.

MicroRNA expression profiling of human tumors has identified signatures associated with diagnosis, staging, progression, prognosis and response to treatment. In addition, profiling has been exploited to identify microRNA genes that may represent downstream targets of activated oncogenic pathways or that are targeting protein coding genes involved in cancer. Recent studies suggest that miRNAs are the primary candidates for the elusive class of cancer predisposing genes, and that other types of non-coding RNAs may play a role in the development of a malignant phenotype. These discoveries could be exploited for the development of useful markers for diagnosis and prognosis, as well as for the development of new RNA-based cancer therapies. The next objective is to further refine the miRNA profiling and to adapt the technique to compliment other methods of analysis for a reliable and practical clinical lung cancer screening method.

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