Analysis of microRNA expression in liquid-based cytological samples may be useful for primary lung cancer diagnosis

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Abstract

Bronchoscopy is frequently performed for patients suspected to have lung cancer. However, we sometimes fail to make a definitive diagnosis, resulting in additional invasive testing. Many studies indicate that microRNAs (miRs) are abnormally expressed in cancers. We examined the diagnostic value of four miRs (miR-21, miR-31, miR-182, and miR-183) extracted from liquid-based cytology samples and validated whether they were diagnostically useful. We collected 18 surgically resected tissue samples and 136 cytological specimens obtained during bronchoscopic examination at Hiroshima University Hospital. We extracted RNA from these samples and compared the expression of four miRs by quantitative reverse transcriptase PCR (qRT-PCR). We confirmed that expression of the four miRs was significantly higher in cancer tissues than in tumor-adjacent normal tissues. We examined the expression of these miRs in 125 (cancer cases = 83, non-cancer cases = 42) of 136 cytological samples. Expression of all four miRs was significantly higher in lung cancer patients than in non-cancer patients. Among samples that were judged as benign or indeterminate, levels of these miRs were also significantly higher in lung cancer patients than in non-cancer patients. The analysis of miR expression in liquid-based cytological samples might be helpful for primary lung cancer diagnosis.

Introduction

Lung cancer is the leading cause of cancer deaths in the world. Bronchoscopy is useful for the diagnosis of lung cancer, so we perform bronchoscopy when we detect nodules, which are suspected as lung cancer by chest imaging. However, we sometimes fail to collect adequate cytological and/or biopsy samples because of tumor location or size. Therefore, it is hard for us to diagnose certain patients as having lung cancer despite the presence of a lung tumor identified by radiological examination. On the other hand, we usually use classification schemes in evaluating cytological specimens. When it is difficult to differentiate atypical cells from carcinoma cells, we judge the cytological specimen to be indeterminate. Therefore, there are some cases that undergo surgical excision for histological diagnosis or additional invasive examinations, and which can result in the diagnosis of benign lesions.

MicroRNAs (miRs) are small noncoding RNAs consisting of approximately 22 nucleotides that play important roles in the regulation of gene expression [1]. Many previous studies have revealed significant differences in miR expression between lung cancer tissue and non-cancerous lung tissue[2] [3], and the diagnostic value of serum miRs [4] [5]. However, there are few studies published regarding the diagnostic value of cytological specimens. Xie et al showed that expression of miR-21 in cancer patients was clearly higher than in cancer-free patients [6]. Huang et al showed that expression

levels of miR-29a and miR-375 of bronchial brush specimens accurately differentiates small cell lung cancer (SCLC) from non-small cell lung cancer (NSCLC), and expression of miR-34a and miR-205 of such brush specimens accurately differentiates squamous cell carcinoma from adenocarcinoma [7]. However, there have been no studies concerning the diagnosis of lung cancer, or the validity or utility of profiles of miRs extracted from liquid-based-cytology (LBC) samples collected by bronchoscopy.

We investigated whether the expression of miRs in cytological samples could be useful diagnostic biomarkers in lung cancer patients. If we could demonstrate that miR expression levels in such specimens are useful for diagnosis, this approach may potentially be applicable to routine samples. In previous studies, it has been reported that miR-21 is up-regulated in various human malignancies [8] [9], and can modulate tumorigenesis, cell proliferation, and metastasis in various cancers. For example, miR-21 targets *PTPN14* in intrahepatic cholangiocarcinoma [10], and *15-PGDH* in gastric cancer [11]. As for lung cancer, miR-21 has been used as a diagnostic marker in many studies [12]. It has been reported that levels of miR-21 increase with oncogenic K-Ras activation, and can modulate NSCLC tumorigenesis [13], by targeting *PTEN* [14], and *EZH2* [15]. Furthermore, high levels of circulating miR-21 in serum have been revealed useful for diagnosis of lung cancer. Thus, miR-21 is considered a very promising biomarker in lung cancer [16] [17].

MiR-31 is also known to be one of various oncogenes involved in colon and uterine cervical cancer [18] [19]. As for lung cancer, Xi Liu et al showed that miR-31 can regulate large tumor

suppressor 2 (*LATS2*) and PP2A regulatory subunit B alpha isoform (*PPP2R2A*) in human lung cancer cell lines [20]. Edmonds et al showed miR-31 is overexpressed in human lung adenocarcinoma and overexpression of miR-31 independently correlates with poor survival, and accelerates lung tumorigenesis in mice [21]. Both miR-182 and miR-183 are members of the miR-183 cluster belonging to the polycistronic miRNA cluster located in the 5-kb region of human chromosome 7q32.3.[22] , Both mir-182 and miR-183 have been reported to be upregulated in human malignancies [23] [24]. Previous studies have shown that tissue and serum levels of miR-182 and miR-183 are elevated in patients with lung cancer compared with healthy controls [25] [26]. MiR-182 is known to promote lung cancer tumorigenesis by modulating pyruvate dehydrogenase kinase 4 [27] and exerts an oncogenic role by targeting *FBXW7* and *FBXW11* [28]. MiR-183 has been reported to be one of various oncogenes by targeting *EGR1* and promoting tumor cell migration [29].

In this study, we examined four miRs, including miR-21, miR-31, miR-182, and miR-183, in surgically resected cancerous and non-cancerous lung tissues and in cytological samples of lung cancers and non-cancerous lesions, and verified whether the accuracy of discrimination between benign and malignant cases can be improved by using the expression profiles of these four miRs extracted from cytological samples.

Materials and Methods

Tissue samples

We collected 18 patient samples of primary lung cancer tissues and paired adjacent normal tissue surgically resected in Hiroshima University Hospital and kept in our lung cancer file. Characteristics of the 18 lung cancer patients are shown in Table 1. Normal and malignant tissue samples were acquired during routine patient therapeutic surgery. We collected tissue samples of approximately 100 mg, which were immediately placed in RNA*later*® (Ambion, Inc., Austin, TX) and stored in a -20℃ freezer until use.

Cytological samples

When we performed bronchoscopy, we submitted bronchial lavage fluid and the washed device fluid as cytological specimens. We assembled 136 bronchial cytological samples kept in our lung cytology file. Patient characteristics are shown in Table 2. Each case was followed until a final diagnosis of cancer or not was made. Submitted specimens were centrifuged at 600×*g* to obtain cell pellets, which were then fixed in Cellprep® (Roche Diagnostics, Basel, Switzerland) to prepare for cytological specimens. Fixed cytological specimens were stored at 26℃. After preparation, approximately half of the residual samples were used for this study within 24 h.

Patient selection of cytological samples.

A total of 136 bronchial cytological samples were collected. We examined the expression of miRs according to the selection criteria (Figure 1). Before performing RT-qPCR, 10 samples were excluded. Cytological examination of the samples was successfully performed in 134 cases; 2 samples were judged as inappropriate because there were few cells present. Four samples involved metastatic cancer, and 5 samples were not used because the total amount of RNA recovered was too low for the present analysis. Finally, 125 samples that could be detected were examined by RT-qPCR. The relationship of examined cases involving histological diagnosis and cytologic classification is shown in Table 3. The number of samples that yielded positive amplification results was different for each miR; miR-21 was detected in all samples, but other miRs were not detected in certain samples. In 29 samples, Ct values for one to three of the four miRs were undetectable within 45 cycles; in 96 samples, Ct values for all four miRs were detected. Expression of the four miRs was compared with pathological diagnosis. Histological examination of surgically resected lung tumors and biopsy specimens was performed in 31 (37.3 %) and 50 cases (60.2 %), respectively. If a case was diagnosed as cancer based on pathological examination, including histological and/or cytological examinations, it was defined as T (tumor group). If a case was diagnosed as having a benign lesion, such as a granuloma or infectious nodule based on pathological diagnosis or on radiological examination showing that, a tumor had become smaller, or on bronchoalveolar lavage testing showing an interstitial pneumonia pattern, it was defined as N (non-tumor group).

RNA extraction and quantitative qRT-PCR

Total RNA was extracted from tissues using *mir*VANA Isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Total RNA was extracted from LBC samples using Magcore® Compact automated nucleic acid extractor (RBC Bioscience, New Taipei City, Taiwan) and Magcore® Total RNA Cultured Cells Kit (RBC Bioscience, New Taipei City, Taiwan) according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop® lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracted RNA samples were stored at -80℃ until use.

Total RNA was diluted to 2 ng/μL with nuclease free water and 10ng of total RNA was reverse-transcribed to complementary DNA (cDNA) using a TaqMan® miR reverse-transcription kit and TaqMan® miR assays (Thermo Fisher Scientific, Waltham, MA, USA). According to the manufacturer's protocol, the reverse transcriptase (RT) reaction was performed in a total volume of 15 μL per reaction and under the following conditions: 16℃ for 30 min, 42℃ for 30 min, 85℃ for 5 min, and then held at 4℃. The following primers were used: *RNU6B* (U6 small nuclear RNA; assay 001093), miR-21 (assay 000397), miR-31 (assay 002279), miR-182 (assay 002334), and miR-183 (assay 002269).

Quantitative RT-PCR was performed using Illumina® Eco Real-time PCR system (Illumina, San Diego, CA, USA). Real-time PCR was performed in a total volume of 20 μL per reaction. After the RT reaction,1.33 μL of the RT product was mixed with 10 μL of TaqMan® Universal Mater Mix II (Thermo Fisher Scientific, Waltham, MA, USA) and 1 μL specific probes of TaqMan® miR Assays (Thermo Fisher Scientific), and nuclease-free water, and subjected to real-time PCR. *RNU6B* was used as an endogenous control for the normalization of expression levels. The relative expression levels of target miRs were calculated by the ΔCt method: ($Ct_{sample} - Ct_{RNU6B\ sample}$) and compared between samples obtained from lung cancer and normal tissues. All experiments were repeated three times.

Statistical analysis

The Data was analyzed using JMP Pro ver.14.1.0 (SAS Institute Inc., Cary, NC, USA). Mann-Whitney test was used to analyze the differences in microRNA expression levels between subjects. Receiver operating characteristic (ROC) curves and the area under curve (AUC) analyses were used to determine the sensitivity and specificity of each microRNA. $P < 0.05$ was considered statistically significant.

RESULTS

Primary lung cancer and non-cancerous lung tissues

We analyzed the expression of four miRs, including miR-21, miR-31, miR-182, and miR-183, in 18 pairs of primary lung cancer tissue and non-cancerous lung tissue. As shown in Figure 2, the relative expression levels of the four miRs in lung cancer were significantly higher than in adjacent, non-cancerous tissues.

Cytological samples

Next, we analyzed the expression of these four miRs in 125 cytology samples. Eighty-two cases of cytological samples were judged as benign or indeterminate, and 40 were finally diagnosed as cancerous after histological examination (Table 3). All cases initially judged as suspicious or malignant were finally diagnosed as cancerous. As shown in Figure 1, the number of samples that could be positively amplified was different for each marker; miR-21 was detected in 125 samples, miR-31 in 123 samples, miR-182 in 109 samples, and miR-183 in 102 samples. For cytological samples, the relative expression of each miR in cancer cases was significantly than those of non-cancer cases (Figure 3). There was no significant correlation between these four miRs and clinical stage (Supplemental figure 2). Considering the expression of each tumor subtype, there was no significant difference between each subtype (Supplemental figure 3). Notably, even for patients whose lesions

were cytologically judged as benign or indeterminate, the relative expression of each miR in cancer cases was significantly higher than those of non-cancer case (Figure 4).

Of the 96 cases in which all four miRs were detected, 54 (56%) were judged as benign, and 24 were finally diagnosed as cancerous. In these 54 cases, ROC analysis was conducted to evaluate the diagnostic value of miR expression in samples diagnosed as benign (Figure 5). The diagnostic values of miR-21, miR-31, miR-182, and miR-183 were 0.676, 0.683, 0.688, and 0.772, respectively. The combined diagnostic value of expression levels of the four miRs was 0.810, better than for each miRNA individually for benign samples.

DISCUSSION

In the present study, we showed that four miRs, including miR-21, miR-31, miR-182, and miR-183, extracted from lung tissue were significantly up-regulated in comparison with non-cancerous lung tissue, and that the expression of the same four miRs extracted from cytological samples of lung cancer cases obtained by bronchial brushing were higher than those of non-lung cancer cases. These results were consistent with those reported in previous studies [12 26 28]. In particular for the samples pre-operatively gained from lung cancer cases but cytologically judged as benign and which did not contain obvious carcinoma cells, the expression of the four miRs was significantly higher than those gained pre-operatively from non-cancerous cases cytologically judged as benign.

To the best of our knowledge, there have been no reports regarding the evaluation of miRs using LBC samples obtained by bronchoscopy. Interestingly, although the expression of miRs extracted from cancer cases was higher than those extracted from non-cancer cases, there were almost no differences in the expression of the investigated miRs among cytology samples judged as benign, indeterminate, suspicious for malignancy, and malignant, irrespective of the presence of malignant cells. In general, when bronchoscopy is performed, we obtain biopsy samples and cytology samples by brushing, while checking the location of the tumor by chest X-ray imaging. Usually, we obtain not only tumor cells, but non-cancerous epithelial, stromal and blood cells. For this reason, the cytology samples contained various cells. Samples obtained from surgically resected lung cancer tissue contained higher proportions of tumor cells than cytology samples obtained from lung cancer cases by bronchoscopy because cytological samples contain various cells, and the proportion of these cells varied between samples. In other words, among the cases judged as malignant by cytology, some samples contained only a few tumor cells, whereas other samples contained many normal epithelial and/or blood cells. It is probably difficult to identify differences in miR expression between samples containing few tumor cells or many normal cells, and in samples obtained from non-tumor cases. We speculate that total RNA extracted from samples containing many blood cells may also contain RNA derived from inflammatory or red blood cells and, consequently, these may affect the detection of

miRs. Some circulating miRs can be affected by hemolysis[30]. Furthermore, the quantity and types of miRs vary among the blood components involved [31].

Pavel et al showed that some miR expression levels in normal bronchial epithelium of smoking patients were higher in lung cancer patients than in non-cancerous ones [32]. Although the four miRs examined in the present study were not included in Pavel and colleagues' earlier study, the results of our research suggested that the expression of certain miRs might be affected in non-cancerous cells by the presence of lung cancer. In the present study, it was considered difficult to eliminate all of these effects in samples composed of various cells.

In addition, although miR-182 and miR-183 are known as oncogenes, miR-182 and miR-183 are also reported to be tumor suppressors [33] [34]. Grofu et al showed that miR-183 regulates migration and invasion via Ezrin in lung cancer cells, and identified miR-183 as a potential inhibitor of metastasis [34]. Levels of other miRs may also be affected by various factors, including clinical ones, and there is a possibility that this may be the reason that it was difficult to identify significant differences in miR expression. Furthermore, the non-cancerous samples may also have affected these results. In the present study, normal controls included cytological samples gained from not only benign nodules, but also bronchoalveolar lavage of interstitial pneumonia, including idiopathic pulmonary fibrosis, chronic hypersensitivity pneumonitis, sarcoidosis, and so on. In general, as bronchoalveolar

lavage of these interstitial pneumonias contains various inflammatory cells, the detected expression levels of miRs can be influenced by the presence of inflammatory cells.

To the best of our knowledge, there have been no reports, which explored the expression of miR extracted from cytological samples fixed with Cellprep® and using automated extraction by Magcore®. Cellprep® is one of the LBC systems developed by Roche Tissue Diagnostics; Cellprep® liquid is a methanol- and formalin-free fixative. In a previous study, fixation time did not affect the detection sensitivity of miRs [35]. It was suggested that Cellprep® liquid does not preclude the feasibility of miRNA analysis, and this method can be easy to apply to routine samples.

There are certain limitations to this study. First, there were many samples in which miR expression was undetectable. We considered two reasons for this problem. First, the detected expression levels of miRs extracted by Magcore® were lower than that detected by an alternative, manual extraction kit (Supplemental Figure.1). It may be suggested that the extraction efficiency for small RNAs, such as miRs, using Magcore® was lower than when a manual extraction kit was employed. Therefore, it was considered that use of the manual extraction kit would be preferable. Another reason is that there was no specific kit available for LBC samples. Because we assumed that the RNA extraction kit for cultured cells could be used for LBC samples, we extracted miRs from LBC samples using an RNA extraction kit intended for cultured cells. Although it seems the extraction method used in the present study was not optimal, Magcore® is a fully automated extraction system,

and the inherent simplicity of Magcore® is a very strong point. Indeed, although the expression of certain miRs was undetectable by RT-qPCR for some samples, many samples were successfully examined in the present study, which suggested that Magcore® Total RNA Cultured Cells Kit could be useful for cytological samples. Secondly, the question arises of whether the choice of internal controls was reasonable or not. Although *RNU6B* is one of the most frequently used reference genes employed as an internal control, use of endogenous controls for miRs extracted from whole blood remains a controversial issue [36]. In the present study, although we thought that epithelial cells had a stronger influence than blood cells, inappropriate choices of internal controls can introduce bias in the comparison of expression of miRs.

In summary, we demonstrated that four miRs could be extracted from LBC samples for classification of their expression levels. Furthermore, the four miRs studied here exhibited significantly higher expression levels in patients with lung cancer compared with non-cancerous patients. There is an opportunity to improve lung cancer diagnostic rates by employing bronchoscopy and cytology, and by performing RT-qPCR in order to examine miR expression levels.

CONFLICT OF INTEREST

All the authors declare that they have no conflict of interest.

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Table 1. Clinical characteristics of 18 NSCLC patients

Table 2. Clinical characteristics of 125 patients

Table 3. Relationship of case numbers examined by RT-qPCR for 4 miRNAs between histological diagnosis and cytologic classification

Figure 1. Selection protocol for liquid-based cytological specimens. (Number of cases histologically diagnosed cancerous (T) or non-cancerous (N))

Figure 2. Relative expression of miR-21(A), miR-31(B), miR-182(C), and miR-183(D) in lung cancer tissues and adjacent non-cancerous tissues. Four miRNAs were significantly up-regulated in cancer tissues. T: lung cancer tissues, N: adjacent non-cancerous tissues. *$P < 0.05$ **$P < 0.01$

Figure 3. Relative expression of each miRNA in cytological samples. Four miRNAs were significantly up-regulated in samples obtained from cases finally diagnosed as cancerous(A-D). T: Cases finally diagnosed as cancer. N: Cases finally diagnosed as non-cancer. *$P < 0.05$ **$P < 0.01$

Figure 4. Relative expression of each miRNA in cytological samples judged as benign or indeterminate. Four miRNAs were significantly up-regulated in samples obtained from cases histologically diagnosed as cancerous in comparison with samples obtained from cases histologically diagnosed as non-cancerous(A-D). T: Cases finally diagnosed as cancer. N: Cases finally diagnosed as non-cancer. *p ＜0.05 **p＜0.01

Figure 5. ROC curve analysis of diagnostic value in cytological samples diagnosed as benign. ROC curve with corresponding the area under the ROC curve for each miRNA expression in LBC from cancer cases vs. non-cancer cases (A-D). ROC curve with corresponding the area under the ROC curve for 4 combined miRNA expression in LBC from cancer cases vs. non-cancer cases (E). The diagnostic value of 4 combined miRNAs was better than each individual miRNA.

Supplemental figure 1. The difference of Ct value between Magcore® and manual isolation kit. The detected expression levels of miRs extracted by Magcore® were lower than that detected by an manual isolation kit

Supplemental figure 2. The correlation between each miR and clinical stage. There was no significant correlation between each miR and clinical stage.

Supplemental figure 3.

The expression of each miR by tumor subtype. There was no significant difference between each tumor subtype.

N: cases finally diagnosed as non-cancer. Ad: cases diagnosed as adenocarcinoma. Sq: cases diagnosed squamous cell carcinoma. Small: cases diagnosed as small cell lung cancer. *$p < 0.05$, **$p < 0.005$.

Table 1. Clinical characteristics of 18 NSCLC patients

Variables	Case no.
Median age (range), years	68(50-89)
Sex	
Male	12
Female	6
Smoking	
Never	6
Former	9
Current	3
Lung cancer histologic type	
Adenocarcinoma	12
Squamous	4
Other	2
pStage	
0	2
I	11
II	0
III	5

Table 2. Clinical characteristics of 125 patients

Variables	Case no.
Median age (range), years	73(21-96)
Sex	
Male	77
Female	48
Smoking	
Never	41
Former	62
Current	22
Cytology classification	
benign	67
indeterminate	15
suspicious of malignancy	15
malignant	28
Diagnosis	
Tumor(T)	83
Non-tumor(N)	42
Lung cancer histologic type	
Adenocarcinoma	52
Squamous cell carcinoma	15
Small cell carcinoma	9
Other	7
cStage	
I	23
II	8
III	16
IV or postoperative recurrence	36

Table 3. Relationship of case numbers examined by RT-qPCR for 4 miRNAs between histological diagnosis and cytologic classification

	Benign	Indeterminate	Suspicious for malignancy	Malignancy	Total
T(lung cancer)	27	13	15	28	83
N(Benign lesion)	40	2	0	0	42
Total	67	15	15	28	125

Figure 1. Selection protocol for liquid-based cytological specimens. (Number of cases histologically diagnosed cancerous (T) or non-cancerous (N))

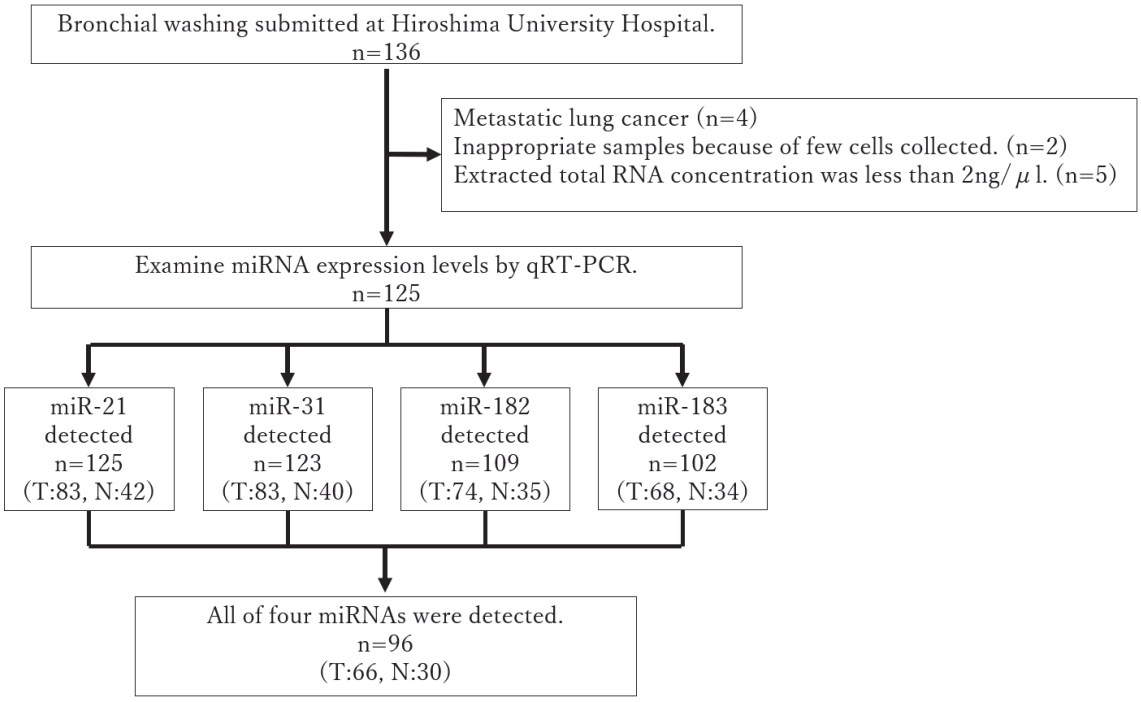


Figure 2. Relative expression of miR-21(A), miR-31(B), miR-182(C), and miR-183(D) in lung cancer tissues and adjacent non-cancerous tissues.

Four miRNAs were significantly up-regulated in cancer tissues.

T: lung cancer tissues, N: adjacent non-cancerous tissues.

*p＜0.05　**p＜0.01

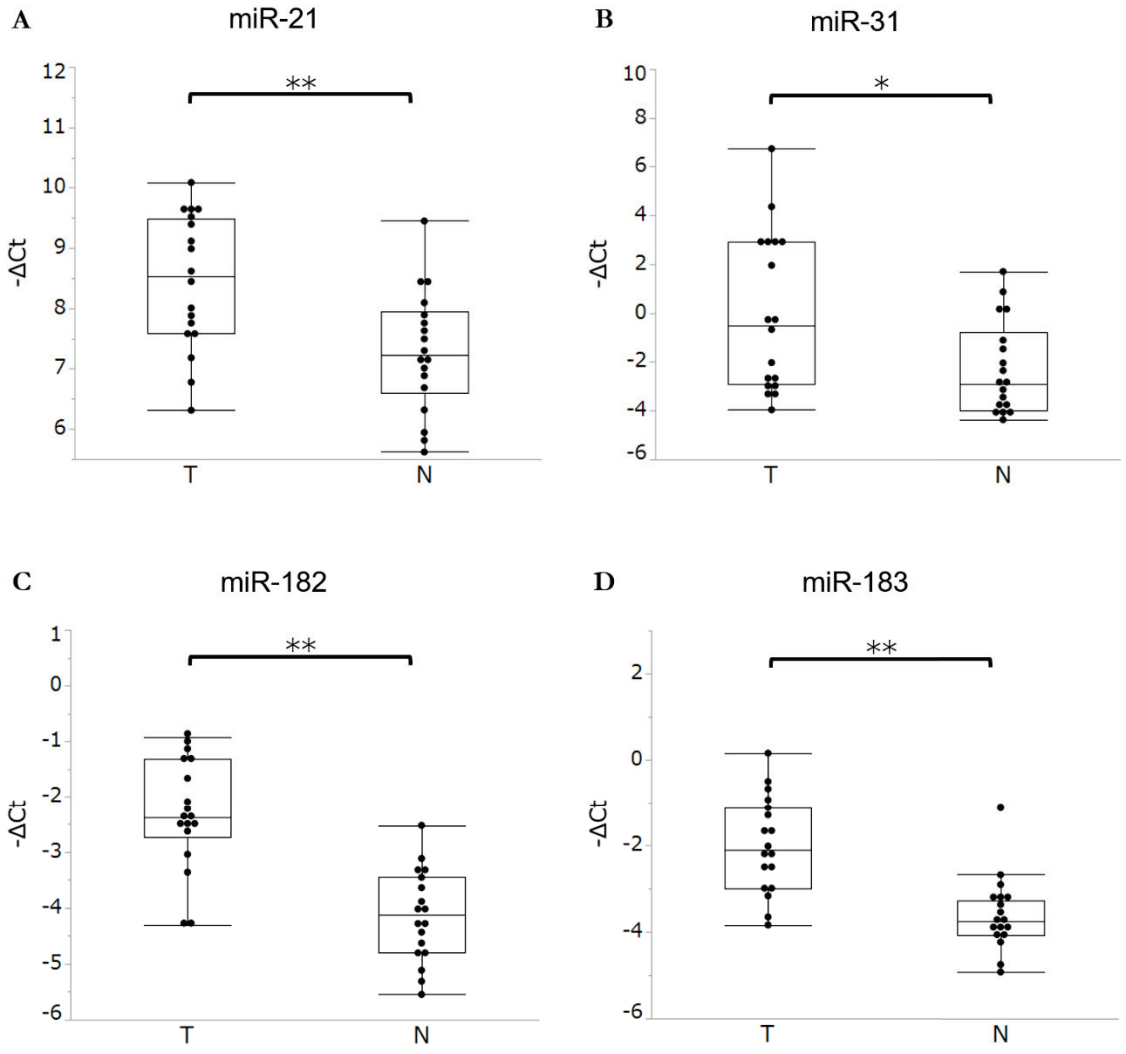


Figure 3. Relative expression of each miRNA in cytological samples.

Four miRNAs were significantly up-regulated in samples obtained from cases finally diagnosed as cancerous(A-D).

T: Cases finally diagnosed as cancer. N: Cases finally diagnosed as non-cancer.

*p＜0.05　**p＜0.01

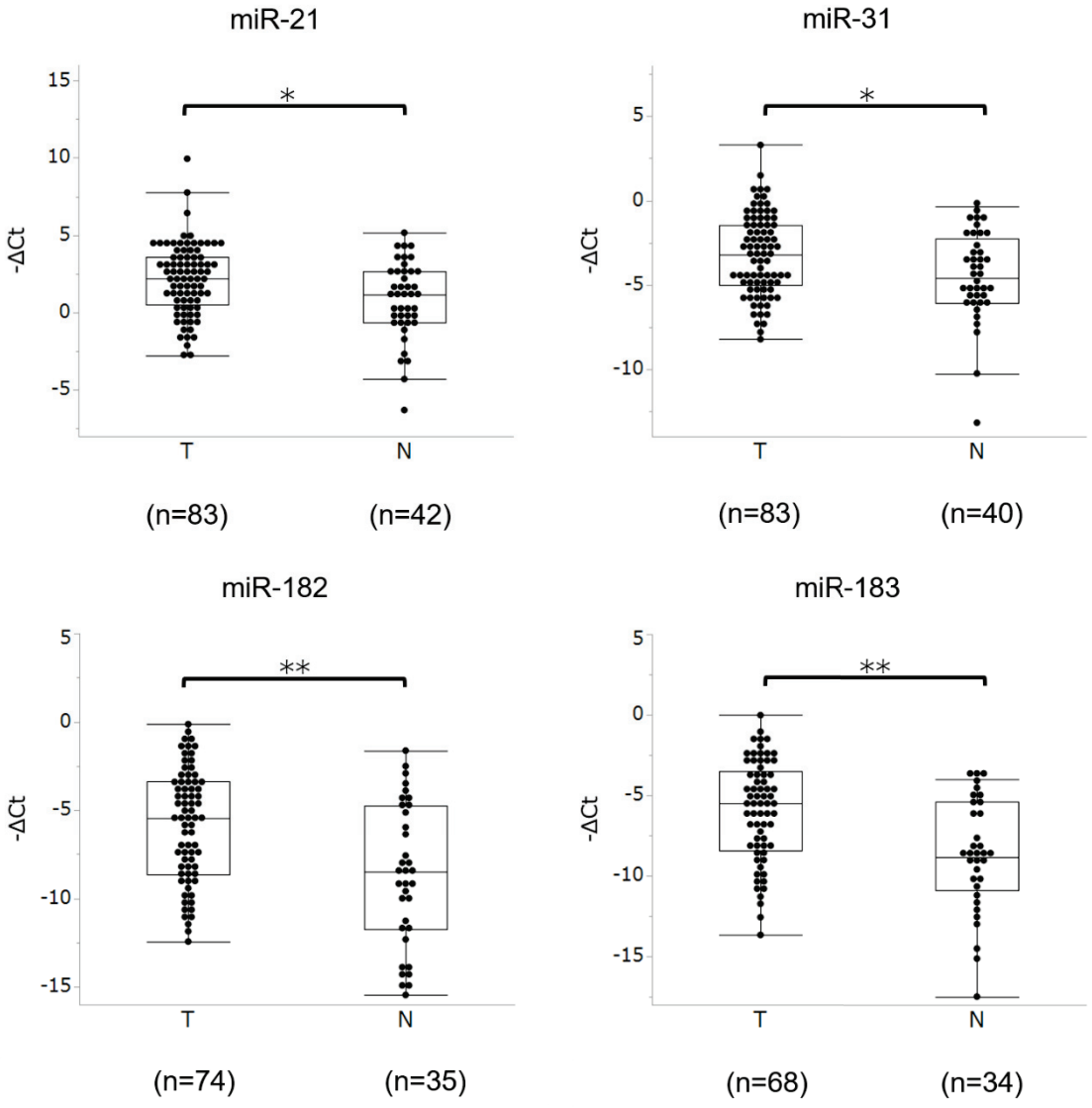


Figure 4. Relative expression of each miRNA in cytological samples judged as benign or indeterminate.

Four miRNAs were significantly up-regulated in samples obtained from cases histologically diagnosed as cancerous in comparison with samples obtained from cases histologically diagnosed as non-cancerous(A-D).

T: Cases finally diagnosed as cancer. N: Cases finally diagnosed as non-cancer.

*p＜0.05　**p＜0.01

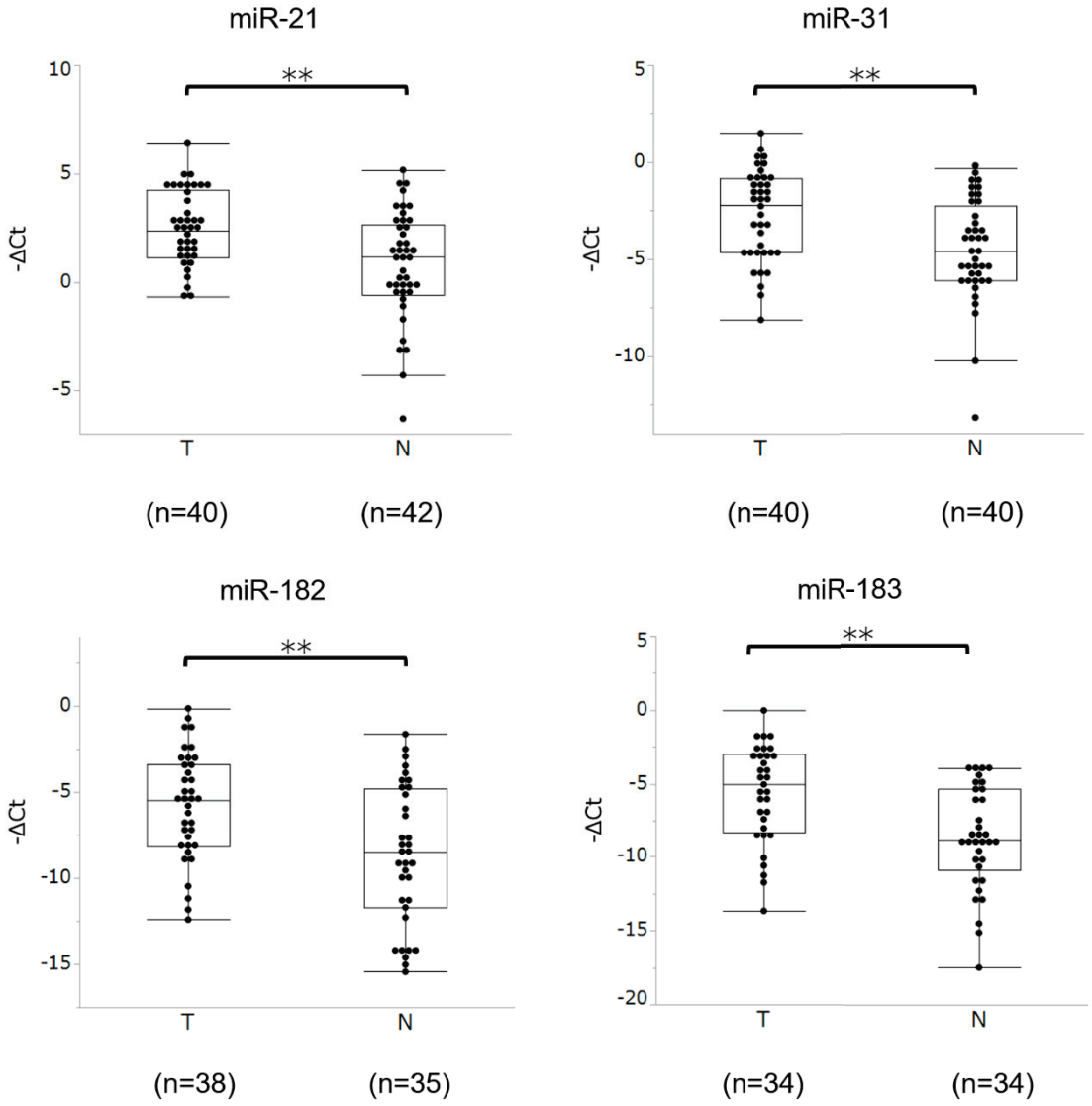
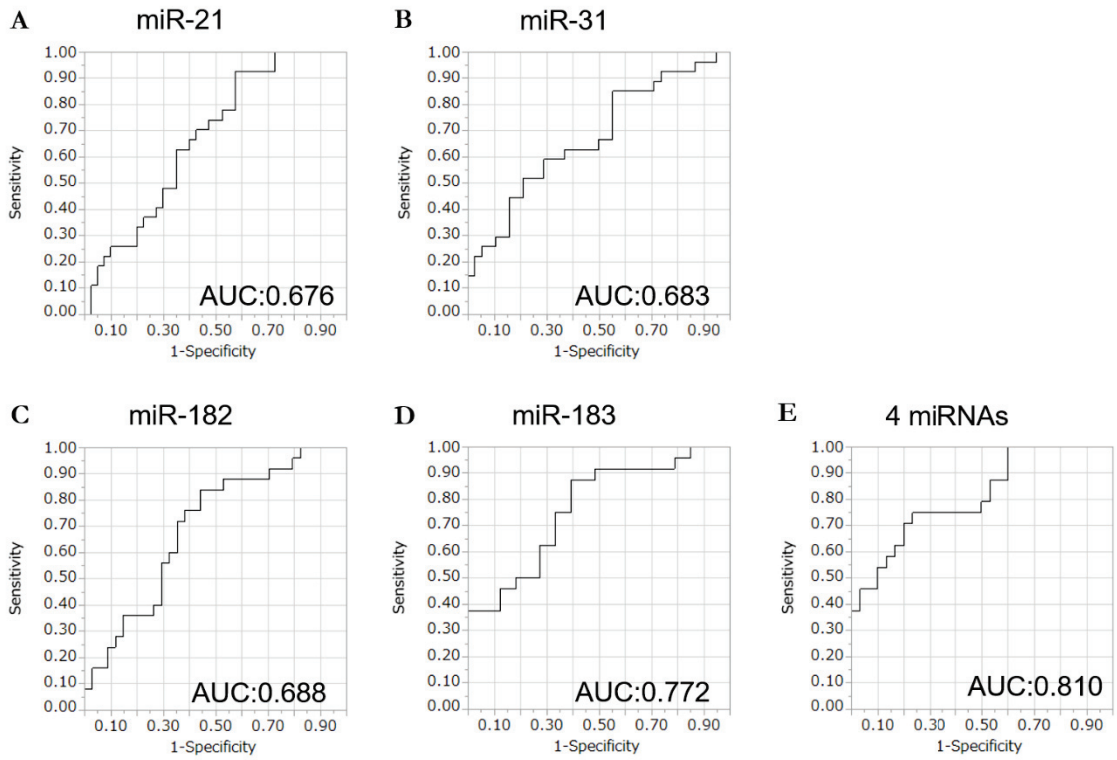


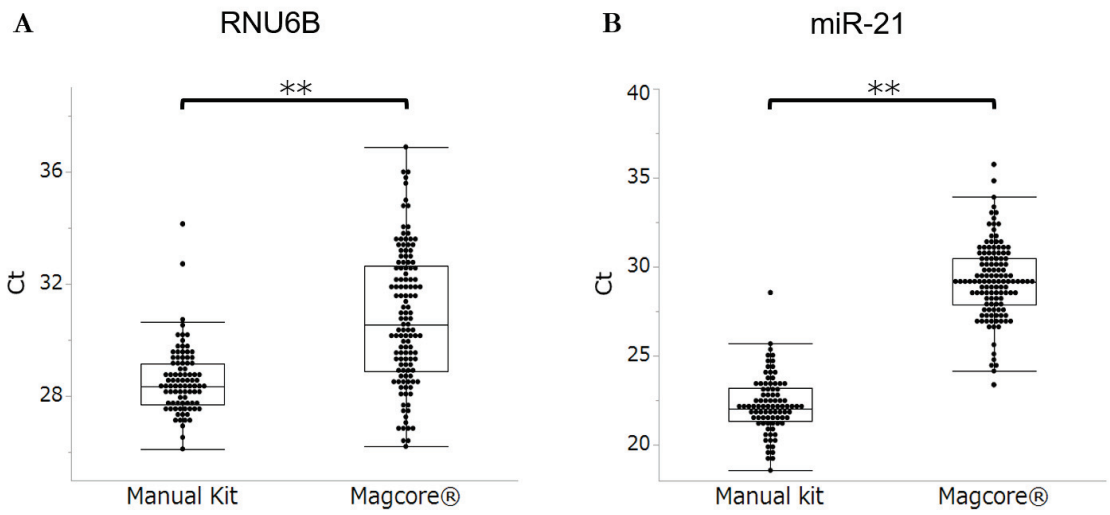
Figure 5. ROC curve analysis of diagnostic value in cytological samples diagnosed as benign.

ROC curve with corresponding the area under the ROC curve for each miRNA expression in LBC from cancer cases vs. non-cancer cases (A-D). ROC curve with corresponding the area under the ROC curve for 4 combined miRNA expression in LBC from cancer cases vs. non-cancer cases (E). The diagnostic value of 4 combined miRNAs was better than each individual miRNA.



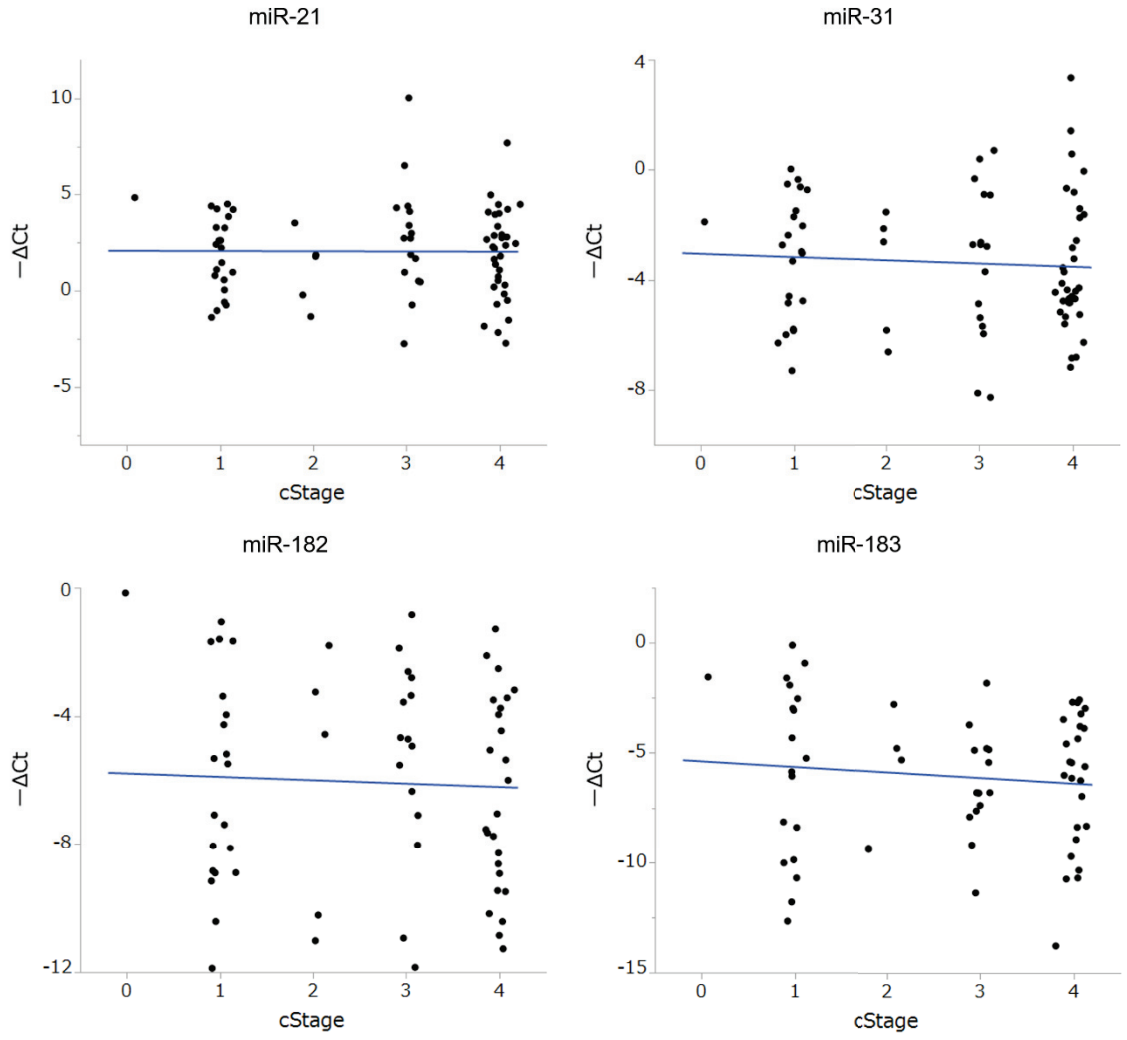
Supplemental figure 1. The difference of Ct value between Magcore® and manual isolation kit.

The detected expression levels of miRs extracted by Magcore® were lower than that detected by a manual isolation kit.



Supplemental figure 2. The correlation between each miR and clinical stage.

There was no significant correlation between each miR and clinical stage.



Supplemental figure 3. The expression of each miR by tumor subtype.

There was no significant difference between each tumor subtype.

N: cases finally diagnosed as non-cancer. Ad: cases diagnosed as adenocarcinoma. Sq: cases diagnosed squamous cell carcinoma. Small: cases diagnosed as small cell lung cancer.

*p＜0.05, **p＜0.005