Matrix metalloproteinase-1 and microRNA-486-5p in urinary exosomes can be used to detect early lung cancer: A preliminary report

WATARU ANDO[1*], MASAYA SOGABE[2-4*], SHIGEMI ISHIKAWA[3,4*], TAKAYUKI UEMATSU[5], HIROYUKI FURUYA[6], HIROAKI YOKOMORI[7], YUTAKA KOHGO[8,9], KATSUYA OTORI[10], TOMOYUKI NAKANO[3,4], SHUNSUKE ENDO[2], HIROYOSHI TSUBOCHI[11] and ISAO OKAZAKI[12]

[1]Department of Clinical Pharmacy, Center for Clinical Pharmacy and Sciences,
Kitasato University School of Pharmacy, Tokyo 108-8641; [2]Department of General Thoracic Surgery,
Jichi Medical University Saitama Medical Center, Saitama 330-0834; [3]Center for Respiratory Diseases;
[4]Department of Chest Surgery, International University of Health and Welfare Hospital,
Nasu-Shiobara, Tochigi 329-2763; [5]Biomedical Laboratory, Division of Biomedical Research,
Kitasato University Medical Center, Kitamoto, Saitama 364-8501; [6]Basic Clinical Science and Public Health,
Tokai University School of Medicine, Isehara, Kanagawa 259-1193;
[7]Department of Internal Medicine, Kitasato University Medical Center, Kitamoto,
Saitama 364-8501; Departments of [8]Internal Medicine and [9]Preventive Medicine,
International University of Health and Welfare Hospital, Nasu-Shiobara, Tochigi 329-2763;
[10]Research and Education Center for Clinical Pharmacy, Division of Clinical Pharmacy,
Laboratory of Pharmacy Practice and Science 1, Kitasato University School of Pharmacy, Sagamihara,
Kanagawa 252-0375; [11]Department of General Thoracic Surgery, Jichi Medical University,
Shimotsuke, Tochigi 329-0498; [12]Department of Health and Welfare, Higashi Nippon International University,
Iwaki, Fukushima 970-8023, Japan

Correspondence to: Dr Wataru Ando, Department of Clinical Pharmacy, Center for Clinical Pharmacy and Sciences, Kitasato University School of Pharmacy, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
E-mail: andow@pharm.kitasato-u.ac.jp

Professor Isao Okazaki, Department of Health and Welfare, Higashi Nippon International University, 37 Suganezawa, Taira Kamata, Iwaki, Fukushima 970-8023, Japan
E-mail: iokazaki@m.tonichi-kokusai.u.ac.jp

*Contributed equally

Abbreviations: AC, adenocarcinoma; AUC, area under the receiver operating characteristic curve; CIC, Capicua; EGFR, epidermal growth factor receptor; Ets, erythroblast transformation specific; ETVs, Ets translocation variants; LC, lung cancer; LCC, large cell carcinoma; miRNA, microRNA; MMP-1, matrix metalloproteinase-1; OR, odds ratio; ROC, receiver operating characteristic; RT-PCR, reverse transcription polymerase chain reaction; SCLC, small cell lung cancer; SC, squamous cell carcinoma; TKI, tyrosine kinase inhibitor

Key words: lung cancer screening, urinary exosome, MMP-1, miRNA-486-5p

Abstract. The present study describes a novel molecular-genetic method suitable for lung cancer (LC) screening in the work-place and at community health centers. Using urinary-isolated exosomes from 35 patients with LC and 40 healthy volunteers, the expression ratio of MMP-1/CD63, and the relative expression levels of both microRNA (miRNA)-21 and miRNA-486-5p were measured. MMP-1/CD63 expression ratio was significantly higher in patients with LC than in the healthy controls {1.342 [95% confidence interval (CI): 0.890-1.974] vs. 0.600 (0.490-0.900); P<0.0001}. The relative expression of miRNA-486-5p in male healthy controls was significantly different from that in female healthy controls, whereas there was no significant difference in miRNA-21. Receiver operating characteristic curve (ROC) analysis of MMP-1/CD63 showed 92.5% sensitivity and 54.3% specificity, whereas miRNA-486-5p showed 85% sensitivity and 70.8% specificity for men, and 70.0% sensitivity and 72.7% specificity for women. The logistic regression model used to evaluate the association of LC with the combination of MMP-1/CD63 and miRNA-486-5p revealed that the area under the ROC curve was 0.954 (95% CI: 0.908-1.000), and the model had 89% sensitivity and 88% specificity after adjusting for age, sex and smoking status. These data suggested that the combined analysis of MMP-1/CD63 and miRNA-486-5p in urinary exosomes may be used to detect patients with early-stage LC in the work-place and at community health centers, although confirmational studies are warranted.

Introduction

Lung cancer (LC) was the leading cause of mortality in 2018 in males and the third in females worldwide (1). In Japan, recent mortality statistics (2021) and incidence rate (2019) revealed that LC accounts for the highest mortality and fourth highest incidence rate in males, and the second highest mortality and third highest incidence rate in females (2). However, LC treatment has improved, which is mainly attributed to the increased detection of early-stage LC, especially in cases where tumors are ≤2 cm in size (3). Low-exposure CT and MRI have majorly contributed to the diagnosis of early LC (4). However, the prognosis of LC remains poor. In Japan, the Act for Health and Safety in Labors strictly requires all workers to undergo a chest X-ray examination to prevent tuberculosis and pulmonary diseases due to occupational hazards. However, chest X-ray examination is not suitable for LC detection because it is difficult to diagnose early-stage LC using plain radiography; the specificity and sensitivity of chest X-ray for LC is also insufficient (5). Hence, there remains a need for develop a novel LC screening test with easy access, high quality, and low price, for the early detection of early-stage patients with LC having high sensitivity and specificity at the work-place and community health centers.

We have previously reported a novel breast-cancer screening method using a combination of microRNA-21 (miRNA-21) and matrix metalloproteinase-1 (MMP-1) in urinary exosomes capable of detecting breast cancer in 95% cases before metastasis (6). All cells secrete exosomes that are small vesicles (30-150 nm in size) containing DNA, mRNA, miRNAs, oncogenic genes, proteins, and lipids among others (7-9). These exosomes relay important cell information between cells, migrate through the blood vessels or other routes, and adhere to the surface of distant cells (7-10). Therefore, they are crucial for maintaining harmony with other organs, not only for physiological growth, development, and cell death, but also for pathogenesis. Exosomes from the cancer cells can invade the distant cells of other organs, eventually resulting in metastasis (7-10). Therefore, urinary exosomes contain sufficient information on the oncogenic genes and their related proteins. However, LC screening using urinary exosomes has not been reported to date. Furthermore, MMP-1 is one of the key enzymes of collagen metabolism (11) involved in the carcinogenesis in LC (12-15). An increased expression of MMP-1 evidenced in early stage of LC (15). However, MMP-1 has not been investigated to serve as a biomarker for LC screening.

We selected miRNA-21 and miRNA-486-5p as candidate miRNAs to serve as biomarkers for LC screening in the present study (16-21). Several studies have reported the usefulness of miRNA-21 and miRNA-486-5p in diagnosing LC using the circulating plasma exosomes (16,20). Both miRNA-21 and miRNA-486-5p have multi-faced functions in cancer diagnosis, tumorigenesis, and prognosis (16,17,20). The function of miRNA-21 inhibits several tumor suppressor genes such as *PTEN* and *HOXD10* (17-19). MiRNA-486-5p targeting the *PIK3R1* gene is involved in the suppression of tumor cell growth (21).

Herein, we analyze the expression of MMP-1/CD63 as well as miRNA-21 and miRNA-486-5p in urinary exosomes of patients with LC and determine their application in early screening and detection of LC in patients.

Materials and methods

Study population. This study was conducted at the International University of Health and Welfare (IUHW) Hospital (Nasu-Shiobara City, Tochigi Japan) and the Kitasato University Medical Center (Kitamoto City, Saitama, Japan) between October 1, 2018 and October 31, 2022. The study was approved by the ethics committee of the two abovementioned institutions. The details were described in Declarations.

Thirty-five patients with LC (24 males and 11 females; mean age, 71.0±8.4 and 64.8±9.5 years, respectively) included in this study were admitted to the Center for Respiratory Diseases, Department of Chest Surgery, IUHW Hospital. The patients were diagnosed with LC after performing chest X-ray, chest CT, chest MRI, and subsequent needle biopsy before surgery, if necessary. The final pathological diagnosis was performed on the basis of surgical resection at the IUHW Hospital. The clinical stage was classified according to the eighth edition of the TNM classification of LC (22).

Urine samples were collected before surgical treatment in the morning before breakfast and stored at -80°C until the exosomes were separated. All samples were collected before patients received surgery, chemotherapy, or radiation.

The control urine samples were obtained from 40 healthy controls (20 males and 20 females) selected among 533 persons (351 males and 182 females) who visited the Department of Preventive Medicine, IUHW Hospital for a health check from January 20, 2020 to February 8, 2020, because only a few visitors were over 70 years of age. The number of selected individuals in the 40-year-old, 50-year-old, 60-year-old, and >70-year-old groups in each sex group was more than five. All selected controls had no complaints or abnormal physical findings such as obesity, hypertension, abnormal peripheral blood examination and blood chemistry, abnormal ECG findings, or abnormal findings on chest radiography, upper GI endoscopy, or abdominal echography. There was no history of cancer, signs of dysplasia, inflammatory disease, autoimmune disease, or chronic diseases such as cardiac, liver, or kidney diseases. All participants provided written informed consent.

Classification of LC clinical stage. The clinical stage was classified according to the eighth edition of the TNM classification of LC (22). The TNM classification of the 35 patients with LC is listed in Table I.

Isolation of the urinary exosomes. All urine samples were transferred to Kitasato University Medical Center at -80°C. A uniform volume of 2 ml urine was collected from all participants for exosome isolation. Before exosome isolation, the thawed urine sample was centrifuged at 3,000 x g for 15 min at 4°C and passed through a 0.22-μm nylon filter. The urinary exosomes isolated using the Exosome Isolation Kit (Cat. 130-110-912, Miltenyi Biotec, Bergisch Gladbach, Germany) were subjected to western blotting using SDS-PAGE and anti-CD63 biotin-conjugated antibody (BioLegend Inc., San Diego, CA, USA) and identified using transmission electron microscopy (Fig. S1) as reported previously (6). The pellet of isolated exosomes was placed over the Transmission Electron Microscopy (TEM) grid coated with carbon/formvar (Oken-Shoji Co., Tokyo, Japan), stained by the 2% uranyl

Table I. Lung cancer cases, TNM classification, and characteristics.

No.	Age/Sex[a]	BMI, kg/m²	Smoking Habit	TNM[b]	Stage[c]	Patho[d]	MMP1/CD63[e]	miR-21, $2^{\Delta\Delta Cqf}$	miR-486-5p, $2^{\Delta\Delta Cqf}$	Family history[g]
1	60/M	22.3	Current	T1aNxM0	IA1	AC	1.25	0.00	2.87	LC
2	85/M	19.6	Former	T2aNxM0	IB	AC	0.89	1.31	1.21	None
3	70/M	22.4	Never	T2bN0M0	IIA	AC	1.80	0.33	5.65	None
4	76/M	25.7	Former	T1cN0M0	IA3	AC	1.92	1.47	5.23	None
5	72/F	20.1	Never	T1bN0M0	IA2	AC	4.03	0.12	0.37	GC
6	70/F	21.5	Never	T1aN0M0	IA1	AC	2.35	0.00	0.32	LiC
7	65/M	19.5	Current	T2bN1M0	IIB	SC	2.42	0.41	0.84	None
8	60/M	24.9	Never	T2aN0M0	IB	AC	4.29	0.01	2.46	LC
9	72/M	21.4	Former	T2aN1M0	IB	AC	2.92	1.32	2.17	None
10	63/M	21.7	Current	TisN0M0	0	AC	0.91	0.00	2.42	LC
11	67/M	25.4	Current	T1cN0M0	IB	SC	0.81	0.00	2.67	None
12	69/M	28.1	Former	T1cN0M0	IA3	AC	0.82	0.04	2.14	None
13	80/M	19.9	Never	T1cN1M1a	IVa	AC	0.41	0.00	0.30	None
14	51/M	19.5	Current	T1cN1M1b	IVb	AC	0.40	1.85	1.21	LC.GC
15	82/M	23.0	Current	T1aNxM0	IA2	SC	0.89	0.00	4.17	LC
16	58/F	18.0	Never	T1cNxM0	IA3	AC	2.32	0.59	0.01	GC
17	83/M	31.2	Never	T4N0M1c	IVb	AC	4.01	10.66	7.59	None
18	72/M	26.9	Current	T1cN2M1b	IVb	SCLC	5.03	1.81	5.52	None
19	74/M	21.3	Current	T2aN2M0	IIIa	SCLC	0.35	1.55	2.83	None
20	69/M	21.5	Current	T2aN1M0	IIB	SC	4.41	3.69	1.80	None
21	62/F	19.2	Former	T2aN3M1c	IVB	AC	0.27	44.43	11.70	None
22	65/F	32.7	Current	T1cN0M0	IA2	SC	1.34	2.40	0.06	None
23	50/F	19.9	Never	T3N2M0	IIIA	AC	6.69	0.01	0.46	None
24	71/M	23.4	Former	T3N0M0	IIB	Pleom	1.49	25.8	6.70	None
25	74/F	24.0	Never	T1miN0M0	IA1	AC	1.97	6.23	2.08	Le
26	70/F	22.7	Never	T1aN0M0	IA1	AC/SC	1.97	1.87	0.91	GC.CC
27	70/F	17.6	Former	T1miNM0	IA1	AC	0.91	1.55	0.45	
28	64/M	23.1	Former	T1bN0M0	IA2	AC	0.59	0.31	0.07	LC
29	65/M	23.6	Former	T1bN1M1b	IVb	AC	0.39	1.30	4.42	None
30	69/M	25.1	Current	T1miN0M0	IA1	AC	0.43	1.87	2.37	None
31	75/M	18.9	Former	T4N3M0	IIIB	SCLC	1.01	0.65	3.27	None
32	82/M	18.8	Current	T3N0M1a	IVa	AC	0.88	1.53	5.44	None
33	47/F	22.2	Never	T1bN0M0	IA2	AC	1.91	1.94	4.01	None

Table I. Continued.

No.	Age/Sex[a]	BMI, kg/m²	Smoking Habit	TNM[b]	Stage[c]	Patho[d]	MMP1/CD63[e]	miR-21, $2^{\Delta\Delta Cqf}$	miR-486-5p, $2^{\Delta\Delta Cqf}$	Family history[g]
34	75/F	22.7	Current	T1bN0M0	IA2	SCLC	4.03	0.56	0.84	CC,LC
35	79/M	21.7	Current	T3N0M0	IIB	LCC	1.11	1.05	1.12	None
					Median		1.34	1.30	2.17	
					(95% CI)		(0.89-1.97)	(0.33-1.55)	(1.12-1.86)	

[a]M, male; F, female. [b]The eighth edition of the TNM classification of lung cancer based on the clinical and pathological diagnosis. [c]Clinical and pathological stage based on TNM classification 8th edition (ref. 47). [d]Patho, pathological diagnosis: AC, adenocarcinoma; SC, squamous cell carcinoma; SCLC, small cell lung cancer; LCC, large cell carcinoma. [e]Number indicates the expression ratio levels of MMP-1/CD63, which was measured using western blotting with antibodies against both proteins. [f]Number indicates the relative expression levels, that is, microRNA (miRNA) copies using RT-PCR in patients divided by the mean number of miRNA copies in healthy controls. [g]Family history (cancer incidence among family member-grandparents, parents, brother(s) and sister(s), and children, of the each case): LC, lung cancer; GC, gastric cancer; LiC, liver cancer; Le, leukemia; CC, colon cancer; 95% CI, 95% confidence interval.

acetate for 2 min at room temperature, and finally exosomes were observed by transmission electron microscopy (Hitachi H-7600, Hitachi Ltd., Tochigi, Japan).

MMP-1/CD63 expression ratio in urinary exosomes. The levels of MMP-1 and CD63 were determined with western blotting using an anti-MMP-1 antibody (Cat. ab134184, Abcam plc., Cambridge, UK) and biotin-conjugated anti-CD 63 antibodies (Cat. 353017, BioLegend Inc., CA, USA). One-sixth of the urinary exosomes extracted from 2 ml was applied to the wells. Anti-MMP-1 antibody (1:1,000) and anti-CD63 antibody (1:1,000) were used. The ratio of MMP-1/CD63 was pixelated in patients with LC and compared to that in healthy controls (Fig. S2). The analysis was performed using the ImageJ 1.52a software (NIH, Bethesda, MD, USA) (23). To validate reproducibility, all experiments were performed twice, and the average or median values were calculated, as described previously (6).

Analysis of miRNA-21 and miRNA-486-5p extracted from the urinary exosomes. RNA for analyzing miRNAs (miRNA-21 and miRNA-486-5p) was extracted from the isolated exosomes using a Total Exosome RNA and Protein Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA); the isolated miRNA was reverse-transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Cat. 4366596, Thermo Fisher Scientific), according to the manufacturer's protocol. RT-qPCR was performed on a Step One Plus® thermal cycler (Applied Biosystems, Foster City, CA, USA) for miRNAs extracted from the urinary exosomes. For determining the miRNA-21 and miRNA-486-5p expression respectively, assays were performed using a commercial miRNA-21 assay kit (Cat. 4427975, Assay ID: 000397, Applied Biosystems) and miRNA-486-5p assay kit (Cat. 4427975, Assay ID 001278, Applied Biosystems) respectively according to the manufacturer's instructions. The assays were performed in duplicate for each sample, as described previously (6). In this study, instead of internal controls, miRNAs were extracted from urinary exosomes and reverse transcription reactions were performed using 5 ng of the recovered miRNAs to correct for miRNA levels between samples, and expression analysis was performed. This method has been proven in previous reports (6).

Relative expression levels of miRNA-21 and miRNA-486-5p in the urine exosomes. Based on the mean number of miRNA-21 and miRNA-486-5p copies in the healthy controls, the copy number of miRNA-21 and miRNA-486-5p in patients with LC was calculated as the relative expression grade, as described previously (6). The $\Delta\Delta Cq$ value was determined by subtracting the mean CT value of miRNA-21 and miRNA-486-5p in the healthy controls from the individual CT value of miRNA-21 and miRNA-486-5p in patients with LC. In the present study, the number of RT-PCR cycles in miRNA-486-5p varied between healthy male and healthy female controls, hence miRNA-486-5p was analyzed by gender. Finally, the copy number of miRNA-21 and miRNA-486-5p were compared as the value of $2^{\Delta\Delta Cq}$.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD) or median [95% confidence interval (CI)]. All statistical analyses were performed using the PRISM 9

software for Mac OS (GraphPad, Inc., La Jolla, CA, USA). Data were tested for normality and equal variance to confirm the appropriateness of the parametric tests, and those that followed a normal distribution were analyzed using the unpaired Student's t-test. Mann-Whitney U-tests were used to compare differences between groups, and Kruskal-Wallis test followed by Dunn's post hoc test were used to compare differences among three or more groups. $P<0.05$ was considered to indicate a statistically significant difference.

Logistic regression analysis was performed with the presence of lung cancer as dependent variable, and the expression ratio for MMP-1/CD63, and relative expression of miRNA-486-5p as independent variable adjusted with age, sex, and smoking status as covariates. ROC analysis was applied for the prediction of logistic regression model, and cut-off value was calculated by the Youden index. Logistic regression analysis and its ROC analysis were performed by IBM SPSS ver 26.0.

Results

Clinical characteristics and histopathological findings in patients with LC. The included patients with LC were relatively early-detected cases since most have visited the Department of Preventive Medicine of IUHW Hospital for health checkups, including LC screening including chest X-ray and cytological examination on sputum, and their suspicious lesions were examined using lung CT and MRI followed by the further examinations (Tables I and SI).

The pathological findings in the surgically resected tissues were as follows: non-small cell lung cancer was evident in 30 patients, adenocarcinoma (AC) in 24 (15 males and 9 females), squamous cell carcinoma (SC) in 5 (4 males and 1 female), and large cell carcinoma (LCC) in 1 (1 male and 0 female). Small cell lung cancer (SCLC) was evident in 4 (3 males and 1 female); pleomorphic carcinoma was evident in one male.

Among the 35 patients with LC, stage 0 was seen in one man, stage I in 19 (10 males and 9 females), stage II in 5 men, stage III in 3 (2 males and 1 female), and stage IV in 7 (6 males and 1 female).

Comparison of expression ratio of MMP-1/CD63 in patients with LC and those in healthy controls. The individual expression ratios of MMP-1/CD63 in patients with LC and healthy controls are listed in Tables I and SII, respectively. The MMP-1/CD63 expression ratio in both male and female healthy controls showed the same distribution trend, and those in both male and female patients with LC was increased (Fig. 1A-C). The median expression ratio in patients with LC was 1.342 (95% CI: 0.890-1.974), which was significantly higher than that in the healthy controls, 0.600 (95% CI: 0.490-0.900) ($P<0.0001$) (Tables I and SII; Fig. 1A).

The MMP-1/CD63 expression ratio in female healthy controls who are under 60 years of age was not significant different from those who are older (Table IIA). Male patients with LC showed higher ratio tendency in both under 60 years and over 60 years of age, especially in those under 60 years patients, than in both healthy males under and over 60 years of age (Table IIA). A significantly higher ratio in both female

patients under and over 60 years was observed compared with the females healthy controls ($P<0.01$ and $P<0.05$, respectively; Table IIA). The MMP-1/CD63 expression ratio of SCLC and those of NSCLC was not statistically different in the limited population.

Expression ratio of MMP-1/CD63 in patients with LC based on the cancer stage. The expression ratio of MMP-1/CD63 in 20 patients with stage 0 and I was 1.342 (95% CI: 0.888-2.319), which was significantly higher than that in 40 healthy controls ($P=0.0074$). The expression ratio of MMP-1/CD63 in 5 patients with stage II was 1.795 (95% CI: 1.105-4.414), which was higher than that in 40 healthy controls ($P=0.0192$), as depicted in Fig. 1D (all), 1E (males), and 1F (females). These data suggest that the expression ratio of MMP-1/CD63 is a useful biomarker for early stage patients with LC including both sex.

Expression ratio of MMP-1/CD63 in 35 patients with LC based on the cancer metastasis. Two cases (No. 13 and 32) revealed M1a, their expression ratio of MMP-1/CD63 were 0.41 and 0.88. Three M1b cases (No. 14, 18 and 29) showed 0.40, 5.03 and 0.39, respectively. The rest two M1c cases (No. 17 and 21) revealed 4.01 and 0.27. Among 7 metastasis cases, two cases showed the marked increased value.

Expression ratio of MMP-1/CD63 based on pathology and smoking habits. The expression ratio of MMP-1/CD63 in 11 patients who were never-smokers was higher (2.32; 95% CI: 1.80-4.29) than that in 14 current smokers (1.01; 95% CI: 0.43-4.03) and in 10 former smokers (0.90; 95% CI: 0.39-1.92), as presented in Table IIIA, Fig. 1G (all), Fig. 1H (males), and Fig. 1I (females), although our preliminary study did not show the statistical significance.

Eleven never-smokers (4 males and 7 females), among whom the expression ratio of MMP-1/CD63 was higher than 1.25 in 9 patients (3 males and 6 females). Interestingly, all had AC and 67% (6/9) were female patients with AC. The ratio was slightly higher in the AC group than that in the SC group (Table IIIA).

Relative expression levels of miRNA-21 and miRNA-486-5p in patients with LC and healthy controls. We selected miRNA-21 and miRNA-486-5p as a candidate screening biomarker as they have been reported to be effective in diagnosis/screening for LC using the circulating plasma exosomes (16-21).

Relative expression levels of miRNA-21 in patients with LC and healthy controls. The relative expression level of miRNA-21 in healthy controls was slightly higher in both of male and female over 60 years healthy controls compared with those in both sex under 60 years healthy controls, although not significantly; the expression level was higher in healthy males than females, although not significantly (Table IIB).

The expression level of miRNA-21 in male patients with LC was not significantly different from those in male healthy controls (Table IIB). The same tendency was seen in female patients with LC compared with those in female healthy controls (Fig. 2A-C; Table IIB). The expression levels of miRNA-21 were not significantly different from those of healthy controls for pathology and smoking habits (Fig. 2D-I).
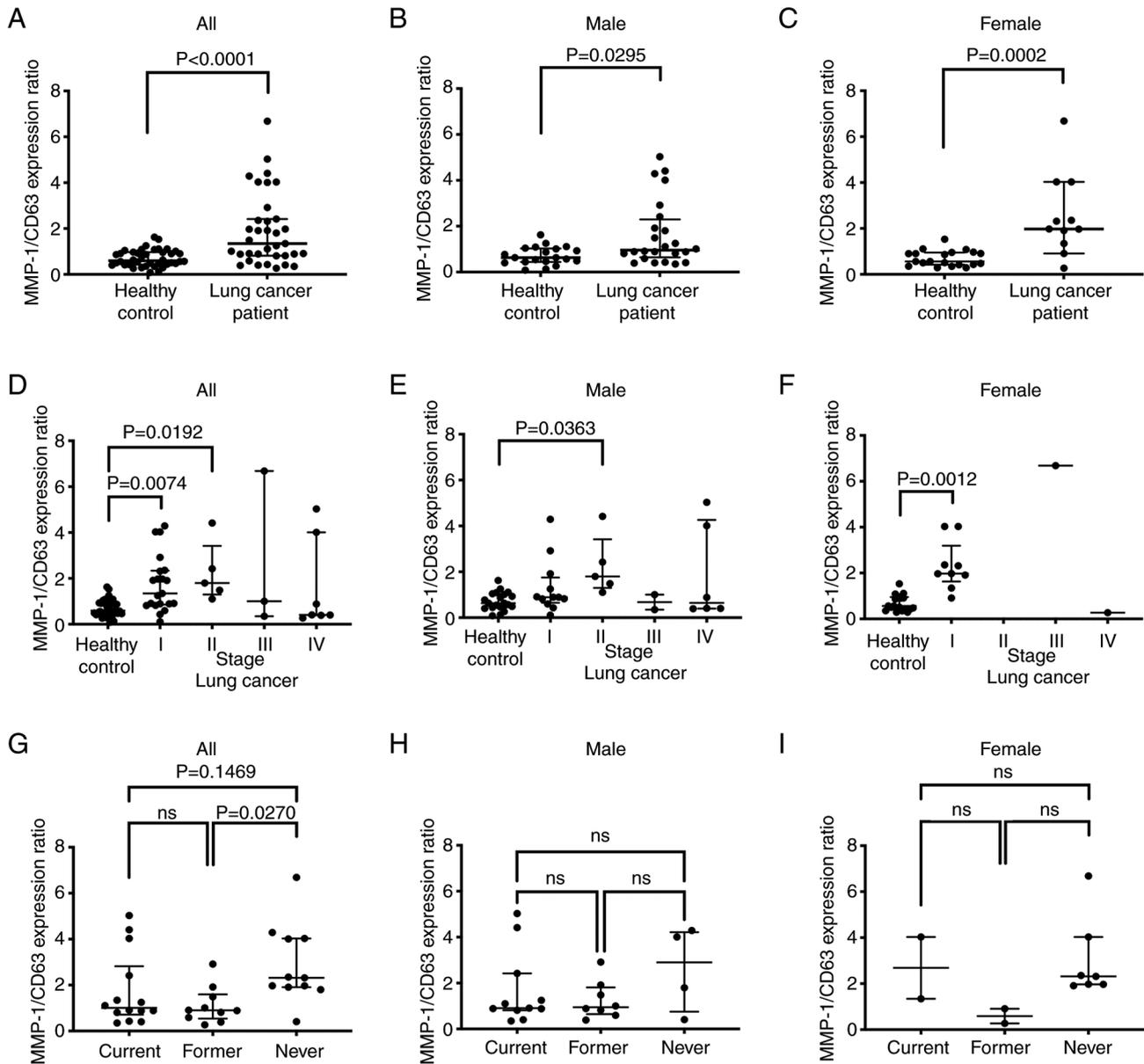
Figure 1. Comparison of the expression ratio of MMP-1/CD63 in 35 patients with LC and 40 healthy controls (20 males and 20 females). (A) Expression ratio of MMP1/CD63 in 35 patients with LC and 40 healthy controls (all). (B) Expression ratio of MMP1/CD63 in 24 male patients with LC and 20 male healthy controls. (C) Expression ratio of MMP1/CD63 in 11 female patients with LC and 20 female healthy controls. (D) Expression Ratio of MMP-1/CD63 in the patients with LC based on cancer stages (all). (E) Expression ratio of MMP1/CD63 in 24 male patients with LC based on cancer stages. (F) Expression ratio of MMP1/CD63 in 11 female patients with LC based on cancer stages. (G) Expression ratio of MMP1/CD63 in all patients with LC based on smoking habits (all). (H) Expression ratio of MMP1/CD63 in 24 male patients with LC based on smoking habits. (I) Expression ratio of MMP1/CD63 in 11 female patients with LC based on smoking habits. Median and 95% confidence intervals for the results were indicated by bars in the plots. LC, lung cancer; MMP-1, matrix metalloproteinase-1; ns, not significant.

Relative expression levels miRNA-486-5p in patients with LC and healthy controls. The relative expression level of miRNA-486-5p in under 60 years healthy males was significantly lower than that in under 60 years healthy females (P<0.01), while significantly higher in over 60 years healthy males than their healthy females counterparts (P<0.05; Table IIC).

The distribution pattern of the relative expression levels of miRNA-486-5p in male patients with LC compared with those in healthy males was up-regulated, while those in females patients with LC compared with those in healthy controls was down-regulated (Fig. 3A-C). These data show the sex difference in the relative expression levels of miRNA-486-5p.

Then we measured the relative expression levels of miRNA-486-5p in healthy controls and patients with LC by sex separately; the individual relative expression levels of miRNA-486-5p are listed in Tables I and SII.

The median expression levels of miRNA-486-5p in male patients with LC was 2.565 (95% CI: 1.800-4.420), which was significantly higher than that in male healthy controls (1.254, 95% CI: 0.790-1.530, P=0.0004). The median relative expression levels in female patients with LC (0.460, 95% CI: 0.060-2.130) tending to be lower than that in female healthy

Table II. Expression ratio of MMP-1/CD63 and relative expression of miRNA-21 and miRNA-486-5p in 40 healthy controls and 35 lung cancer patients.

A, Expression ratio of MMP-1/CD63

Characteristic Age	Healthy control			LC		
	Total	Male	Female	Total	Male	Female
≤60 years	0.87 (0.48-1.22)	0.63 (0.46-1.04)	0.56 (0.43-0.91)	2.12 (1.04-4.89)[a]	1.25 (0.40-4.29)	2.32 (1.91-6.69)[b]
>60 years	0.57 (0.39-1.00)	0.63 (0.42-1.02)	0.50 (0.35-1.03)	1.11 (0.82-2.39)[b]	0.91 (0.70-2.17)	1.97 (1.02-3.61)[a]
Total	0.60 (0.08-1.62)	0.63 (0.46-1.01)	0.56 (0.43-0.91)	1.34 (0.89-1.97)[c]	0.96 (0.81-1.92)[d]	1.97 (0.91-4.01)[c]

B, Relative expression of miRNA-21

Characteristic Age	Healthy control			LC		
	Total	Male	Female	Total	Male	Female
≤60 years	1.08 (0.48-1.94)	1.22 (0.89-1.95)	0.87 (0.43-3.07)	0.30 (0.01-1.87)	0.001 (0-1.85)	0.59 (0.01-1.94)
>60 years	1.23 (0.50-2.25)	1.39 (0.39-2.68)	0.90 (0.52-1.75)	1.31 (0.22-1.87)	1.30 (0.17-1.68)	1.71 (0.23-5.27)
Total	1.16 (0-12.8)	1.29 (0.75-1.99)	0.87 (0.49-1.68)	1.30 (0.33-1.55)	1.18 (0.04-1.55)	1.55 (0.01-6.23)

C, Relative expression of miRNA-486-5p

Characteristic Age	Healthy control			LC		
	Total	Male	Female	Total	Male	Female
≤60 years	1.11 (0.77-2.24)	0.85 (0.57-0.94)	2.51 (1.32-3.27)[e]	1.84 (0.35-3.16)	2.46 (1.21-2.87)[a]	0.46 (0.01-4.01)
>60 years	0.96 (0.32-1.63)	1.52 (0.83-2.35)	0.44 (0.18-1.06)[f,g]	2.17 (0.84-4.83)[a]	2.67 (1.51-5.34)[a]	0.65 (0.33-1.78)[g]
Total	1.04 (0.02-8.13)	0.93 (0.79-1.53)	1.25 (0.47-2.51)	2.17 (1.12-1.86)	2.565 (1.80-4.42)[d]	0.46 (0.06-4.01)

Median (95% CI). Statistical differences: [a]P<0.05, [b]P<0.01, healthy control vs. LC in same sex; [c]P<0.01, healthy control vs. LC in total; [d]P<0.05, healthy control vs. LC in same group; [e]P<0.01 male vs. female in same group; [f]P<0.01 ≤60 years vs. >60 years; [g]P<0.05 male vs. female in same group. miRNA, microRNA; LC, lung cancer.

Table III. Comparison of MMP-1/CD63 and miRNA-486-5p expression based on smoking status and cancer type in lung cancer patients.

A, Comparison of MMP-1/CD63 expression based on smoking status and cancer type in lung cancer patients

Types of lung cancer	Total n	Total Median (95% CI)	Current n	Current Median (95% CI)	Former n	Former Median (95% CI)	Never n	Never Median (95% CI)
Non-small cell lung cancer	30	1.30 (0.84-2.35)	10	1.01 (0.88-1.32)	8	0.85 (0.54-1.16)	12	2.15 (1.88-4.01)
Adenocarcinoma	23	1.25 (0.71-2.34)	4	0.90 (0.76-0.99)	8	0.85 (0.54-1.16)	11	2.32 (1.85-4.02)
Squamous cell carcinoma	6	1.66 (1.01-2.31)	5	1.34 (0.89-2.42)	0		1	1.97
Large cell carcinoma	1	1.11	1	1.11	0		0	
Small cell carcinoma	4	2.52 (0.84-4.28)	3	4.03 (2.19-4.53)	1	1.01	0	
Sarcoma (pleomorphic)	1	1.41 (0.98-2.82)	0		1	1.49	0	

B, Comparison of miRNA-486-5p expression based on smoking status and cancer type in lung cancer patients

Types of lung cancer	Total n	Total Median (95% CI)	Current n	Current Median (95% CI)	Former n	Former Median (95% CI)	Never n	Never Median (95% CI)
Non-small cell lung cancer	30	2.11 (0.56-3.72)	10	2.11 (1.14-2.82)	8	2.16 (1.02-4.62)	12	1.50 (0.36-2.85)
Adenocarcinoma	23	2.17 (0.45-4.21)	4	2.64 (2.11-3.51)	8	2.16 (1.02-4.62)	11	2.08 (0.35-3.23)
Squamous cell carcinoma	6	1.36 (0.86-2.45)	5	1.80 (0.84-2.67)	0		1	0.91
Large cell carcinoma	1	1.12	1	1.12	0		0	
Small cell carcinoma	4	3.05 (2.33-3.83)	3	2.83 (1.83-4.17)	1	3.27	0	
Sarcoma (pleomorphic)	1	6.70	0		1	6.70	0	

miRNA, microRNA; MMP-1, matrix metalloproteinase-1; 95% CI, 95% confidence interval.
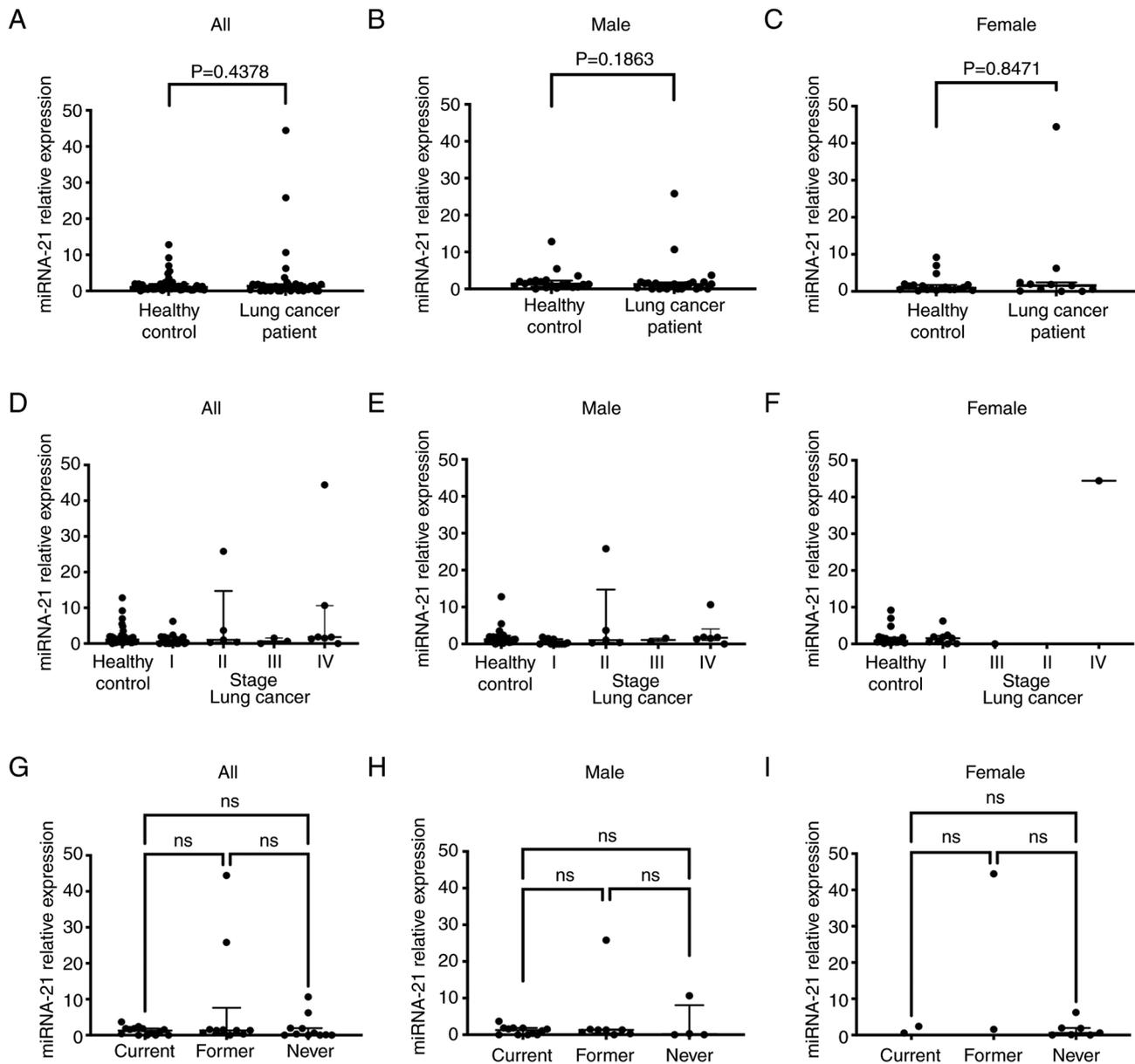
Figure 2. Comparison of relative expression of miRNA-21 in 35 patients with LC and 40 healthy controls (20 males and 20 females). (A) Relative expression of miRNA-21 in 35 patients with LC and 40 healthy controls (all). (B) Relative expression of miRNA-21 in 24 male patients with LC and 20 male healthy controls. (C) Relative expression of miRNA-21 in 11 female patients with LC and 20 female healthy controls. (D) Relative expression of miRNA-21 in the patients with LC based on cancer stages (all). (E) Relative expression of miRNA-21 in 24 male patients with LC based on cancer stages. (F) Relative expression of miRNA-21 in 11 female patients with LC based on cancer stages. (G) Relative expression of miRNA-21 in all patients with LC based on smoking habits (all). (H) Relative expression of miRNA-21 in 24 male patients with LC based on smoking habits. (I) Relative expression of miRNA-21 in 11 female patients with LC based on smoking habits. Median and 95% confidence intervals for the results were indicated by bars in the plots. LC, lung cancer; miRNA, microRNA; ns, not significant.

controls (1.250, 95% CI: 0.470-2.510, P=0.1566), as shown in Fig. 3A-C.

The results of miRNA-21 and miRNA-486-5p significantly varied; we concluded that the relative expression of miRNA-486-5p is a useful screening method for LC at work-place and community health centers.

Signature of miRNA-486-5p in patients with LC based on cancer stages. The expression levels of miRNA-486-5p in stage I and IV all patients with LC were shown in Fig. 3D, and those of male patients slightly higher than those in the healthy male controls (Fig. 3E), whereas the relative

expression of miRNA-486-5p tended to be lower in stage I female patients with LC than in healthy female controls (Fig. 3F). That is, miRNA-486-5p showed sex-difference, up-regulation in males and down-regulation in female patients with LC.

Relative expression of miR-486-5p in 35 Patients with LC based on the cancer metastasis. Two cases (No. 13 and 32) revealed M1a, their expression ratio of MMP-1/CD63 were 0.30 and 5.44. Three M1b cases (No. 14, 18 and 29) showed 1.21, 5.52 and 4.42, respectively. The rest two M1c cases (No. 17 and 21) revealed 7.59 and 11.7. Among 7 metastasis
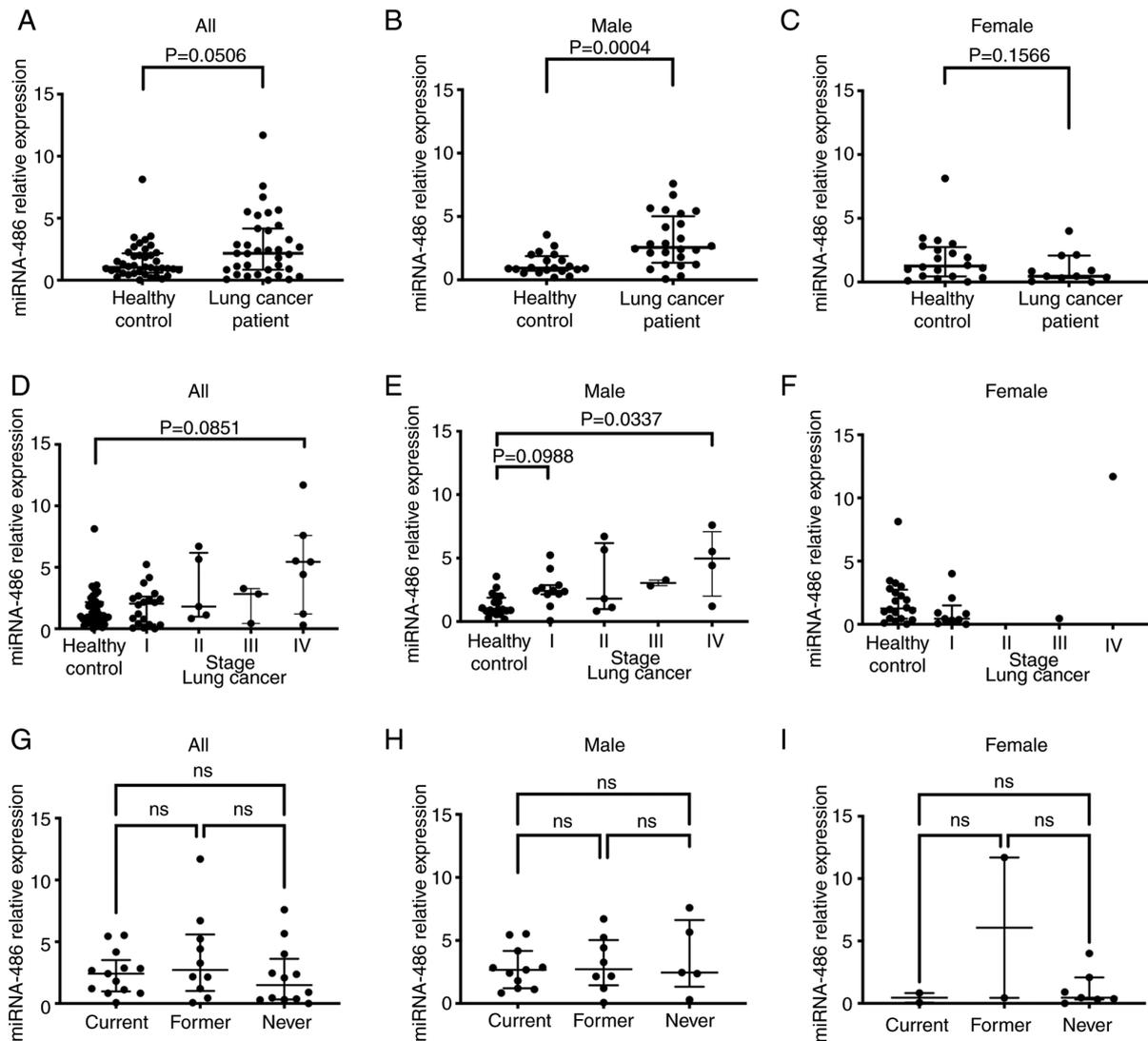
Figure 3. Comparison of the Relative Expression of miRNA-486-5p in 35 patients with LC and 40 healthy controls (20 males and 20 females). (A) Relative expression of miRNA-486-5p in 35 patients with LC and 40 healthy controls (all). (B) Relative expression of miRNA-486-5p in 24 male patients with LC and 20 male healthy controls. (C) Relative expression of miRNA-486-5p in 11 female patients with LC and 20 female healthy controls. (D) Relative expression of miRNA-486-5p in 40 patients with LC based on cancer stages (all). (E) Relative expression of miRNA-486-5p in 24 male patients with LC based on cancer stages. (F) Relative expression of miRNA-486-5p in 11 female patients with LC based on cancer stages. (G) Relative expression of miRNA-486-5p in all patients with LC based on smoking habits (all). (H) Relative expression of miRNA-486-5p in 24 male patients with LC based on smoking habits. (I) Relative expression of miRNA-486-5p in 11 female patients with LC based on smoking habits. Median and 95% confidence intervals for the results were indicated by bars in the plots. LC, lung cancer; miRNA, microRNA; ns, not significant.

cases, six cases showed the extremely very high increased value.

Expression levels of miRNA-486-5p based on smoking habits. Never-smoker female patients with LC showed low expression levels of miRNA-486-5p, except one case. In contrast, among the 4 never-smoker male patients with LC, two had high and two had low miRNA-486-5p expression levels. The relative expression of miRNA-486-5p was not related to smoking habits (Fig. 3G-I).

Receiver operating characteristics analysis
The sensitivity and specificity of MMP-1/CD63 as a primary screening approach for LC. The AUC of MMP-1/CD63 in the patients with LC was 0.755 (95% CI 0.640-0.871), and the cut-off value was 1.25 (Fig. 4A). The sensitivity and

specificity of MMP-1/CD63 expression were 0.925 and 0.543, respectively.

The sensitivity and specificity of miRNA-486-5p as a primary screening approach for LC based on sex. The AUC of miRNA-486-5p in male patients with LC was 0.801 (95% CI 0.669-0.939), that in female patients with LC was 0.627 (95% CI 0.396-0.858); the cutoff values were 2.14 and 0.91, respectively (Fig. 4B and C). The sensitivity and specificity of miRNA-486-5p in the male patients with LC were 0.850 and 0.708, respectively, and those in the female patients with LC were 0.700 and 0.727, respectively.

ROC analysis of combined biomarkers of MMP-1/CD63 and miRNA-486-5p. Both the biomarkers of the expression ratio of MMP-1/CD63 and the relative expression of miRNA-486-5p in the urinary exosomes improved ROC analysis: 86% sensitivity and 85% specificity calculated using
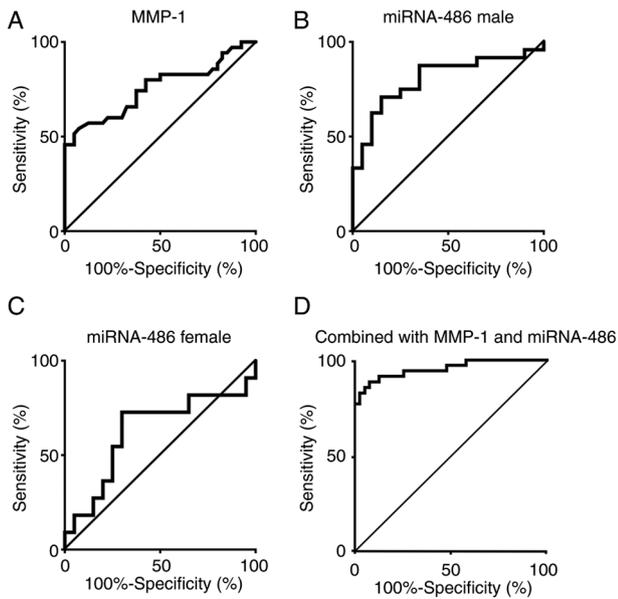
Figure 4. ROC analysis. (A) ROC curves of the expression ratio of MMP-1/CD63 in urinary exosomes from 35 patients with LC and 40 healthy controls. (B) ROC curves of relative expression of miRNA-486-5p in urinary exosomes from 24 male patients with LC and 20 male healthy controls. (C) ROC curves of relative expression of miRNA-486-5p in urinary exosomes from 11 female patients with LC and 20 female healthy controls. mir-486; microRNA-486-5p. (D) ROC analysis for the usefulness of both biomarkers in LC screening via logistic regression analysis. AUC was 0.954 (95% CI: 0.908-1.000). AUC, area under the ROC curve; miRNA, microRNA; MMP-1, matrix metalloproteinase-1; LC, lung cancer; ROC, receiver operating characteristic.

the cut-off value of 1.25 for MMP-1/CD63, 2.14 in males and 0.91 for females for miRNA-486-5p against 35 patients with LC and 40 healthy controls in the present study as one cohort.

Logistic regression analysis for the usefulness of both biomarkers in LC screening: The association between the presence of lung cancer and the expression ratio for MMP-1/CD63, and relative expression of miRNA-486-5p. The association between the expression ratio for MMP-1/CD63 and the presence of lung cancer showed an odds ratio (OR)=31.70 (95% CI: 4.12-243.67), while that between the relative expression of miRNA-486-5p and the presence of lung cancer had an OR=1.56 (95% CI:1.01-2.42) after adjusting with age, sex, and smoking status (Table IV). AUROC of the logistic regression model including MMP-1/CD63 and miRNA-486-5p was 0.954 (95% CI: 0.908-1.000), with 89% sensitivity and 88% specificity using the cut-off value of 0.48 (Fig. 4D).

The relative expression of miRNA-486-5p is a supportive biomarker for the expression ratio of MMP-1/CD63, which may contribute to the better specificity.

Discussion

To the best of our knowledge, this is the first study that used urinary exosome-derived MMP-1 and miRNA for early LC screening. We demonstrated that the combined screening for both biomarkers of MMP-1/CD63 and miRNA-486-5p targeting urinary exosomes is useful in detecting early-stage LC. Our purpose was to develop a universal and practical

screening method for early-stage LC for job workers and community residents to enable a simple, easy to access, cost-effective screening of LC and requires only a small volume of urine.

The expression ratio of MMP-1/CD63 did not show a sex-based difference in the present study; the ratio increased in both male and female patients with LC, as observed in breast cancer patients (6). In 1998, Rutter *et al* reported a single nucleotide polymorphism (SNP) at -1607 bp in the *MMP-1* promoter region, where an additional guanine (G) created an erythroblast transformation specific (Ets) binding site, 5'-GGA-3' (12). This SNP was associated with transcriptional and protein/DNA binding activities; hence its frequency was investigated in tumor cells. Subsequent studies reported a close relationship between the *MMP-1* promoter SNP and the risk of LC (13,14). Sauter *et al* found that *MMP-1* SNPs are related to an increased risk of early onset of LC; this risk is further worsened by smoking in patients who are younger than 51 years of age (15). Moreover, increased MMP-1 levels in serum or plasma (24-26) and increased expression of MMP-1 in the tumor tissues of patients with LC was observed by immunohistochemical staining (26).

Therefore, we consider that the expression ratio of MMP-1/CD 63 in the urinary exosomes of the patients with LC is a useful biomarker for LC screening, and the present study reports the upregulation of MMP-1 in the urinary exosomes in early-stage patients with LC. MMP-1 upregulation in LC can be affected by environmental and/or genetic factors, such as signal transducer and activator of transcription 3 (27), extracellular signal-regulated kinases/mitogen-activated protein kinases pathways in the epidermal growth factor receptor (EGFR) ligands, and their signaling pathways (28). The Capicua (CIC) repressor (29-34) is a similar factor. The missense, nonsense, insertion, deletion, and splice sites for the CIC repressor have been reported (29-34), but the direct relationship between CIC and upregulation of MMP-1 expression in LC has not yet been determined. CIC is a high mobility group-box transcriptional repressor, and its target genes include the polyoma enhancer activator 3, whose group Ets translocation variants (ETVs) have been well characterized (29-32). A recent report revealed the progression of hepatocellular carcinoma related to the CIC-ETV4-MMP-1 axis (33). We have previously reported the increased expression ratio of MMP-1/CD63 in breast cancer patients including early-stage of cancer (6), while the data after operation were low, although high value was observed in the case with metastasis after operation (6). The CIC repressor involvement in Ewing sarcoma, melanoma, prostate-, breast-, lung-, gastric-, or colon cancer has not been fully verified. Since CIC in *Drosophila* plays an important role in terminal and dorsoventral patterning (30), similar genes are supposed in human (33,34).

Saito *et al* demonstrated that the EGFR-tyrosine kinase inhibitor (TKI)-resistant AC showed strong MMP-1-positive staining with a significantly independent poor prognosis (35). However, once MMP-1 expression is upregulated, the cell phenotype may change in many organs associated with the hedgehog protein and/or epithelial-mesenchymal transition, thereby leading to carcinogenesis (35,36). This suggests that MMP-1-positive cancer patients have a poor prognosis, as reported in the case of colorectal cancer (37). Our screening tests for early-stage LC

Table IV. Logistic regression analysis between lung cancer, and smoking status, MMP-1/CD63 and microRNA-486-5p.

Characteristic	Regression coefficient	SE	Wald	P-value	Odds ratio	95% CI
Age (continuous variable)	0.108	0.045	5.677	0.017	1.11	1.02-1.22
Sex (male)	-0.380	1.069	0.127	0.722	0.68	0.08-5.55
Smoking status (current and former smoking)	4.158	1.361	9.327	0.002	63.94	4.44-921.85
MMP-1/CD63 (continuous variable)	3.456	1.041	11.031	0.001	31.70	4.12-243.67
microRNA-486-5p (continuous variable)	0.444	0.224	3.932	0.047	1.56	1.01-2.42
const	-13.663	3.935	12.053	0.001	0.000	

const, constant; miRNA, microRNA; MMP-1, matrix metalloproteinase-1; SE, standard error; 95% CI, 95% confidence interval of odds ratio.

illustrated the usefulness of urinary exosomes and can clarify the relationship between CIC and LC occurrence and metastases in future studies. Furthermore, combined screening of MMP-1/CD 63 and miRNA-486-5p using urinary exosomes as a target could detect LC more accurately.

The miRNA-21 was useful for breast cancer screening and can modulate the expression of cancer suppressor genes such as *PTEN*, *HOXD10*, *TPM1* and *mapin* (a tumor-suppressing serpin protein) (6,17-19). However, several patients with LC are over the age of 70 years; therefore; hence, relative expression of miRNA-21 in such patients could not be discriminated from those of the healthy controls among the aged individuals. Therefore, we focused on miRNA-486-5p, which targets the *PIK3R1* gene, a suppressor of tumor growth (20,21). Surprisingly, miRNA-486-5p showed sex differences here but not in previous reported studies. The obtained signature of miRNA-486-5p in this study is not consistent with the literature (20,21). ElKhouly *et al* (20) reported that miRNA-486-5p is multifaceted, thereby reflecting the disease conditions on the date of examination. These high-age limitation have not been previously reported in studies on miRNAs in patients with LC (38-44). However, recent reports on miRNA-486 suggest that dysregulation of miRNAs may affect sex hormone signaling via modulation of estrogen receptors and through miRNAs (45,46). Our results suggest that the relative expression of miRNA-486-5p was a useful biomarker for LC screening at work-place and community health centers, with a sensitivity and specificity of nearly 90%. Moreover, the relative expression of miRNA-486-5p increased at the early stage of LC. Although miRNA-486-5p was increased in AC and SCC, the association with smoking habits was not expected. Saito *et al* reported that the number of cases with high MMP-1 immunoreactivity was significantly higher in smokers (26/54 cases) than non-smokers (8/27 cases) (35), which is inconsistent with our findings, showing higher expression of MMP-1 in never-smoker patients with AC. However, Pao *et al* reported frequently observed point mutations in codon 858 (exon 21) in EGFR-TKI-sensitive never-smokers patients (47). The results of the ROC analysis for logistic regression model suggests that MMP-1/CD63 and miNA-486-5p could predict lung cancer, although further studies are warranted.

Isolation of exosomes from urine and miRNA analysis may be less methodologically established than isolation from serum or plasma. Hence, it may be difficult to normalize for urine-derived exosomes. One reason for this is the difficulty in selecting appropriate miRNAs as controls for body fluid samples, including urine (48,49). In this study, we did not use internal controls such as small RNAs such as U6 and 5S rRNA, which are commonly used in miRNA analysis (50,51). Since these small RNAs are structurally different from miRNAs in urinary exosome-derived miRNA expression analysis, it is unclear whether they can be used as generic internal controls like GAPDH and β-actin in mRNA expression analysis. In our preliminary study, the measurement of internal standard miRNAs in exosomes did not work well. We next investigated the use of externally added miRNAs as internal standards. However, since RNA added externally as an internal control was not derived from exosomes extracted from patients but from RNA synthesized during RNA extraction (50,51), we considered the external addition of RNA to be insufficient as a control in this study. For example, U6 RNA is not appropriate in patient with liver fibrosis, suggesting that it may not function as an intrinsic control depending on the type of disease complicating the patient (52). Furthermore, we believe that externally added miRNAs cannot eliminate the effects of contamination and the risk of degradation. Furthermore, simple analysis using an electron microscope revealed no differences in the shape, size, or number of urine-derived exosomes per urine volume. Therefore, we believe that there are no major differences in the number of exosomes or the variety of miRNA pools between each sample. Therefore, in this study, exosomes were extracted from 2 ml of urine and all RNA was recovered; 5 ng of RNA was used for reverse transcription, after which the CT values of the target miRNAs were measured; individual samples from LC and healthy controls were normalized using the mean CT values of healthy controls. In other words, they were compared to patients based on the healthy control mean expression levels of the target miRNAs. Although this method has been reported in previous reports (6), this study, which did not use internal controls for miRNAs, is more controversial in the normalization of

urine-derived exosomes. It is hoped that more appropriate methods for internal control of urinary exosomes will be established in the future.

This study has several limitations. The study is preliminary, the population is not large, and it has not been conducted on exosome characteristics other than EM. Future studies should identify more precise characteristics on exosomes.

Global statistics estimate that approximately 25% of patients with LC are never smokers (53). Therefore, our screening method can help identify numbers of the early-staged patients with LC that are never-smokers but exposed to passive smoking. In Asian countries, there are never-smoker female patients with LC, which is a major challenge that our novel screening method can address.

Our study proposed a new screening method useful for workers who cannot visit a health-check clinic for cancer screening due to busy work schedules and/or economic limitations.

We are presently investigating the collection of information on the false-positive cases in at least 500 visitors for the annual health checks. Further studies with a larger cohort of patients with LC are needed to validate our findings.

Acknowledgements

Not applicable.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

WA, MS, TU, SI and IO designed and conducted the study. WA and MS analyzed the data. WA, MS and IO confirm the authenticity of all the raw data. HY confirmed morphological analysis. WA and MS performed statistical analysis. Logistic regression analysis was done by HF. WA, MS, SI, HY, YK, KO, TN, SE, HT, and IO interpreted and discussed the data. WA, MS, HF and IO wrote the draft of this manuscript. SI, HY, YK, KO, TN, SE and HT edited the paper for English grammar and writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by 'The Ethics Committee of IUHW Hospital' (#13-B-299 IUHW on May 24, 2018; last updated August 7, 2020), and 'The ethical committee of Kitasato University Medical Center' (#28-51 Kitasato University Medical Center on March 27, 2017) and conformed to the principles of the 1975 Declaration of Helsinki, as reflected by its prior approval by the institution's human research committee.

All the study participants provided written informed consent. All participants signed informed consent for participation in this study.

Patient consent for publication

All participants signed informed consent to publish the results in peer review journal.

Authors' information

IO published a research paper in Nature (1974) entitled 'Collagenase activity in experimental hepatic fibrosis', and continued matrix metalloproteinase study. The year before last year he published original paper as a first author entitled 'Sequential matrix metalloproteinase-1 expression triggered by infiltrating monocytic lineage cells modulates pathophysiological aspects of human nonalcoholic steatohepatitis, Metalloproteinases in Medicine 2020:7 1-13'.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
2. National Cancer Center: Latest Cancer Statistics. https://ganjoho.jp/reg_stat/statistics/stat/summary.html Accessed February 24, 2023. (In Japanese).
3. Koike T, Yamato Y, Asamura H, Tsuchiya R, Sohara Y, Eguchi K, Mori M, Nakanishi Y, Goya T, Koshiishi Y, et al: Improvements in surgical results for lung cancer from 1989 to 1999 in Japan. J Thorac Oncol 4: 1364-1369, 2009.
4. Wood DE, Kazerooni EA, Baum SL, Eapen GA, Ettinger DS, Hou L, Jackman DM, Klippenstein D, Kumar R, Lackner RP, et al: Lung cancer screening, version 3.2018, NCCN clinical practice guidelines in oncology. J Natl Compr Canc Netw 16: 412-441, 2018.
5. Gavelli G and Giampalma E: Sensitivity and specificity of chest X-ray screening for lung cancer: Review article. Cancer 89 (11 Suppl): S2453-S2456, 2000.
6. Ando W, Kikuchi K, Uematsu T, Yokomori H, Takaki T, Sogabe M, Kohgo Y, Otori K, Ishikawa S and Okazaki I: Novel breast cancer screening: Combined expression of miR-21 and MMP-1 in urinary exosomes detects 95% of breast cancer without metastasis. Sci Rep 9: 13595, 2019.
7. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ and Lötvall JO: Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9: 654-659, 2007.
8. Anastasiadou E and Slack FJ: Cancer. Malicious exosomes. Science 346: 1459-1460, 2014.
9. Kalluri R: The biology and function of exosomes in cancer. J Clin Invest 126: 1208-1215, 2016.
10. Zhang L and Yu D: Exosomes in cancer development, metastasis, and immunity. Biochim Biophys Acta Rev Cancer 1871: 455-468, 2019.
11. Okazaki I and Nabeshima K: Introduction: MMPs, ADAMs/ADAMTSs research products to achieve big dream. Anticancer Agents Med Chem 12: 688-706, 2012.
12. Rutter JL, Mitchell TI, Butticè G, Meyers J, Gusella JF, Ozelius LJ and Brinckerhoff CE: A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. Cancer Res 58: 5321-5325, 1998.
13. Zhu Y, Spitz MR, Lei L, Mills GB and Wu X: A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter enhances lung cancer susceptibility. Cancer Res 61: 7825-7829, 2001.

14. Su L, Zhou W, Park S, Wain JC, Lynch TJ, Liu G and Christiani DC: Matrix metalloproteinase-1 promoter polymorphism and lung cancer risk. Cancer Epidemiol Biomarkers Prev 14: 567-570, 2005.

15. Sauter W, Rosenberger A, Beckmann L, Kropp S, Mittelstrass K, Timofeeva M, Wölke G, Steinwachs A, Scheiner D, Meese E, *et al*: Matrix metalloproteinase 1 (MMP1) is associated with early-onset lung cancer. Cancer Epidemiol Biomarkers Prev 17: 1127-1135, 2008.

16. Shen J, Liu Z, Todd NW, Zhang H, Liao J, Yu L, Guarnera MA, Li R, Cai L, Zhan M and Jiang F: Diagnosis of lung cancer in individuals with solitary pulmonary nodules by plasma microRNA biomarkers. BMC Cancer 11: 374, 2011.

17. Xu LF, Wu ZP, Chen Y, Zhu QS, Hamidi S and Navab R: MicroRNA-21 (miR-21) regulates cellular proliferation, invasion, migration, and apoptosis by targeting PTEN, RECK and Bcl-2 in lung squamous carcinoma, Gejiu City, China. PLoS One 9: e103698, 2014.

18. Zhu S, Si ML, Wu H and Mo YY: MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 282: 14328-14336, 2007.

19. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A and Lund AH: Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 283: 1026-1033, 2008.

20. ElKhouly AM, Youness RA and Gad MZ: MicroRNA-486-5p and microRNA-486-3p: Multifaceted pleiotropic mediators in oncological and non-oncological conditions. Noncoding RNA Res 5: 11-21, 2020.

21. Tian F, Wang J, Ouyang T, Lu N, Lu J, Shen Y, Bai Y, Xie X and Ge Q: MiR-486-5p serves as a good biomarker in nonsmall cell lung cancer and suppresses cell growth with the involvement of a target PIK3R1. Front Genet 10: 688, 2019.

22. Goldstraw P, Chansky K, Crowley J, Rami-Porta R, Asamura H, Eberhardt WE, Nicholson AG, Groome P, Mitchell A, Bolejack V, *et al*: The IASLC lung cancer staging project: Proposals for revision of the TNM stage groupings in the forthcoming (eighth) edition of the TNM classification for lung cancer. J Thorac Oncol 11: 39-51, 2016.

23. Schneider CA, Rasband WS and Eliceiri KW: NIH image to ImageJ: 25 Years of image analysis. Nat Methods 9: 671-675, 2012.

24. Schutz A, Schneidenbach D, Aust G, Tannapfel A, Steinert M and Wittekind C: Differential expression and activity status of MMP-1, MMP-2 and MMP-9 in tumor and stromal cells of squamous cell carcinomas of the lung. Tumour Biol 23: 179-184, 2002.

25. An HJ, Lee YJ, Hong SA, Kim JO, Lee KY, Kim YK, Park JK and Kang JH: The prognostic role of tissue and serum MMP-1 and TIMP-1 expression in patients with non-small cell lung cancer. Pathol Res Pract 212: 357-364, 2016.

26. Li M, Xiao T, Zhang Y, Feng L, Lin D, Liu Y, Mao Y, Guo S, Han N, Di X, *et al*: Prognostic significance of matrix metalloproteinase-1 levels in peripheral plasma and tumour tissues of lung cancer patients. Lung Cancer 69: 341-347, 2010.

27. Schütz A, Röser K, Klitzsch J, Lieder F, Aberger F, Gruber W, Mueller KM, Pupyshev A, Moriggl R and Friedrich K: Lung adenocarcinomas and lung cancer cell lines show association of MMP-1 expression with STAT3 activation. Transl Oncol 8: 97-105, 2015.

28. Park S, Jung HH, Park YH, Ahn JS and Im YH: ERK/MAPK pathways play critical roles in EGFR ligands-induced MMP1 expression. Biochem Biophys Res Commun 407: 680-686, 2011.

29. Tanaka M, Yoshimoto T and Nakamura T: A double-edged sword: The world according to Capicua in cancer. Cancer Sci 108: 2319-2325, 2017.

30. Jiménez G, Shvartsman SY and Paroush Z: The Capicua repressor-a general sensor of RTK signaling in development and disease. J Cell Sci 125: 1383-1391, 2012.

31. Lee Y, Fryer JD, Kang H, Crespo-Barreto J, Bowman AB, Gao Y, Kahle JJ, Hong JS, Kheradmand F, Orr HT, *et al*: ATXN1 protein family and CIC regulate extracellular matrix remodeling and lung alveolarization. Dev Cell 21: 746-757, 2011.

32. Okimoto RA, Breitenbuecher F, Olivas VR, Wu W, Gini B, Hofree M, Asthana S, Hrustanovic G, Flanagan J, Tulpule A, *et al*: Inactivation of Capicua drives cancer metastasis. Nat Genet 49: 87-96, 2017.

33. Kim E, Kim D, Lee JS, Yoe J, Park J, Kim CJ, Jeong D, Kim S and Lee Y: Capicua suppresses hepatocellular carcinoma progression by controlling the ETV4-MMP1 axis. Hepatology 67: 2287-2301, 2018.

34. Jiménez G, Guichet A, Ephrussi A and Casanova J: Relief of gene repression by torso RTK signaling: Role of capicua in Drosophila terminal and dorsoventral patterning. Genes Dev 14: 224-231, 2000.

35. Saito R, Miki Y, Ishida N, Inoue C, Kobayashi M, Hata S, Yamada-Okabe H, Okada Y and Sasano H: The significance of MMP-1 in EGFR-TKI-resistant lung adenocarcinoma: Potential for therapeutic targeting. Int J Mol Sci 19: 609, 2018.

36. Niland S, Riscanevo AX and Eble JA: Matrix metalloproteinases shape the tumor microenvironment in cancer progression. Int J Mol Sci 23: 146, 2021.

37. Murray GI, Duncan ME, O'Neil P, Melvin WT and Fothergill JE: Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. Nat Med 2: 461-462, 1996.

38. Cazzoli R, Buttitta F, Di Nicola M, Malatesta S, Marchetti A, Rom WN and Pass HI: microRNAs derived from circulating exosomes as noninvasive biomarkers for screening and diagnosing lung cancer. J Thorac Oncol 8: 1156-1162, 2013.

39. Zhou X, Wen W, Shan X, Zhu W, Xu J, Guo R, Cheng W, Wang F, Qi LW, Chen Y, *et al*: A six-microRNA panel in plasma was identified as a potential biomarker for lung adenocarcinoma diagnosis. Oncotarget 8: 6513-6525, 2017.

40. Zhang L, Shan X, Wang J, Zhu J, Huang Z, Zhang H, Zhou X, Cheng W, Shu Y, Zhu W and Liu P: A three-microRNA signature for lung squamous cell carcinoma diagnosis in Chinese male patients. Oncotarget 8: 86897-86907, 2017.

41. Dejima H, Iinuma H, Kanaoka R, Matsutani N and Kawamura M: Exosomal microRNA in plasma as a non-invasive biomarker for the recurrence of non-small cell lung cancer. Oncol Lett 13: 1256-1263, 2017.

42. Shan X, Zhang H, Zhang L, Zhou X, Wang T, Zhang J, Shu Y, Zhu W, Wen W and Liu P: Identification of four plasma microRNAs as potential biomarkers in the diagnosis of male lung squamous cell carcinoma patients in China. Cancer Med 7: 2370-2381, 2018.

43. Feng M, Zhao J, Wang L and Liu J: Upregulated expression of serum exosomal micrornas as diagnostic biomarkers of lung adenocarcinoma. Ann Clin Lab Sci 48: 712-718, 2018.

44. Zhang Y, Zhang Y, Yin Y and Li S: Detection of circulating exosomal miR-17-5p serves as a novel non-invasive diagnostic marker for non-small cell lung cancer patients. Pathol Res Pract 215: 152466, 2019.

45. Wang R, Bhat-Nakshatri P, Zhong X, Zimmers T and Nakshatri H: Hormonally regulated myogenic miR-486 influences sex-specific differences in cancer-induced skeletal muscle defects. Endocrinology 162: bqab142, 2021.

46. Thakur L and Thakur S: The interplay of sex steroid hormones and microRNAs in endometrial cancer: Current understanding and future directions. Front Endocrinol (Lausanne) 14: 1166948, 2023.

47. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, *et al*: EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 101: 13306-13311, 2004.

48. Matsuzaki K, Fujita K, Jingushi K, Kawashima A, Ujike T, Nagahara A, Ueda Y, Tanigawa G, Yoshioka I, Ueda K, *et al*: MiR-21-5p in urinary extracellular vesicles is a novel biomarker of urothelial carcinoma. Oncotarget 8: 24668-24678, 2017.

49. Yazarlou F, Mowla SJ, Oskooei VK, Motevaseli E, Tooli LF, Afsharpad M, Nekoohesh L, Sanikhani NS, Ghafouri-Fard S and Modarressi MH: Urine exosome gene expression of cancer-testis antigens for prediction of bladder carcinoma. Cancer Manag Res 10: 5373-5381, 2018.

50. Occhipinti G, Giulietti M, Principato G and Piva F: The choice of endogenous controls in exosomal microRNA assessments from biofluids. Tumour Biol 37: 11657-11665, 2016.

51. Xu D, Di K, Fan B, Wu J, Gu X, Sun Y, Khan A, Li P and Li Z: MicroRNAs in extracellular vesicles: Sorting mechanisms, diagnostic value, isolation, and detection technology. Front Bioeng Biotechnol 10: 948959, 2022.

52. Benz F, Roderburg C, Vargas Cardenas D, Vucur M, Gautheron J, Koch A, Zimmermann H, Janssen J, Nieuwenhuijsen L, Luedde M, *et al*: U6 is unsuitable for normalization of serum miRNA levels in patients with sepsis or liver fibrosis. Exp Mol Med 45: e42, 2013.

53. Okazaki I, Ishikawa S, Ando W and Sohara Y: Lung adenocarcinoma in never smokers: Problems of primary prevention from aspects of susceptible genes and carcinogens. Anticancer Res 36: 6207-6224, 2016.