J Cancer Res Clin Oncol (2014) 140:145–150
DOI 10.1007/s00432-013-1555-5

ORIGINAL PAPER

Digital PCR quantification of miRNAs in sputum for diagnosis of lung cancer

Ning Li · Jie Ma · Maria A. Guarnera · HongBin Fang · Ling Cai · Feng Jiang

Received: 26 September 2013 / Accepted: 16 November 2013 / Published online: 27 November 2013

Abstract
Purpose MicroRNAs (miRNAs) play important roles in the initiation and progression of lung cancer. Measuring miRNA expression levels in sputum could provide a potential approach for the diagnosis of lung cancer. The emerging digital PCR is a straightforward technique for precise, direct, and absolute quantification of nucleic acids. The objective of the study was to investigate whether digital PCR could be used to quantify miRNAs in sputum for lung cancer diagnosis.
Methods We first determined and compared dynamic ranges of digital PCR and conventional quantitative reverse transcriptase PCR (qRT-PCR) for miRNA quantification using RNA isolated from sputum of five healthy individuals. We then used digital PCR to quantify copy number of two lung cancer-associated miRNAs (miR-31 and miR-210) in 35 lung cancer patients and 40 cancer-free controls.
Results Copy number of the miRNAs measured by digital PCR displayed a linear response to input cDNA amount in a twofold dilution series over seven orders of magnitude. miRNA quantification determined by digital PCR assay was in good agreement with that obtained from qRT-PCR analysis in sputum. Furthermore, combined quantification of miR-31 and miR-210 copy number by using digital PCR in sputum of the cases and controls provided 65.71 % sensitivity and 85.00 % specificity for lung cancer diagnosis.
Conclusion As digital PCR becomes more established, it would be a robust tool for quantitative assessment of miRNA copy number in sputum for lung cancer diagnosis.

Keywords Digital PCR · miRNAs · Sputum · Diagnosis · Lung cancer

Introduction

Lung cancer is responsible for 29 % of cancer deaths, causing more deaths than breast cancer, colon cancer, and prostate cancer combined (Siegel et al. 2013). Approximately, 85 % of lung cancers are non-small-cell lung cancer (NSCLC). The early detection of NSCLC followed by suitable treatments can reduce lung cancer-related deaths (Aberle et al. 2011; Greenlee et al. 2001; Siegel et al. 2013). Sputum is one of the most noninvasively accessible body fluids and contains exfoliated airway epithelial cells (Thunnissen 2003). Molecular analysis of sputum can indicate the specific source of the abnormal cells in the lungs, thus providing a noninvasive and organ-specific diagnostic approach for lung cancer (Thunnissen 2003). MicroRNAs (miRNAs) contribute to the regulation of key cellular processes, including cellular development, differentiation, proliferation, cell death, and metabolism (Ambros 2003). The

Ning Li and Jie Ma have equally contributed to this article.

N. Li · J. Ma · M. A. Guarnera · F. Jiang (✉)
Department of Pathology, The University of Maryland Greenebaum Cancer Center, University of Maryland School of Medicine, 10 South Pine Street, MSTF 7th Floor, Baltimore, MD 21201-1192, USA
e-mail: fjiang@som.umaryland.edu

N. Li
School of Traditional Chinese Materia Medica, Key Laboratory of Structure-Based Drug Design and Discovery (Shenyang Pharmaceutical University), Ministry of Education, Wenhua Road 103, 110016 Shenyang, China

H. Fang · L. Cai
Division of Biostatistics, The University of Maryland Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD, USA

dysregulation of miRNAs has important roles in tumor initiation and progression (Galasso et al. 2010; Lu et al. 2005). miRNA expression profiles offer molecular signatures for the classification, diagnosis, and progression of cancer, and thus could be developed as cancer biomarkers (Lu et al. 2005; Shen et al. 2011a, b, 2013a, b; Shen and Jiang 2012; Yanaihara et al. 2006). We previously showed that miRNAs were stably present in sputum and reliably measurable by using quantitative reverse transcriptase PCR (qRT-PCR) assay (Anjuman et al. 2013; Xie et al. 2010; Xing et al. 2010; Yu et al. 2010). We recently found that combined analysis of two miRNAs (miR-31 and miR-210) in sputum using qRT-PCR could yield 65.2 % sensitivity and 89.7 % specificity for lung cancer diagnosis (Fan et al. 2007).

Droplet digital PCR is a direct method for quantitatively measuring nucleic acids and more sensitive in resolving copy number changes of DNA and RNA targets compared to qRT-PCR (Day et al. 2013; Vogelstein and Kinzler 1999). In this study, we evaluated dynamic range of digital PCR for miRNA quantification in sputum and investigated its potential to be used as a tool for lung cancer diagnosis. This study presents the earliest assessment of digital PCR as a viable means for quantitative detection of miRNAs in sputum for lung cancer diagnosis.

Table 1 Demographic and clinical data of lung cancer patients and cancer-free controls

Parameter	Lung cancer patients	%	Cancer-free controls	%
	Mean		Mean	
Total no.	35		40	
Age, years	68.9		65.7	
	8.3		8.5	
Gender				
Men	22	62.9	26	65
Women	13	37.1	14	35
Race				
White American	26	74.3	29	72.5
African-American	9	25.7	11	27.5
Smoking, pack-years	48.4		20.8	
	26.5		11.6	
Histology				
Adenocarcinoma	19	54.3		
Squamous cell carcinoma	16	45.7		
All are stage I NSCLC				

NSCLC non-small-cell lung cancer

Materials and methods

Subjects and sputum collection and preparation

This study was performed under a protocol approved by the Institutional Review Board of University of Maryland Baltimore. Written informed consent was obtained from all enrolled subjects: 35 stage I NSCLC patients and 40 cancer-free smokers. Geographic and clinical characteristics of the cases and controls are shown in Table 1. The 35 eligible cancer patients were stage I NSCLC patients before receiving surgical treatment, preoperative adjuvant chemotherapy, and radiotherapy. Inclusion criteria for cancer-free controls were individuals who had no a history of cancer in the last 3 years at the time of enrolment. Clinical diagnosis of lung cancer was made with histopathological examinations of specimens obtained by CT-guided transthoracic needle biopsy, transbronchial biopsy, videotape-assisted thoracoscopic surgery, or surgical resection. The surgical pathologic staging was determined according to the TNM classification of the International Union Against Cancer with the American Joint Committee on Cancer and the International Staging System for Lung Cancer. Histopathological classification was determined according to the World Health Organization classification.

Sputum was collected from the participants as described in our previous reports (Anjuman et al. 2013; Jiang et al. 2009, 2010; Li et al. 2007; Qiu et al. 2008; Xie et al. 2010; Xing et al. 2010; Yu et al. 2010). The samples were processed on ice in four volumes of 0.1 % dithiothreitol (Sigma-Aldrich Corporation, St. Louis, MO, USA), and the mixture was vortexed for 15 min. A double volume of a phosphate-buffered saline solution was then added, and the mixture was briefly vortexed. After filtration through two layers of a sterile gauze to remove mucous and debris, sputum was centrifuged for 10 min at 800×*g*. The cell pellet was resuspended in a phosphate-buffered saline solution.

miRNA quantification in sputum by using digital PCR and qRT-PCR

Total RNA from the cell pellets of sputum was purified by using miRVana™ PARIS™ Kit (Ambion, Austin, TX, USA) as described in our previous reports (Fan et al. 2007; Shen et al. 2011a, b; Xie et al. 2010; Xing et al. 2010; Yu et al. 2010). The qualification and quantification of RNA were assessed by using Biospectrometer (Hutchinson Technology Inc., Hutchinson, MN, USA) and Electrophoresis Bioanalyzer (Agilent Technologies, Foster City, CA, USA). cDNA was reversely transcribed from 100 ng RNA using miRNA-specific primers for miR-31 and 210 in 15 µl reaction mixture, respectively. Digital PCR was performed in the samples as previously described (Heredia et al. 2013; Hindson et al. 2011, 2013). Briefly, TaqMan™ reaction mix containing sample cDNA was partitioned into aqueous

droplets in oil via the QX100 Droplet Generator and then transferred to a 96-well PCR plate. A two-step thermocycling protocol [95 °C × 10 min; 40 cycles of (94 °C × 30 s, 60 °C × 60 s), 98 °C × 10 min] was undertaken in a Bio-Rad C1000 (Bio-Rad, Pleasanton, CA, USA). The PCR plate was then transferred to the QX100 Droplet Reader for automatic reading of samples in all wells. To determine dynamic range of miRNA quantification by digital PCR in sputum, twofold dilution series of cDNA derived from sputum RNA samples of five cancer-free individuals were assessed by digital PCR for miRs-31 and 210, respectively.

Conventional qRT-PCR analysis was performed as described in our previous reports (Shen et al. 2011a, b, Xie et al. 2010; Xing et al. 2010; Yu et al. 2010). Briefly, cDNA was combined with PCR reagents to produce a PCR reaction in a total volume of 5.0 µl. qPCR was carried out on an Applied BioSystems 7900HT thermocycler (Bio-Rad) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. qPCR data were analyzed by using Bio-Rad's manager software (Bio-Rad) with automatic threshold cycle (C_t) setting for assigning baseline and threshold for C_t determination. C_t values of the miRNAs were normalized in relation to that of U6. All assays were performed in triplicates, and one no-template control and two interplate controls were carried along in each experiment.

Statistical analysis

We used *t* test to determine significant differences of values between groups. We applied Pearson's correlation analysis to assess relationship between copy number of the miRNAs and demographic characteristics of the patients and cancer-free controls. We used Kappa statistics to evaluate agreement between the different individuals for quantification of miRNAs. We used clinicopathologic diagnoses as reference standards to estimate sensitivity and specificity of the miRNAs. We applied the receiver-operator characteristic (ROC) curve and area under the curve (AUC) analyses to determine the accuracy of each miRNA in a specimen. All P values shown were two-sided, and a P value of <0.05 was considered statistically significant.

Results

A dynamic range of digital PCR for miRNA quantification in sputum

To determine performance characteristics of digital PCR for the assessment of miRNAs in sputum, cDNA was transcribed from RNA of cellular pellets of sputum obtained from five healthy individuals who were nonsmokers. The generated cDNA was then diluted by twofold across seven orders of magnitude ranging from 1:8 to 1:512. The serially diluted samples were run for quantification of miR-31 and miR-210 by using digital PCR. As shown in Fig. 1a, each well of the samples contained at least 10,000 droplets, implying that the specimens could be successfully "read" by passing the droplets through a fluorescence detector. There was excellent linearity between the cDNA input and values measured by digital PCR across seven orders of magnitude for the miRNA targets (Fig. 1b, c). To determine reproducibility between digital PCR assays for miRNA quantification, the panel of diluted samples was independently analyzed by two research staff. There was a high agreement between the results created from the independent digital PCR assays (the kappa statistic for concordance was 0.89, $P < 0.01$). Furthermore, to compare dynamic ranges of digital PCR and qPCR for miRNA quantification in sputum, the serially diluted specimens were also analyzed by using qRT-PCR assay. The relative expression of each miRNA by qRT-PCR was calculated and normalized to U6 using a C_t method. As shown in Fig. 1d, there was a good agreement between copy numbers of the miRNAs determined by digital PCR and the expression levels measured by qRT-PCR across the serially diluted samples.

Diagnostic performance of miRNA quantification in sputum by digital PCR

To investigate the potential of using digital PCR for assessment of miRNAs in sputum for lung cancer diagnosis, we evaluated absolute copy number of two miRNAs, miR-31 and miR-210, in a cohort of 35 NSCLC patients and 40 cancer-free subjects. miR-31 and miR-210 were selected, as they were previously identified as potential sputum miRNA biomarkers for lung cancer diagnosis (Anjuman et al. 2013). Copy number of miR-31 per µl in sputum of cancer-free individuals and lung cancer patients was 73.78 ± 10.81 and 173.2 ± 24.52, respectively ($P < 0.01$) (Fig. 2a). Copy number of miR-210 per µl in sputum of cancer-free individuals and lung cancer patients was 303.6 ± 34.14 and 737.8 ± 100.00, respectively ($P < 0.01$) (Fig. 2b). Overall, both miR-31 and miR-210 had significantly higher copy number in sputum of lung cancer patients compared with cancer-free controls. Furthermore, assaying copy number of the two individual miRNAs generated AUC value of 0.75 and 0.73, respectively, in distinguishing NSCLC patients from the controls (Fig. 3a, b). Combined quantification of the two miRNAs yielded 0.86 AUC that was statistically higher than that of individual one used alone (Fig. 3c) (all $P < 0.05$). Given a specificity of 85.00 %, the two miRNAs used in conjunction revealed a sensitivity of 65.71 % in differentiating NSCLC patients from the cancer-free subjects. The prevalence of the miRNA copy number in sputum was related with pack-years of smoking ($P < 0.05$), however,

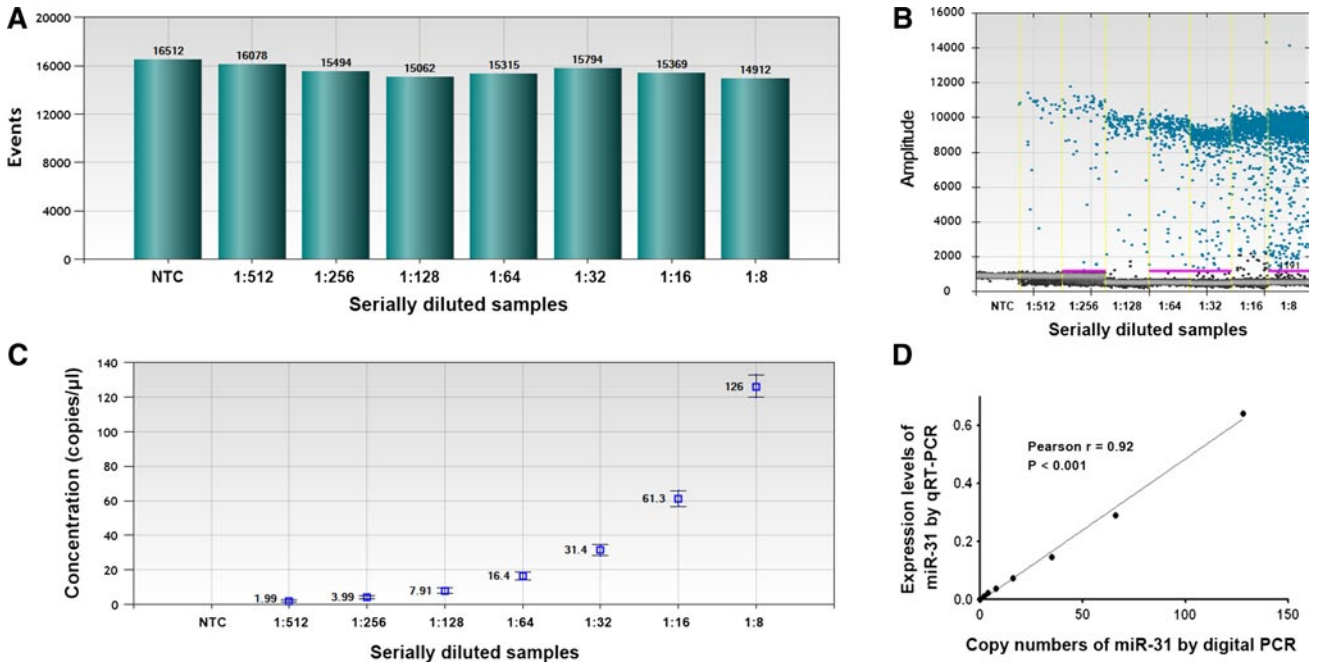

Fig. 1 The dynamic range of digital PCR for miR-31 quantification in sputum of healthy individuals. The generated cDNA was diluted by twofold across seven orders of magnitude ranging from 1:8 to 1:512 and run for miRNA quantification by using digital PCR. **a** Each well of the samples and a negative template control (NTC) sample contained at least 10,000 droplets, suggesting that sputum could successfully be analyzed by digital PCR. **b** Intensity plots of DX100 QuantaSoft results for quantification of miR-31 in the serially diluted sputum samples. **c** Concentration represented by copy number of miR-31 per µl of PCR reaction measured by digital PCR assay. The error bars associated with each point represent the 95 % confidence interval. **d** Comparison of digital PCR and qRT-PCR using the serially diluted samples for quantification of miR-31 suggested high correlation of copy number obtained from digital PCR with expression levels determined by qRT-PCR. The dynamic range of quantification of miR-210 by using digital PCR and qPCR displayed similar results (data not shown)

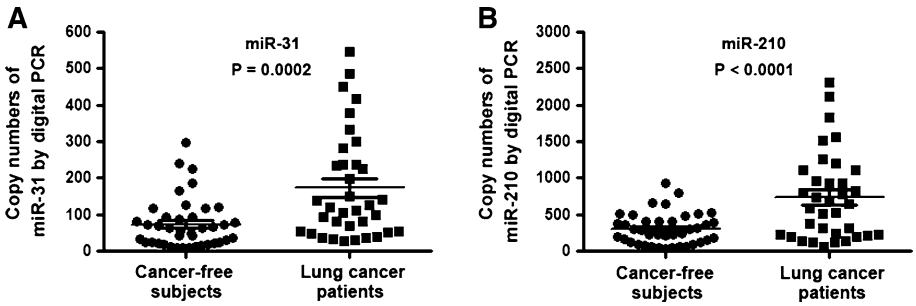

Fig. 2 Comparison of copy number of miRNAs in 40 cancer-free controls and 35 stage I NSCLC patients. **a** Copy number of miR-31 in the cancer-free controls and stage I NSCLC patients. Horizontal lines indicate mean values. **b** Copy number of miR-210 in the cancer-free controls and stage I NSCLC patients

not associated with patient age, gender, histological tumor type and stage, and location of the tumors (all $P > 0.05$).

Discussion

qRT-PCR uses Ct as a metric for analysis of miRNA expression levels, in which Ct values for miRNA targets are referenced to endogenous small RNA controls across samples and used for normalization. However, expression levels of the endogenous controls and their transcripts may differ between samples (Dodd et al. 2013; Whale et al. 2012). Therefore, qRT-PCR is an indirect method for analysis of RNA targets and does not provide exact quantitation of miRNAs.

Digital PCR is a direct method for absolute nucleic acid quantification based on the partitioning of individual analyte molecules into many replicate reactions at limiting dilution, resulting in one or zero molecules in most reactions (Day et al. 2013; Vogelstein and Kinzler 1999). After endpoint PCR, the starting concentration of template is determined by Poisson statistical analysis of the number

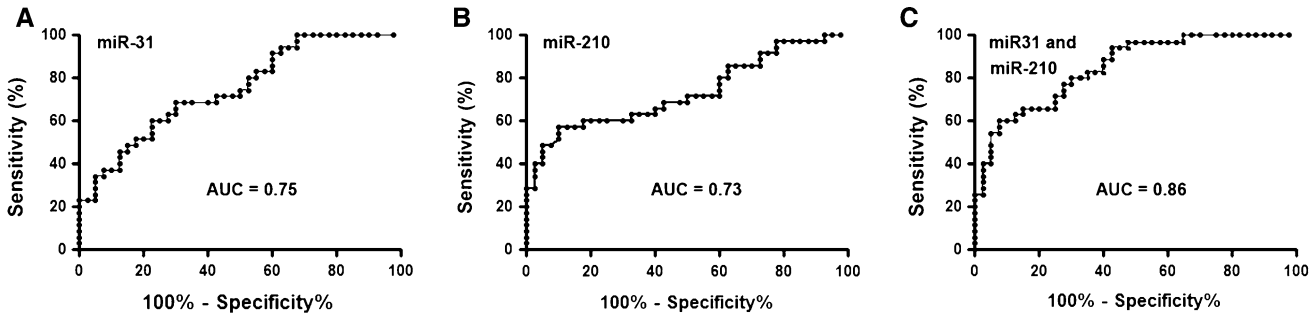

Fig. 3 Diagnostic values of miRNA quantification by digital PCR in sputum. **a** Receiver-operator characteristic (ROC) curve analysis of copy numbers of the miR-31 in sputum of the 35 lung cancer patients and 40 cancer-free individuals. The area under the ROC curve (AUC) for each miRNA conveyed its accuracy in differentiating lung cancer patients from the control subjects with respect to sensitivity and specificity. miR-31 produced 0.75 AUC value. **b** miR-210 produced 0.73 AUC value. **c** Combined quantification of miR-31 and miR-210 by digital PCR produced 0.86 AUC that was statistically higher than that of individual one used alone

of positive (containing amplified target) and negative (no amplified target detected) reactions (Day et al. 2013; Vogelstein and Kinzler 1999). Thus, notable benefits of digital PCR over qPCR are the capability to obtain absolute quantification without external references and robustness to variations in PCR efficiency. Herein, we find that each well of each sputum sample contains at least 10,000 droplets, and digital PCR produces a direct, high-confidence measurement of the original target miRNA concentration. Furthermore, rapid analysis of at least 10,000 of droplets per sputum indicates that absolute quantification of miRNA copy number by digital PCR is highly efficient. In addition, the digital nature of the results means that data handling is relatively straightforward. Therefore, digital PCR would overcome the weakness of the conventional qRT-PCR technique for absolute assessment of miRNAs in sputum.

Our head-to-head comparison of digital PCR and qRT-PCR in the same set of specimens reveals a good agreement between copy numbers of the miRNAs by digital PCR and the expression levels by qRT-PCR. Furthermore, digital PCR displays a high reproducibility in measuring copy number of the sputum miRNAs. In addition, the combed analysis of the two miRNAs by digital PCR offers higher sensitivity and specificity for lung cancer diagnosis compared with a single one used alone. Altogether, digital PCR could provide a viable means for absolute quantification of miRNAs in sputum whose changes are the hallmarks of lung cancer.

This is a pilot study to investigate the feasibility of using digital PCR for miRNA quantification in sputum, as such some limitations exist. First, the sample size is small. Second, the sensitivity (65.71 %) and specificity (85.00 %) by quantifying the two miRNAs are not efficient for diagnosis of NSCLC. In our ongoing project, we are evaluating digital PCR for sputum miRNA quantification in large cohort of cases and controls. We are also analyzing more lung tumor-associated miRNAs defined from our and others' groups (Shen et al. 2011a, b, 2013a, b; Yanaihara et al. 2006) to identify additional sputum-based miRNA biomarkers that can be added to the current ones, so that the diagnostic efficacy of digital PCR could be improved.

Conclusion

This work demonstrates that digital PCR can absolutely quantify copy number of miRNAs and potentially provide a viable means for the detection of sputum miRNAs for lung cancer diagnosis. Nonetheless, the continued development of this new technology and further exploring its value for routine use in diagnostic testing for lung cancer would be required.

Acknowledgments This work was supported in part by NCI R01CA161837, VA merit Award I01 CX000512, LUNGevity/Upstage Foundation Early Detection Award, and University of Maryland Cancer Epidemiology Alliance Seed Grant (F.J.).

Conflict of interest The authors declare no conflict of interest.

References

Aberle DR, Adams AM, Berg CD, Black WC, Clapp JD, Fagerstrom RM et al (2011) Reduced lung-cancer mortality with low-dose computed tomographic screening. N Engl J Med 365:395–409

Ambros V (2003) MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell 113:673–676

Anjuman N, Li N, Guarnera M, Stass SA, Jiang F (2013) Evaluation of lung flute in sputum samples for molecular analysis of lung cancer. Clin Transl Med 2:15

Day E, Dear PH, McCaughan F (2013) Digital PCR strategies in the development and analysis of molecular biomarkers for personalized medicine. Methods 59:101–107

Dodd DW, Gagnon KT, Corey DR (2013) Digital quantitation of potential therapeutic target RNAs. Nucleic Acid Ther 23:188–194

Galasso M, Sana ME, Volinia S (2010) Non-coding RNAs: a key to future personalized molecular therapy? Genome Med 2:12

Greenlee RT, Hill-Harmon MB, Murray T, Thun M (2001) Cancer statistics, 2001. CA Cancer J Clin 51:15–36

Heredia NJ, Belgrader P, Wang S, Koehler R, Regan J, Cosman AM et al (2013) Droplet Digital PCR quantitation of HER2 expression in FFPE breast cancer samples. Methods 59:S20–S23

Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ et al (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 83:8604–8610

Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ et al (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods 10:1003–1005

Jiang F, Todd NW, Qiu Q, Liu Z, Katz RL, Stass SA (2009) Combined genetic analysis of sputum and computed tomography for noninvasive diagnosis of non-small-cell lung cancer. Lung Cancer 66:58–63

Jiang F, Todd NW, Li R, Zhang H, Fang H, Stass SA (2010) A panel of sputum-based genomic marker for early detection of lung cancer. Cancer Prev Res (Phila) 3:1571–1578

Li R, Todd NW, Qiu Q, Fan T, Zhao RY, Rodgers WH et al (2007) Genetic deletions in sputum as diagnostic markers for early detection of stage I non-small cell lung cancer. Clin Cancer Res 13:482–487

Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D et al (2005) MicroRNA expression profiles classify human cancers. Nature 435:834–838

Qiu Q, Todd NW, Li R, Peng H, Liu Z, Yfantis HG et al (2008) Magnetic enrichment of bronchial epithelial cells from sputum for lung cancer diagnosis. Cancer 114:275–283

Shen J, Jiang F (2012) Applications of microRNAs in the diagnosis and prognosis of lung cancer. Expert Opin Med Diagn 6:197–207

Shen J, Liu Z, Todd NW, Zhang H, Liao J, Yu L et al (2011a) Diagnosis of lung cancer in individuals with solitary pulmonary nodules by plasma microRNA biomarkers. BMC Cancer 11:374

Shen J, Todd NW, Zhang H, Yu L, Lingxiao X, Mei Y et al (2011b) Plasma microRNAs as potential biomarkers for non-small-cell lung cancer. Lab Invest 91:579–587

Shen J, Stass SA, Jiang F (2013a) MicroRNAs as potential biomarkers in human solid tumors. Cancer Lett 329:125–136

Shen J, Guarnera MA, Cai L, Fang H, Jiang F (2013b) Analysis of microRNAs in sputum improves CT for diagnosis of lung cancer. J Thorac Oncol (in press)

Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63:11–30

Thunnissen FB (2003) Sputum examination for early detection of lung cancer. J Clin Pathol 56:805–810

Vogelstein B, Kinzler KW (1999) Digital PCR. Proc Natl Acad Sci USA 96:9236–9241

Whale AS, Huggett JF, Cowen S, Speirs V, Shaw J, Ellison S et al (2012) Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic Acids Res 40:e82

Xie Y, Todd NW, Liu Z, Zhan M, Fang H, Peng H et al (2010) Altered miRNA expression in sputum for diagnosis of non-small cell lung cancer. Lung Cancer 67:170–176

Xing L, Todd NW, Yu L, Fang H, Jiang F (2010) Early detection of squamous cell lung cancer in sputum by a panel of microRNA markers. Mod Pathol 23:1157–1164

Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M et al (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9:189–198

Yu L, Todd NW, Xing L, Xie Y, Zhang H, Liu Z et al (2010) Early detection of lung adenocarcinoma in sputum by a panel of microRNA markers. Int J Cancer 127:2870–2878