microRNAs Derived from Circulating Exosomes as Noninvasive Biomarkers for Screening and Diagnosing Lung Cancer

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Introduction: Lung cancer is the highest cause of mortality among tumor pathologies worldwide. There are no validated techniques for an early detection of pulmonary cancer lesions other than low-dose helical computed tomography scan. Unfortunately, this method has some negative effects. Recent studies have laid the basis for development of exosomes-based techniques to screen/diagnose lung cancers. As the isolation of circulating exosomes is a minimally invasive procedure, this technique opens new possibilities for diagnostic applications.

Methods: We used a first set of 30 plasma samples from as many patients, including 10 patients affected by lung adenocarcinomas, 10 with lung granulomas, and 10 healthy smokers matched for age and sex as negative controls. Wide-range microRNAs analysis (742 microRNAs) was performed by quantitative real time polymerase chain reaction. Data were compared on the basis of lesion characteristics, using WEKA software for statistics and modeling. Subsequently, selected microRNAs were evaluated on an independent larger group of samples (105 specimens: 50 lung adenocarcinomas, 30 lung granulomas, and 25 healthy smokers).

Results: This analysis led to the selection of four microRNAs to perform a screening test (miR-378a, miR-379, miR-139-5p, and miR-200b-5p), useful to divide population into two groups: nodule (lung adenocarcinomas + carcinomas) and non-nodule (healthy former smokers). Six microRNAs (miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, and miR-154-3p) were selected for a second test on the *nodule* population to discriminate between lung adenocarcinoma and granuloma.

Conclusions: The screening test showed 97.5% sensitivity, 72.0% specificity, and area under the curve receiver operating characteristic of 90.8%. The diagnostic test had 96.0% sensitivity, 60.0% specificity, and area under the curve receiver operating characteristic of 76.0%. Further evaluation is needed to confirm the predictive power of these models on larger cohorts of samples.

Key Words: Exosome, Screening test, Diagnostic test, Lung adenocarcinoma, microRNA.

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Lung cancer is the leading cause of cancer-related deaths worldwide. Most lung lesions are diagnosed at advanced stages with an overall 5-year survival rate of 15%.[1] With the exception of the recently published U.S. National Lung Screening Trial[2] there are no validated population-based screening procedures. Furthermore, there are no serum/plasma biomarkers to determine whether a low-dose helical computerized tomogram should be performed in high-risk individuals.

Exosomes are microvesicles specialized in transporting different types of molecules currently seen as a short-/long-range communication system.[3] microRNAs are small molecules with capacity of posttranscriptional regulation, and a single microRNA has the abilities to bind with several mRNAs through a suppressor complex and block an entire biological pathway. Numerous proteins may be found within exosomes but RNA, with a strong percentage of microRNAs,[4–6] seems to predominate in these structures.

Several articles have demonstrated the importance of circulating exosomes and microRNAs regarding: lung cancer detection,[6,7] discrimination among histotypes,[8] development and prognosis,[9] and early detection.[10] Furthermore, it has been suggested that cells can produce specific microRNAs that respond to stimuli or messages from adjacent cells.[11]

Tumor cells are a perfect illustration of this paradigm because they produce exosomes containing microRNAs completely different from those present in normal cells from which neoplastic cells originated.[12] Cancer cells use the exosomes for many purposes, including the control of adjacent cells without inducing any mutation or confusion in the immune

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system, by facilitating the migration of metastases over long distances, evading detection by the targeted stromal cells in a *Trojan-horse*–like process.[13]

The aim of the present study was to develop two plasma-based tests, one for screening and one for diagnosis of lung adenocarcinoma. Plasma-based diagnostics could fit, by their nature, in a prevention policy based on periodic checks.

MATERIALS AND METHODS

Plasma Samples

Training Set

Thirty frozen plasma samples were selected for the study group from the NYU plasma bank and grouped into the following three categories: 10 lung adenocarcinomas, 10 lung granulomas, and 10 healthy former smokers. Samples were matched for age, sex, and smoking history. A total of 500 µl of plasma was taken from each sample. This group was analyzed on the microRNA Ready-to-Use PCR, Human panel I+II, V2.M (Exiqon, Vedbæk, Denmark).

Validation Set

A subsequent quantitative real time polymerase chain reaction (RT-PCR) validation group that was matched for age, sex, and smoking history, consisted of 50 lung adenocarcinomas, 30 lung granulomas, and 25 healthy former smokers. For this second analysis group was used 250 µl of plasma each sample.

Selection Criteria

To select the training set samples we decided to use restrictive selection criteria regarding the patients: age ranging from 40 to 80 years, being smokers at the time of sampling, low racial variability, equal mix of both sexes, and early-stage nodule (Ia or Ib) with regard to lung adenocarcinomas and granulomas.

Selection criteria for the validation set were less restrictive. We maintained the same age range (40–80 years), being smokers, having tumors, lung granulomas in early stage, and no allowance was made for sex and race variability (Table 1).

Exosome Precipitation and microRNA Extraction Protocol

First, 126 µl of ExoQuick exosome precipitation solution (System Biosciences, Mountain View, CA) was added to the 500 µl of stored plasma to precipitate exosome pellet, as described by manufacturer. This exosome pellet was lysed in 300 µl of RNeasy Lysis Buffer RLT (Qiagen, Milano, Italy), and then a Trizol (Life Technologies, Grand Island, NY) RNA extraction was performed with addition of MS2 phage RNA carrier (Roche, Basel, Switzerland); 800 µl of Trizol + 1.25 µl MS2 RNA carrier; ratio of Trizol to chloroform was 4:1. All concentrations were halved for the subsequent RNA extraction of validation group.

TABLE 1. Overview of Population Characteristics

Variable	Adenocarcinomas (n = 50)	Granulomas (n = 30)	Healthy (n = 25)	*p*
Study set	(n = 10)	(n = 10)	(n = 10)	
Age, mean ± SD	66.1 ± 14.0	64.8 ± 13.7	65.6 ± 7.4	0.971[a]
Sex, n (%)				0.861[b]
Male	3 (30.0)	3 (30.0)	4 (40.0)	
Female	7 (70.0)	7 (70.0)	6 (60.0)	
Smoking habit, n (%)				0.024[b]
Yes	5 (50.0)	5 (50.0)	10 (100.0)	
No	5 (50.0)	5 (50.0)	—	
Nodule size, mean ± SD	1.42 ± 0.24	1.34 ± 0.56	—	0.683[c]
Validation set				
Age, mean ± SD	70.5 ± 9.2	65.6 ± 9.5	66.7 ± 8.8	0.050[a]
Sex, n (%)				0.126[b]
Male	25 (50.0)	11 (36.7)	16 (64.0)	
Female	25 (50.0)	19 (63.3)	9 (36.0)	
Smoke habits, n (%)				0.151[b]
Yes	41 (82.0)	16 (53.3)	3 (12.0)	
No	9 (18.0)	11 (36.7)	22 (88.0)	
Unknown	—	3 (10.0)	—	
Nodule size, mean ± SD	1.49 ± 0.45	1.34 ± 0.56	—	0.192[c]

[a]One-way ANOVA test.
[b]χ^2 test.
[c]Student's *t* test for unpaired data.
ANOVA, analysis of variance.

cDNA Synthesis, microRNAs Plates, and Quantitative RT-PCR

Seven microliters of Trizol-extracted RNA, in 20 μl of total volume, were subjected to reverse transcription with miRCURY LNA Universal cDNA synthesis kit (Exiqon), incubated for 60 minutes at 42°C followed by enzyme heat inactivation for 5 minutes at 95°C. Wide-range microRNAs analysis was performed using microRNA Ready-to-Use PCR, Human panel I+II, V2.M (Exiqon) according to manufacturer's protocol.

Quantitative RT-PCR was carried out in total volume of 10-μl reaction mixture (384-well format) using miRCURY LNA Universal RT microRNA PCR, SYBR Green master mix (Exiqon) according to the manufacturer's protocol. Amplification was performed as follows: 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds, and 60°C for 10 seconds, ramp rate 100% under standard condition.

microRNAs expression was determined using the ABI 7900HT and was quantified using SDS software version 2.4 (Life Technologies, Grand Island, NY), setting a threshold of 1.0 and a manual baseline from one to 13 cycles. The validation cohort was analyzed in triplicate.

Evaluation of Appropriate Housekeeping microRNA

Because of the absence of reference genes in the plasma samples, it was critical to choose an appropriate housekeeping microRNA. Ten samples were chosen at random in the training set (3 lung granulomas, 3 lung adenocarcinomas, and 4 healthy). Let-7a, mir-20a, mir-221a,[12] mir-16,[14] and let-7b were tested by RT-PCR (every sample in duplicates). glyceraldehyde 3-phosphate dehydrogenase was also examined. Resulting data were analyzed using Normfinder software Version 0.953.[15]

Evaluation of the Stability of microRNAs in Plasma Samples

To validate whether our plasma samples storage resulted in stable levels of the microRNAs, we also evaluated microRNA concentrations in plasma over time and after freeze–thaw. A single blood sample was selected randomly and the plasma was divided into several aliquots from which RNA extractions were performed. These aliquots included fresh plasma and frozen plasma for five different periods: 24 hours, 48 hours, 1 week, 1 month, and 2 months. The following microRNAs were tested in these aliquots using quantitative RT-PCR: let-7a, mir-20a, and mir-221. One spiked-in synthetic control (reagent content in cDNA synthesis kit; Exiqon) was added artificially to the cDNA synthesis in a known amount to evaluate the reaction quality.

Wide-Range microRNAs Profiling

The two Exiqon plates (microRNA Ready-to-Use PCR, Human Panel I + II, V2.M; Exiqon) allowed quantitative RT-PCR of 742 human microRNAs, six reference genes, and relative internal controls/calibrators. To avoid any kind of contamination, the plates were dispensed using the Biomek 3000 laboratory automation workstation (Beckman Coulter, Brea, CA).

Statistical Method of microRNA Selection

The quantitative variables were summarized as mean ± SE. The qualitative variables were summarized as frequency and percentage. Statistical analysis was performed using parametric tests because the distribution of the variables was normal, calculated with Shapiro-Wilk test.

The first step of the analysis was conducted on the training set to develop a potential screening test, samples were regrouped into two categories to compare differences in microRNAs: nodules (10 lung adenocarcinomas + 10 granulomas) versus nonnodules (10 healthy former smokers). To develop a diagnostic test, aimed to describe the malignancy of nodules group samples, only the 10 lung adenocarcinomas (cancers group) were compared with the 10 lung granulomas (granulomas group).

One-way analysis of variance or Student's *t* test for unpaired, when appropriate, was performed to evaluate statistically significant differences of quantitative variables among groups; χ^2 test was applied on qualitative variables.

CfsSubsetEval (class attributes) function of WEKA software version 3.6.4 (University of Waikato, Hamilton, New Zealand)[16] was used to select the more informative microRNAs for the discrimination between lung adenocarcinomas, lung granulomas, and healthy former smokers groups in the training set. This method assesses the predictive ability of each attribute, as well as the degree of redundancy among each microRNA. It prefers attributes that are highly correlated within each class, but that have low intercorrelation. The choice of attributes was performed using 10-fold cross-validation, and selection of the 14 best attributes was based on their being selected at least 50% of the time.[17]

To evaluate the statistically significant differences between nodules versus nonnodules and granulomas versus cancers in the selected microRNAs of the entire validation cohort (105 samples: 50 lung adenocarcinomas, 30 lung granulomas, and 25 healthy former smokers), a Student's *t* test for unpaired data with Benjamini-Hochberg correction for multiple comparisons was used; false-discovery rate was set to 0.05.

Statistical Modeling and Validation

Starting from the previously selected microRNAs a stepwise logistic regression analysis (class SimpleLogistic in the WEKA software package) was conducted, on training set, to choose a best model to classify nodules versus nonnodules. Analysis was performed with 20-fold cross-validation, 500 max boosting interactions, 50 heuristic stop. The same procedure was conducted to choose the best model to classify granuloma versus cancer. Screening models were applied on the entire validation cohort of 105 samples.

Diagnostic models were applied on lung adenocarcinoma and lung granuloma samples taken from the validation set, 80 samples. Alpha (α) values were evaluated for each sample. To quantify the goodness of screening and diagnostic models, the sensitivity, specificity, positive and negative

predictive values, and receiver operating characteristic curve analysis were evaluated separately.

RESULTS

Before starting with microRNA profiling, some critical issues were addressed, including: selection of an appropriate housekeeping microRNA to normalize RT-PCR results and assessment of stability of exosomes in frozen archival plasma samples. The main aim of this study was to select the lowest number of microRNAs useful to create a strong screening and diagnostic model.

Housekeeping microRNA Selection

According to previously published articles,[12,14] and the Exiqon RT-PCR manual, five microRNAs (Let-7a, mir-20a, mir-221a, mir-16, and let-7b) and GAPDH were selected to be evaluated as possible housekeeping microRNA on a random cohort of 10 samples. Normfinder software was used to give a numerical evaluation of stability with relative SEs.

Let-7a expression showed the lowest output value and error, proving to be the best housekeeping microRNA among those analyzed (Fig. 1 supplementary data, Supplemental Digital Content 1, http://links.lww.com/JTO/A468); this in accordance with the findings of Chen et al.[12] GAPDH expression, instead, was found in only two of 10 samples (data not shown).

microRNA (and Exosome) Stability in Frozen Plasma Samples

To assess the stability of microRNAs extracted from plasma conserved for different period of time at −80°C, various aliquots of the same sample (fresh plasma and 5 frozen plasma aliquots, stored for: 24 hours, 48 hours, 1 week, 1 month, and 2 months after preparation) were subjected to RNA extraction and RT-PCR using mir-20a, mir-221a, and Let-7a primers. The raw Ct are showed in Figure 1, there were no detectable oscillations in the total concentration of examined microRNAs.

Spiked-in RNA was used to evaluate the quality of the whole PCR reaction, it showed a low statistical deviation from arithmetic mean (<0.25 Ct) evidence of good quality PCR reactions, which allow us to directly compare different samples of raw Ct.

This evidence confirmed that exosomes remained unaltered in plasma when properly stored at −80°C.[17]

microRNAs Profiling and Selection of *Interesting* microRNAs

Wide-range microRNA analysis has shown that 278 microRNAs were expressed in all three studied groups. On these 278 microRNAs, statistical analysis was performed via WEKA software (CfsSubsetEval analysis) as described in Materials and Methods section. The 14 best microRNAs

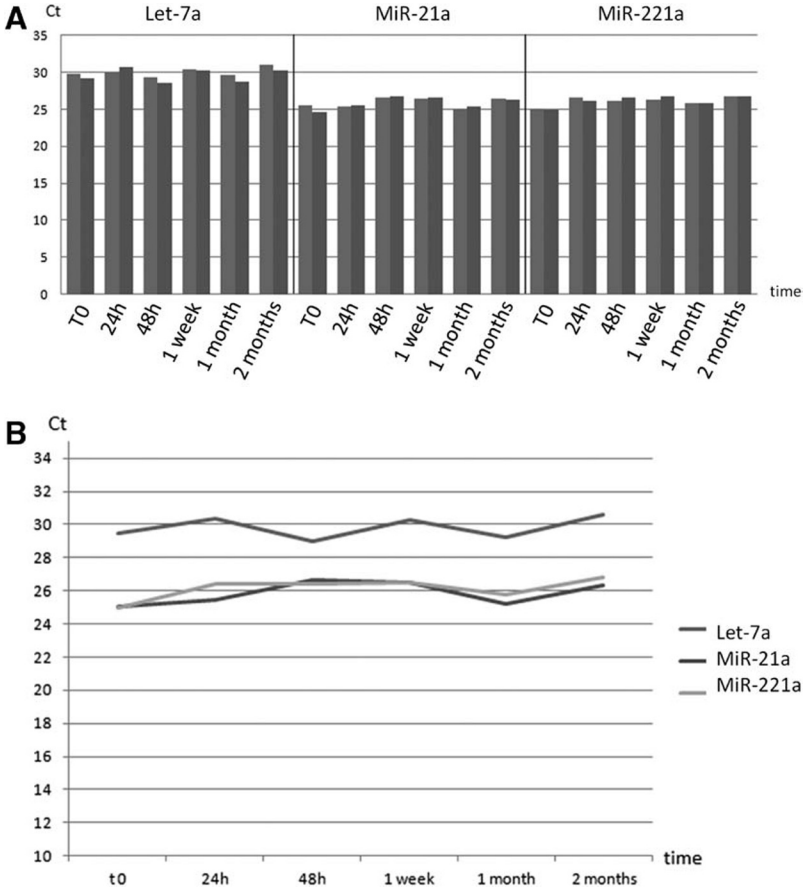

FIGURE 1. *A* and *B*, Exosome stability in time. We analyzed exosome stability in frozen samples using raw Ct of mir-20a, mir-221, and Let-7a. Graphs show reverse-transcriptase polymerase chain reaction of resulting raw Ct of the same plasma sample, divided in several aliquots and RNA extracted from fresh plasma aliquot and frozen plasma aliquots at 24 hours, 48 hours, 1 week, 1 month, and 2 months. Graph (*A*) shows raw Cts of the various aliquots and the duplicate tests divided by microRNAs. Graph (*B*) compares microRNA trends in time. There is no detectable oscillation of microRNA raw Cts from fresh aliquot to the one frozen for 2 months.

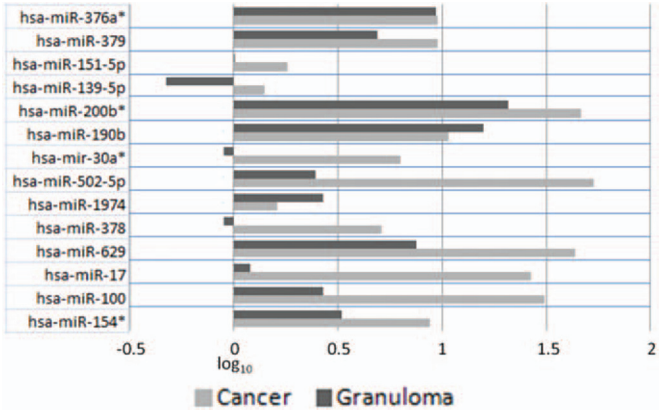

FIGURE 2. Overview of microRNA expression levels. After data set normalization, the $\log_{10}$ of $2^{(-\Delta\Delta Ct)}$ was taken each microRNA. The graph shows expression level value of each microRNA, one for lung adenocarcinomas from healthy controls (reported as *cancer* bars in the graph) and another for lung granulomas from healthy controls (reported as *granuloma* bars in the graph). Concerning lung granuloma expression levels, three to 14 microRNAs showed slight down-regulation, 10 microRNAs were found up-regulated. miR-151a-5p showed no expression levels compared with normal donors. All 14 microRNAs resulted in up-regulation in lung adenocarcinoma expression levels.

(characterized by the highest CfsSubsetEval value) were selected to be further evaluated in the validation cohort.

miR-502-5p, miR-376a-5p, miR-1974, miR-378a, miR-379, miR-151a-5p, miR-139-5p, miR-200b-5p, miR-190b, miR-30a-3p, miR-629, miR-17, miR-100, and miR-154-3p were found to be the best microRNAs by CfsSubsetEval analysis; the results ranged between a higher value of 100% and a lower value of 50% (Fig. 2 supplementary data, Supplemental Digital Content 2, http://links.lww.com/JTO/A469). The quantitative RT-PCR data obtained on the validation series were used to double check these 14 microRNAs by fold-changes technique and *t* tests. The acquired *p* values were for nodules versus nonnodules and for granulomas versus adenocarcinomas.

Concerning lung granulomas expression levels, only three to 14 microRNAs were slightly down-regulated (miR-139-5p, miR-30a-3p, miR-378a) with less than −0.5 expression level value. Ten microRNAs were found up-regulated: miR-502-5p, miR-1974, miR-17, miR-100, and miR-154-3p were slightly up-regulated (≤0.5); miR-376a-5p, miR-378a, miR-379, miR-139-5p, miR-30a-3p, and miR-629 were moderately up-regulated (expression levels value between 0.5 and 1); miR-200b-5p and miR-190b were highly up-regulated (>1). miR-151a-5p Has Shown no Expression Levels Regarding Lung Granulomas.

Regarding lung adenocarcinomas there was no down-regulation, all 14 microRNAs were up-regulated: miR-151a-5p, miR-1974, and miR-139-5p were slightly up-regulated (≤0.5); miR-376a-5p, miR-378a, miR-379, miR-30a-3p, and miR-154-3p were moderately up-regulated (expression levels value between 0.5 and 1); miR-200b-5p, miR-190b, miR-502-5p, miR-629, miR-17, and miR-100 were highly up-regulated (>1). Expression levels are shown in Figure 2.

p Values, obtained by testing the validation cohort ΔΔCts, were used to evaluate whether data were statistically significant and useful for the construction of the screening test, the diagnostic test, or both. Box-whiskers graphs of selected microRNAs are available in supplementary data Figure 3 (Supplemental Digital Content 3 [http://links.lww.com/JTO/A470] and 4 [http://links.lww.com/JTO/A471]).

Student's *t* test nodule versus nonnodule confirmed that three to 14 microRNAs were useful for the statistic modeling of lung cancer screening test: miR-151a-5p, miR-139-5p, and miR-1974; five other microRNAs, miR-379, miR-200b-5p, miR-502-5p, miR-378a, and miR-100 could be considered significant for *p* value less than 0.05. Student's *t* test lung granulomas versus lung adenocarcinoma has shown the importance of eight microRNAs for the development of the diagnostic test: miR-17, miR-30a-3p, miR-378a, miR-151a-5p, miR-502-5p, miR-154-3p, miR-100, miR-139-5p, in the same way that miR-200b-5p and miR-629 could be considered significant for *p* value less than 0.05 (Table 1 supplementary data, Supplemental Digital Content 5, http://links.lww.com/JTO/A472).

microRNA Evaluation and Test Modeling Results

The models were created with WEKA software as described in Materials and Methods section. Starting from the previously described 14 microRNAs, modeling software attempted to use the smallest number of microRNAs to describe the data set. Regarding the screening test, selected microRNAs are: miR-378a, miR-379, miR-139-5p, and miR-200-5p. Concerning the diagnostic test, selected microRNAs are: miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, and miR-154-3p. The specific results for both typologies of test are reported below in Table 2; Figures 3 and 4 show receiver operating characteristic curves of the two tests.

TABLE 2. Overview of Test Characteristics

Test	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	AUC ROC
Screening test, %	97.5	72	92	90	90.8
Screening algorithm	α = 0.86 + (miR378a) (−0.3) + (miR379) (0.19) + (miR-139-5p) (0.37) + (miR-200b-5p) (−0.17)				
Diagnostic test, %	96	60	80	90	76
Diagnostic algorithm	α = −0.89 + (miR-151a-5p) (−0.15) + (miR-200b-5p) (0.07) + (miR-30a-3p) (0.05) + +(miR629) (−0.17) + (miR100) (0.04) + (miR-154-3p) (−0.05)				

Shows the tests characteristics: sensibility, specificity, positive predictive value, negative predictive value, and AUC ROC. It also shows the tests algorithms.
AUC ROC, area under the curve receiver operating characteristic.

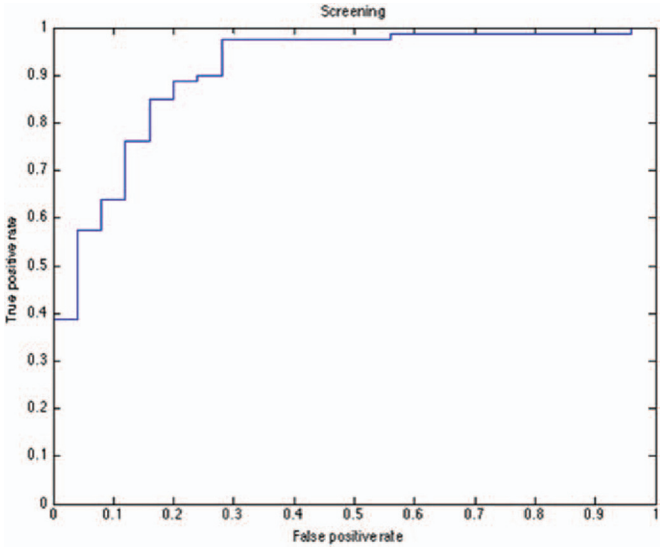

FIGURE 3. Screening model ROC curve. ROC plot for screening model microRNA set. The screening model distinguishes between control subjects and patients with any kind of nodules (lung adenocarcinomas and granulomas) with an area under the curve = 0.908 ($p < 0.001$). ROC, receiver operating characteristic.

DISCUSSION

This study was designed for a very specific purpose, the development of two tests useful in a two-step policy of diagnosis for lung adenocarcinoma. Prevention and periodic tests have revealed that mortality can be reduced for colon and rectum, breast, and uterine cervix cancers.[18]

Currently, lung cancer has the highest mortality rate and is the second most common cancer in both men (after prostate cancer) and women (after breast cancer). About 28% of all cancer deaths can be ascribed to this pathology; about 15% of all new cancers are non–small-cell lung cancer or small-cell lung cancer.[18–22]

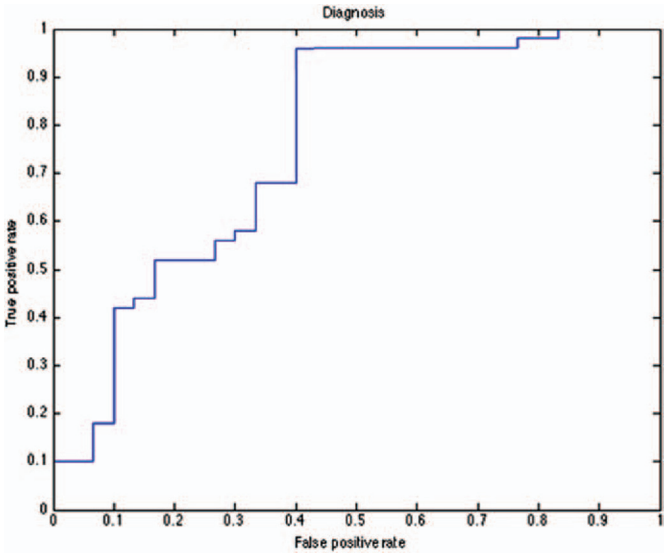

FIGURE 4. Diagnosis model ROC curve. ROC plot for diagnostic model microRNA set. The diagnostic model distinguishes between lung adenocarcinoma and granuloma patients with an area under the curve = 0.760 ($p < 0.001$). ROC, receiver operating characteristic.

Although low-dose spiral computed tomography (CT or helical CT) has shown some promise in detecting early lung cancers in heavy smokers and former smokers,[2,23] we are still far from getting results comparable with those of breast cancer.

This technique is useful in detecting small abnormalities in the lung, but it is known to have some downsides and impacts on the patient overall health.[23,24]

Circulating exosomes, and their microRNA content, have opened a new field for molecular diagnostics. microRNAs could suppress specific protein synthesis and entire biological pathways in exosome-targeted cells.[25]

Exosome-based diagnostic techniques have the potential for having high reproducibility and require only a blood sampling. It is our opinion that any alteration in the *healthy* status could lead to a change of microRNAs pattern in the bloodstream. A high-throughput study, as the one presented in this article, could be a step to decode the exo/miR-code by which cells exchange messages and influence each other's biological cycle, without affecting the general structure of their genomes.

Correlations between microRNAs deregulated in tissues compared with bloodstream have rarely been observed. Plasma samples could be useful independently of tissue specimens.[12]

In this study we moved from a wide-range analysis of 746 microRNAs to a narrow selection of 14 microRNAs known to have different functions. miR-200b-5p was used as a biomarker to assess the recurrence of small-cell lung tumors after surgical resection.[26] miR-100, miR-378a, and miR-629 primarily regulate the expression of PPP3CA and FZD, two important oncogenes in colorectal cancer and basal cell carcinoma, having at the same time a lower affinity with genes of the map-kinase cascade.[27–29] Some of the microRNAs are important in the long-term potentiation pathway (mir-30a, miR-139-5p). The mir-30a is involved in metastasis and cellular invasion, targeting the gene Snai1 and inhibiting the epithelial/mesenchymal transition.[30] miR-139-5p is involved in kidney and colorectal cancer and has been used for molecular diagnosis of these forms.[31,32] miR-151a-5p acts mainly on the response of the map-kinase cascade, important in cell migration and invasion,[33] it also varies in response to ionizing radiation.[34] Mir-154 has privileged target genes in UBE2D2, UBE2D3, and CUL2, which are involved in ubiquitin-mediated proteolysis.[35] The miR-379 has a strong affinity with the gene HLCS involved in the metabolism of biotin, and genes involving regulation of cell adhesion, also important in patient response to drugs and their relative resistance.[36,37]

This study must be confirmed with a larger data set. We do not yet know the affects of sex, age, and cancer treatment on microRNAs levels in plasma. Our data suggest that exosomes analysis in plasma samples are better suited to screen a high-risk population than discriminate between malignant and nonmalignant lesions. Currently, with the exception of CT scan, there is no screening technique for lung cancer that can compete with other techniques implemented for different types of cancer. CT scan showed a tendency to overestimate

significantly the number of positives. A total of 96.4% of the positive screening results in the low-dose CT group, in a recent U.S. National screening trial,[2] were false positive. Having also a number of negative effects on the patient, the CT scan is a test not repeatable in the short term. Molecular tests can be repeated a virtually infinite number of times, without any damage to patients. This pilot study showed a specificity of 72%; false-positive rate is therefore significantly reduced when compared with that of the CT scan.

The importance of the biological role of exosomes is growing exponentially in recent years of research. Further studies are needed to fully understand this *new* way of cell to cell communication. We strongly believe in the opportunity to choose exosomes as screening–diagnostic tools.

REFERENCES

1. Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin* 2011;61:212–236.
2. The National Lung Screening Trial Research Team. Reduced lung-cancer mortality with low-dose computed tomographic screening. *N Engl J Med* 2011;365:395–409.
3. Zomer A, Vendrig T, Hopmans ES, van Eijndhoven M, Middeldorp JM, Pegtel DM. Exosomes: fit to deliver small RNA. *Commun Integr Biol* 2010;3:447–450.
4. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008;105:10513–10518.
5. Cortez MA, Calin GA. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. *Expert Opin Biol Ther* 2009;9:703–711.
6. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. *Annu Rev Med* 2009;60:167–179.
7. Shen J, Todd NW, Zhang H, et al. Plasma microRNAs as potential biomarkers for non-small-cell lung cancer. *Lab Invest* 2011;91:579–587.
8. Lebanony D, Benjamin H, Gilad S, et al. Diagnostic assay based on hsa-miR-205 expression distinguishes squamous from nonsquamous non-small-cell lung carcinoma. *J Clin Oncol* 2009;27:2030–2037.
9. Boeri M, Verri C, Conte D, et al. MicroRNA signatures in tissues and plasma predict development and prognosis of CT detected lung cancer. *Proc Natl Acad Sci USA* 2011;108(9):3713–3718.
10. Zheng D, Haddadin S, Wang Y, et al. Plasma microRNAs as novel biomarkers for early detection of lung cancer. *Int J Clin Exp Pathol* 2011;4:575–586.
11. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9:654–659.
12. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008;18:997–1006.
13. Yang C, Robbins PD. The roles of tumor-derived exosomes in cancer pathogenesis. *Clin Dev Immunol* 2011;2011:842849.
14. Juan W, Wen G, Cheng-Jun Z, et al. Identification of plasma microRNA-21 as a biomarker for early detection and chemosensitivity of non-small cell lung cancer. *Chin J Cancer* 2011;30(6):407–414.
15. Jensen J, Ørntoft T. Normalization of real-time quantitative RT-PCR data: a model based variance estimation approach to identify genes suited for normalization—applied to bladder- and colon-cancer data-sets. *Cancer Research* 2004;(64):5245–5250.
16. Witten IH, Frank E. Data Mining: Practical Machine Learning Tools and Techniques, 2nd Ed. San Francisco, CA: Morgan Kaufmann, 2005.
17. Lodes MJ, Caraballo M, Suciu D, et al. Detection of cancer with Serum microRNAs on an Oligonucleotide Microarray. *PLoS One* 2009;4(7):e6229.
18. National Cancer Institute. Statistical tools and data (Cancer.gov web site). May, 2011. Available at: http://www.cancer.gov/statistics. Accessed may 5, 2011.
19. Heisey R, Clemons M, Granek L, et al. Health care strategies to promote earlier presentation of symptomatic breast cancer: perspectives of women and family physicians. *Curr Oncol* 2011;18:e227–e237.
20. Majek O, Danes J, Skovajsova M, et al. Breast cancer screening in the Czech Republic: time trends in performance indicators during the first seven years of the organised programme. *BMC Public Health* 2011;11:288.
21. Arolfo S, Teggia PM, Nano M. Gastrointestinal stromal tumors: thirty years experience of an institution. *World J Gastroenterol* 2011;17:1836–1839.
22. Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin Lung Cancer* 2009;10:42–46.
23. Bach PB, Jett JR, Pastorino U, Tockman MS, Swensen SJ, Begg CB. Computed tomography screening and lung cancer outcomes. *JAMA* 2007;297:953–961.
24. Pastorino U. Lung cancer screening. *Br J Cancer* 2010;102:1681–1686.
25. Davalos V, Esteller M. MicroRNAs and cancer epigenetics: a macrorevolution. *Curr Opin Oncol* 2010;22:35–45.
26. Patnaik SK, Kannisto E, Knudsen S, Yendamuri S. Evaluation of microRNA expression profiles that may predict recurrence of localized stage I non-small cell lung cancer after surgical resection. *Cancer Res* 2010;70:36–45.
27. Navon R, Wang H, Steinfeld I, Tsalenko A, Ben-Dor A, Yakhini Z. Novel rank-based statistical methods reveal microRNAs with differential expression in multiple cancer types. *PLoS One* 2009;4:e8003.
28. Gao W, Shen H, Liu L, Xu J, Xu J, Shu Y. MiR-21 overexpression in human primary squamous cell lung carcinoma is associated with poor patient prognosis. *J Cancer Res Clin Oncol* 2011;137:557–566.
29. Eichner LJ, Perry MC, Dufour CR, et al. miR-378(*) mediates metabolic shift in breast cancer cells via the PGC-1β/ERRγ transcriptional pathway. *Cell Metab* 2010;12:352–361.
30. Kumarswamy R, Mudduluru G, Ceppi P, et al. MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snai1 and is downregulated in non-small cell lung cancer. *Int J Cancer* 2012;130:2044–2053.
31. Fridman E, Dotan Z, Barshack I, et al. Accurate molecular classification of renal tumors using microRNA expression. *J Mol Diagn* 2010;12:687–696.
32. Chang KH, Miller N, Kheirelseid EA, et al. MicroRNA signature analysis in colorectal cancer: identification of expression profiles in stage II tumors associated with aggressive disease. *Int J Colorectal Dis* 2011;26:1415–1422.
33. Ding J, Huang S, Wu S, et al. Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDIA. *Nat Cell Biol* 2010;12:390–399.
34. Niemoeller OM, Niyazi M, Corradini S, et al. MicroRNA expression profiles in human cancer cells after ionizing radiation. *Radiat Oncol* 2011;6:29.
35. Wang W, Peng B, Wang D, et al. Human tumor microRNA signatures derived from large-scale oligonucleotide microarray datasets. *Int J Cancer* 2011;129:1624–1634.
36. Haenisch S, Laechelt S, Bruckmueller H, et al. Down-regulation of ATP-binding cassette C2 protein expression in HepG2 cells after rifampicin treatment is mediated by microRNA-379. *Mol Pharmacol* 2011;80:314–320.
37. Guo L, Liu Y, Bai Y, Sun Y, Xiao F, Guo Y. Gene expression profiling of drug-resistant small cell lung cancer cells by combining microRNA and cDNA expression analysis. *Eur J Cancer* 2010;46:1692–1702.