A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer

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Lung cancer is the first cause of cancer mortality worldwide, and its early detection is currently the main available strategy to improve disease prognosis. While early diagnosis can be successfully achieved through tomography-based population screenings in high-risk individuals, simple methodologies are needed for effective cancer prevention programs. We developed a test, based on the detection of 34 microRNAs (miRNAs) from serum, that could identify patients with early stage non-small cell lung carcinomas (NSCLCs) in a population of asymptomatic high-risk individuals with 80% accuracy. The signature could assign disease probability accurately either in asymptomatic or symptomatic patients, is able to distinguish between benign and malignant lesions, and to capture the onset of the malignant disease in individual patients over time. Thus, our test displays a number of features of clinical relevance that project its utility in programs for the early detection of NSCLC.

INTRODUCTION

Non-small cell lung carcinoma (NSCLC) is the first cause of cancer mortality worldwide (Parkin et al, 2005), largely due to the lack of effective tools for early diagnosis. Patients diagnosed with early stage disease (stage I) have a reasonably favourable prognosis (60–80% survival at 5 years, Jemal et al, 2008). Unfortunately, only 20–25% of all NSCLCs are diagnosed early

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(Greenlee et al, 2000; Porter & Spiro, 2000). Tobacco exposure is the major risk factor for lung cancer (Peto et al, 1996). Even in former smokers, the tobacco-associated risk remains high for a long time (Jemal et al, 2009). Thus, effective tools for the early detection of NSCLC, especially in high-risk individuals, are critically needed.

Low dose spiral computed tomography (LD-CT) has been tested in prospective observational trials and has proven to be an effective detection method for small nodules (even <5 mm) as well as for the diagnosis of asymptomatic lung cancers, most of which (67–85%) are at stage I (Henschke et al, 2006; Pastorino et al, 2003; Swensen et al, 2003; Veronesi et al, 2008). There is debate over whether annual LD-CT screening reduces the number of diagnoses of advanced stage disease (stages III–IV) and lung cancer deaths (Bach et al, 2007; Chien & Chen, 2008; McMahon et al, 2008), and the possibility has been raised that LD-CT screening may lead to over-diagnosis and over-treatment of biologically indolent cancers, which would rarely reach clinical relevance or pose a health hazard for patients (Reich, 2008; Welch et al, 2007). However, the effectiveness of LD-CT has recently been confirmed, as a large randomized trial (The National Lung Screening Trial, Aberle et al, 2011) was prematurely stopped on the basis that 'the accumulated data now provide a statistically convincing

answer to the study's primary question' (http://www.cancer.gov/newscenter/pressreleases/NLSTresultsRelease).

While early diagnosis is indeed an effective method to reduce lung cancer deaths, concerns remain regarding the feasibility of large-scale population screening by LD-CT: the technique is cumbersome, it requires specialized health centres, and the recruitment of at-risk individuals with no symptoms of disease is difficult. Therefore, the development of diagnostic blood tests becomes of paramount relevance for the execution of effective population screenings. The detection of specific circulating microRNAs (miRNAs) might serve this purpose. Indeed there is evidence to support the idea that circulating miRNAs with diagnostic potential exist for almost every type of malignant (Lawrie, 2010; Skog et al, 2008; Tanaka et al, 2009) and non-malignant disease (Laterza et al, 2009; Redell et al, 2010; Rong et al, 2011; Wang et al, 2009; Zhang et al, 2010), including lung cancer (Wittmann & Jack, 2010). Here, we describe a blood test,

based on the detection of serum miRNAs, that identifies early NSCLC patients in a population of asymptomatic high-risk individuals with ~80% accuracy.

RESULTS

Serum miRNAs in asymptomatic NSCLC patients and healthy smokers

We took advantage of a large study (the COSMOS study, Veronesi et al, 2008), in which 5203 high-risk individuals were screened by annual LD-CT to detect lung cancer (Fig 1A, clinical and pathological information for all patients in this study are summarized in Table SI of Supporting Information). Ninety-three NSCLCs were diagnosed in the first 2 years of screening (55 at baseline and 38 at annual screening). Sera—collected before surgery—were available from 59 of these patients
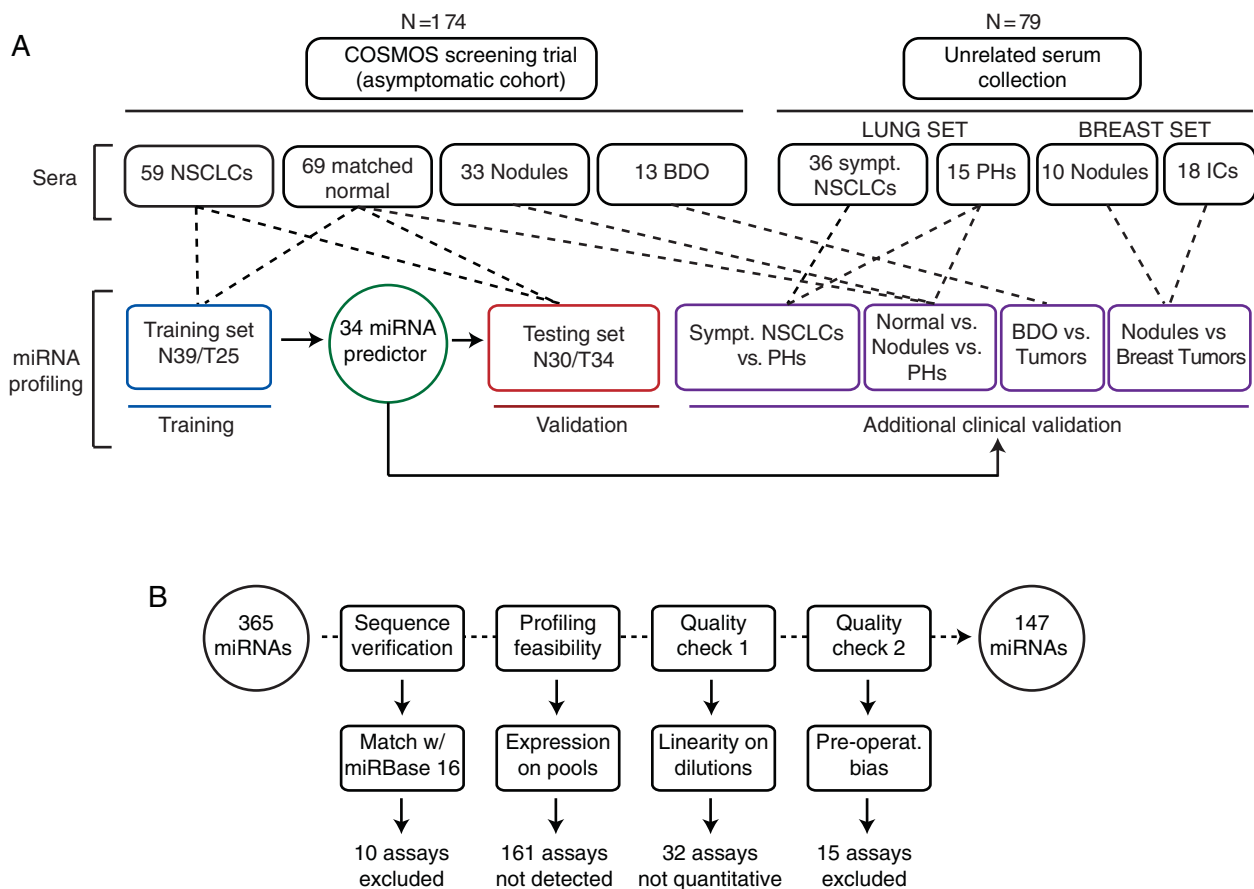

Figure 1. Study design.

A. Sera were obtained from two independent collections: (i) from the COSMOS study [59 non-small cell lung carcinomas (NSCLCs), 69 normal, 33 nodules, 13 sera before disease onset (BDO)], and (ii) from an unrelated serum collection of symptomatic cases [36 NSCLCs (from symptomatic patients), 15 PHs]. COSMOS NSCLC and normal sera were divided into a training set and a testing set (N, normal; T, tumour). Additional sets were used for other clinical validations, as shown in the figure and explained in the main text.

B. A total of 365 miRNA assays (Table SII of Supporting Information) were employed in the screening. A series of tests was implemented to exclude those miRNAs that did not meet a number of stringent criteria for inclusion in the final analysis (see Fig S1 and Table SII of Supporting Information for details). This led to the selection of 147 miRNAs that were used for all analyses presented in the study.

(Fig 1A). At the time of serum collection, all patients were asymptomatic, and the detection of cancer was solely a consequence of their enrolment in the observational project. We selected control sera (matched for confounding factors, such as gender, age, and smoking exposure) from 69 individuals enrolled in the same study, in whom no cancer was found by LD-CT during the entire study (healthy smokers, henceforth 'normal') (Fig 1A). Sera from the COSMOS study were divided into two sets: a training set (39 normal subjects and 25 with adenocarcinomas—AC) and a testing set (30 normal subjects, 22 AC and 12 squamous cell carcinomas—SCC) (Fig 1A, and Table 1).

A total of 365 miRNA assays were employed in the study (listed in Table SII of Supporting Information). A series of calibration tests (schematized in Fig 1B and described in Fig S1 and Table SII of Supporting Information) were performed to select a pool of 147 miRNAs that were informative (see Materials and Methods Section and Fig S1 for details) for the purpose of our study. The levels of these 147 miRNAs were evaluated in RNAs extracted from all the training/testing sera (and also from all other sera used in this study). After miRNA profiling and normalization (see Materials and Methods Section, Table SIII and Fig S2 of Supporting Information), we used the training set to derive a 34-miRNA signature capable of separating tumour from normal sera (see Materials and Methods Section, and Table SIV of Supporting Information). One-way hierarchical clustering analysis showed that the 34-miRNA signature is composed of two main groups of miRNA, showing increased or decreased expression in tumour *versus* normal sera, respectively (Fig S3 and Table SIV of Supporting Information). Importantly, the trend of regulation in tumour *versus* normal of these two groups of miRNAs was maintained between the training and the testing set, thus confirming stability of the expression profile (Fig S3).

A 34-miRNA model for NSCLC diagnosis in asymptomatic high-risk individuals

We developed a multivariate risk predictor, using the weighted linear combination of the 34 miRNA expression values (see Materials and Methods Section and Table SI of Supporting Information), to assign each patient to a high or low risk category (with a cut-off score set at 3.235 during the training of the classifier, which was performed on the training set). In the training set, the risk algorithm displayed an accuracy of 78% and an area under the curve (AUC) of 0.92 (Table 2, Fig 2A). The predictor was remarkably stable when applied to the testing set, displaying an accuracy of 80% and an AUC of 0.89 (Table 2, Fig 2A). Importantly, the algorithm, which was derived on a training set containing only ACs, performed well in both SCCs and ACs in the testing set (AUC: 0.85 and 0.94 for AC and SCC, respectively; Table 2, Fig 2A). In addition, fairly accurate predictions could also be obtained by employing models containing fewer miRNAs. As shown in Fig S5, a 5-miRNA model displayed an AUC of 0.77 when applied to the testing set. By increasing the number of miRNAs, the predictor became increasingly more accurate, reaching an AUC of 0.89 when the 34-miRNA model was employed.

Finally, it is of note that, while our training set was intentionally enriched in stage I patients (one of our main goals was to develop a signature sensitive enough to detect also early stage cancers, in which surgery is most effective), the testing set contained a relevant proportion of more advanced cancers (Table 1). In the testing set, the predictor performed well for cancers of all stages (I–IV), with an AUC of 0.89 for stage I tumours and 0.88 for stage II–IV (Table 2, Fig 2A), arguing for its ability to detect all stages of NSCLCs in asymptomatic patients. Indeed, a sensitivity analysis showed that the 34-miRNA signature remained a strong predictor of risk independently of the subgroup of patients considered (Fig 2B). Finally, it is worth mentioning that comparable results (in the above-described and in all subsequent analyses) were obtained using two different methods for data normalization (see Fig S4 of Supporting Information).

Table 1. Clinical characteristics of patients of the training and testing sets

Categories	Training set (N = 64)	Testing set (N = 64)	Symptomatic set (N = 36)
Tumour	25	34	36
Normal	39	30	0
Male	45	43	30
Female	19	21	6
Non-smokers	0	0	8
Age (years)	58 ± 6	60 ± 6	66 ± 8
Pack-years	54 ± 18	49 ± 17	45 ± 37
Tumour subtype			
AC	25	22	23
SCC	0	12	13
Tumour Stage			
IA	19	18	7
IB	2	4	9
II–IV	4	12	20

AC, adenocarcinoma; SCC, squamous cell carcinoma.

The clinical and pathological characteristics are reported for the training and testing sets of patients. The average and relative standard deviation (±SD) is indicated for age (years) and smoking status (pack-years).

Table 2. Performance of the predictive model in various sets

Set	AUC	ACC %	SEN %	SPE %
Training set (39 N, 25 AC)	0.92	78[a]	69[a]	84[a]
Testing set (30 N, 22AC, 12 SCC)	0.89	80	71	90
Testing set—AC only (30 N, 22 AC)	0.85	79	64	90
Testing set—SCC only (30 N, 12 SCC)	0.94	88	83	90
Testing set—stage I only (30 N, 22 T)	0.89	77	59	90
Testing set—stage II–IV only (30 N, 12 T)	0.88	90	92	90

Performances of the 34-miRNA predictor model. The area under curve (AUC, from receiver operating characteristic curves, see Fig 2A) is shown together with the overall accuracy (ACC), the sensitivity (SEN, the probability for a tumour to be correctly predicted as 'tumour') and specificity (SPE, the probability for a normal sample to be correctly predicted as 'normal').

[a]Accuracy, sensitivity and specificity in the training set are based on K-fold (K = 5) cross-validation result of the DLDA classifier (see Materials and Methods Section).
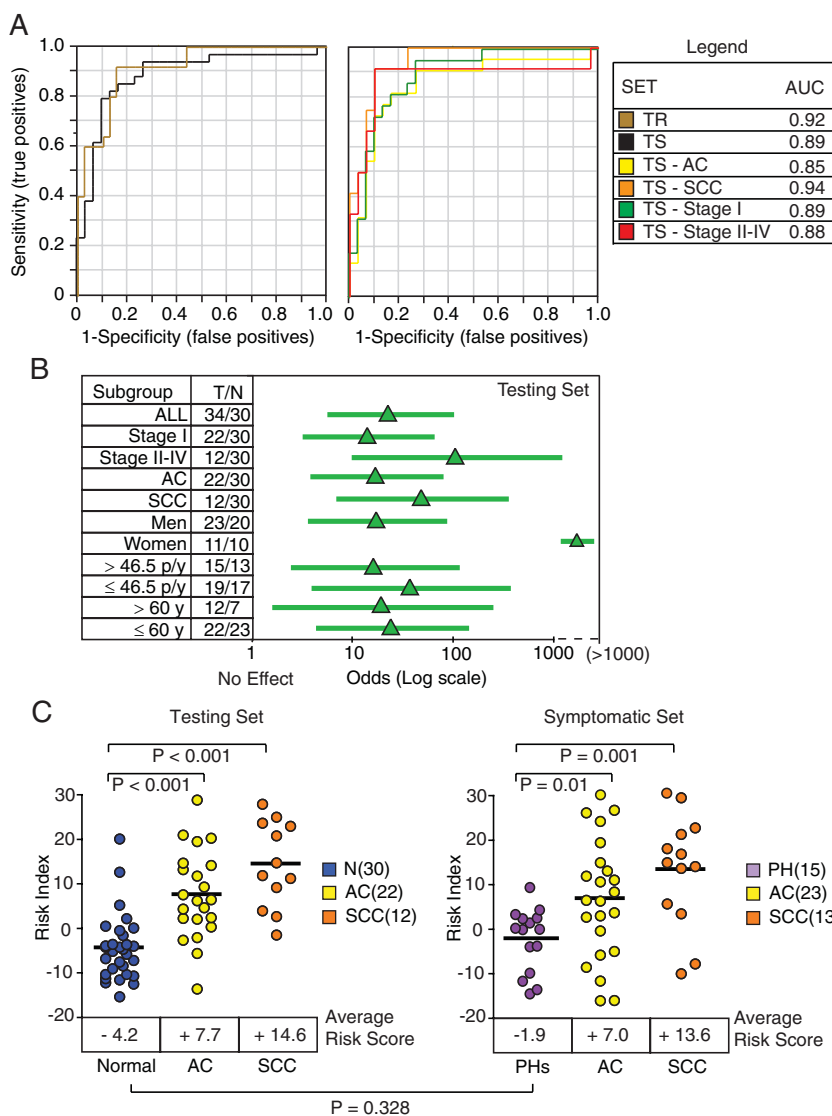
Figure 2. The 34-miRNA diagnostic model.

A. Receiver operating characteristic (ROC) curves of the 34-miRNA diagnostic model (curves are presented in two separate panels solely for reasons of clarity). Colour codes are as per the 'legend'. TR, training set; TS, testing set; TS-AC, testing set considering only ACs; TS-SCC, testing set considering only SCCs; TS-Stage I, testing set considering only stage I tumours; TS-Stage II–IV, testing set considering all other tumour stages.

B. Forest plot showing the 34-miRNA model prediction strength in the testing set (all, 30 normal and 34 tumours) stratified by available clinical-pathological parameters. Triangles represent the odds ratios; lines represent the relative 95% confidence intervals (nominal logistic regression). Age (years) and packs/year (p/y) cut-offs were defined by the relative averages in the 64 patients. p-Value were <0.01 in all analyses (two-tails Fisher's exact test).

C. Risk of cancer based on the 34-miRNA risk model in NSCLC patients (separately for ACs and SCCs) from the testing set (left panel) and symptomatic cases (right panel). Average risk scores and p-value (ANOVA) are also shown.

Additional clinical validations

We performed a series of analyses to further validate the 34-miRNA predictor and to gain further insights into its potential value in the clinical setting. Initially, we wanted to exclude the possibility that the performance of our predictor could be linked to some unknown 'study bias'. Such a bias could be hypothetically represented by the fact that tumours detected by LD-CT might be intrinsically different from those that present themselves symptomatically, or to other systematic biases in the management of the individuals enrolled in the COSMOS trial.

To address this issue, we employed pre-operative sera from an independent cohort of symptomatic NSCLC patients, who underwent surgery at the European Institute of Oncology across a number of years as part of the routine clinical activity of the Institute (the Symptomatic set, 23 AC and 13 SCC, Fig 1A and Table 1). As a stringent control for the symptomatic set, we screened pre-operative sera from patients harbouring benign pulmonary hamartomas (PHs, 15 sera, Fig 1A). When the 34-miRNA predictor was applied to evaluate the risk in the symptomatic set and in the PH set, it performed remarkably well (Fig 2C). The average risk index of NSCLCs patients was virtually indistinguishable, both for ACs and SCCs, between the symptomatic set and the testing set of the COSMOS trial (Symptomatic set: AC = 7.0, SCC = 13.6; COSMOS set: AC = 7.7, SCC = 14.6; Fig 2C). In addition, the average risk index of patients from the symptomatic set was clearly higher, in a statistically significant manner, than that of PH-harbouring patients (average risk score PHs = −1.9, AC = 7.0, SCC = 13.6, p-value <0.01 and <0.001 between PHs and AC or SCC, respectively, Fig 2C, right). Finally, the risks of normal individuals from the testing set of the COSMOS trial and of PH-harbouring patients were similar and the difference was not statistically significant (Cosmos set Normal = −4.2, Symptomatic set PHs = −1.9, p = 0.328, Fig 2C, see also Fig S4 of Supporting Information).

We next took advantage of sera from a group of 33 patients, who were detected at baseline LD-CT with benign lung nodules and did not develop lung cancer during the entire period of the study (nodules, Fig 1A). This provided us with the opportunity to test whether our predictor could distinguish between benign and frankly malignant lung disease in asymptomatic patients.
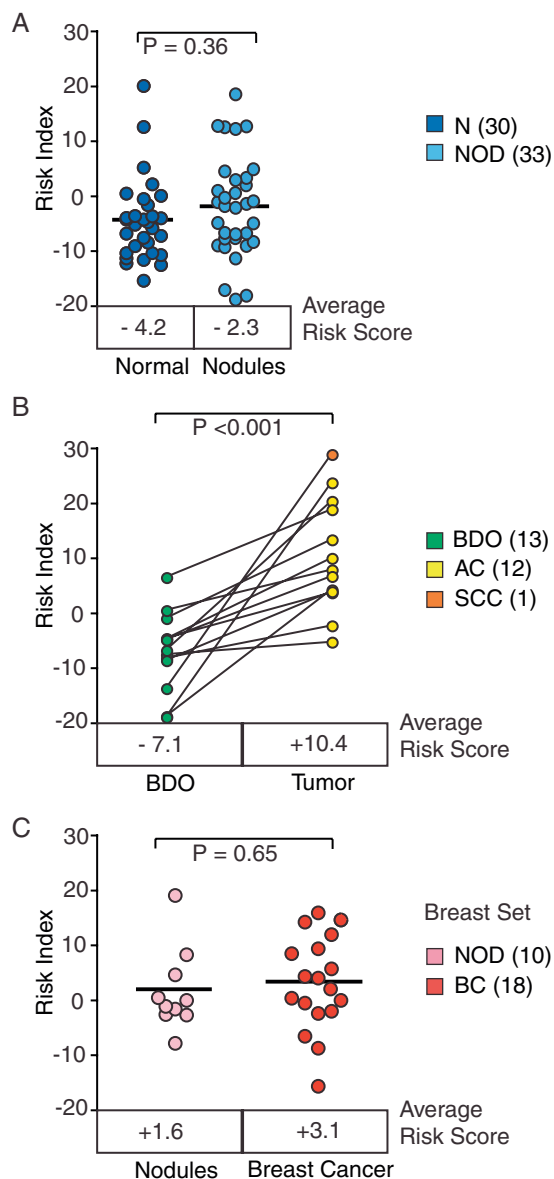

Figure 3. Performance of the 34-miRNA diagnostic model under various conditions of clinical interest.

A. Risk index in patients with LD-CT detected benign nodules (NOD), or normal individuals (N). Average risk scores and *p*-value (Welch's *t*-test) are also shown.

B. Risk index in patients before disease onset (BDO) and after the onset of disease (AC, SCC). Average risk scores and *p*-value (one-tailed paired *t*-test) are also shown.

C. Risk index in patients with breast cancer (BC) or benign breast nodules (NOD). Average risk scores and *p*-value (Welch's *t*-test) are also shown.

We therefore compared the performance of the predictor in the 'normal' sera of the testing set and in the sera of patients with the LD-CT-detected benign nodules. There were no significant differences in the average risk of the normal and nodule categories in spite of the fact that the 34-miRNA model and the risk algorithm were derived by training on a dataset (the training set) that did not include nodules (average risk score: normal $= -4.2$, nodules, -2.3, $p = 0.36$; Fig 3A). Indeed, the specificity of the predictor in scoring patients with benign nodules as 'normal' was 79% (26 out of 33 samples), whereas in normal high-risk volunteers it was 90% (27 out of 30 samples). In the COSMOS trial, the percentage of screened individuals who displayed non-calcified nodules was 53% (Veronesi et al, 2008). Thus, we predict that the false positive rate of our test applied to a population of high-risk individuals (which comprises both normal individuals and those with benign nodules) would be ~16%.

Next, we analyzed a group of sera collected before the onset of NSCLC (*i.e.* from patients who were negative at the screening round but who developed lung cancer >1 year after). For 13 of such cases, we had both the sera harvested before disease onset (BDO) and the tumour sera that were already included in the training or testing sets. When the risk predictor algorithm was applied, it indicated a significantly increased average risk index for sera collected after the onset of the disease (average risk BDO, -7.1; tumour, 10.4; $p < 0.001$, paired *t*-test; Fig 3B). Thus, at least in the cases analyzed, the 34-miRNA model was capable of detecting the conversion from a normal to a malignant state.

Finally, we tackled the question of the specificity of the 34-miRNA predictor for NSCLC detection, as opposed to other types of cancer, by screening sera from a cohort of 18 patients with invasive ductal breast carcinoma and 10 with breast benign nodules. In unsupervised hierarchical clustering, using all 147 miRNAs, the expression profile of these samples did not show major differences with respect to all other sera used in this study (Fig S2 of Supporting Information). When the 34-miRNA risk predictor algorithm was applied, it could not discriminate between breast tumours and benign breast nodules ($p = 0.65$; Fig 3C).

DISCUSSION

We have developed a serum test that can identify patients with early stage NSCLC in asymptomatic high-risk individuals. The test displays several characteristics that are desirable in a clinical setting: (i) it can be performed on modest amounts of serum (0.5–1 ml) without any need for a pre-amplification step, (ii) it is likely going to be considerably cheaper, easier and more immediately implementable (particularly from the point of view of patient accrual and compliance) than current screening procedures, (iii) it assigns disease probability accurately in high-risk asymptomatic individuals, (iv) it can also distinguish malignant lesions from benign nodules that are frequently found by LD-CT in high risk population, (v) it captures the onset of the malignant disease in asymptomatic individuals. When applied to a high-risk population screened by LD-CT, the most powerful

tool for early diagnosis available, our test showed an accuracy of 80% and was remarkably stable between training and testing sets.

A number of signatures have been reported, which were developed for the diagnosis of NSCLCs (Chen et al, 2008; Foss et al, 2011; Heegaard et al, 2011; Hu et al, 2010; Shen et al, 2011). The majority of these signatures were obtained (and validated to various degrees) on symptomatic lung cancers, and it is not presently known whether they can be useful in the diagnosis of asymptomatic patients, for the purpose of diagnostic anticipation. A study by Boeri et al (2011), however, was published while we were completing the present manuscript, portraying a study design similar to ours. Among various signatures, these authors identify a diagnostic signature, which is conceptually comparable to ours in that it allows diagnosis in asymptomatic subjects. Their diagnostic signature is composed of 13 miRNAs, five of which overlap with our signature, with concordant direction of variation (upregulated or downregulated). Although there is a partial overlap between the two signatures, which may suggest that the two studies capture the core of circulating miRNA differences between normal and cancer individuals, the majority of the miRNAs are different. The reason for this difference is presently unclear. As observed for cancer messenger RNA (mRNA) signatures derived from similar studies, differences could be due to intrinsic genetic heterogeneity between groups of patients. It is also to be noted that Boeri et al performed their analyses in plasma, while we analyzed sera, and that it is known that differences in the analyte can have an impact on the results of studies of this nature (Heegaard et al, 2011; Kroh et al, 2010; McDonald et al, 2011). Finally, the signatures could be different as the result of their intrinsically different properties. Our signature was trained on a high number of controls, and upon validation showed a remarkable specificity both on normal subjects and benign lung nodules. It will be interesting to determine whether the signature of Boeri et al possesses similar features when tested on a high number of screening relevant controls, such as benign lung nodules, which are detected at high frequency in high-risk populations by LD-CT scan (Veronesi et al, 2008). Specificity is a relevant issue when developing a signature for diagnostic anticipation in high-risk individuals, and it becomes even more relevant in the case of benign nodules. Our signature seems also specific for lung cancer when compared to another type of cancer (*i.e.* breast cancer).

An important question relates to the tissue origin of the serum miRNA of our signature. In principle these miRNAs can originate from the tumour itself or from host responses to the tumour. In the former case, an additional question is whether the miRNAs of the signature play any role in the process of lung tumourigenesis. Our data do not allow us to distinguishing between these possibilities. However, it is interesting to note that several members of our predictor are directly involved in lung cancer. Members of the Let-7 family (let-7 a/b/e are present in the predictor and are downregulated in our tested tumour sera) are often downregulated in lung cancer with diagnostic and prognostic value (Yanaihara et al, 2006). These miRNAs regulate lung tumour growth in NSCLC cell lines and mouse models (Trang et al, 2010), by targeting several oncogenes

including rat sarcoma viral oncogene homolog (RAS) (Johnson et al, 2005). Members of the 17–92 cluster, the first oncomiR identified (He et al, 2005), are also present in our predictor (miR-17 and miR-92a). Interestingly, the 17–92 cluster, which is under the control of the myelocytomatosis viral oncogene homolog (MYC) oncogene (He et al, 2005; O'Donnell et al, 2005), has a role in lung development (Ventura et al, 2008) and interacts with multiple cancer-relevant pathways (reviewed in Mendell, 2008). Finally, miR-126 and miR-486-5p have both been found to be downregulated in primary cancer and in the sera of NSCLC patients in several independent studies (Boeri et al, 2011; Hu et al, 2010; Shen et al, 2011; Volinia et al, 2006; Yanaihara et al, 2006). In addition, miR-126 inhibits cancer cell growth and metastasis *in vitro* and *in vivo* (Crawford et al, 2008; Liu et al, 2009; Tavazoie et al, 2008). In the case of miR-486-5p, while this miRNA has not been directly mechanistically implicated in lung cancer, it is of note that it is expressed in stem-like precursors bronchoalveolar stem cells (BASCs) from mouse lung (Qian et al, 2008). Thus, the potential role of our signature miRNAs in the pathogenesis of NSCLC deserves further experimental attention.

While a larger study that systematically and prospectively compares the results of LD-CT and our serum test will be needed to optimize our blood test, we anticipate that the composition of the 34-miRNA model will not change substantially, given its remarkable stability. However, large-scale validation might improve the predictive power of the test by allowing fine-tuning of the predictive algorithm.

We envision that our serum test for NSCLC will find its main application in the clinic as a 'first line screening test' for high-risk individuals, to identify those who should undergo further testing, including by LD-CT. Such a test might prove very useful for high-risk population screening, as its implementation in the clinic is cheap and minimally invasive. Furthermore, the simplicity of the procedure avoids 'medicalization' of asymptomatic individuals, which could encourage population compliance to large-scale screening programmes.

MATERIALS AND METHODS

Patient selection criteria

Patients with NSCLC and healthy individuals were selected from a consecutive series of 5203 patients enrolled in the COSMOS (Veronesi et al, 2008). All were smokers or former smokers with a smoking exposure of more than 20 pack-years, aged over 50 (see Table 1). Tumour stage at the time of diagnosis was determined according to guidelines of the American Joint Committee on Cancer (http://www.cancerstaging.org/). Informed consent was obtained from all patients. Other cohorts of patients employed in the study are described in the main text. A summary of the clinical and pathological information for all patients is in Table SI of Supporting Information.

miRNA profiling and data normalization

Serum preparation and RNA extraction are described in Fig S1 of Supporting Information. RNAs were isolated from 1 ml of serum (0.5 ml for sera of the symptomatic set). MiRNA levels were analyzed with the TaqMan® Low Density Array microRNA Signature Panel (v1.0)

The paper explained

PROBLEM:

Non-small cell lung carcinoma is the leading cause of cancer mortality worldwide, largely due to a lack of effective tools for early diagnosis. Patients diagnosed with early stage disease (stage I) have a reasonably favourable prognosis (60–80% survival at 5 years). Unfortunately, only 20–25% of all NSCLCs are diagnosed early. Effective tools for the early detection of NSCLC, especially in high-risk individuals (*i.e.* smokers), are critically needed to reduce diagnoses of advanced stage disease (stages III–IV) and lung cancer deaths. Several studies have reported the usefulness of LD-CT, which is effective for the detection of nodules (even when these are small) and which can be used to diagnose asymptomatic lung cancers, most of which are at stage I. There is, however, concern regarding the feasibility of large-scale population screening by LD-CT: the technique is cumbersome, it requires specialized health centres, and the recruitment of at-risk individuals with no signs of disease is difficult. A blood test, on the other hand, could be administered easily, would be fast and cheap, would not require specialized health centres and would encourage the recruitment of high-risk individuals for the diagnostic anticipation of lung cancer.

RESULTS:

The authors developed a blood test for lung cancer diagnosis in asymptomatic high-risk individuals (heavy smokers, aged over 50) based on the detection of miRNAs from serum. Sera were collected from high-risk subjects enrolled in a large prospective early detection trial (the COSMOS study) for lung cancer by annual LD-CT. A diagnostic signature of 34 serum miRNAs was identified. The signature can identify patients with early stage NSCLC with 80% overall accuracy. In addition, the signature can distinguish between benign lung nodules (which are a frequent occurrence in at-risk individuals) and frank NSCLCs. Finally, the signature can capture the time of disease onset in individual patients over time.

IMPACT:

The authors report a blood test for the diagnosis of NSCLC of potential impact in the design of screening programmes for early detection in at-risk individuals, with perspective improvement in the prognosis of the disease.

(Applied Biosystems), as described in Fig S1. Data were analyzed with SDS Relative Quantification Software version 2.2.2 (Applied Biosystems). Ct values were exported into Excel software (Microsoft) for data analysis.

According to the analyses described in Fig S1, only 147 assays (listed in Table SII of Supporting Information) were suitable for the purpose of our study, thus only these assays were considered for data normalization and further analyses. In the training set, we selected six miRNAs behaving as 'housekeeping (HK) serum-miRNAs' (miR-197, miR-19b, miR-24, miR-146, miR-15b, miR-19a, Table SIII of Supporting Information) with the following criteria: (i) they passed all the quality tests described in Fig S1, in particular, they were optimal quantitative variables ($R^2 = 0.99$), (ii) they were expressed at high levels and in all samples (median Ct < 30; 100% of detection); (iii) they were not statistically different among the analyzed classes (Welch's *t*-test, $p > 0.1$); iv) they were the least variable miRNAs among all samples (SD < 0.9 Ct). Raw data were therefore normalized by the geometric mean of the 'housekeeping (HK) serum-miRNAs'. Only values below a minimal threshold (Ct < 36) were normalized in order to avoid artefactual regulation due to sample normalization. Values for the testing and symptomatic sets were normalized using the same HK serum-miRNAs. Expression values for all serum samples used in the study, before and after normalization, are shown in Fig S2 of Supporting Information. In addition, to exclude the possibility that our results were influenced by the normalization method, we repeated all the analyses with a different method, *i.e.* the median normalization method in which each sample was centred based on the median value of all the 147 expressed miRNAs. The two different normalization methods yielded almost identical results ($R^2 = 0.8$, *p*-value <0.0001; Fig S4 of Supporting Information).

Clustering and class prediction analyses

Hierarchical clustering analysis was performed using Cluster 3.0 for Mac OSX (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). Expression data were clustered using uncentred correlation and average linkage. Tree pictures were generated using Java TreeView software (http://jtreeview.sourceforge.net).

For class prediction, $\log_2$ median centred expression data were analyzed using BRB-ArrayTools Version: 3.8.0-Beta_1 Release (April 2009) (http://linus.nci.nih.govBRB-ArrayTools.html). MiRNAs significantly different between case and control classes in the training set, at 0.05 significance level (parametric *t*-test, random variance model), were used for class prediction. The misclassification rate of the classifier was computed by diagonal linear discriminant analysis (DLDA) and *K*-fold ($K = 5$) cross-validation method repeated 100 times. Statistical significance of the DLDA classifier was assessed by 1000 random permutations of the class labels. The classification of patients in the testing or symptomatic sets was performed blinded, using the following prediction rule from the Diagonal linear Discriminant predictor: a sample is classified 'high risk' if the inner sum of the weights (wi) (see Table SI) and expression (xi) of the 34 miRNAs is greater than the threshold (determined in the training set); that is, Σ wi xi > 3.235.

To ensure that the 34 miRNA composition of our model was optimal we repeated the entire analysis using four additional classifiers built by using 5, 10, 15, 30 miRNAs of the initial list of 34 miRNAs (Fig S5). Indeed, the best performance in terms of AUC was obtained by using the 34-miRNA classifier (Fig S5).

Statistical significance of the differences of the average risk index in the various sets of patients was calculated using ANOVA (in the case of more than two groups) or Welch's *t*-test using Prism (GraphPad Software, Inc.). Statistical significance of the differences of the average

risk index between BDO and relative matched tumour sera was calculated using the one-tailed paired *t*-test (GraphPad Software, Inc.). Sensitivity analyses and Forest Plots were prepared using the statistical software JMP IN (SAS), and *p* values calculated with the Fisher's exact test.

Author contributions

Acknowledgements

The authors declare that they have no conflict of interest.

References

Aberle DR, Berg CD, Black WC, Church TR, Fagerstrom RM, Galen B, Gareen IF, Gatsonis C, Goldin J, Gohagan JK, et al (2011) The National Lung Screening Trial: overview and study design. Radiology 258: 243-253

Bach PB, Jett JR, Pastorino U, Tockman MS, Swensen SJ, Begg CB (2007) Computed tomography screening and lung cancer outcomes. JAMA 297: 953-961

Boeri M, Verri C, Conte D, Roz L, Modena P, Facchinetti F, Calabro E, Croce CM, Pastorino U, Sozzi G (2011) MicroRNA signatures in tissues and plasma predict development and prognosis of computed tomography detected lung cancer. Proc Natl Acad Sci USA 108: 3713-3718

Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, et al (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18: 997-1006

Chien CR, Chen TH (2008) Mean sojourn time and effectiveness of mortality reduction for lung cancer screening with computed tomography. Int J Cancer 122: 2594-2599

Crawford M, Brawner E, Batte K, Yu L, Hunter MG, Otterson GA, Nuovo G, Marsh CB, Nana-Sinkam SP (2008) MicroRNA-126 inhibits invasion in non-small cell lung carcinoma cell lines. Biochem Biophys Res Commun 373: 607-612

Foss KM, Sima C, Ugolini D, Neri M, Allen KE, Weiss GJ (2011) miR-1254 and miR-574-5p: serum-based microRNA biomarkers for early-stage non-small cell lung cancer. J Thorac Oncol 6: 482-488

Greenlee RT, Murray T, Bolden S, Wingo PA (2000) Cancer statistics, 2000. CA Cancer J Clin 50: 7-33

He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, et al (2005) A microRNA polycistron as a potential human oncogene. Nature 435: 828-833

Heegaard NH, Schetter AJ, Welsh JA, Yoneda M, Bowman ED, Harris CC (2011) Circulating microRNA expression profiles in early stage non-small cell lung cancer. Int J Cancer advance online publication 4 May 2011. DOI: 10.1002/ijc.26153

Henschke CI, Yankelevitz DF, Libby DM, Pasmantier MW, Smith JP, Miettinen OS (2006) Survival of patients with stage I lung cancer detected on CT screening. N Engl J Med 355: 1763-1771

Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, Chen Y, Xu L, Zen K, Zhang C, et al (2010) Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J Clin Oncol 28: 1721-1726

Jemal A, Thun MJ, Ries LA, Howe HL, Weir HK, Center MM, Ward E, Wu XC, Eheman C, Anderson R, et al (2008) Annual report to the nation on the status of cancer, 1975-2005, featuring trends in lung cancer, tobacco use, and tobacco control. J Natl Cancer Inst 100: 1672-1694

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ (2009) Cancer statistics, 2009. CA Cancer J Clin 59: 225-249

Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ (2005) RAS is regulated by the let-7 microRNA family. Cell 120: 635-647

Kroh EM, Parkin RK, Mitchell PS, Tewari M (2010) Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods 50: 298-301

Laterza OF, Lim L, Garrett-Engele PW, Vlasakova K, Muniappa N, Tanaka WK, Johnson JM, Sina JF, Fare TL, Sistare FD, et al (2009) Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. Clin Chem 55: 1977-1983

Lawrie CH (2010) microRNA expression in erythropoiesis and erythroid disorders. Br J Haematol 150: 144-151

Liu B, Peng XC, Zheng XL, Wang J, Qin YW (2009) MiR-126 restoration down-regulate VEGF and inhibit the growth of lung cancer cell lines in vitro and in vivo. Lung Cancer 66: 169-175

McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimnich A (2011) Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem advance online publication 12 April 2011. DOI: 10.1373/clinchem.2010.157198

McMahon PM, Kong CY, Johnson BE, Weinstein MC, Weeks JC, Kuntz KM, Shepard JA, Swensen SJ, Gazelle GS (2008) Estimating long-term effectiveness of lung cancer screening in the Mayo CT screening study. Radiology 248: 278-287

Mendell JT (2008) miRiad roles for the miR-17-92 cluster in development and disease. Cell 133: 217-222

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT (2005) c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435: 839-843

Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. CA Cancer J Clin 55: 74-108

Pastorino U, Bellomi M, Landoni C, De Fiori E, Arnaldi P, Picchio M, Pelosi G, Boyle P, Fazio F (2003) Early lung-cancer detection with spiral CT and positron emission tomography in heavy smokers: 2-year results. Lancet 362: 593-597

Peto R, Lopez AD, Boreham J, Thun M, Heath C, Jr, Doll R (1996) Mortality from smoking worldwide. Br Med Bull 52: 12-21

Porter JC, Spiro SG (2000) Detection of early lung cancer. Thorax 55: S56-S62

Qian S, Ding JY, Xie R, An JH, Ao XJ, Zhao ZG, Sun JG, Duan YZ, Chen ZT, Zhu B (2008) MicroRNA expression profile of bronchioalveolar stem cells from mouse lung. Biochem Biophys Res Commun 377: 668-673

Redell JB, Moore AN, Ward NH, III, Hergenroeder GW, Dash PK (2010) Human traumatic brain injury alters plasma microRNA levels. J Neurotrauma 27: 2147-2156

Reich JM (2008) A critical appraisal of overdiagnosis: estimates of its magnitude and implications for lung cancer screening. Thorax 63: 377-383

Rong H, Liu TB, Yang KJ, Yang HC, Wu DH, Liao CP, Hong F, Yang HZ, Wan F, Ye XY, et al (2011) MicroRNA-134 plasma levels before and after treatment for bipolar mania. J Psychiatr Res 45: 92-95

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

Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT, Jr, Carter BS, Krichevsky AM, Breakefield XO (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 10: 1470-1476

Swensen SJ, Jett JR, Hartman TE, Midthun DE, Sloan JA, Sykes AM, Aughenbaugh GL, Clemens MA (2003) Lung cancer screening with CT: Mayo Clinic experience. Radiology 226: 756-761

Tanaka M, Oikawa K, Takanashi M, Kudo M, Ohyashiki J, Ohyashiki K, Kuroda M (2009) Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. PLoS One 4: e5532

Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massague J (2008) Endogenous human microRNAs that suppress breast cancer metastasis. Nature 451: 147-152

Trang P, Medina PP, Wiggins JF, Ruffino L, Kelnar K, Omotola M, Homer R, Brown D, Bader AG, Weidhaas JB, et al (2010) Regression of murine lung tumors by the let-7 microRNA. Oncogene 29: 1580-1587

Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR, et al (2008) Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell 132: 875-886

Veronesi G, Bellomi M, Mulshine JL, Pelosi G, Scanagatta P, Paganelli G, Maisonneuve P, Preda L, Leo F, Bertolotti R, et al (2008) Lung cancer screening with low-dose computed tomography: a non-invasive diagnostic protocol for baseline lung nodules. Lung Cancer 61: 340-349

Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, et al (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 103: 2257-2261

Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, Hood LE, Galas DJ (2009) Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci USA 106: 4402-4407

Welch HG, Woloshin S, Schwartz LM, Gordis L, Gotzsche PC, Harris R, Kramer BS, Ransohoff DF (2007) Overstating the evidence for lung cancer screening: The International Early Lung Cancer Action Program (I-ELCAP) Study. Arch Intern Med 167: 2289-2295

Wittmann J, Jack HM (2010) Serum microRNAs as powerful cancer biomarkers. Biochim Biophys Acta 1806: 200-207

Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, et al (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9: 189-198

Zhang Y, Jia Y, Zheng R, Guo Y, Wang Y, Guo H, Fei M, Sun S (2010) Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. Clin Chem 56: 1830-1838