Plasma DNA Quantification in Lung Cancer Computed Tomography Screening

Five-Year Results of a Prospective Study

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***Rationale*: Free circulating plasma DNA has emerged as a potential biomarker for early lung cancer detection. In a previous case–control study we have shown that high levels of plasma DNA are a strong risk factor for lung cancer.**

***Objectives*: To assess the diagnostic performance and prognostic value of plasma DNA levels in a cohort of 1,035 heavy smokers monitored by annual spiral computed tomography (CT) for 5 years.**

***Methods*: Plasma DNA levels were determined through real-time quantitative PCR at baseline and at time of lung cancer diagnosis. Screening performance of the assay was calculated through the area under the receiver-operating characteristic curve (AUC-ROC). Kaplan-Meier analyses were computed for association with prognosis.**

***Measurements and Main Results*: Median baseline concentration of plasma DNA was not different in individuals who developed CT-detected lung cancers in the 5-year period (n = 38) versus cancer-free control subjects (AUC-ROC, 0.496; $P = 0.9330$), and only slightly higher at the time of cancer diagnosis (AUC-ROC, 0.607; $P = 0.0369$). At surgery, plasma DNA was higher in tumors detected at baseline (AUC-ROC, 0.80; $P < 0.0001$) and in Stage II to IV tumors detected during the first 2 years of screening (AUC-ROC, 0.87; $P < 0.0001$). A longitudinal study of plasma DNA levels showed increased values approaching to lung cancer diagnosis ($P = 0.0010$). Higher plasma DNA was significantly associated with poorer 5-year survival ($P = 0.0066$).**

***Conclusions*: Baseline assessment of plasma DNA level does not improve the accuracy of lung cancer screening by spiral CT in heavy smokers. Higher levels of plasma DNA at surgery might represent a risk factor for aggressive disease.**

Keywords: lung cancer; circulating DNA; spiral computed tomography

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The development of spiral computed tomography (CT) has opened new perspectives for identification of small tumors. Investigation of biomarkers associated with these lesions might have importance for future clinical management.

What This Study Adds to the Field

Plasma DNA assay can identify tumors with a higher growth rate, while it is not sensitive for slowly growing lesions. Five-year survival analysis demonstrates that a higher amount of plasma DNA at surgery is indicative of a poorer prognosis.

Lung cancer is the leading cause of cancer deaths in the world due to its high incidence and mortality, with 5-year survival estimates around 10% for non–small cell lung cancer (NSCLC). Lung cancer etiology is primarily linked to genetic and epigenetic damage caused by tobacco smoke, and mortality from this disease can be reduced by primary prevention through smoking cessation. Currently, at the time of diagnosis only one-third of patients with NSCLC have a disease amenable to curative surgery, still the most effective treatment for NSCLC, and early-stage detection has the theoretical potential to improve resectability and reduce mortality.

Past experience showed that screening of heavy smokers by chest X-ray and sputum cytology did not improve disease-specific survival, but recent development of spiral computed tomography (CT) has opened new perspectives. Low-dose CT scan of the chest has been very effective in detecting small tumors, with a high proportion of resectable and Stage I disease (1, 2). The benefits of lung cancer screening are still controversial (3), and ongoing randomized trials will demonstrate the real impact of early detection on mortality of high-risk individuals.

In parallel, there is growing interest in noninvasive screening based on the identification of biomarkers in biological fluids before the onset of clinically detectable cancer. Analysis of biomarkers in the context of screening trials is especially relevant for the potential in contributing to the diagnostic algorithm or to patients' stratification for inclusion in the screening programs. Furthermore, since the nature and life-threatening potential of CT-detected nodules is currently debated due to the potential issue of overdiagnosis (4, 5), investigation of biomarkers associated with these lesions might have importance for future clinical management.

A prospective pilot trial of early lung cancer detection that was launched in Milan in 2000, applying yearly low-dose spiral CT and selective use of positron emission tomography (PET) to a cohort of 1,035 high-risk heavy smoker volunteers aged 50 years or older, also included systematic plasma sampling for free circulating DNA testing.

The methodology of plasma DNA quantification was tested in a prior case–control study that demonstrated higher levels of plasma DNA in 69% of 100 patients with lung cancer versus

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only 2% of 100 matched control subjects (6), with a strong diagnostic power and a receiver operating characteristic (ROC) curve of 0.94. Given the high sensitivity and specificity of the qPCR test, which proved to be independent from tumor size, we decided to test the value of plasma DNA quantification in combination with spiral CT for early lung cancer detection.

The present study reports the results of plasma DNA quantification by real-time quantitative PCR in the entire cohort of 1,035 volunteers enrolled in the early detection trial, based on a minimum follow-up of 5 years. Statistical analysis was performed to investigate the diagnostic performance and the prognostic value of plasma DNA levels in a lung cancer screening setting. Some of the results of these studies have been previously reported in the form of an abstract (7).

METHODS

Cohort Description

The INT/IEO prospective study accrued 1,035 volunteers aged 50 years or older, current or former smokers with a minimum pack-year index of 20, to be investigated with low-dose spiral CT every year for 5 years. Informed consent was obtained from all participants. The study was approved by a local institutional review board.

Of the 1,035 subjects, 956 remained cancer free over the 5 years of the study, 38 developed lung cancer, and 41 developed other tumors (3, 8). Two subjects who developed both lung cancer and an additional tumor in another site in the course of the study are included in the group of lung cancer. Clinical outcome of lung cancer patients was evaluated for a median follow-up period of 62 months. Series characteristics for the three groups of subjects are shown in Table 1. The prevalence of very heavy smokers ($>$ 40 packs/yr) was significantly higher ($P = 0.0037$) in patients with lung cancer.

Samples Collection and DNA Isolation

Plasma samples (separated from 7.5 ml of peripheral blood in EDTA) were collected at first year of screening (baseline) and at the second year of annual screening (first annual spiral CT repeat) for all the 1,035 individuals. Additional blood samples were collected from the screened individuals when CT was indicative of lung cancer, as well as at the time of lung cancer resection throughout the study.

Plasma separation and DNA extraction were performed as previously reported (9). The DNA purified from 1 ml of plasma was eluted in a final volume of 50 μl of water. Testing of plasma DNA was performed without knowledge of the case/control status.

Time elapsed from plasma collection to DNA extraction/quantification was narrowed down to a median time of 14 months for both the baseline plasma DNA quantification (that was analyzed in a prospective fashion, close to the time of accrual) and for the quantification of plasma samples in patients with lung cancer at the time of surgery or of a indicative CT result, thus minimizing the issue of storage-related DNA degradation that we identified during the course of the study (10). Analyses were therefore performed only on samples with comparable storage. Systematic testing of all plasma samples obtained at the first annual repeat was not performed at the time of collection, and samples were stored for later examination. Unfortunately the possibility to retrospectively analyze the samples was hampered by the above-mentioned degradation issue.

DNA Quantification in Plasma

To quantify free circulating DNA in plasma we used a real-time quantitative PCR approach based on the amplification of the human telomerase reverse transcriptase locus (hTERT) as previously described (6). Plasma DNA levels were assessed for all baseline samples and at the time of cancer diagnosis and resection.

All the data were analyzed using the Sequence Detection System software (Applied Biosystems, Milan, Italy) to interpolate the standard amplification curve of DNA at known quantity with amplification curve of the unknown target sample in triplicates, thus obtaining the amount of DNA in the experimental sample.

TABLE 1. SERIES CHARACTERISTICS ACCORDING TO DISEASE STATUS

	Disease					
	Lung Cancer		Other Tumors		Control Subjects	
	No.	%	No.	%	No.	%
Overall	38	100	41	100	956	100
Sex						
Female	9	23.7	13	31.7	274	28.7
Male	29	76.3	28	68.3	682	71.3
Age, yr						
Median (min–max)	58 (50–73)		62 (50–76)		58 (50–84)	
Smoking habit						
Former smoker	3	7.9	8	19.5	152	15.9
$\leq$ 40 packs/yr	9	23.7	16	39.0	450	47.1
$>$ 40 packs/yr	26	68.4	17	41.5	354	37.0
Presence of concomitant disease						
No	31	81.6	32	78.0	753	78.8
Yes	7	18.4	9	22.0	203	21.2

For the follow-up study, all the consecutive plasma samples for each patient were simultaneously analyzed in the same real-time PCR experiment to allow comparative quantification of samples along the observation time.

Statistical Methods

Standard descriptive statistics were calculated for categorical data (relative frequency, percentage) and continuous data (median, minimum-maximum, or interquartile range), as shown in the tables. Median DNA levels were compared nonparametrically among distinct groups of subjects by means of Wilcoxon Two-sample test or Kruskal-Wallis test, as appropriate. To evaluate the diagnostic performance of plasma DNA concentrations, the area under receiver-operating characteristic curve (AUC-ROC) was calculated nonparametrically as proposed by Hanley and McNeil (11). An AUC-ROC equal to 1 denotes perfect discrimination between patients with cancer and patients without cancer, a value equal to 0.5 denotes the lack of discrimination, and values in between indicate a degree of discrimination between strong and poor. Finally, longitudinal DNA measurements along follow-up appointments were analyzed by means of linear mixed models, so as to account for the correlation between distinct measurements within the same subject. Overall survival curves in patients with lung cancer diagnosis, according to the tertiles of the DNA distribution, were computed with the Kaplan-Meier method and compared with the logrank test.

Statistical analyses were performed using SAS. Two-sided P values below 0.05 were considered statistically significant.

RESULTS

Plasma DNA Levels in Patients with Lung Cancer and in Control Subjects

DNA levels according to cancer status are shown in Table 2. Baseline DNA levels of individuals that developed lung cancer throughout the study were not different from those of cancer-free control subjects, nor from the levels detected in patients with other tumors (whose individual characteristics are described in Table E1 in the online supplement). Accordingly, estimates of the AUC-ROC failed to reach statistical significance, being equal to 0.496 ($P = 0.93$) and 0.492 ($P = 0.87$), respectively, and indicating lack of predictive value of plasma DNA quantification in this setting. The median baseline value (3.9 ng/ml) was consistent with that previously reported for control subjects in the case–control study (3.1 ng/ml) (6), indicating reproducibility in the measurement of plasma DNA levels in different cohorts of healthy subjects. Throughout the study, care was taken to ensure that analyses were performed

TABLE 2. DNA LEVELS ACCORDING TO DISEASE STATUS AND AREAS UNDER THE RECEIVER OPERATING CHARACTERISTIC CURVES FOR ASSESSING THE DISCRIMINATION BETWEEN PATIENTS WITH TUMORS AND CONTROL SUBJECTS

	No. Measures	Median DNA Level (*ng/ml*)	Interquartile Range	AUC-ROC (*P* Value)
Control subjects, overall*	947	3.9	2.1–6.1	—
Other tumors	41	3.9	1.7–6.2	0.492 (0.8746)
Lung cancer				
Baseline	38	3.6	2.5–5.4	0.496 (0.9330)
At surgery	34	4.8	3.4–8.0	0.607 (0.0369)

Definition of abbreviation: AUC-ROC = areas under the receiver operating characteristic curves.

* Data were not available in nine control subjects.

only on samples with comparable storage to avoid issues related to time-dependent degradation (10).

Since in our previous study we had identified higher DNA levels in patients with lung cancer compared with healthy control subjects, we also analyzed DNA levels at the time of resection of CT-detected tumors. Although a significant result was still achieved by considering DNA levels at surgery, in which the AUC-ROC for the discrimination versus control subjects was equal to 0.607 ($P = 0.0369$), the median value observed (4.8 ng/ml) was considerably lower compared with that reported with the same technique in 100 consecutive patients with clinically detected NSCLC (24.3 ng/ml). ROC curves for patients with lung cancer are plotted in Figure 1A.

Additional analyses were performed to investigate the factors affecting DNA levels and possibly the diagnostic performance.

DNA levels in control subjects were evaluated according to age, sex, smoking habit, and the presence of concomitant diseases (Tables E2 and E3). DNA levels were fairly homogeneous in all the investigated subgroups, and the only significant result obtained would suggest higher levels in males compared with females. Prompted by such a finding, the diagnostic performance of plasma DNA at surgery in patients with lung cancer was assessed according to sex. AUC-ROC estimates were 0.565 in males and 0.724 in females, but the difference between the two estimates was not statistically significant ($P = 0.20$). The possible influence of concomitant diseases was also investigated more in detail, by comparing DNA levels in distinct types of disease (systems or organs involved), with particular interest toward inflammatory diseases of the lung and benign tumors. The results obtained (Table E3) did not suggest that concomitant diseases might influence plasma DNA or its diagnostic performance.

DNA Levels in Patients with Lung Cancer According to Clinical Characteristics

Plasma DNA levels in patients with lung cancer were also evaluated according to the year of diagnosis, as well as histologic and stage disease characteristics (Table 3). Although a limited number of observations are available, thus making comparisons less reliable, the only clear-cut finding was that DNA levels at surgery were much higher for tumors detected in the first year of screening (median 10.1 ng/ml), with a trend toward a reduction in Year 2 (4.9 ng/ml) and Years 3 to 5 (3.3 ng/ml, $P = 0.0141$ for the overall comparison). Though not supported by statistical significance, trends were evident also for histology and tumor stage, inasmuch DNA levels at baseline or at surgery consistently tended to be higher for tumors in Stage II to IV or other than adenocarcinoma. In terms of diagnostic performance, these patterns translated into relatively high AUC-ROC figures when considering plasma DNA at surgery in tumors detected in the first year of screening (AUC-ROC, 0.802; $P < 0.0001$), or Stage II to IV tumors detected during the first 2 years of screening (AUC-ROC, 0.866; $P < 0.0001$). Corresponding ROC curves are shown in Figure 1B.

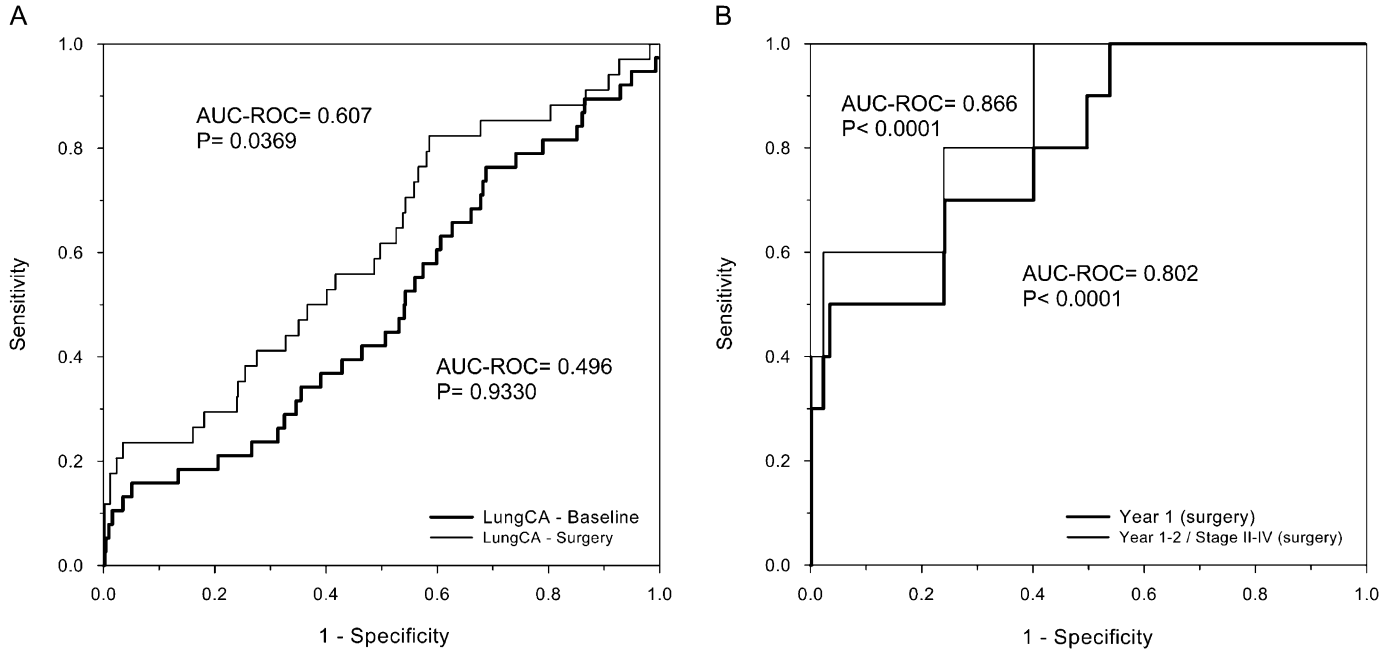

Figure 1. (*A*) Receiver-operating characteristics (ROC) curve analysis for plasma DNA concentration in patients with lung cancer versus control subjects, at baseline and at time of surgery, respectively; for each curve, area under the curve (AUC) estimate and *P* value for testing the significance of difference from 0.5 (lack of discrimination) are indicated. (*B*) ROC curve analysis for plasma DNA concentration (at time of surgery) in patients with lung cancer detected at Year 1 versus control subjects, and in patients with Stage II to IV lung cancer detected at Years 1 or 2 versus control subjects, respectively; for each curve, AUC estimate and *P* value for testing the significance of difference from 0.5 (lack of discrimination) are indicated.

TABLE 3. DNA LEVELS IN PATIENTS WITH LUNG CANCER ACCORDING TO INDIVIDUAL CHARACTERISTICS

	No. Measures	Median DNA Level (*ng/ml*)	Interquartile Range	*P* Value*
Baseline				
Diagnosis Year				0.1356
1	11	4.5	3.1–13.8	
2	11	2.7	1.1–3.7	
⩾ 3	16	4.0	2.6–6.2	
Histology				0.2517
Adenocarcinoma	26	3.2	2.2–5.4	
Other	12	4.6	3.1–11.6	
Stage				0.5153
I	24	3.5	2.0–5.4	
II – IV	14	4.4	2.6–5.9	
At surgery				
Diagnosis Year				0.0141
1	10	10.1	4.7–36.7	
2	11	4.9	3.5–6.0	
⩾ 3	13	3.3	1.6–5.0	
Histology				0.1844
Adenocarcinoma	25	4.1	3.4–6.3	
Other	9	6.3	5.3–19.9	
Stage				0.2008
I	22	4.3	3.4–6.0	
II–IV	12	7.0	3.7–18.0	

* *P* value for testing homogeneity in DNA levels across distinct patient subgroups.

Plasma DNA Concentrations According to Time Before Surgery

Thirty-three patients had two to four plasma samples collected at different time points during the 5 years of the screening up to the time of surgery, for a total of 93 measurements. A significant difference ($P = 0.0010$) was observed in the amount of circulating DNA in plasma samples taken at all time points before surgery (n = 60; median 2.9 ng/ml) and those at the time surgery (n = 33; median 4.7 ng/ml). The difference was still significant ($P = 0.0071$) when comparing DNA values in plasma samples collected within 12 months from surgery (n = 57; median 4.6 ng/ml) versus more than 12 months preceding lung cancer diagnosis (n = 36; median 2.4 ng/ml), thus showing that plasma DNA tends to increase as the time to lung cancer diagnosis decreases. A graphical representation of longitudinal plasma DNA measurements is supplied in Figure 2.

Association of Plasma Circulating DNA Levels with Survival

Five-year overall survival in the whole series of patients with lung cancer was 62% (95% confidence limits, 45–76%). Tertile stratification showed that long-term survival (Figure 3) was significantly worse (33% at 5 yr) in patients with DNA concentration in the upper tertile (⩾ 6.3 ng/ml, $P = 0.0066$).

Because of the small overall number of deaths (n = 11), it was not possible to estimate the adjusted prognostic effect of plasma DNA with multivariable analysis. However, patterns similar to that described above were observed in the subgroups of patients with tumors detected in Years 1 to 2, Years 3 to 5, or Stage IB to IV. Little information was available for Stage IA patients (just one death in a subject with plasma DNA ⩾ 6.3 ng/ml), for whom 5-year survival was 91%.

DISCUSSION

In the last decade numerous studies have investigated the molecular characteristics of free circulating DNA in plasma and serum of patients with cancer with the intent of explaining its origin and mechanism of release, as well as to establish the clinical relevance of free circulating DNA for cancer diagnosis (12, 13). A possible role for noninvasive diagnosis of lung cancer has been suggested for several biomarkers and for the quantification of the total amount of circulating DNA in plasma (6, 9, 14–21). However, a prospective investigation of the relationship between amount of DNA in plasma, KRAS2 and TP53 mutations, and cancer risk in a large unselected European population (European Prospective Investigation into Cancer and nutrition, EPIC) failed to show any predictive value of these markers on lung cancer development (22, 23).

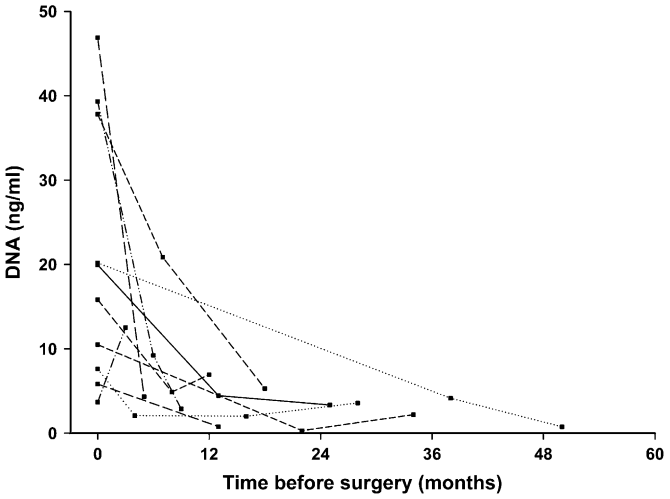

Figure 2. Plasma DNA concentrations according to time before surgery. The *lines* represent the patterns observed in nine patients with lung cancer with peak plasma DNA greater than or equal to 5 ng/ml, which varied considerably (200% at least) before surgery.

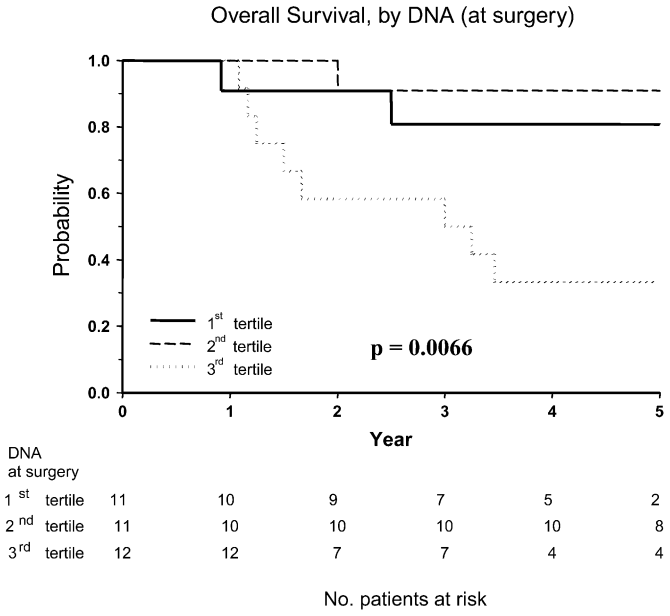

Figure 3. Kaplan-Meier plots of overall survival according to plasma DNA at surgery. Plasma DNA values were classified on the basis of the tertiles of their distribution, with cutoffs at 3.59 ng/ml and 6.3 ng/ml (1st tertile: 0–3.59 ng/ml; 2nd: > 3.59–6.3 ng/ml; 3rd: > 6.3 ng/ml).

In the present study we evaluated the diagnostic performance and the prognostic value of the amount of plasma circulating DNA, previously validated in a case–control series of clinically detected lung cancers (6), in heavy-smoking volunteers enrolled in a CT screening program conducted in Milan from the years 2000 to 2006. To our knowledge, this study is the first attempt to investigate circulating DNA in a large cohort of volunteers monitored by annual chest CT for 5 years.

No predictive value of baseline plasma DNA levels on tumor onset was observed either in patients diagnosed in the first and second year of the screening separately or throughout the entire screening program. In particular the assay had very poor performance in detecting the small adenocarcinomas that represent a typical finding of CT-screening protocols in contrast to unscreened tumors. This lack of difference could be explained by the long interval between sampling and cancer detection, with almost 40% of tumors presenting beyond 3 years from the initial plasma collection. In fact, baseline plasma DNA values were slightly higher, although not statistically significant, in cases diagnosed during the first year of screening. These findings are consistent with previously published studies, suggesting that DNA release in plasma could be a relatively late result of tumor–host interactions. Although CT-detected tumors have been shown to harbor many molecular features associated with clinically detected cancers (24, 25), some differences with unscreened cancers have also been reported, including expression of genes involved in tumor growth (24), central scar and invasion foci size of adenocarcinomas, and tumor diameter (26). All these factors might also contribute to the lack of predictive value of free circulating DNA in the context of CT-detected cancers. Furthermore, in our previous study we observed that plasma DNA was associated with age and microvessel density in younger patients (< 60 yr), suggesting that the assay might not perform well in patients detected at a younger age or in tumors with low angiogenetic potential.

Interestingly, a significant association was detected when analyses were limited to subjects who developed lung cancer within 12 months after blood drawing. In fact the quantification of multiple plasma samples collected during follow-up appointments showed that DNA levels significantly increased as the time to lung cancer diagnosis decreased. Such a time trend, while showing a limited role of plasma DNA levels in early lung cancer screening, suggests that changes in plasma DNA levels might be useful in the follow-up of resected cancers.

Significantly higher amounts of circulating plasma DNA were found in cancer patients at the time of CT detection than in cancer-free controls at baseline, but the overall discriminatory power of the plasma assay proved to be quite poor. Nonetheless, plasma DNA levels were considerably higher in patients detected at the first year of the screening compared with those identified during the following years of the trial. The higher DNA levels observed in Stage II to IV tumors during the first 2 years of screening (AUC-ROC value, 0.866) are in contrast to our previous reports on the lack of association of plasma DNA levels with tumor stage in symptomatic patients (6). These discrepancies could be related to specific biological features of screening detected tumors, and potential selection of slow-growing disease. As a matter of fact, even the magnitude of the difference observed in our previous case–control study (eightfold higher levels) is far greater than values reported here (6).

The natural history of subcentimetric CT-detected lung cancers is still largely unknown. In the Mayo Clinic study (27) the mean volumetric doubling time of screening-detected lung cancer was significantly longer than the one of lung tumors diagnosed before the advent of CT screening. Moreover, a recent meta-analysis by Bach and coworkers (3) of three concurrent screening trials, including the Mayo CT trial and the present cohort, showed a threefold increase in the number of detected lung cancers compared with the expected number of cases in the three cohorts (and 10-fold increase in surgical resections) supporting the concept of an excess of lung cancers detected by CT screening. It is possible to hypothesize that this plasma DNA assay can identify only tumors with a higher growth rate, while it is not sensitive enough for slowly growing lesions. The higher DNA levels observed among prevalent cases (first year of screening) or advanced disease (Stage II–IV), as well as the difference in long term survival, is consistent with such a hypothesis.

Moreover, 5-year survival analysis, based on extended follow-up, demonstrates that a higher amount of plasma DNA at surgery is indicative of a poorer prognosis, and could provide clinically relevant information.

Future studies using more accurate quantitative or qualitative assays will clarify if analysis of circulating DNA can provide a fingerprint of different metastatic behavior, and improve the clinical management of CT detected lung cancer.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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