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Advances in Brief

Analysis of Circulating Tumor DNA in Plasma at Diagnosis and during Follow-Up of Lung Cancer Patients[1]

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Abstract

We evaluated whether the amount of circulating DNA in plasma could discriminate between lung cancer patients and healthy individuals and whether it is related to disease progression, and we analyzed the kinetics of plasma DNA in disease-free, surgically resected patients. Plasma DNA quantification and analysis of microsatellite alterations were performed in a consecutive series of 84 patients with non-small cell lung cancer, who were studied during follow-up, and 43 healthy controls. In patients, the mean values of plasma DNA concentration were higher than in controls even considering stage Ia patients. Sensitivity and specificity estimates were calculated as the area under the receiver operating characteristic curve (AUC-ROC) curve and showed a value of 0.844. Variations in DNA level and in microsatellite changes correlated with the clinical status of 38 patients monitored during follow-up. The data suggest that quantification and molecular characterization of plasma DNA in lung cancer patients are valuable noninvasive diagnostic tools for discriminating patients from unaffected individuals and for detecting early recurrence during follow-up.

Introduction

Of the more than 150,000 new cases of lung cancer diagnosed in Europe every year, 35,000 of which are in Italy, no more than 10% can be cured and gain a long-term survival. For these reasons, there is a critical need for new research approaches aimed at improving lung cancer management (1). Even with smoking cessation, the risk of lung cancer remains elevated more than 15 years later, in specific high-risk groups, because smoking cessation cannot reverse the carcinogenic transformation attributable to the accumulation of genetic changes in the airways of former smokers. In fact, chromosomal and molecular alterations in apparently normal bronchial cells of patients, and in current or former smokers have been identified (2–4).

Identification and characterization of the genetic changes that drive lung cancer development and progression have provided us with a variety of molecular markers that may ultimately redefine the criteria for cancer diagnosis and provide new tools for early detection through the development of sensitive procedures aimed at detecting these alterations in easily accessible samples, such as blood and sputum.

In fact, genetic alterations, including LOH[3] at *FHIT* and other loci on 3p, 9p, 17p; microsatellite instability; *p16* and other TSG promoter methylation; K-*ras* and *p53* mutations, have been detected in bodily fluids as found in cytological samples of the sputum and bronchial lavage of lung cancer patients and chronic smokers (5–8). Circulating tumor DNA carrying several of these molecular changes has also been reported in the plasma or serum of patients with various malignancies including SCLC and NSCLC (9–11) and in head & neck (12, 13) esophageal, breast (14, 15), liver (16), colon (17), pancreatic (18), and renal cancer (19). In a previous study (20), we have reported that 61% of the NSCLC patients showing allele shift and LOH at FHIT and other genomic loci in tumor samples also displayed a microsatellite change in plasma, irrespective of tumor size and stage, thus suggesting that circulating tumor DNA is associated with early phases of lung tumor development.

Although the exact mechanism of the release of circulating DNA remains to be proved, an active release of DNA from highly proliferating cells has been proposed (21). However, most of the thus far published studies have used, for the detection of circulating tumor DNA, cumbersome methods for routine clinical use. In addition, they did not evaluate sensitivity and specificity of the molecular assays in a large series of patients with respect to control groups and did not analyze the kinetics of circulating tumor DNA in the follow-up of radically resected patients.

The purpose of our study was to determine whether the amounts of circulating DNA could discriminate between lung cancer patients and healthy individuals by using both DNA quantification assay and molecular characterization of tumor plasma DNA through the analysis of microsatellite alterations; whether the presence and quantity of tumor DNA in plasma have any relationship with stage, histotype, and recurrence of disease during follow-up; and, finally, to determine the kinetics of circulating plasma DNA in surgically treated patients.

Materials and Methods

Samples Collection and DNA Isolation. Patients with a confirmed diagnosis of NSCLC at the Istituto Nazionale Tumori of Milan gave their informed consent to be included in the study. Tumor specimens were surgically resected and immediately stored at −80°. Peripheral blood was extracted from each patient on the day before surgery and collected in lithium-heparin. Plasma was immediately separated from the cellular fraction by centrifugation two times at 2500 rpm for 10 min at 4°. The resulting supernatant (plasma) and 2 ml of whole blood were frozen at −80°. DNA was extracted from tissue, plasma, and blood cell samples by using QIAamp DNA Mini kit (Qiagen, Italy) according to the "tissue protocol" and "blood and body fluids protocol." Plasma (1000 μl) was purified by five passages on the same column (Qiagen), and the resulting DNA was eluted in 50 μl of sterile bidistillated water and stored at −20°. Tumor and whole blood DNA concentrations were estimated by spectrophotometry.

DNA Quantification Assay in Plasma. Quantification of the circulating plasma DNA was performed by using DNA DipStick TM Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The assay provides linear results from 0.1 to 10 ng of nucleic acid. Briefly, appropriate dilutions (undiluted, 1:10, and 1:100) of the control (plasmid DNA) and samples DNAs were prepared with sterile water and 1 μl each of the sample, and its dilutions

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[3] The abbreviations used are: LOH, loss of heterozygosity; FHIT, fragile histidine triad; TSG, tumor suppressor gene; SCLC, small cell lung cancer; NSCLC, non-SCLC; FAL, fractional allelic loss; ROC, receiver operating characteristic (curve); AUC-ROC; area under the ROC curve; ADC, adenocarcinoma; SQC, squamous carcinoma; LC, large cell carcinoma.

were spotted onto membranes and allowed to dry. After serial dipping in coupling and developing solutions, the sticks were dried, and color intensities of the sample spots on the membranes were compared with the control DNA. When comparing the sample dots to the standards, if the sample concentrations had not fallen within the range of standards and were of intermediate intensity compared with those on the standard, additional dilutions were prepared based on the initial result, and the assay was repeated. At least three independent quantification assays were performed for each plasma sample.

DNA values of 5, 50, and 500 ng/ml of plasma were assigned to the samples corresponding to concentrations 0.1, 1, and 10 ng/μl, respectively, estimated on color intensity of the spots, considering that DNA obtained from 1 ml of plasma was eluted in 50 μl. Intermediate DNA values were obtained by evaluation of intermediate dilutions of the samples harvested for more accurate reading of the assay.

This allowed us to categorize DNA values into the following classes: 0–5, 6–25, 26–125, 126–250, 251–500, and >500 ng/ml.

PCR Amplification. The analysis of microsatellite instability and LOH was performed by studying microsatellite alterations at loci located at 3p14.2 (*D3S1300*, *FHIT* locus), 3p21 (*D3S1289*), 3p23 (*D3S1266*), 3p24.2 (*D3S2338*), and 3p25–26 (*D3S1304*), which are hot-spots of deletions in lung cancer. The sequences of nucleotide markers for microsatellite analysis are available through the Genome Database.[4] Thirty ng of tumor and lymphocyte DNA were used for the analysis. Two to 30 ng of purified DNA was used for PCR amplification of plasma by using primer pairs synthesized with FAM, HEX, or NED fluorescent label (ABI Prism Linkage mapping set; PE Applied Biosystems). PCR protocol was as follows: 1.5 μl of 10× Buffer II gold PE, 1.5 μl of 2.5 mM $MgCl_2$, 0.2 μl of 2.5 mM dNTP mix, 1 μl of 10 μM labeled primer mix, 0.12 μl of 5 units/μl Ampli-Taq Gold, and 9.8 μl of sterile water. Final volume of the reaction was 15 μl. Samples were processed in a GeneAmp PCR system 9700 thermal cycler through 45 cycles, each cycle consisting of 10 s at 96°, 30 s at 55° annealing temperature, 3 min at 70°. Pools of the fluorescent PCR products for each clinical specimen were separated electrophoretically on a 5% polyacrylamide gel and analyzed by laser fluorescence using ABI Prism DNA Sequencer (377 PE-Applied Biosystem) equipped with GeneScan TM 2.1 software. LOH and the presence of allele shifts indicating genomic instability were recorded in the various samples and compared with the profiles obtained in DNA from normal peripheral lymphocytes. LOH was scored when a reduction of at least 30% of allele intensity in the experimental sample was seen. FAL value was calculated for each sample as (loci scored with allelic imbalances) ÷ (total informative loci). All of the DNA samples with microsatellite alterations were amplified at least twice to rule out PCR artifacts or sample contamination. In the presence of allelic imbalance in plasma, increasing amounts of plasma DNA were used in the PCR reaction to exclude unreliable allelotyping.

Statistical Analyses. DNA values were analyzed as a discrete variable according to the previously specified categories. Weighted means were computed for descriptive purposes, using class frequencies as weights and midpoints of each category as the values to be averaged, whereas comparisons were based on Pearson's χ^2 tests.

To assess whether circulating DNA might discriminate between lung cancer patients *versus* healthy individuals, we computed sensitivity and specificity estimates for different DNA tresholds, and the AUC-ROC according to Hanley and McNeil (22). The corresponding 95% confidence limits were obtained through the bias-corrected and accelerated (BC_a) bootstrap procedure described by Efron and Tibshirani (23).

Time-to-tumor relapse and time to death were computed from the date of surgery to the date of event occurrence, or the date of the last follow-up assessment available for event-free patients. Relapse-free and overall survival curves according to the baseline DNA values were compared with log-rank test for trend (24).

Results

A consecutive series of 84 patients with radically resected primary NSCLC (stages I-III) and 43 healthy blood donor controls were included in the study.

[4] Internet address: www.gdb.org.

Quantification Test of Circulating Plasma DNA in Patients and Controls. Table 1 summarizes the results of the quantification assay in patients and controls. (Table 1) The mean age was 63 years (range, 39–81 years) for cases and 41 years (range, 21–61) for controls. In the latter group 34 were males and 8 individuals were smokers.

DNA levels were measurable in 81 (96%) of 84 patients and in 32 (74%) of 43 controls. In patients, the mean value of plasma DNA concentration was 318 ng/ml, and significantly differed from the mean amount of 18 ng/ml observed in controls. Age and sex did not correlate with plasma DNA values in either group. Among tumor patients, no significant association was observed among plasma DNA values and sex, age, histotype, and tumor stage. DNA levels were already high even in stage Ia patients when compared with control individuals. Higher levels of circulating DNA did not correlate with patients prognosis, in terms of relapse-free survival ($P = 0.675$) or overall survival ($P = 0.548$).

Sensitivity and specificity estimates over a range of cutoff points on plasma DNA levels are reported in Table 2. The value of the AUC-ROC was 0.844 (Table 2; 95% confidence interval, 0.767–0.898), indicating a good discrimination power of the test.

Quantification Test of Circulating Plasma DNA in the Follow-Up. Circulating plasma DNA and the analysis of microsatellite alterations during follow-up could be assessed in a subgroup of 38 patients (Table 3). In 35 clinically relapse-free individuals (Table 3), DNA concentrations in the follow-up plasma sample (34 ng/ml on average) were significantly lower than those recorded in the sample at the time of surgery (345 ng/ml on average; $P < 0.001$) and comparable with the value observed in the control group ($P = 0.929$ for the test for trend). Circulating tumor DNA levels in relapse-free individuals showed a trend toward reduction with time but were already low in the 1–6-month interval. Conversely, four individuals showed a 2- to 20-fold increase in the amount of plasma DNA in the second plasma samples (two patients) and in a third (two patients) plasma samples available 7–23 months after surgery. Two of them presented liver metastases and died 2 months later; one patient displayed a local recurrence of carcinoma after 2 years. The fourth patient displayed a new primary tumor of the liver (colangiocarcinoma) 7 months after lung resection.

Analysis of Microsatellite Alterations in the Followed-Up Patients and in Controls. The analysis of microsatellite alterations was performed in the plasma samples of 38 patients and of the 43 controls. In these individuals, we analyzed the presence of microsatellite alterations (genetic instability and LOH) at loci located at 3p14.2 (*D3S1300*, *FHIT* locus), 3p21 (*D3S1289*), 3p23 (*D3S1266*), 3p24.2

Table 1 *Quantification (weighted means) of plasma DNA in patients and controls, and Ps comparing DNA levels according to main patient and disease characteristics*

	No. of cases	DNA (ng/ml)	*P*
Patients	84	318	
Age			0.396
39–59	31	305	
60–69	34	261	
≥70	19	441	
Sex			0.403
F	12	222	
M	72	334	
Type			0.120
SQC	25	288	
ADC	47	325	
Other	12	353	
Stage			0.355
Ia	14	320	
Ib	32	344	
II	15	304	
III	23	290	
Controls	43	18	

Table 2 *Sensitivity and specificity of DNA quantification assay, and corresponding AUC-ROC (with 95% confidence interval)*

DNA (ng/ml)	No. of patients	No. of controls	Sensitivity %	Specificity %
0–5	11	20	87	47
6–25	10	17	75	86
26–125	18	6	54	100
126–250	7	0	45	100
251–500	27	0	13	100
>500	11	0	0	100
	AUC ROC = 0.844 (0.767–0.898)			

Table 3 *Quantification (weighted means) of circulating DNA in plasma at baseline and during follow-up*

	No. of cases	DNA (ng/ml) baseline	DNA (ng/ml) follow-up
Patients	38	333	61
Outcome			
NED[a]	35	345	34
Time interval			
1–6	14	216	45
7–12	4	501	39
13–18	5	318	32
19–24	6	440	27
>24	6	471	15
REC	3	193	375
Controls	43	18	

[a] NED, no evidence of disease; REC, recurrence of disease.

(*D3S2338*), 3p25–26 (*D3S1304*), which are hot-spots of deletions in lung cancer. LOH and the presence of allele shifts indicating genomic instability were recorded in the serial plasma samples and compared with the profiles obtained in DNA from the matched previously resected tumor. Twenty (61%) of 33 informative analyzed patients displayed LOH at least at one locus on 3p in the tumor specimens (12 ADC, 7 SQC, 1 LC) with an average FAL of 0.4 (Table 4). Nine (45%) of these 20 patients (Table 4) showed the presence of the same allelic imbalance observed in the tumor in the corresponding plasma sample taken at the time of surgery. Seven of these patients had ADC; two had SQC. Seven tumors were in stage I, one in stage II, and one in stage IIIa. In six patients, the DNA value in plasma was in the 251–500-ng/ml class, whereas the remaining three patients showed DNA values falling in classes >500, 126–250, and 6–25 ng/ml, respectively. To study the persistence of these alterations in plasma, these patients were followed up; and a second, and in few cases a third, plasma sample was scored for allelic imbalances. Seven patients resulted negative in the second plasma specimen collected 4–24 months after surgery when they did not have any evidence of disease. In the remaining two patients, the same alteration persisted after 22 and 23 months from surgery, respectively: one patient had metastasis of the liver, and one patient had a local recurrence of the disease. The two patients showed a significant increase in the amounts of circulating DNA. None of the 43 control individuals displayed genetic changes in plasma.

Discussion

The results of this study provide straightforward evidence of an increased amount of tumor circulating DNA in the plasma of lung cancer patients. To evaluate the accuracy of the DNA quantification assay, we have constructed a ROC curve. The test discriminated between the two groups; the best results were obtained with the classes 6–25 ng/ml and 26–125 ng/ml plasma DNA concentration, for which a sensitivity of 75 and 54% and a specificity of 86 and 100% were recorded, respectively.

In addition, the molecular characterization of plasma circulating DNA by the analysis of microsatellite alterations at multiple loci on 3p, although of not great sensitivity (estimated treshold of the assay is 1:100–1:200) has proved the tumor origin of the released plasma DNA in several patients. Of interest, all of the patients except three who showed microsatellite imbalances in the plasma had a stage I tumor, thereby suggesting a possible use of genetic analysis of plasma DNA for lung cancer screening. Moreover, for the first time, a group of 38 disease-free surgically treated patients was followed up and monitored for variation of circulating DNA levels and for the presence of the specific microsatellite changes identified in the corresponding tumor specimens in serial plasma samples taken at various time points after tumor removal (between 1 and 28 months). The results showed that, in the follow-up, both variations in DNA level and the persistence of genetic changes correlate with the clinical status of the patients.

Data collected on the kinetics of circulating tumor DNA in the plasma of disease-free patients indicate that this phenomenon is not long lasting, because a relevant drop in DNA levels is already visible at 1–6 months after tumor removal and reaches the level observed in normal healthy subjects. These data suggest that in tumor-free patients, plasma DNA either is released at lower rates or is rapidly degraded. The test proved to be sensitive because all of the patients with recurrent or metastatic tumor showed reappearance of high levels of circulating DNA at the time of the clinical detection of their disease. The molecular follow-up of the other patients of our series is ongoing to establish whether variations in plasma circulating tumor DNA might anticipate clinical diagnosis.

In the past, several studies reported increased levels of free DNA in the serum of patients with various types of cancer as determined by indirect radioimmunological methods (25), by direct nick translation DNA labeling (26), or by spectrophotometry (27). The data suggested that increased amount of circulating DNA in advanced-stage disease correlated with prognosis (26, 28) and response to treatment (25, 27). However, these studies used either radioactive or low sensitivity, time-consuming techniques, did not perform subsequent determinations during the course of the disease in patients, and the tumor origin of serum/plasma DNA was not proven. More recently, molecular studies have provided evidence that circulating DNA in plasma and serum has the same genetic markers as the corresponding tumor in various malignancies (8–19). In lung cancer, the presence of both microsatellite alterations (9, 20) and promoter hypermethylation of TSGs (10) have been reported in plasma/serum of SCLC and NSCLC patients, thus supporting the tumor origin of circulating DNA and the possibility of using molecular tests for its characterization. However, accurate quantification of the amounts of DNA present in the plasma/serum of lung cancer patients with respect to a series of healthy control individuals, as well as an estimate of the persistence of circulating tumor DNA and of the associated molecular changes during follow-up of patients have not been provided. In addition, a major problem in these studies is the still low sensitivity of molecular analyses in plasma/serum ranging from 28 to 73% for microsatellite changes and methylation assays (9–11, 20). For oncogene or TSG mutations screening (*i.e.*, *p53*, K-*ras*), enrichment techniques that raise the sensitivity of the molecular analyses are available, but they

Table 4 *Microsatellite alterations (3p)[a] in tumors and plasma samples of followed-up individuals*

	n	Tumor	FAL[b]	Plasma 1	Plasma 2[c]
Patients	35	20/33 (61%)	0.4	9/20 (45%)	2/20 (10%)[d]
Controls	43			0/43	

[a] 3p14.2 (*D3S1300*, *FHIT* locus), 3p21 (*D3S1289*), 3p23 (*D3S1266*), 3p24.2 (*D3S2338*), 3p25–26 (*D3S1304*).
[b] Average FAL.
[c] 4–24-month interval after surgery.
[d] Two relapsing cases.

are rather expensive and time consuming and require prior knowledge of the mutation present in the tumor specimen. Thus, they are not feasible in the absence of tumor DNA such as in screening programs including heavy smokers without cancer. Because of the differences in assay sensitivity for distinct markers, to optimize the results, it will be essential to obtain a panel of genetic markers capable of detecting the molecular changes in plasma DNA for every single tumor.

The data here provided suggest that accurate quantification of DNA amounts in the plasma of lung cancer patients, once a cutoff value is established, is a valuable tool to discriminate between patients with disease and unaffected individuals and may be proposed as an early detection test as well as a complementary, noninvasive assay to follow up patients and high-risk individuals such as symptomatic chronic smokers. Increased tumor DNA in the plasma of these subjects might prompt more accurate and specific clinical examinations.

Lung cancer screening remains a leading problem throughout the world, and efforts to establish diagnostic platforms able to identify the early clonal phase of progression of lung cancer by minimally invasive procedure in plasma or other biological fluids are needed (1). The combination of molecular testing with conventional and newer diagnostic approaches, such as low-dose spiral computed tomography and positron emission tomography, should be evaluated for early diagnosis and screening for lung cancer.

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