*Advances in Brief*

# Detection of Microsatellite Alterations in Plasma DNA of Non-Small Cell Lung Cancer Patients: A Prospect for Early Diagnosis[1]

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## Abstract

**A major problem in lung cancer is the lack of clinically useful tests for early diagnosis and screening of an asymptomatic population by noninvasive diagnostic procedures. Recent studies have demonstrated the possibility to detect genetic alterations in plasma or serum DNA from patients with various cancers. However, these data rely on small series of aggressive tumors with advanced-stage disease. To determine whether genetic changes in plasma are also detectable in patients with limited disease and thereby potentially useful for early detection, we looked for microsatellite instability (allele shift) and loss of heterozygosity in plasma DNA of 87 stage I-III non-small cell lung cancers and 14 controls. Combining two markers with a high rate of instability (*D21S1245*) and loss of heterozygosity (*FHIT* locus), a microsatellite alteration was observed in 49 of 87 (56%) non-small cell lung cancer tumors and in 35 of 87 (40%) plasma samples. Thirty of 49 (61%) of the cases showing tumor alterations also displayed a change in plasma DNA; in addition, 5 patients displayed alterations in plasma samples only. None of the control individuals had genetic changes in plasma. No association was found between the frequency of microsatellite alterations in plasma and tumor stage or histology. Of interest, plasma DNA abnormalities were detectable in 43% of pathological stage I cases and in 45% of tumors up to 2 cm in maximum diameter. These findings highlight new prospects for early tumor detection by noninvasive screening procedures based on the analysis of genetic changes in plasma.**

## Introduction

Lung cancer is a major cause of morbidity and mortality in developed countries and a rapidly expanding epidemic worldwide (1). Despite optimal use of therapeutic resources, the improvement of cure rates has been modest during the last decade, and overall survival of incident cases is only10–15% (2). When lung cancer is detected in an early stage, surgical resection can achieve a 60–80% 5-year survival, but the majority of cases are unresectable at the time of diagnosis and are no longer curable. In the past, screening procedures with conventional sputum cytology and chest radiography have been unable to decrease lung cancer mortality. However, more sensitive tools based on specific biological markers may provide new opportunities for screening of early detection of this disease (3).

Inactivation of tumor suppressor genes *p53*, *FHIT*, and *p16INK4/MST1*; deregulated expression of *EGFR* and *HER2/neu* oncogenes and of proteins involved in apoptosis control such as Bcl2; and point mutations in *KRAS2* represent the most frequent alterations in NSCLC.[3] In addition, LOH at multiple chromosomal loci and microsatellite (DNA repeat sequences) instability occur at various frequencies in NSCLC and may serve as clonal markers for cancer detection (4, 5). The possibility of using such genetic markers for early detection of lung cancer has been suggested primarily in tissue biopsies (6–9) and exfoliated cells in sputum and bronchoalveolar lavage (10–14).

Recent studies have demonstrated that genetic alterations are detectable in circulating DNA in the plasma or serum of patients with various malignancies such as SCLC and head and neck, colon, and pancreatic cancer. However, these studies were restricted to a small group of patients with extended disease (15–18).

Here we investigated the frequency and the extent of microsatellite alterations (shift and LOH) in plasma DNA of NSCLC patients with limited disease to gain insight into their possible use for early detection of lung cancer. We selected a group of 87 individuals with stage I-III NSCLC and 14 controls. Markers were chosen to detect shifts or LOH as follows: (*a*) a tetranucleotide repeat (*D21S1245*) recognized as being prone to microsatellite instability in various cancer types (11); and (*b*) dinucleotides *D3S1234*, *D3S1300*, or *D3S4103*, according to their informativeness, all located in introns of the *FHIT* gene, a tumor suppressor that overlaps the common fragile site *FRA3B* and shows a high rate of LOH in lung cancer (19, 20), particularly in smokers (21).

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; LOH, loss of heterozygosity; SCLC, small cell lung cancer; SQC, squamous cell carcinoma; ADC, adenocarcinoma.

*Table 1* Frequency of microsatellite alterations (shift and LOH) in tumor and plasma samples according to main clinicopathological features

| | | Microsatellite alterations | | | |
|---|---|---|---|---|---|
| | *n* | Tumor | *P* | Plasma | *P* |
| Patients | 87 | 49 (56%) | | 35 (40%) | 0.03 |
| Age (yr) | | | | | |
| 40–59 | 30 | 15 (50%) | 0.44 | 9 (30%) | 0.35 |
| 60–69 | 34 | 22 (65%) | | 15 (44%) | |
| 70+ | 23 | 12 (52%) | | 11 (48%) | |
| Sex | | | | | |
| Male | 55 | 37 (67%) | 0.013 | 21 (38%) | 0.65 |
| Female | 32 | 12 (36%) | | 14 (44%) | |
| Type | | | | | |
| SQC | 45 | 30 (67%) | 0.40[a] | 16 (36%) | 0.12 |
| ADC | 32 | 13 (41%) | | 12 (37%) | |
| Other | 11 | 6 (55%) | | 7 (64%) | |
| Stage | | | | | |
| I | 40 | 24 (60%) | 0.52 | 17 (43%) | 0.64 |
| II | 25 | 11 (44%) | | 8 (32%) | |
| IIIA | 19 | 12 (63%) | | 8 (42%) | |
| IIIB–IV | 3 | 2 (67%) | | 2 (67%) | |
| Controls[b] | 14 | 1 (7%) | | 0 | |

[a] Overall value; SQC *versus* ADC = 0.02.
[b] Patients with infectious or inflammatory lesions, carcinoids, or other benign tumors.

## Materials and Methods

**Samples Collection and DNA Isolation.** Patients with a confirmed diagnosis of NSCLC at the Royal Brompton Hospital gave their informed consent to be included in the study. Tumor specimens were surgically resected and immediately stored at −140°C. Peripheral blood was extracted from each patient on the day of admission and collected in lithium-heparin. Plasma was separated from the cellular fraction by centrifugation at 3500 rpm for 10 min at 4°C. The resulting supernatant (plasma) and pellet (cellular fraction) were frozen at −80°C. DNA was extracted from tissues, plasma, and blood cells samples by using QIAamp tissue and blood kits (Qiagen) according to the tissue protocol and the blood and body fluids protocol. Fifty ng of tumor and lymphocytes DNA were used for the analysis. Plasma (200–400 μl) was purified on a column, and 5 μl of the eluted DNA were usually sufficient for PCR amplification.

**PCR Amplification.** The sequences of nucleotide markers for microsatellite analysis, *D21S1245*, *D3S1300*, *D3S4103*, and *D3S1234*, are available through the Genome Database. PCR amplification was carried out in a 50-μl final volume with 50 ng of genomic DNA template; 100 ng of each unlabeled primer; 25 μM dGTP, dATP, and dTTP; 2.5 μM dCTP; 1 μCi of [α-$^{32}$P]dCTP (Amersham); 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.4); and 2.5 units of Taq polymerase (Perkin-Elmer). Samples are processed through 25–30 cycles, with each cycle consisting of 30 s at 94°C, 30 s at an annealing temperature of 57°C to 60°C, as appropriate for each primer, and 30 s at 72°C.

Denatured PCR products are electrophoresed on 6% urea-polyacrylamide gels at room temperature in a vertical Bio-Rad apparatus. The gels are dried and exposed to autoradiography.

For informative cases, allelic loss is scored if the autoradiographic signal of one allele is reduced approximately 30% in the tumor and sputum DNA compared with the corresponding normal allele by visual inspection of independent observers. The loci displaying microsatellite instability (shift) are not scored for allelic loss. All of the DNA samples with microsatellite alterations were amplified twice to rule out PCR artifacts or sample contamination.

*Table 2* Frequency of microsatellite alterations (shift and LOH) in tumor and plasma samples for each microsatellite marker studied

| Markers | LOH tumor | Shift tumor | LOH plasma | Shift plasma |
|---|---|---|---|---|
| *D21S1245* | 26/76[a] (34%) | 4/87 (5%) | 15/76 (20%) | 9/87 (10%) |
| *FHIT*[b] | 38/80 (47%) | 2/87 (2%) | 14/76 (18%) | 1/85 (1%) |

[a] Number of evaluable and informative samples.
[b] One of three microsatellites (*D3S1234*, *D3S1300*, or *D3S4103*) was analyzed in each patient according to its informative status.

## Results

**Molecular Analysis of Tumor and Plasma DNA.** Table 1 records the molecular findings and clinicopathological features of the 87 NSCLC patients and 14 control individuals carrying infectious or inflammatory lesions, carcinoids, or other benign tumors. The median age was 63 years (range, 41–82 years) for cases and 51 years (range, 29–73 years) for controls. In the latter group, 10 (71%) were males, and 10 individuals were smokers.

We found that 56% (49 of 87) of NSCLC tumors exhibited microsatellite alterations (shift or LOH) in at least one locus [34% with marker *D21S1245* and 47% using markers within *FHIT* (Table 2)]. In cancer patients, microsatellite alterations in paired plasma and tumor DNA samples were compared with the corresponding repeat sequences in normal lymphocytes. Shifts were recognized as the appearance of a new allele(s) in tumor DNA that was absent in normal DNA, and LOH was scored if the allele signal was reduced to less than 30% of normal intensity. Thirty of 49 (61%) of the patients with microsatellite alterations in tumor DNA also showed changes in plasma DNA. These alterations matched in all cases, except for six patients in whom the alterations found in tumor and plasma were of different types (*i.e.*, loss of a different allele, or LOH in one sample and a shift in the other one). This phenomenon may be related to differential shedding of DNA in the plasma from malignant cells and suggests either the presence of heterogeneous clones in the tumor or the presence of other occult lesions in these patients. In addition, five patients displayed microsatellite changes in plasma DNA that were not detectable in the corresponding tumors. This could reflect the implication of several clones in some tumors or the presence of a mixture of normal stromal and inflammatory cells in the tumor specimen that, in the absence of microdissection procedures, could have precluded the identification of the clonal genetic change. As reported previously (15–18), plasma samples show selective enrichment of tumor DNA, demonstrated here by the possibility to see clear LOH in most plasma specimens (Fig. 1).

**Correlation with Patient's Clinicopathological Features.** In tumor samples, microsatellite alterations were revealed to be more frequent in male patients ($P = 0.013$) and in

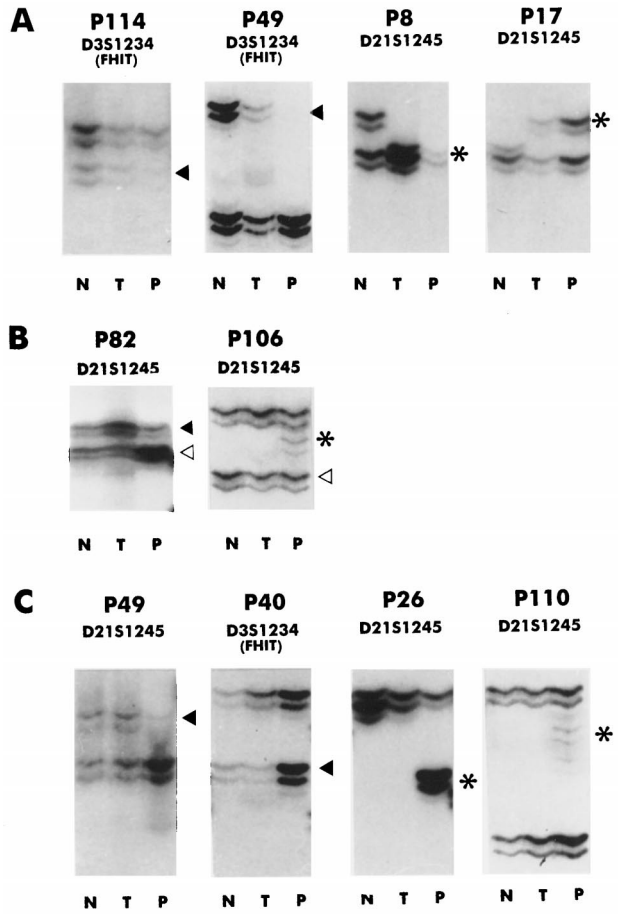


*Fig. 1* Representative microsatellite analysis of plasma (*P*), tumor (*T*), and lymphocyte (*N*) of NSCLC patients. Patient number and microsatellites markers are indicated above each block. Loss of an allele is designated by an *arrowhead*, and allele shifts are indicated by an *asterisk*. *A*, examples of microsatellite alterations occurring in tumor and plasma DNA. *P114*, loss of the lower allele in tumor and plasma (◀); *P49*, loss of the upper allele in tumor and plasma (◀); *P8*, shift of the upper allele in tumor and plasma (∗); *P17*, shift (presence of new top allele; ∗). *B*, different types of microsatellite alterations in plasma *versus* tumor DNA. *P82*, loss of the lower allele in tumor (◁) and loss of the upper allele in plasma (◀); *P106*, loss of the lower allele in tumor (◁) and shift in plasma (presence of new alleles; ∗). *C*, microsatellite alterations in plasma DNA only (*P49*, *P26*, and *P110*) or tumor DNA only (*P40*). *P49*, loss of the upper allele in plasma (◀); *P40*, loss at lower allele in tumor (◀); *P26*, shift of the lower allele in plasma (∗); *P110*, shift in plasma (presence of new alleles; ∗).

SQCs ($P = 0.02$), a finding likely related to the prevalent occurrence in this series of the squamous histotype in men (58% SQC *versus* 39% ADC). However, the frequency of such alterations in tumors was evenly distributed among the various stages (Table 1).

On the contrary, the occurrence of microsatellite alterations in plasma was not associated with sex, tumor type, or stage (Table 1). As a matter of fact, plasma DNA abnormalities were detectable in 43% (17 of 40) of pathological stage I tumors and in 45% (9 of 20) of tumors up to 2 cm in maximum diameter.

Within the subset of cases with abnormal tumors, the frequency of microsatellite changes in plasma DNA was somewhat higher for ADC compared with the SQC (69% *versus* 50%).

After a median observation time of 11 months, cancer recurrence was detected in 15 cases. The frequency of relapses was the same in patients with and without microsatellite alterations in the tumor (16% *versus* 18%, respectively) or in the plasma (17% in each group). Although these subgroups are small, and the follow-up is limited, it is unlikely that significant differences may become evident in the future, given the present distribution.

## Discussion

This study shows that the occurrence of allele shift and LOH in plasma DNA is a frequent and relatively early event that is not related to the tumor burden (size or pathological stage) or predictive of a higher risk of recurrence. These findings are in keeping with our previous observation, based on the analysis of 515 cases of stage I NSCLC, that higher microvessel density (or neoangiogenesis) is not associated with the risk of tumor progression and occurs independently of tumor size (22, 23).

Previous studies have used microsatellite analysis at various loci to detect genetic alterations in plasma or serum in a small number of patients with SCLC (15) and head and neck cancer (16). Altered microsatellite plasma DNA was found in 15 of 21 (71%) SCLC patients without a significant difference between patients with limited or extended disease. Six of 21 (29%) head and neck cancer patients, all of whom had advanced disease and poor clinical outcome, were reported to have microsatellite changes in serum DNA.

In the present study, 61% of the NSCLC patients showing allele shift and LOH 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 the early phases of lung tumor development. Moreover, shift and LOH were already detectable in plasma but were not yet detectable in the corresponding tumor in a small additional group of patients, suggesting selective enrichment of circulating tumor DNA.

These findings have important clinical implications. In fact, given the high prevalence of plasma DNA alterations in stage I and stage II NSCLC (a potentially curable disease) and the large amount of tumor DNA released from these tumors, genetic analysis of plasma DNA could be used for diagnostic purposes and could form the basis for lung cancer screening. The sensitivity of the plasma test could be further increased by the simultaneous search for other, distinctly different genetic alterations within the same specimen. In fact, we have demonstrated by immunohistochemical studies that loss of function of *FHIT* and *p53* genes are independent events, and 83% of early-stage NSCLCs show at least one of these two abnormalities (24). *KRAS* mutations have been reported in plasma DNA from patients with colorectal and pancreatic cancer (17, 18), indicating that a wide range of genetic changes could be found in plasma DNA. The analysis of mutations in *p53* and *KRAS* genes, as well an extended analysis of LOH of *FHIT* and other loci on the short arm of chromosome 3, characterizing most, if not all, lung cancer patients, could increase the diagnostic range of

plasma DNA analysis and thus increase the clinical usefulness of this noninvasive test.

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