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Original article

Microsatellite alterations and TP53 mutations in plasma DNA of small-cell lung cancer patients: Follow-up study and prognostic significance

R. Gonzalez,[1] J. M. Silva,[1] A. Sanchez,[1] G. Dominguez,[1] J. M. Garcia,[1] X. qi Chen,[2] M. Stroun,[2] M. Provencio,[1] P. España,[1] P. Anker[2] & F. Bonilla[1]

[1]*Department of Medical Oncology, Clinica Puerta de Hierro, Madrid, Spain;* [2]*Laboratory of Plant Biochemistry and Physiology, Faculty of Science of Geneva University, Pavillon des Isotopes, Geneva, Switzerland*

Summary

Background: Small-cell lung cancer (SCLC), one of the major types of lung cancer, is associated with many different somatic molecular genetic changes. These alterations, observed in tumor DNA, have also been identified in the plasma DNA of patients. We undertook the present study to make a prospective investigation into the correlation between abnormal plasma DNA and patient survival.

Patients and methods: Thirty-five patients with SCLC were selected after histological diagnosis. Polymorphic markers (ACTBP2, UT762 and AR) were chosen for their reported high rate of alterations in SCLC and analyzed in tumor tissue, normal blood cells and plasma DNA. Furthermore, we looked for mutations of the TP53 gene in tumor and plasma DNA.

Results: In 25 patients (71%) at least one molecular change precisely matching that of the primary tumor was detected in the plasma DNA. No difference in survival was observed between patients with aberrant plasma DNA and patients without plasma DNA alterations. However, patients with microsatellite modifications and TP53 mutations concomitantly, showed a significant difference ($P = 0.02$) in survival compared with patients bearing only one of these molecular changes. In 15 cases it was possible to find a correlation either between tumor response and disappearance of abnormal plasma DNA, or tumor progression and persistence of plasma DNA alterations.

Conclusions: Free plasma DNA with molecular alterations is present to a high degree in plasma DNA of SCLC patients and may have a role as a prognostic factor.

Key words: circulating DNA, plasma DNA alterations, prognostic factor, SCLC, tumor DNA alterations

Introduction

Small-cell lung cancer (SCLC) represents about 20% of all primary lung cancers [1, 2]. The predominant risk factor by far is cigarette smoking [3]. The tumor arises from basal neuroendocrine cells, infiltrating the submucosa in the early stages of the disease [4]. Despite the diversity and intensity of current therapies, survival is usually short. In SCLC several clinical parameters have been established as prognostic factors. Long-term survival has been associated to age, 65 years old or less at diagnosis; performance status, ECOG 0–1; and limited disease (LD) [5]. The overall survival rate of patients treated with combination chemotherapy, with or without chest irradiation, is about 7% at two years, ranging from 2% for patients with extensive disease (ED) to 13% with LD [5]. More recently, concurrent chemotherapy and radiotherapy has been reported, in the case of LD, to allow a 44% survival at two years [6].

SCLC is associated with different types of molecular gene aberrations including somatic mutations of oncogenes such as c-*myc* [7] or mutations of tumor suppressor genes such as TP53 and RB [8, 9] as well as allelic loss and microsatellite alterations in several chromosomal regions [2, 10–14]. Detection of these molecular changes may help define histological diagnosis or be used as a prognostic factor.

Increased quantities of DNA have been found in the plasma of patients suffering from different malignancies [15–17]. This circulating extracellular DNA exhibits tumor related alterations such as decreased strand stability [17], ras or TP53 mutations [18–24], microsatellite alterations [25–30] or aberrant promoter hypermethylation of tumor suppressor genes [31–33].

Several studies seem to indicate that aberrations in plasma/serum DNA may be used as a prognostic factor. All patients suffering from head and neck cancer with microsatellite alterations in the serum matching those of the primary tumor had advanced disease [27]. Two reports on pancreatic carcinoma provide evidence that K-ras mutations in plasma DNA are linked to overall survival [19, 34]. Nakamori and his group found that patients showing ras mutations in plasma were more likely to respond poorly to treatment, and those who still harbored a mutation in the plasma, even after seemingly successful surgery, had a poor prognosis [34]. However, no prospective studies have been designed to analyze the value of free plasma DNA during

patient follow-up and its real implication as a predictive factor.

In relation to lung cancer, quantitative studies show a definite correlation between plasma DNA levels and prognosis [35, 36]. Plasma DNA levels were increased in patients with advanced disease and correlated with other enzymatic tumor markers [35].

Moreover, microsatellite instability, expressed either as a new allele or the loss of one allele [26], or aberrant DNA methylation [31] have been shown to occur in serum DNA of patients suffering from non-small-cell lung cancer (NSCLC). In SCLC, 76% of the patients presented a microsatellite alteration in the tumor DNA and 71% in plasma DNA [25].

The purpose of this prospective phase II study was to identify plasma DNA with similar features to tumor DNA in SCLC patients, using microsatellite alterations and TP53 mutations as molecular markers, and correlate them with patient survival, trying to investigate the predictive value in the outcome of aberrant plasma DNA.

Patients and methods

Patient population

Between May 1997 and January 1999, 35 patients with histologically confirmed diagnosis of SCLC were recruited for this phase II study. Informed consent was obtained from all participants after explaining the nature of the study, which was approved by the Research Ethics Board of our hospital. The median age of the patients was 61 years (range 38–79 years). Thirty-one were male and four female. The disease was classified as limited disease (LD) when tumor was restricted to one hemithorax, including ipsilateral supraclavicular fossa, and any wider disease including distant metastasis was classified as extensive disease (ED). The 113-week follow-up period lasted from May 1997 until June 1999; the median follow-up time for all patients was 57 weeks. Patient survival was calculated as the interval between the date of diagnosis and date of death or date of the last follow-up visit, and was the major clinical endpoint.

Tissue samples

A blood sample was collected from each patient with histological diagnosis of SCLC on the day prior to the first chemotherapy course. The diagnosis of SCLC was based on histological analysis of tumor samples collected at bronchoscopy or on cytological examination of sputum. None of the patients underwent surgical extirpation of the primary tumor or metastasis. Sixteen bronchial biopsy specimens were available for molecular study. These were snap-frozen in liquid nitrogen until processed. In addition, 13 healthy controls with no history of cancer or other known disease were recruited from blood donor volunteers.

DNA extraction

Blood cell and plasma DNA was extracted immediately. Tumor tissue and blood cell DNA was purified by a nonorganic method (S-4520 Kit, Oncor Inc., Gaithersburg, Maryland). Plasma DNA was extracted using Qiagen columns (Qiamp Blood Kit, Qiagen Inc., Hilden, Germany) according to the protocol for blood and body fluids with the following modifications. Up to 12 ml of plasma previously heated on a heat block to 99 °C for 5 min were used. The heated sample was then centrifuged at 14000 rpm for 30 min, after which the clear supernatant was collected (25). Proteinase K (20 mg/ml) (Boehringer Mannheim, Mannheim, Germany) and buffer AL (Qiagen Inc.) were added in a 1 : 10 proportion with respect to the collected supernatant, and incubated overnight at 55 °C.

Clinical characteristics of the patients

Clinical staging procedures included chest X-rays, blood cell counts and biochemical profiles, computed tomography of chest and abdomen, and finally bone scintigraphy. Computed tomography of the brain was performed only when there was clinical suspicion of central nervous system involvement. This study was designed as a prospective phase II study with all patients undergoing the same treatment. Patients with ED received four chemotherapy (CT) cycles based on carboplatin 300 mg/m^2 intravenously on day 1 plus etoposide 100 mg/m^2, intravenously on day 1 and orally on days 2, 3, 4 and 5. Patients with LD were submitted to the same treatment followed by chest irradiation and two additional series of the same CT. Following treatment, which usually lasted 28 weeks, clinical follow-up took place every 12 weeks. Plasma was collected and the DNA analyzed on each occasion. A median of 4 plasma samples (range 1–10) were examined for each patient. Disease recurrence was treated with CT (cyclophosphamide, 1000 mg/m^2 i.v. on day 1; adriamycin, 45 mg/m^2 i.v. on day 1; and vincristine 2 mg/m^2 iv on day 1) until a second complete response or disease progression was observed.

Microsatellite analysis and PCR conditions

Three microsatellite markers were used to determine loss of heterpzygosity (LOH) or microsatellite shift on chromosomes 5 (ACTBP2), 12 (UT762) and X(AR). These markers were previously reported to show a high rate of microsatellite alterations in SCLC patients [10]. PCR was performed in 25 µl volumes using 0.75 units of Ampli Taq Gold DNApolymerase (Perkin-Elmer, Roche Molecular Systems, Inc, Branchburg, New Jersey), 2.5 µl of 10X PCR buffer, 200 µM dNTP, each primer at 0.6 µM with different concentrations of $MgCl_2$ depending on the polymorphic marker. A 35-cycle amplification was performed in a thermal cycler (Perkin-Elmer, Cetus, Foster City, California). The annealing temperatures were 59 °C, 62 °C and 57 °C, respectively. The alleles were separated by mixing 25 µl of PCR products with a 10 µl volume of loading buffer (total volume 35 µl), 0.02% xylene cyanol and 0.02% bromophenol blue. Electrophoresis was run on nondenaturing 8%–12% polyacrylamide gels for 12–15 h at 500 v. After gel electrophoresis, the allelic band intensity was detected by a nonradioisotopic technique using a commercially available silver staining method [37]. We analyzed the allelic intensities by densitometry. The gel image was captured by a GS-690 Imaging Densitometer (Bio-Rad laboratories, Hercules, California) digitized in 400 dpi, and analyzed by using Multi-Analyst/PC (Bio-Rad Laboratories, Hercules, California).

Ten of the results obtained were checked by laser fluorescence. One primer of each set was labeled with a fluorescent dye at the 5' end and all primers were purified by HPLC. Ten ng and 20 ng of plasma DNA, 20 ng of blood cell and tumor DNA were used as a template in a hot-start PCR in a 25 µl reaction mixture: 1.5 mM $MgCl_2$, 20 µM dNTP, 0.6 u Taq polymerase, 2–10 pmol of each primer (depending on the primer). Each PCR reaction was carried out in the same conditions as described above. PCR products were separated electrophoretically on 8% polyacrylamide gels and detected by laser fluorescence using an automated gene sequencer (Alfexpress, Amersham-Pharmacia). Fluorescent gel data was analyzed with the Allele-link 2 program. PCR products from lymphocytes, corresponding blood plasma and tumor tissue were analyzed on the same gel. The size in base pairs of the microsatellite alleles was automatically calculated using internal size markers. Automatic analysis of peak areas allowed relative quantifica-

tion of PCR products and determination of allelic ratios. LOH was scored if the allelic ratios of tumor or plasma PCR products and corresponding lymphocyte PCR products were below a cut-off value of 70%. Results indicating LOH were repeated at least twice.

Point mutations of the *p53* gene

Since up to 50% of SCLC patients were reported to exhibit TP53 mutations we also looked for the presence of point mutations in exons 5, 6, 7, and 8 of TP53 in the plasma DNA of SCLC patients. PCR-SSCP analysis was performed according to a modification of the method reported by Orita et al. [38]. The primers used for amplification of the different exons were: 5-TCCTTCCTCTTCCTACAG and 5-ACCCTGGGCAACCAGCCCTGT for exon 5; 5-ACAGGGCTGGTTGCCCAGGGT, and 5-AGTTGCAAACCAGACCTCAGGCG for exon 6; 5-TCCTAGGTTGGCTCTGACTGT and 5-AGTGGCCCTGACCTGGAGTCT for exon 7; and 5-GGGACAGGTAGGACCTGATTTCCTT and 5-ATCTGAAGGCATAACTGCACCCTTGG for exon 8 (Source: J. Weissenbach, Genethon, Whitehead Institute Center for Genome Research). The annealing temperatures were 65 °C, 67 °C, 62 °C and 68 °C, respectively. PCR was performed under standard conditions in 25 µl that contained 2 µl (100 ng) of DNA template (tumor, normal or plasma DNA); 2.5 µl of 10 X PCR buffer and 0.75 U of Ampli Taq Gold (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, New Jersey); 200 µM dideoxynucleoside triphosphate (dNTP) mix, each primer at 0.6 µM, different concentrations of magnesium chloride depending on the primer, and distilled H_2O needed to reach the total volume. For PCR amplification, the samples underwent 40 cycles at 94 °C for 1 minute, and were then subjected to different annealing temperatures depending on the primer, and 70 °C for 1 minute. The amplified products were denatured by mixing with 15 µl of denaturing stop solution that contained 98% formamide, 10 mmol/l edathamil (pH 8.0), 0.02% xylene cyanol and 0.02% bromophenol blue. heated to 95 °C for 5 minutes and rapidly cooled on ice. Electrophoresis was run on nondenaturing 8%–12% polyacrylamide gels for 12–15 hours at 250 v. The allelic band intensity on the gels was detected by a nonradioisotopic method using a commercially available silver staining method [37]. The specimens that showed a differential band at SSCP were amplified to obtain templates for DNA sequencing. These amplifications were independent from those used for SSCP analysis. Amplified DNA fragments were purified from 0.9% agarose gels using a Geneclean Kit (Bio-101, Inc., La Jolla, California), and used for direct DNA sequencing with the dNTP method with a Sequenase Kit (United States Biochemical Corp. Cleveland, Ohio).

Statistical analysis

Given the sample size of the study, it was considered that the statistical power of the study was higher than 75%. The statistical study was performed using SPSS version 7.5 for Windows (SPSS, Inc., Chicago, Illinois) and included Kaplan-Meier for estimation of survival curves. Differences between two curves were examined by the log-rank and Breslow tests. The level of significance was set at < 0.05.

Results

Molecular alterations detected

We detected at least one molecular alteration, LOH or shift, with microsatellite markers UT762, ACTBP2 and AR (X) (Figures 1–3), or a mutation on TP53 gene (Figure 4) in the plasma DNA of 25 of 35 patients (71%). Twenty-two showed molecular alteration at the time of diagnosis, for the remaining three it appeared later during follow-up. In eight cases (23%), microsatellite alterations coincided with a TP53 mutation. Twelve patients (34%) exhibited only microsatellite alterations, and TP53 mutations alone were detected in five (14%).

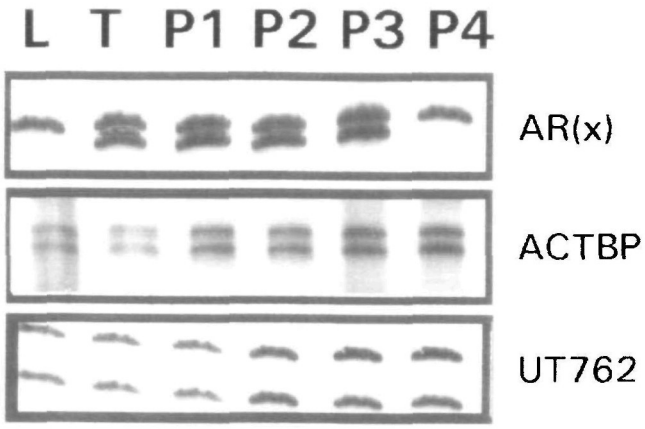

Figure 1. Photograph of the gels taken under normal light after staining with a (NO3)Ag method showing microsatellite alterations at the AR(X) marker in lymphocyte (L), tumor (T) and plasma (P) DNA of patient #22. The sequence of plasma determinations (P1-4) demonstrates the persistence of microsatellite shift at the AR(X) marker at the time of diagnosis (P1), after 4 courses of chemotherapy (P2) and after combined treatment chemotherapy plus radiotherapy (P3), and the disappearance of the microsatellite shift at 52 weeks (P4) coincident with complete disease remission. No alterations were detected at markers ACTBP and UT762 throughout follow-up.

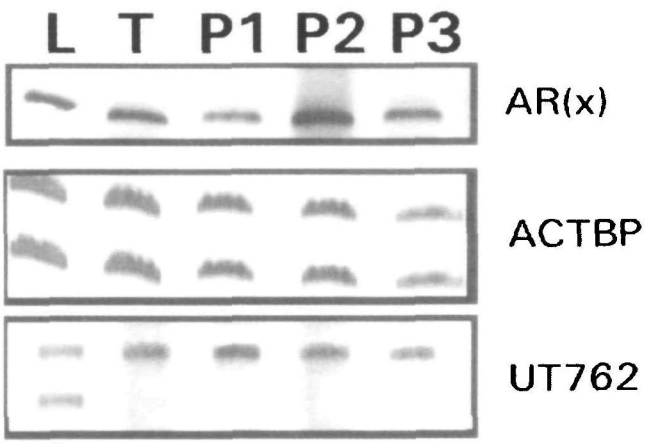

Figure 2. Representative photograph of the gels taken under normal light after staining with a (NO3)Ag method, showing microsatellite alterations at the UT762 marker in lymphocyte (L), tumor (T) and plasma (P) DNA of patient #11. A loss of heterozygosity was observed at marker UT762 throughout follow-up of the patient, from diagnosis (P1) until death, 28 weeks later (P3) with no response to treatment. There were no changes at markers AR(X) (homozygous in male patients) or ACTBP.

Out of 16 available tumor samples, 12 (75%) presented microsatellite alterations and/or TP53 mutations, 10 of which (83%) showed similar molecular changes in the plasma (Table 1). Analysis of microsatellite patterns by laser fluorescence using an automated gene sequencer (Figure 3) confirmed the results obtained with the silver stained gels.

No difference in DNA pattern was detected between plasma DNA samples and normal blood cell DNA from 13 healthy controls.

Table 1. Clinical features and microsatellite alterations and TP53 gene mutations in plasma and tumor DNA in SCLC patients.

Patient	Age/sex	Stage	UT762		ACTBP2		AR(X)		TP53		Alteration, Dg. time	Alteration, follow-up
			Tum	Pl	Tum	Pl	Tum	Pl	Tum	Pl		
1	70/M	ED	–	–	–	–	–	–	–	–	N	N
2	72/M	LD	ND	–	ND	–	ND	–	ND	+	N	Y
3	45/M	LD	ND	–	ND	–	ND	–	ND	–	N	N
4	60/F	LD	–	–	–	–	–	–	–	–	N	N
5	52/M	LD	S	S	–	–	S	S	+	+	Y	Y
6	59/M	LD	ND	–	ND	–	ND	–	ND	–	N	N
7	79/M	LD	LOH	LOH	–	–	–	–	+	+	N	Y
8	60/M	LD	ND	–	ND	S	ND	–	ND	–	Y	Y
9	70/M	LD	LOH	LOH	–	–	–	–	–	–	N	Y
10	60/M	ED	ND	LOH	ND	–	ND	–	ND	–	Y	N
11	38/F	LD	LOH	LOH	–	–	–	–	–	–	Y	Y
12	42/M	LD	–	–	–	–	–	S	–	–	Y	N
13	63/M	ED	–	–	LOH	LOH	–	–	–	–	Y	Y
14	60/M	ED	ND	S	ND	–	ND	–	ND	–	Y	Y
15	42/F	LD	ND	LOH	ND	–	ND	–	ND	+	Y	Y
16	68/M	LD	–	–	–	–	–	–	–	–	N	N
17	67/M	ED	S	S	S	–	–	–	–	–	Y	Y
18	61/M	LD	–	S	LOH	LOH	–	–	+	+	Y	Y
19	65/M	LD	ND	–	ND	–	ND	–	ND	+	Y	N
20	69/M	LD	ND	–	ND	LOH	ND	S	–	–	Y	N
21	70/M	LD	ND	–	ND	LOH	ND	–	ND	–	Y	Y
22	69/F	LD	–	–	–	–	S	S	+	+	Y	Y
23	67/M	LD	ND	–	ND	–	ND	–	ND	–	N	N
24	50/M	ED	ND	–	ND	–	ND	–	ND	–	N	N
25	50/M	LD	–	–	–	–	–	–	+	+	Y	N
26	75/M	ED	–	–	–	–	S	S	+	+	Y	Y
27	76/M	ED	ND	LOH	ND	–	ND	–	ND	+	Y	Y
28	55/M	ED	ND	–	ND	–	ND	–	ND	–	N	N
29	64/M	LD	ND	–	ND	–	ND	–	ND	+	Y	Y
30	57/M	LD	–	–	LOH	–	–	–	–	–	N	N
31	68/M	LD	ND	–	ND	–	ND	–	ND	–	N	N
32	48/M	LD	ND	S	ND	–	ND	S	ND	–	Y	N
33	65/M	LD	–	LOH	–	–	–	–	+	–	Y	N
34	50/M	ED	ND	–	ND	S	ND	–	ND	+	Y	Y
35	56/M	ED	ND	–	ND	–	ND	–	ND	+	Y	N

Abbreviations: F – female; M – male; ND – not done; N – absence of molecular alterations at diagnosis time or during the follow-up; Y – Presence of molecular alterations at diagnosis time or during the follow-up; – – absence of S, LOH or TP53 mutations; + – presence of TP53 mutations; Tum – tumor DNA; Pl – plasma DNA.

Survival data

According to stage at diagnosis, 24 patients had LD and 11 patients ED, with an equivalent proportion of LD or ED patients having abnormal plasma DNA in both subgroups. The overall median survival at the end of the follow-up period was 42 weeks (range 4–97 weeks). Nine patients remained alive, with a median follow-up of thirty-three weeks (range 24–73 weeks), all of them displayed molecular alterations in their plasma DNA, and twenty-six patients died after a median survival time of forty-three weeks (range 4–97 weeks). The 10 patients

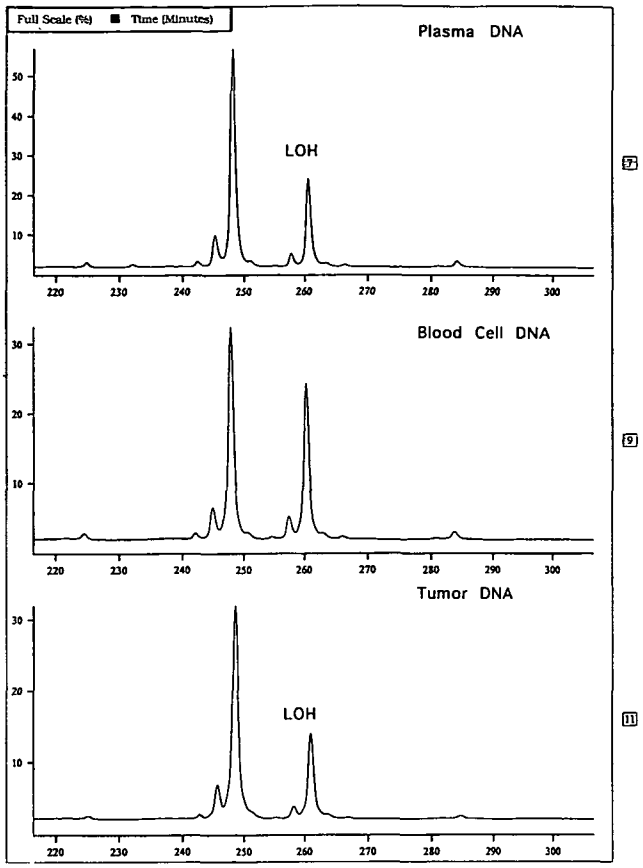

Figure 3. Original tracings from microsatellite analysis from plasma, blood cell and tumor DNA of patient 9, with marker UT762. Plasma and tumor DNA reveal LOH.

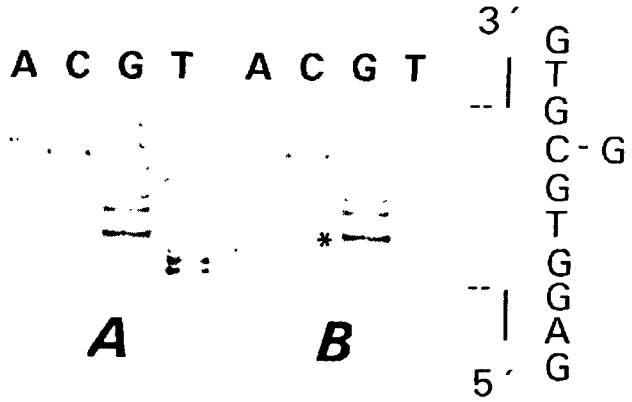

Figure 4. Sequencing analysis in plasma DNA (B) of patient #29 shows a nucleotide change at codon 273 (C to G) in the direct sequencing in exon 8 of the TP53 gene. This nucleotide change causes an amino acid change (Arg-Gly). Panel (A) control sequence.

that showed no aberrant plasma DNA were dead at the end of the study. All deaths were tumor related and non-toxic deaths were registered.

Statistical analysis of survival in the different subgroups revealed the following results: (a), the comparison between patients with aberrant plasma DNA at the time of diagnosis (n = 22; median survival 57 weeks) *versus* those patients with no DNA alterations (n = 10; median survival 46 weeks), showed no statistically significant difference (P = 0.4); (b), patients in whom abnormal plasma DNA disappeared following treatment (n = 11; median survival 33 weeks) and patients with persistence of plasma DNA changes (n = 10; median survival 57 weeks) did not differ significantly (P = 0.5); (c), the survival of patients with only microsatellite alterations (n = 12; median survival 28 weeks) or TP53 gene mutations (n = 5; median survival 59 weeks), did exhibit a statistically significant difference (P = 0.02) regarding patients presenting both types of DNA changes concomitantly (n = 8; median survival 70 weeks) (Figure 5).

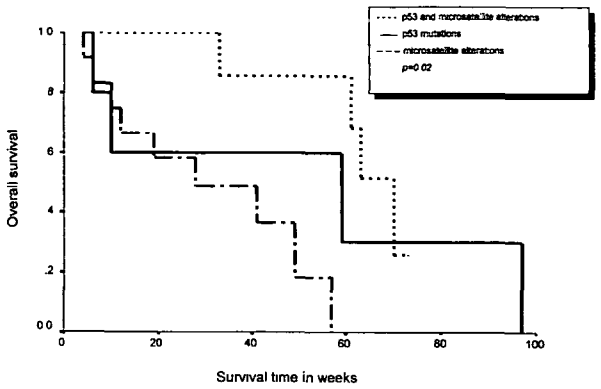

Figure 5. Kaplan–Meier survival curves for patients with only microsatellites changes, or TP53 gene mutations, *versus* patients with both type of alterations concomitantly, in free plasma DNA.

Correlation between clinical development and molecular events

In 15 patients (60%) of the 25 with abnormal plasma DNA, a correlation was observed between the clinical outcome and the presence of plasma DNA changes (Table 2). Among these cases, three different subgroups were distinguished: the first included four patients (patients 5, 15, 29, 34) with partial or complete response of their tumor to treatment and concomitantly the disappearance of the plasma DNA molecular changes, subsequently, with the persistence of partial response or disease recurrence, new appearance of abnormal plasma DNA was observed. The second group consisted of eight patients (patients 8, 11, 13, 14, 17, 21, 26, 27) with no response to treatment and no clearance of the plasma DNA molecular changes. The third group, involved three patients (patients 2, 7, 9) with no molecular alterations at diagnosis and late appearance of plasma DNA changes coincident with disease recurrence. In one of these cases, the presence of *de novo* TP53 gene mutation was detected 12 weeks prior to clinical relapse.

Discussion

Our study confirms the presence of microsatellite alterations similar to those found in tumors in the plasma DNA of SCLC patients [25]. However, it is the first

Table 2. Clinical and molecular profiles of the 15 patients that showed a correlation between plasma DNA alterations and clinical outcome.

Patient	Age/ sex	Stage	Alter. at diag.	Survival time in weeks							
				16	28	40	52	64	76	88	100	112
2	72/M	LD	–	CR/–	CR/–		CR/●		DR/●		DOD	
5	51/M	LD	■●	CR/–	CR/–		DR/■	DR/■	DOD			
7	79/M	LD	–	CR/–		DR/■●		DOD				
8	60/M	LD	■	NR/■	DOD							
9	70/M	LD	–	CR/–		DR/■	DOD					
11	38/F	LD	■	NR/■	NR/■		DOD					
13	63/M	ED	■	NR/■	NR/■		DOD					
14	60/M	ED	■	NR/■	DOD							
15	42/F	LD	■	PR/–	PR/■		DR/■●	DOD				
17	67/M	ED	■	NR/■	DOD							
21	70/M	LD	■	NR/■		NR/■	DOD					
26	73/M	ED	■●	NR/■	NR/■		NR/■●	DOD				
27	76/M	ED	■●	NR/■●	NR/■●		DOD					
29	64/M	LD	●	CR/–		CR/●	DR/●	AWD				
34	50/M	ED	■	PR/–		PR/■●	DR/■●	AWD				

Abbreviations: AWD – alive with disease; DOD – died of the disease; ED – extensive disease; LD – limited disease; ■ – microsatellite alterations; NED – no evidence of disease; NR – no response; CR – complete response; PR – partial response; DR – disease recurrence; M – male; F – female; ● – TP53 mutations.

report of SCLC patients where TP53 mutations were detected in the same patients studied for microsatellite alterations. The presence or absence of plasma DNA with either microsatellite alterations alone, or the presence or absence of TP53 alone, had no influence on patient survival. Only when microsatellite alterations and TP53 were present together was the median survival statistically higher than for patients with no plasma DNA changes.

Although based on relatively small numbers, this apparently paradoxical finding could be analogous to the phenomenon observed on hereditary non-polyposis colorectal cancer, where it was shown that patients with replication errors (RER) had a better prognosis and survival rate than those without RER [39]. A similar correlation between better prognosis and RER positive cancers has also been found in sporadic colorectal carcinoma [40, 41], gastric [42, 43], endometrial carcinoma [44], or cancer in the papilla of Vater [45]. Microsatellite alterations and similarly TP53 mutations, especially in exon 7 or 8, have been found to be associated with poor prognosis in NSCLC [46–49] but to our knowledge no report has been published on joint studies of *p53* mutations and microsatellite instability or LOH on other chromosomes. It does not seem illogical to assume that cells with several genetic defects may be weaker and more easily destroyed by, or be more accessible to, chemotherapy [50]. In conclusion, the role of this association, which demonstrated a statistically significant difference, as a factor for prognosis remains to be established.

Regarding the correlation between the clinical outcome of patients and the variations of aberrant plasma DNA, in 60% of patients the type of response to treatment was in parallel with persistence or disappearance of abnormal plasma DNA, and moreover, in one patient the reappearance of plasma DNA alterations preceded the recurrence of the disease by 12 weeks. The predictive value of a molecular marker as a possible early recurrence indicator that might be of some use in monitoring the response to treatment, also of use in post-treatment follow-up and complete response, is an important aspect to consider.

Our study in small-cell lung cancer shows that aberrations in plasma DNA could sometimes be used as a prognostic factor, an event which has been observed in other malignancies [19, 34], however, larger studies should be made to assess the real value of this fact.

Acknowledgements

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References

1. Ries L, Hankey B, Miller B et al. Cancer Statistics Review 1973–1988. Bethesda: National Cancer Institute 1991 (Table VI-21).
2. Bonfill X, Moreno C, Prada G. Lung cancer mortality among males of Catalonia and Spain compared with other European countries between 1975–1977 and 1987–1989. Int J Cancer 1996; 65; 751–4.
3. El-Torky M, El-Zeky F, Hall JC. Significant changes in the distribution of histologic types of lung cancer. Cancer 1999; 65: 2361–8.
4. Devesa SS, Shaw GL, Blot WJ. Changing patterns of lung cancer incidence by histological type. Cancer Epidemiol Biomark Prev 1999, 1: 29–37.
5. Ihde DC, Pass HI, Glatstein E. Small-cell lung cancer. In de Vita VT, Hellman S, Rosenberg SA (eds): Cancer: Principles and Practice of Oncology. Philadelphia: Lippincott 1997; 911–43.
6. Turrisi AT III, Kim K, Blum R et al. Twice-daily compared with once-daily thoracic radiotherapy in limited small-cell lung cancer treated concurrently with cisplatin and etoposide. N Engl J Med 1999; 340: 265–71.
7. Gazzeri S, Brambilla E, Caron de Fromentel C et al. *p53* genetic abnormalities and *myc* activation in human lung carcinoma. Int J Cancer 1994; 58: 24–32.
8. Przygodzki RM, Finkelstein SD, Langer JC et al. Analysis of *p53*, K-ras-2, and C-raf-1 in pulmonary neuroendocrine tumors. Correlation with histological subtype and clinical outcome. Am J Pathol 1996; 148: 1531–41.
9 Salgia R, Skarin AT. Molecular abnormalities in lung cancer. J Clin Oncol 1998; 16: 1207–17.
10. Mao L, Lee DJ, Tockman MS et al. Microsatellite alterations as clonal markers for the detection of human cancer. Proc Natl Acad Sci USA 1999; 91: 9871–5.
11. Kim SK, Ro JY, Kemp BL et al. Identification of three distinct tumor suppressor loci on the short arm of chromosome 9 in small-cell lung cancer. Cancer Res 1997; 57: 400–3.
12. Merlo A, Gabrielson E, Mabry M et al. Homozygous deletion on chromosome 9p and loss of heterozygosity on 9p, 6p, and 6q in primary human small-cell lung cancer. Cancer Res 1994; 54: 2322–6.
13. Shivapurkar N, Virmani AK, Wistuba II et al. Deletions of chromosome 4 at multiple sites are frequent in malignant mesothelioma and small-cell lung carcinoma. Clin Cancer Res 1999; 5: 17–23.
14. Kim SK, Ro JY, Kemp BL et al. Identification of two distinct tumor-suppressor loci on the long arm of chromosome 10 in small-cell lung cancer. Oncogene 1998; 17: 1749–53.
15. Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer 1983; 51: 2116–20.
16. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977; 37: 646–50.
17. Stroun M, Anker P, Maurice P et al. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology 1989; 46: 318–22.
18. Mulcahy HE, Lyautey J, Lederrey C et al. A prospective study of K-ras mutations in the plasma of pancreatic cancer patients. Clin Cancer Res 1998; 4: 271–5.
19. Castells A, Puig P, Mora J et al. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: Diagnostic utility and prognostic significance. J Clin Oncol 1999; 17: 578–84.
20. Anker P, Lefort F, Vasioukhin V et al. K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. Gastroenterology 1997; 112: 1114–20.
21. Hibi K, Robinson CR, Booker S et al. Molecular detection of genetic alterations in serum of colorectal cancer patients. Cancer Res 1998; 58: 1405–7.
22. Kopreski MS, Benko FA, Kwee C et al. Detection of mutant K-ras DNA in plasma or serum of patients with colorectal cancer. Br J Cancer 1997; 76: 1293–9.
23. Vasioukhin V, Anker P, Maurice P et al. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol 1994; 86: 774–9.
24. Silva JM, Gonzalez R, Dominguez G et al. TP53 gene mutations in plasma DNA of cancer patients. Genes Chromosomes Cancer 1999; 24: 160–1.
25. Chen XQ, Stroun M, Magnenat JL et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nature Med 1996; 2: 1033–5.
26. Sanchez-Cespedes M, Monzó M, Rosell R et al. Detection of chromosome 3p alterations in serum DNA of non-small-cell lung cancer patients. Ann Oncol 1998; 9: 113–6.
27. Nawroz H, Koch W, Anker P et al. Microsatellite alterations in serum DNA of head and neck cancer patients. Nature Med 1996; 2: 1035–7.
28. Goessl C, Heicapell R, Münker R et al. Microsatellite analysis of plasma DNA from patients with clear-cell renal carcinoma. Cancer Res 1998; 58: 4728–32.
29. Silva JM, Dominguez G, Garcia JM et al. Presence of tumor DNA in plasma of breast cancer patients: Clinicopathological correlations. Cancer Res 1999; 59: 3251–6.
30. Chen XQ, Bonnefoi H, Diebold-Berger S et al. Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. Clin Cancer Res 1999; 5: 2297–303.
31. Esteller M, Sanchez-Cespedes M, Rosell R et al. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small-cell lung cancer patients. Cancer Res 1999; 59: 67–70.
32. Wong IHN, Lo YMD, Zhang J et al. Detection of aberrant *p16* methylation in the plasma and serum of liver cancer patients. Cancer Res 1999; 59: 71–3.
33. Silva JM, Dominguez G, Villanueva JM et al. Aberrant DNA methylation of tha $p16^{INK4a}$ gene in plasma DNA of breast cancer patients. Br J Cancer 1999; 80: 1262–4.
34. Yamada T, Nakamori S, Ohzato H et al. Circulating DNA K-ras mutation in pancreatic adenocarcinoma. Clin Cancer Res 1998; 4: 1527–32.
35. Fournié GJ, Courtin JP, Laval F. Plasma DNA as a marker of cancerous cell death. Investigation in patients suffering from lung cancer and in nude mice bearing human tumour. Cancer Lett 1995; 2: 221–7.
36. Maebo A. Plasma DNA level as a tumor marker in primary lung cancer. Jap J Thoracic Dis 1990; 28: 1085–91.
37. Oto M, Miyake S, Yuasa Y. Optimization of nonradioisotopic single strand conformation polymorphism analysis with a conventional minislab gel electrophoresis apparatus. Anal Biochem 1993; 213: 19–22.
38. Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 1989; 5: 874–9.
39. Jass JR, Pokos V, Arnold JL et al. Colorectal neoplasms detected colonoscopically in at-risk members of colorectal cancer families stratified by the demonstration of DNA microsatellite instability. J Mol Med 1996; 74: 547–52.
40. Iacopette BJ, Welch J, Soong R et al. Mutation of the transforming growth factor-beta type II receptor gene in right-sided colorectal cancer: Relationship to clinicopathological features and genetic alterations. J Pathol 1998; 184: 390–405.
41. Lukish JR, Muro K, DeNobile J et al. Prognostic significance of DNA replication errrors in young patients with colorectal cancer. Ann Surg 1998; 227: 52–6.
42. dos Santos NR, Seruca R, Constancia M et al. Microsatellite instability at multiple loci in gastric carcinoma: Clinicopathologic implications and prognosis. Gastroenterology 1996; 110: 38–44.
43. Seruca R, Santos NR, David L et al. Sporadic gastric carcinomas with microsatellite instability display a particular clinicopathologic profile. Int J Cancer 1995; 64: 32–6.

44. Kobayashi K, Sagae S, Kudo R et al. Microsatellite instability in endometrial carcinomas: Frequent replication errors in tumors of early onset and/or of poorly differentiated type. Genes Chromosomes Cancer 1995; 14: 128–32.
45. Achille A, Biasi MO, Zamboni G et al. Cancers of the papilla of vater: Mutator phenotype is associated with good prognosis. Clin Cancer Res 1997; 3: 1841–7.
46. Rosell R, Pifarré A, Monzó M et al. Reduces survival in patients with stage-I non-small-cell lung cancer associated with DNA-replication errors. Int J Cancer 1997; 74: 330–4.
47. Mitsudomi T, Oyama T, Nishida K et al. Loss of heterozygosity at 3p in non-small-cell lung cancer and is prognostic implication. Clin Cancer Res 1996; 2: 1185–9.
48. Huang C, Taki T, Adachi M et al. Mutations in exon 7 and 8 of *p53* as poor prognostic factors in patients with non-small-cell lung cancer. Oncogene 1998; 16: 2469–77.
49. Fong KM, Kida Y, Zimmerman PV et al. Loss of heterozygosity frequently affects chromosome 17q in non-small-cell lung cancer. Cancer Res 1995; 55: 4268–72.
50. Claij N, de Riele H. Microsatellite instability in human cancer: A prognostic marker for chemotherapy? Exp Cell Res 1999; 246: 1–10.

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Correspondence to:
F. Bonilla, MD
Molecular Genetics Unit
Department of Medical Oncology
Clinica Puerta de Hierro
C/San Martin de Porres, 4
28035 Madrid
Spain
E-mail: felixbv@stnet.es