

Mutations of p53 gene can be detected in the plasma of patients with large bowel carcinoma

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Abstract

Aims—To attempt to detect p53 gene mutations in the plasma of patients with large bowel carcinoma.

Methods—Plasma was collected from 20 control patients with no history of cancer and from 17 patients with large bowel carcinoma. Corresponding tumour and benign lymph node (control) samples for each of the carcinoma patients were obtained from paraffin blocks. A Dukes' stage was determined for each tumour. DNA was extracted from the plasma samples and the paraffin embedded tissue using previously described methods. A nested primer polymerase chain reaction protocol was used for the amplification of exons 5 to 8 of the p53 gene. "Cold" single strand conformational polymorphism (SSCP) was performed on mini gels and silver stained. Abnormal bands were excised, the DNA eluted, and reamplified for automated dye termination sequencing. Any sample showing an apparent mutation was rechecked from the original extracted DNA sample at least three times.

Results—p53 gene mutations were not found in the control specimens. They were found in both the primary tumour and the plasma in three cases, in the primary tumour alone in one case, and in the plasma alone in two cases. One of the latter two cases also had metastatic transitional cell carcinoma of the bladder and the other had widespread metastatic deposits. One of the cases with mutant DNA in both the plasma and the primary was a Dukes' stage B tumour. The others were Dukes' C and Dukes' D.

Conclusions—p53 gene mutations can be detected in the plasma of some patients with large bowel carcinoma and these are concordant with those in the primary carcinomas.

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Since the 1970s it has been recognised that small quantities of "free" DNA occur in peripheral blood and may be increased many-fold in cancer patients.¹⁻⁴ Supported by the observation that high levels were associated with a poor prognosis and that the levels tended to decrease with effective treatment, it was suggested that this increased DNA was derived from cancer cells.^{5,6} In 1995 N-ras

mutations were reported in the plasma DNA of leukaemic and myelodysplastic patients.⁶ In 1996 two reports showed concordance of the pattern of microsatellite alterations between the DNA of carcinomas of the head, neck and lung, and the DNA in the plasma.^{7,8} Then in 1997 a report demonstrated concordance of K-ras mutations between colorectal carcinomas and plasma DNA.⁹ Mutations of the p53 gene are probably the commonest gene mutations in human cancers.¹⁰ In this study we attempted to detect them in the plasma of patients with large bowel carcinoma.

Methods

COLLECTION OF SAMPLES

Approximately 30 ml of plasma were collected from 20 control patients under the age of 25 years with no history of cancer, and from 17 patients with large bowel carcinoma. These were stored at -70°C . Corresponding tumour and benign lymph node (control) samples for each of the carcinoma patients were obtained from blocks of 10% formol saline fixed, paraffin embedded tissue. In most cases the paraffin embedded tissue was derived from surgery undertaken close to the time that the plasma was taken, but in a few cases surgery had been several years before. The clinical notes and histology for each case were reviewed to assign an up to date Dukes' stage.

DNA EXTRACTION FROM PLASMA

Plasma (900 μl) was placed into 1.5 ml Eppendorf tubes and Triton X-100 (BDH Chemicals, Poole, UK) added to a final concentration of 1%. The Eppendorf tubes were incubated at 95°C for 20 minutes, then cooled briefly in an ice bath and centrifuged for 20 minutes at 17 800 *g*. The supernatant was recovered and the protein "pellet" discarded.¹¹ Aliquots of this supernatant (1-5 μl) provided a template for subsequent polymerase chain reactions (PCR).

EXTRACTION OF DNA FROM PARAFFIN BLOCKS

Tissue was shaved from the desired region of the paraffin block with a scalpel blade. It was dewaxed by washing twice in xylene and then twice in ethanol. DNA was extracted from the dewaxed tissues according to Gemmell and Akiyama,¹² with some minor alterations. Briefly, tissue was resuspended in 200 μl of 0.8% NaCl and 250 μl of lysis solution with 100 μg of proteinase K and incubated overnight at 56°C . Next, 120 μl of 6 M NaCl and an equal volume of chloroform were added, the samples mixed on a rotating wheel for 20 minutes, and then centrifuged (17 800 *g*) for 10 minutes. The supernatant was retrieved and the

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DNA precipitated by the addition of a half volume of 7.5 M ammonium acetate and 2.5 volumes of 100% ethanol. Finally, the samples were centrifuged for 20 minutes and the DNA pellet washed in 70% ethanol and resuspended in 50 µl of TE buffer.

POLYMERASE CHAIN REACTION

A nested PCR protocol was used for the amplification of exons 5 to 8 of the p53 gene. This was based on a method previously described by Wang *et al* in 1996.¹³ It involved two sets of PCR primers for each of the exons amplified. PCR was performed with 0.8 µM forward and reverse of the first primer sets, 200 µM dNTPs, 3 mM MgCl₂, and 1.25 U of Taq polymerase (Boehringer Mannheim, Mannheim, Germany) in a final reaction volume of 50 µl. An MJ Research DNA Engine (MJ Research, Watertown, Massachusetts, USA) was used with the following thermal cycling conditions: first round at 94°C for two minutes, followed by 25 cycles of 94°C for one minute, 55°C for 30 seconds, and 72°C for one minute. Finally, the tubes were incubated at 72°C for five minutes. The second round, using the second "nested" primer sets, differed only in that a higher annealing temperature of 56°C and only 20 cycles of PCR were used; 1–2 µl of first round PCR product was used as a template in the second round of amplification.

SINGLE STRAND CONFORMATIONAL POLYMORPHISM

Single strand conformational polymorphism (SSCP) was performed as described by Wang *et al* with only minor alterations.¹³ Briefly, 5–10 µl of the PCR product was mixed with 15 µl of 95% formamide stop buffer and denatured at 95°C for 10 minutes. Following denaturation, the sample tubes were plunged into an ice bath for two minutes. The samples were loaded onto a 0.8 mm thick 8 × 8 cm native 15% acrylamide gel with 10% glycerol and electrophoresed for five hours at 200 V (30 mA) on a Hoefer Mighty Small 2 Minigel Apparatus (Hoefer Scientific Instruments, San Francisco, California, USA) at 19°C. Following electrophoresis

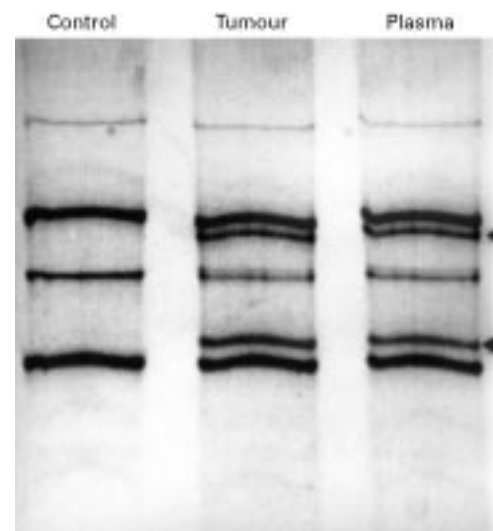


Figure 1 A silver stained polyacrylamide SSCP gel from case 1 (exon 5). The lanes from the tumour and plasma contain identical bands that are not present in the control lane. Sequencing showing a mutation TGC > TAC at base 141.

the gels were silver stained using a previously described method.¹⁴

Bands showing conformational polymorphism were excised from the SSCP gels, destained, the DNA eluted and reamplified with the second round PCR primers using the PCR conditions described above.¹⁵ These PCR products were purified using Promega Wizard Prep Kits (Promega, Madison, Wisconsin, USA) and an aliquot used as a template for dichlororhodimine terminator chemistry on an ABI prism automated sequencer (Perkin-Elmer, Melbourne, Australia).

The analysis for each case was repeated at least twice and in those in which a mutation was found the analysis was repeated from the original extracted DNA sample at least three more times.

Results

The results are presented in table 1. None of the plasma samples from the 20 control patients without cancer showed a mutation, and none of the benign lymph nodes of the carcinoma patients showed a germline mutation. Mutations were identified in at least one of the samples from six of the cases. All samples showing a conformational polymorphism on SSCP were found to have a mutation on sequencing and in those cases in which conformational polymorphisms were found in the primary tumour and in the plasma, the pattern of polymorphism and the sequenced mutation were identical (fig 1).

Discussion

Mutations of the p53 gene were found in the plasma of patients with colorectal carcinoma and these were concordant with those found in the primary tumours on both SSCP and sequencing.

For a marker to be useful it must have a high degree of specificity and sensitivity. Plasma p53 gene mutations appear to be highly specific, as mutations were not detected in control patients

Table 1 The detection of p53 mutations in the primary tumours and plasma of 17 patients with large bowel carcinoma

Patient	Dukes stage	Mutation				
		Sample	Exon	Base	Change	Consequence
1	B	T+P	5	141	TGC→TAC	Cys→Try
2	C	T+P	7	248	CGG→CTG	Arg→Leu
3	A	–	–	–	–	–
4	B	–	–	–	–	–
5	A*	P	5	147	GTT→ATT	Val→Ile
6	D	T+P	5	175	CGC→CAC	Arg→His
7	C	T	7	241	C deletion	Frame shift
8	C	–	–	–	–	–
9	D	–	–	–	–	–
10	D	–	–	–	–	–
11	D*	P	8	279	GGG→GAG	Gly→Glu
12	B	–	–	–	–	–
13	D	–	–	–	–	–
14	A	–	–	–	–	–
15	D	–	–	–	–	–
16	A	–	–	–	–	–
17	D	–	–	–	–	–

P, plasma; T, primary tumour.

*Case 5 had other neoplasms including poorly differentiated transitional carcinoma of the bladder. Case 11 had metastatic disease from a primary four years earlier.

and the mutations detected in the plasma of the carcinoma patients were exactly concordant with those detected in the primary tumours. However, the theoretical maximum sensitivity is poor, as p53 gene mutations occur in only 50% of human cancers. The most powerful approach would probably be to screen for a panel of genetic abnormalities including p53 mutations, Ras mutations, and microsatellite alterations.

Mutations could be detected in the plasma of patients with Dukes' stage B carcinoma, indicating the potential to detect small tumour masses before they present clinically. A previous study was able to detect K-ras mutations in stage B carcinomas.⁹

Case 5 showed a mutation in the plasma but not in the primary (Dukes' stage A) tumour. This patient was known to also have metastatic poorly differentiated carcinoma that was thought to have arisen from the bladder.

Case 11 had a mutation only in the plasma sample. We repeated the analysis of the case many times but we were not able to show a mutation in the corresponding tumour sample. A possible explanation is that a subclone of metastatic tumour has acquired a p53 mutation that was not present in the primary tumour four years previously. However, this seems not to be a common event as a previous study found no evidence of subclonality in examining the pattern of p53 gene mutation in various metastatic deposits.¹⁶ It was suggested that this was because p53 gene mutations are a relatively early event in the development of carcinomas. Another explanation could be that the patient has another tumour, such as a lung carcinoma, that has not yet been detected clinically.

The complexity of the method that we used in our study made it time consuming and expensive. It was necessary to perform SSCP, band excision, elution, and reamplification because most tumours with p53 gene mutations have one normal p53 allele and this, together with the heavy contamination of the plasma with normal DNA, would have made it impossible to sequence the mutant PCR product directly. However, having demonstrated the validity of SSCP in the detection of mutations in the plasma one could justifiably use SSCP alone. Another approach would be to screen the primary carcinoma for a p53 gene mutation and, if present, use it to design a primer set incorporating the mutation at the 3' end of one primer. These primers could then be used to detect the presence of mutant DNA in the plasma as a test for tumour recurrence after surgery or other treatment. The plasma assay would then be reduced to a simple PCR test. Alternatively, the usual primers could be used

and then the product could be dot blotted with a radioisotope labelled oligonucleotide probe specific for the mutation found in the primary tumour.

Techniques have been described for the detection of gene mutations (particularly ras) in faecal material.¹⁷⁻¹⁹ These have been advocated as possible screening tests for early colonic carcinomas and adenomas. Analysis of gene mutations in the plasma is unlikely to detect preinvasive neoplasms, as sufficient DNA is unlikely to enter the blood stream. Nevertheless, plasma DNA mutations will probably be found in association with malignancies in a wide variety of organs, and the analysis of plasma may prove to be a comprehensive strategy for the investigation of patients with a suspected malignant neoplasm.

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