Microsatellite abnormalities in plasma of patients with breast carcinoma: concordance with the primary tumour

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Abstract

Aims—To detect microsatellite abnormalities in the primary tumours and plasma of patients with breast carcinoma.

Methods—Plasma was obtained from 17 breast carcinoma patients before surgery. Corresponding tumour and benign lymph node (control) samples for each of the carcinoma patients were obtained from paraffin blocks. DNA was extracted from the plasma samples and the paraffin embedded tissue using previously described methods.

Results—The 17 primary tumours showed two examples of loss of heterozygosity and three examples of microsatellite instability; the 17 plasma samples showed three and one, respectively. Many of the longer microsatellites (over 200 base pairs) were difficult to amplify from plasma. The investigations suggested that this was because of the highly fragmented nature of plasma DNA. Only one example of loss of heterozygosity and one example of microsatellite instability showed a concordant pattern in both primary tumour and plasma. These were both in the same patient.

Conclusions—DNA mutations concordant with those in the primary carcinomas can occasionally be detected in the plasma of patients with breast carcinoma. However, the frequency would have to be markedly improved before this could be of any diagnostic value.

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Keywords: tumour markers; breast carcinoma; polymerase chain reaction; microsatellite

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The aim of our present study was to analyse the plasma of patients with breast carcinoma to see if there was evidence of mutant DNA from the tumour in the plasma. We did this by examining a set of microsatellite loci for evidence of loss of heterozygosity and evidence microsatellite instability.

Methods

COLLECTION OF SAMPLES

Plasma samples (approximately 1.5 ml) were collected from 17 patients with breast 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% formal saline fixed, paraffin embedded tissue. The paraffin embedded tissue was derived from surgery that was performed one or two days after the plasma was taken. There were no specific selection criteria for the patients in the study other than they were female, had a definite preoperative diagnosis of breast carcinoma, and were prepared to volunteer for the study.

PLASMA DNA EXTRACTION

We attempted two methods of DNA extraction from plasma. In the first method 150 μl of a 5% Chelex 100 solution (Sigma-Aldrich) was added to 50 μl of plasma, and incubated at 56°C for 30 minutes. The solution was then vortexed at high speed for 10 seconds and incubated in a boiling water bath for eight minutes. Again the solution was vortexed at high speed for 10 seconds, centrifuged for 10 minutes at 15 000 g, and stored at 4°C . The supernatant was removed as required for polymerase chain reaction (PCR) reactions.

In the second method, 630 μl of plasma was placed into 1.5 ml Eppendorf tubes and triton X-100 (BDH Chemicals) was added to a final concentration of 1%. The Eppendorf tubes were incubated at 95°C for 20 minutes and centrifuged for 20 minutes at 17 800 g. The supernatant was recovered and the protein pellet discarded. Aliquots of this supernatant (2-5 μl) provided a template for subsequent PCR reactions. We used the Chelex 100 method for most of the samples, for reasons outlined in Discussion.

EXTRACTION OF DNA FROM PARAFFIN BLOCKS

Tissue was cut from the desired region of the paraffin block with a scalpel blade. This was dewaxed by washing twice in xylene and then twice in ethanol. DNA was extracted from the

The small quantities of "free" DNA in plasma are often increased in cancer patients.¹⁻⁴ It was thought that this increased DNA could be derived from cancer cells. High levels were associated with poor prognosis, and the levels decreased with treatment.^{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} Concordance of K-ras mutations between colorectal carcinomas and plasma DNA was described in 1997.⁹ In 1998 our research group described concordant p53 gene mutations in plasma and large bowel carcinoma.¹⁰

Table 1 Primer sequences and annealing temperatures used in the screening of breast cancer DNA

Primer name	Primer sequence: forward (F), reverse (R)	Annealing temperature
AR (X)	5'-TCCGCGAAGTGATCCAGAAC-3' (F) 5'-CTTGGGGGAGAACCATTCTCA-3' (R)	56°C
ACTBP2	5'-AATCTGGGGACAAGAGTGA-3' (F) 5'-ACTTCTCCCTACCGCTATA-3' (R)	60°C
UT762	5'-GTCAGTATTACCCTGTACCA-3' (F) 5'-GTTGAGGATTTTTCATCAGT-3' (R)	57°C
D14	5'-AACACCCCTAATTACCACT-3' (F) 5'-ATGATTCCACAAGATGGCAG-3' (R)	54°C
DRLPA	5'-CACAGTCTCAACACATC-3' (F) 5'-CCTCCAGTGGGTGGGAAATGCTC-3' (R)	53°C
FgA	5'-CCATAGGTTTTGAACCTACAG-3' (F) 5'-CTTCTCAGATCCTCTGACAC-3' (R)	55°C
15q11	5'-TTGACCTGAATGCACCTGTGA-3' (F) 5'-TTCCATACCTGGGAACGAGT-3' (R)	55°
GGAA4D07	5'-GAGAGGGCAAGACTTGAAG-3' (F) 5'-ATGGAAGAGCGTTCTAAAACA-3' (R)	57°C
GGA2E02	5'-AGGAAAGAGAAAGAAAGGAAG-3' (F) 5'-TATATGATGAAAGTATATTGGGG-3' (R)	57°C
D18S34	5'-CAGAAAATTCTCTCTGGCTA-3' (F) 5'-CTCATGTTCTTGGCAAGAAT-3' (R)	57°
D17S513	5'-CATTCACTTGTGGGCTGCTGTC-3' (F) 5'-TGTAAGAAAGGCTCCACAAAGCA-3' (R)	57°C

dewaxed tissues according to Gemmell *et al.*,¹¹ 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 and the samples were mixed on a rotating wheel for 20 minutes and then centrifuged (17 800 g) for 10 minutes. The supernatant was retrieved and the 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, the DNA pellet washed in 70% ethanol, and resuspended in 50 µl of TE buffer (Tris HCL 10 mM plus EDTA 1 mM).

POLYMERASE CHAIN REACTION

PCR was performed to amplify microsatellites that had shown high incidences of abnormali-

Case 10: FgA

Figure 1 Denaturing polyacrylamide gel analysis for case 10. Lane N is the product from the lymph node DNA, T is from the primary tumour, and P is from the plasma. There is loss of heterozygosity for ACTBP2 and a band shift for FgA, indicating microsatellite instability.

ties in previous studies, many of them on breast carcinomas (table 1).¹²⁻¹⁷ The reaction mix consisted of 2 µl of DNA solution, 0.8 µM of the reverse primer, 0.13 µM of end labelled ³³P forward primer, 200 µM each dNTP, 0.625 U of Taq polymerase (Boehringer Mannheim), and 25 µl of amplification buffer. A DNA engine (MJ Research) was used, with the following thermal cycling conditions: first round at 94°C for five minutes, followed by 35 cycles of 94°C for 30 seconds, 53–60°C (depending on the primer set used) for 30 seconds, and 72°C for 30 seconds. Finally, the tubes were incubated at 72°C for five minutes then stored at 4°C for polyacrylamide gel analysis.

DENATURING POLYACRYLAMIDE GEL ANALYSIS

Samples were run on a 6% denaturing polyacrylamide gel as described by Mao *et al.*¹⁸ Briefly, the PCR products were mixed with an equal volume of 95% gel loading buffer and denatured at 95°C for five minutes. A 5 µl aliquot of each sample was loaded onto a 0.2 mm thick, preheated, denaturing 6% polyacrylamide gel and electrophoresed at 60 W (1500 V) for 90 minutes. Following electrophoresis the gel was transferred to Whatman No 4 blotting

Table 2 Results of PCR of 11 microsatellite loci in samples of normal DNA, tumour, and plasma from 17 breast carcinoma patients

	1	2	3	4	5	6	7	8	9	10	11
Type	ILC	IDC	ITC	IDC	IDC	IDC	IDC	IDC	IDC	ILC	IDC
Size (mm)	14	11	4	40	4	12	62	30	22	15	60
Metastatic deposits				Ax N		Ax N	Ax N				Ax N
AR(X)	N T P	N T P	N T P	N T P	N T P	N T P	N T P	N T P	N T P	N T P	N T P
ACTBP2	+ L -	+ + +	+ - +	+ + -	- + +	+ + -	+ + -	+ + -	+ + -	+ L L	+ L -
UT762	+ + -	+ + +	+ + L	+ + +	0 0 0	0 0 0	+ + -	+ + -	+ + -	+ + +	- - -
D14	+ + +	+ + +	+ + +	+ + +	- + +	+ + +	+ + -	+ + +	+ + -	+ + +	- + -
DRLPA	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	- - -
FgA	0 0 0	+ - -	0 0 0	0 0 0	0 0 0	+ + -	+ + -	+ + -	+ + -	+ M M	- - -
D17S513	0 0 0	+ + +	0 0 0	0 0 0	+ + -	+ + -	+ + -	+ + -	+ + -	+ + +	+ - -
D18S34	0 0 0	+ + +	0 0 0	0 0 0	+ + +	+ + +	+ + -	+ + +	+ + -	+ + +	+ + -
GGA2E02	0 0 0	+ + +	- + 0	0 0 0	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
GGA4D07	0 0 0	+ + -	0 0 0	0 0 0	- + +	0 0 0	0 0 0	+ + +	0 0 0	+ - -	- + +
15q11	0 0 0	+ + +	0 0 0	0 0 0	+ + +	+ + +	+ + -	+ + +	+ + +	+ + +	+ + -

Ax N, axillary lymph node metastases; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IMC, invasive mucinous carcinoma; ITC, invasive tubular carcinoma; L, loss of heterozygosity; M, microsatellite instability; N, benign lymph node sample; P, plasma sample; T, tumour sample; +, successful amplification; -, unsuccessful amplification; 0, amplification not attempted.

Figure 2 Denaturing polyacrylamide gel analysis for case 3. There is loss of heterozygosity in the plasma only for ACTBP2 for UT762.

paper and dried at 80°C for one hour on a gel drier. Dried gels were then exposed to Kodak scientific imaging film (type [X-OMAT] AR) for up to four days. The film was developed using Kodak developer and fix/stop solutions, then analysed for microsatellite instability and/or loss of heterozygosity.

Results

The results are presented in table 2. Briefly of the 495 analyses attempted, 227 failed either because the PCR repeatedly failed to give a product (147) or because there was insufficient sample to attempt an analysis (80). Often repeated attempts at PCR for one microsatellite resulted in there being insufficient plasma sample to complete all of the other microsatellite PCR analyses. The larger microsatellites such as FgA had a particularly high frequency of failed PCR. Most of the failed PCR analyses occurred in the plasma samples.

Microsatellite abnormalities were found in the tumour alone in three cases. In the tumour and plasma of case 10 there was loss of heterozygosity for ACTBP2 and a band shift for FgA (fig 1). In case 3 there was an loss of heterozygosity in the plasma only for ACTBP2 for UT762 (fig 2).

Table 2 continued

12			13			14			15			16			17		
IDC 65			IDC 15			IDC 61			IMC 32			IDC 12			IDC 13		
Ax N																	
N	T	P	N	T	P	N	T	P	N	T	P	N	T	P	N	T	P
-	+	+	0	0	0	0	0	0	+	+	-	+	+	+	+	+	+
-	+	+	0	0	0	+	+	0	+	M	-	+	+	+	+	+	+
0	0	0	+	+	0	+	+	0	+	+	-	+	+	+	+	+	+
-	-	+	+	+	0	+	+	0	+	+	-	+	+	+	+	+	+
0	0	0	+	+	0	+	+	0	+	+	-	+	+	+	+	+	+
0	0	0	+	+	0	+	+	0	+	+	-	+	+	+	+	+	+
+	-	-	0	0	0	+	+	0	+	+	-	+	+	+	+	+	+
-	+	+	0	0	0	+	+	0	+	+	-	+	+	+	+	+	+
-	-	+	+	+	0	+	+	0	+	+	+	+	+	+	+	+	+
-	+	+	+	M	0	-	+	0	0	0	0	+	+	+	0	0	0
+	+	+	+	+	0	+	+	0	+	+	+	+	+	+	+	+	+

Discussion

A major problem in our study was the failure of PCR amplification, particularly from the plasma samples. We tried to investigate the reason for this, as one might expect the plasma to have yielded better quality template than the formalin fixed, paraffin embedded tissue. We found that the plasma samples often had quite high concentrations of DNA on fluoroscopic measurement (typically 10 to 40 µg/ml of plasma) but Southern blotting using a probe for the human specific repetitive Alu sequence (300 base pairs) suggested that this DNA was highly fragmented compared with DNA extracted from tissue samples. This was supported by a serial dilution experiment comparing template DNA extracted from paraffin embedded tissue with template from plasma (fig 3). During this study we had examined the efficiency of a triton method and also a standard sodium dodecyl sulphate/proteinase K method, and found that our 5% Chelex 100 method yielded 10 to 100 times that of the other two methods. However, when we compared the PCR products of the Chelex 100 and triton extracts we found little difference, suggesting that much of the “extra” DNA obtained by the Chelex 100 method was of poor quality. The particularly poor results for FgA may be because it was one of the longest targets (approximately 270 base pairs). On the other hand 15q11, with a target of about 90 base pairs, performed much better. In a previous study looking for p53 gene mutations in plasma we found the amplifications from plasma samples to be relatively unproblematic and it was the formalin fixed, paraffin embedded tissue that was hard to get good results from. A nested primer reaction had to be used for the paraffin embedded tissue but not for the plasma.¹⁰ This may be because all of the p53 exons that were amplified were quite short.

Arising from our investigation of the frequent failure of the PCR from the plasma samples came the observation that at low template concentrations one could observe artefactual loss of heterozygosity (fig 3). This “allele drop out” is a potential source of confusion in the interpretation of PCR amplification from poor quality templates in this and other studies. This may be the explanation for the loss of heterozygosity seen in ACTBP2 and UT762 for case 3. Alternative explanations for the findings in case 3 include the development of a subclone in metastatic tumour with a microsatellite abnormality that was not present in the primary tumour that we sampled. However, it is very unlikely that metastatic disease was present, given that the primary was a small tubular carcinoma. Another possibility was that the patient could have an entirely separate tumour in another organ but a review of the clinical notes a year after the excision of the breast lesion showed no evidence of a second tumour. Chen *et al* described a similar finding in the plasma of one of their cases and explained it as being a likely consequence of the development of a subclone.⁷ One should also be aware that the administration of donor blood products could also give rise to artefactual microsatellite abnormalities

Figure 3 The amplification products of serial dilutions of DNA extracts from formalin fixed, paraffin embedded tissue and from plasma. The first lane of the paraffin extract is the polymerase chain reaction (PCR) product for ACTBP2 (approximately 290 base pairs) from 22 ng of template DNA, and the first lane of the product from plasma is from 24 ng of template. There are five subsequent lanes for paraffin and plasma, with approximately fourfold increases in the template dilution. It appears that there is markedly less efficient amplification from the plasma DNA. In addition there appears to be artefactual loss of heterozygosity in the sixth lane of the paraffin samples, owing to unequal amplification of the two alleles at low template concentrations.

in the plasma samples. None were given to case 3 around the time of her operation.

There is wide variation in the reported frequency of microsatellite abnormalities in breast carcinoma. In 1997 Dillon *et al* reviewed published reports and found that microsatellite instability was detected in a total of 36 of 276 tumours (13%)¹⁹; the number of instances of microsatellite instability was only 64 of 2499 (2.5%). Some studies found none at all and others found microsatellite instability in 85% of tumours. In their own study of a large series of breast carcinomas, Dillon *et al* found microsatellite instability in 13 of 733 invasive carcinomas (1.8%) but loss of heterozygosity in 75/164 specimens (46%).¹⁹ We tried to select microsatellite loci that had been shown to have a high frequency of abnormalities in previous studies, particularly in breast carcinomas. For example Paulson *et al* found 22 of 39 breast carcinomas had microsatellite abnormalities for either GGAA2E02 or GGAA4D07 loci.¹⁵ We found only one abnormality for GGAA4D07 and none for GGAA2E02. Yang *et al* found abnormalities for D17S513 in three of 11 breast carcinomas under 2 cm and nine of 10 carcinomas greater than 2 cm.¹² We had hoped that by selecting the microsatellites in our study carefully we would obtain a similarly high frequency of abnormalities in the primary tumours that would then make the examination of the plasma potentially informative. However, we found only six abnormalities in 17 cases, despite examining up to 11 different microsatellites. Another frustration was that corresponding plasma samples could be successfully amplified for only two of these. These two both showed identical abnormalities in the plasma. These two abnormalities both occurred in the same tumour (case 10), which was a lobular carcinoma measuring 1.5 cm across. One showed loss of heterozygosity and the other microsatellite instability. This patient has no evidence of metastatic tumour eight months after surgery, although she did have anaemia of unknown cause. She died about a year after surgery from numerous other medical problems.

The discovery of concordant abnormalities in the plasma and the primary tumour in this case shows that if the technical aspects could be

improved then an examination of the plasma for microsatellite abnormalities might have a role in the assessment of breast lesions. However, there would have to be quite a marked increase in the frequency of detectable microsatellite abnormalities in the plasma. Selection of short microsatellites targets, less than about 120 base pairs, would probably improve the frequency of successful amplification from plasma DNA.

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