

ORIGINAL ARTICLE

Limitations of the use of single base changes in the p53 gene to detect minimal residual disease of breast cancer

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In recent years, breast cancer has been the most common indication for peripheral blood progenitor cell (PBPC) transplantation in the USA. Despite this, few randomised studies have been performed to assess the efficacy of this procedure. As demonstrated by gene marking studies,¹ malignant cells may contaminate progenitor cell harvests and be reinfused during transplantation to cause disease relapse. Several methods to detect such minimal residual disease are available, and broadly divided under categories of immunocytochemistry, tissue culture, and molecular or polymerase chain reaction (PCR) based techniques, all with a wide range of sensitivities. The last of these can potentially achieve the highest sensitivity because it can exponentially amplify an extremely small amount of DNA. The methods studied more extensively in breast cancer use lineage associated markers, namely members of the cytokeratin (CK) family, particularly CK19, to detect epithelial cells not normally found within bone marrow or PBPC harvests. Although the sensitivities of these methods are high, false positives can occur. For example, using reverse transcriptase PCR (RT-PCR) to detect transcripts of the CK19 gene, false positive results occurred in bone marrow from patients with chronic myeloid leukaemia² and in normal subjects.³ Another approach is to use a marker specific to the tumour rather than the lineage. In theory, methods using tumour specific markers should benefit from a high degree of specificity, but are unable to determine the viability of the tumour cells, which can only be demonstrated by tissue culture based techniques.^{4,5}

"Malignant cells may contaminate progenitor cell harvests and be reinfused during transplantation to cause disease relapse"

Background/Aims: Peripheral blood progenitor cell (PBPC) transplantation is frequently used in the treatment of malignant diseases, but contamination of the graft by tumour cells is a real concern and may lead to disease relapse. The feasibility of applying heterogeneous single base genetic changes as tumour specific markers to detect minimal residual disease in PBPC harvests was studied, using the p53 gene and breast cancer as models.

Methods: Tumour tissues from 51 patients with cellular aliquots from PBPC harvests available were studied. Thirty eight patients had metastatic disease or were at high risk of metastasis, and 13 had high risk stage II/III disease with four or more involved axillary lymph nodes. Tumour DNA was screened for p53 mutations in exons 5 to 9, using denaturing gradient gel electrophoresis, followed by sequencing. Based on sequence information, allele specific primers were designed for each mutation and the non-radioisotopic, amplification refractory mutation system (ARMS) was used to screen DNA from PBPC harvests for minimal residual disease. Attempts were made to optimise each system, based on parameters determined using the T47D breast cancer cell line with a confirmed point mutation in codon 194.

Results: Twelve different somatic mutations were found, two of which could not be sequenced. The remainder were point mutations. Only five of the 10 ARMS systems were successfully optimised, and minimal residual disease detection sensitivities ranged from one copy of tumour DNA in 10² to 10³ copies of wild-type DNA. Using ARMS, three of five patients and eight of 12 of their PBPC harvests showed minimal residual disease.

Conclusions: These results suggest that the use of single base genetic changes in minimal residual disease detection is relatively insensitive and is limited to a small number of patients and to certain mutations. In addition, it is labourious and therefore unlikely to play an important role in clinical practice.

Mutations in the p53 gene in breast cancer are relatively common and tumour specific. We studied the application of a PCR based technique, the amplification refractory mutation system (ARMS), to amplify this genetic marker and to enable the detection of minimal residual disease in PBPC harvests. p53 mutations have been used to study minority tumour clones during clonal expansion or evolution,⁶⁻⁹ but, unlike ras,¹⁰ p53 mutation is used infrequently to detect minimal residual disease by means of the ARMS technique. We hypothesised that this approach would combine a high sensitivity with broad applicability to this group of patients. In essence, the study involved screening primary breast tumours for p53 mutations using denaturing gradient gel electrophoresis (DGGE), and sequencing the mutations of those demonstrating an aberrant banding pattern. From this information, allele specific primers were designed to amplify the mutant but not the wild-type (WT) allele in ARMS.

ARMS is a useful, PCR based technique for the detection of known mutations. Essentially, it involves the use of allele specific primers that amplify mutant but not WT DNA sequences, or vice versa. In most applications, two sets of PCRs using one common primer and two allele specific primers corresponding to WT or mutant alleles, respectively, are used. In our study, specifically for the detection of mutant tumour sequences, we designed allele specific primers for the mutant sequences only.

Abbreviations: ARMS, ARMS, amplification refractory mutation system; CK, cytokeratin; DGGE, denaturing gradient gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; PBPC, peripheral blood progenitor cell; RT-PCR, reverse transcriptase polymerase chain reaction; Ta, annealing temperature; WT, wild-type

The use of p53 mutations in breast cancer served as a model to assess the feasibility of using subtle genetic alterations as markers for the detection of minimal residual disease that could be applied to other genes and other malignancies. Here, we describe our experience with this approach using breast cancer and p53 mutations as models, and discuss the limitations encountered.

METHODS

Patients

Two groups of patients with breast cancer were studied: group 1 consisted of 37 patients with known metastatic disease; and group 2 included 13 patients randomised into a prospective Anglo-Celtic Cooperative Oncology Group study, which compared high dose chemotherapy and PBPC transplantation with conventional chemotherapy as adjuvant treatment. One additional patient (B16), who was deemed to be at high risk of metastasis because of regional recurrence after primary treatment, was studied. Patients in the metastatic group have a poorer prognosis by nature of their disease stage, and often have a higher incidence of p53 mutations, which are an independent marker of poor prognosis.¹¹ Patients were selected from the second group by the fact that they were randomised to receive high dose chemotherapy so that PBPC harvests were available to be studied. The number of patients in this group is relatively small because cases were accrued at an early stage of our study. Written informed consent was obtained from all patients.

Of the 51 primary breast tumours studied, 22 were frozen sections taken at the time of initial surgery and stored at -70°C and 29 were paraffin wax embedded sections. Progenitor cell mobilisations were performed using cyclophosphamide (4 gm/m^2 body surface area) and filgrastim ($300\text{ }\mu\text{g}$), as described previously.¹² Cellular aliquots from each harvest procedure were stored at -70°C and DNA was extracted from these.

Mutation detection

DNA was prepared from the primary tumour tissue of each patient by a spin column/membrane DNA adsorption technique (Qiagen, Crawley, UK). The p53 gene was screened for mutations by GC clamped DGGE after PCR amplification of five individual DNA fragments corresponding to exons 5–8, using primer sequences modified from Borresen *et al.*¹³ Exon 5 was screened in two fragments (5a and 5b; table 1). All reactions were performed in 35 cycles of 94°C , followed by 59°C (exon 5a) or 55°C (exons 5b, 6, 7, and 8) and 72°C with $0.25\text{ }\mu\text{M}$ of each primer, $200\text{ }\mu\text{M}$ of each deoxynucleotide triphosphate (dNTP), and 1 U of Taq polymerase in a $50\text{ }\mu\text{l}$ reaction volume. DGGE was performed on a polyacrylamide gel with a linear denaturing gradient with concentrations from 30% to 80%, where 100% denaturant corresponded to 7M urea and 40% formamide,¹⁴ and crosslinked with N,N'-diallyltartardiamide. Electrophoresis was performed at 200 V and 60°C for six hours. For samples showing an aberrant banding pattern, the mutant homoduplex band was excised directly from the gel, the DNA eluted,¹⁵ reamplified, purified, and sequenced commercially by the Sanger dideoxy technique on an automated ABI Prism sequencer (Oswel, Southampton, UK).

ARMS

For tumours in which a p53 mutation was characterised, ARMS primers (20 bp), incorporating the mutation at the 3' base, were designed for PCR. Depending on the position of the mutation, one of the primers used to amplify the initial exon fragment was chosen as the second primer. ARMS products were size separated by polyacrylamide gel electrophoresis (PAGE) and visualised by ethidium bromide staining. Using a breast cancer cell line, T47D, which has a missense mutation CTT to TTT in codon 194, various parameters were adjusted to optimise the

Table 1 PCR primers for the amplification of p53 exons 5 to 8

Exon amplified	Primer sequence
5aGC	5'-*GTG TGA CTG CTT GTA GAT-3'
5a	5'-CTC TGT CTC CTT CCT CTT-3'
5bGC	5'-*GTT CCA CAC CCC CGC CCG GCA-3'
5b	5'-GCC CCA GCT GCT CAC CAT CG-3'
6GC	5'-*GGA GAG ACG ACA GGG CTG GTT-3'
6	5'-AGT TGC AAA CCA GAC CTC AGG C-3'
7GC	5'-**CTA CCT CGC TTA GTG CTC CCT-3'
7	5'-CAC CAT CCA CTA CAA CTA CA-3'
8GC	5'-**CTA CCT CGC TTA GTG CTC CCT-3'
8	5'-ATC CTG AGT AGT GGT AAT CT-3'

*GC clamp sequence (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC-3') was added to the 5' end of exons 5aGC, 5bGC, and 6GC primers; **GC clamp sequence (5'-GCG GGC GGC GCG GGG CGC GAG GGC GGC GGC GGC GGC GGC GGC-3') was added to the 5' end of exons 7GC and 8GC primers.

Table 2 Sequence information on tumours containing p53 mutations

Patient	Exon	Codon no.	Codon change	Amino acid change
B3	5b	171	GAC → TAG	Glu → End
B11	5b	175	CGC → CAC	Arg → His
B12	6	213	CGA → CGG	Arg → Arg
B15	6	213	CGA → CGG	Arg → Arg
B16	6	193	CAT → GAT	His → Asp
B27	8	267	CGG → CAG	Arg → Glu
B28	7	248	CGG → CAG	Arg → Gln
B30	6	198	GAA → TAA	Glu → End
B33	7	248	CGG → CAG	Arg → Gln
B37	5b	179	CAT → CGT	His → Arg
B45	8	273	CGT → TGT	Arg → Cys
B46	6	216	GTG → TTG	Val → Leu
B50	6	216	GTG → ATG	Val → Met

ARMS reaction. These were: annealing temperature (T_a), magnesium concentration, primer concentration, dNTP concentration, the addition of glycerol or formamide reaction cofactors, and an additional mismatch at the 3' end of the ARMS primer. ARMS was applied to clinical samples from each patient in whom a p53 mutation was detected. Each system was optimised on the above parameters, and used to screen PBPC harvests for evidence of minimal residual disease. Reactions were performed in triplicate using $1\text{ }\mu\text{g}$ of DNA.

RESULTS

Mutation detection

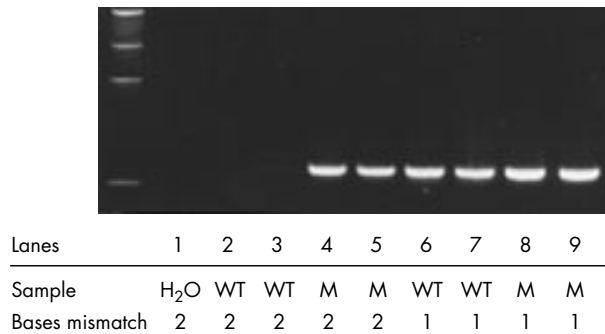
An abnormal banding pattern on DGGE was detected in 14 individual patients, one of whom had an abnormal pattern in two different p53 fragments. Of these 15 positive lanes on DGGE, 13 had a clearly defined four band pattern, representing mutant and WT homoduplexes and two mutant–WT heteroduplexes. The remaining two cases showed a distinct band that co-migrated with WT on DGGE and two extra bands with greatly different mobilities. These were rescreened after TaqI restriction endonuclease digestion of the GC clamped PCR products to reveal shorter DNA fragments with significantly different melting profiles, representing mutant homoduplexes (data not shown). The distribution of mutations among the five fragments were: five in exon 5b, six in exon 6, two in exon 7, and two in exon 8. Twelve were found in the metastatic group and three in the adjuvant group. Despite repeated sequencing, two of 15 samples gave inconclusive results (patients B1 and B15), and clear mutant sequences could not

Table 3 Optimised ARMS conditions for 11 tumours and the T47D cell line with a p53 mutation

Patient/cell line	3' mismatch	2nd mismatch	Product size	Ta (°C)	Mg conc. (mM)	No. of PCR cycles
T47D	T-G	A-A	127 bp	64	1.75	35
B3	A-G	A-A	87 bp	64	2	35
B11	G-T	C-T	140 bp	67	1.5	35
B16	G-G	C-C	130 bp	64	2	35
B27	A-C	A-G	143 bp	60	2	35
B28*	G-T	G-G	78 bp			
B30	T-C	C-C	115 bp	56	2	35
B33*	G-T	G-G	78 bp			
B37	A-C	C-T	112 bp	69	1.75	33
B45	T-G	C-C	125 bp	60	1.75	35
B46*	A-G	C-T	170 bp			
B50*	G-T	T-C	170 bp			

*ARMS failed to optimise.

ARMS, amplification refractory mutation system; Ta, annealing temperature.

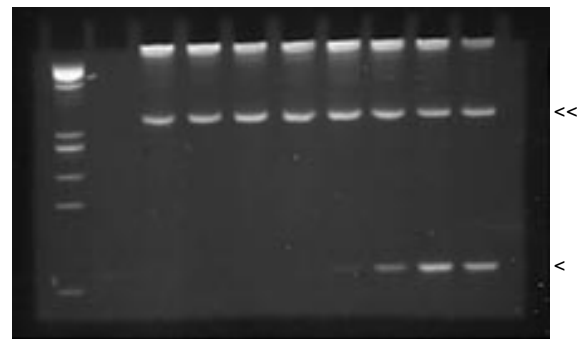
**Figure 1** Comparison of the amplification refractory mutation system (ARMS) using primers with a one or two base mismatch. ARMS primers specific for the p53 mutation in the T47D cell line were used under identical reaction conditions and the PCR products from the one or two base mismatched primers were analysed by polyacrylamide gel electrophoresis and visualised by ethidium bromide staining. Lane 1 represents the water control (H₂O) without DNA. The lane on the left contains ϕ X174/HaeIII molecular weight markers. M, mutant tumour DNA; WT, wild-type DNA.

be determined. Of the remaining 13, two were found to have an identical constitutional silent polymorphism in codon 213 (CGA → CCG, arg → arg).¹⁶ This was confirmed by DGGE screening of PBPC samples (representative of “normal” tissues) from these patients indicating the same abnormal banding (data not shown). Therefore, 11 positive samples were able to serve as markers for minimal residual disease. Two patients had an identical mutation in codon 248, hence 10 individual mutations were recognised, all of which were single base, missense mutations resulting in an amino acid change (table 2).

Optimisation of ARMS conditions

To improve the specificity of the ARMS reaction for the mutant sequence, several PCR conditions were optimised to enhance the differential yield of mutant over WT amplicons. Optimisation was carried out on T47D DNA and on all patient mutant sequences. Each mutant sequence required a unique set of reaction conditions (table 3). These could not be predicted from the position of the base pair mismatch or from the type of base involved. However, it was noted that certain reaction conditions were more effective than others at improving the specificity of the ARMS reaction. In descending order of importance they were as follows.

(1) Second mismatch at the 3' end of the ARMS primer. The choice of both mismatches was based on the destabilisation ranking described by Newton *et al.*¹⁷ A second base mismatch

**Figure 2** Sensitivity of the amplification refractory mutation system (ARMS) using a primer with a two base mismatch. DNA from mixtures of T47D cells in normal peripheral blood leucocytes was amplified by a T47D ARMS primer with a two base mismatch and the reaction products separated by polyacrylamide gel electrophoresis and visualised by ethidium bromide staining. A 360 bp internal standard PCR control was included to monitor the efficiency of the amplification reaction. The detection limit was 1/10³ leucocytes. The lane on the left contains the ϕ X174/HaeIII molecular weight markers. The blank lane represents the water control (H₂O) without DNA. < indicates the 127 bp ARMS product; << indicates the 360 bp internal PCR control product. T, T47D tumour DNA; WT, wild-type DNA.

improved the specificity of the T47D ARMS reaction substantially (fig 1) and was applied subsequently to all ARMS primers designed for clinical samples (table 3).

(2) Further reduction in WT DNA amplification during the ARMS reaction was achieved through adjustment of Ta in the range 56°C to 69°C (table 3).

(3) Reaction specificity was improved by adjusting the magnesium concentration over a range 1.5mM to 2.5mM (table 3).

(4) Titration of other reagents within the PCR reaction including primer concentration over a range 0.25 μ M to 0.025 μ M, and dNTP concentration over a range 200 μ M to 3.125 μ M did not improve the specificity of the ARMS reaction.

(5) The addition of reaction cofactors—formamide (2.5–10%) or glycerol (5–20%)—was either inhibitory or did not improve the specificity of the ARMS product.

In summary, the most crucial condition was the introduction of an additional mismatch at the penultimate base of the

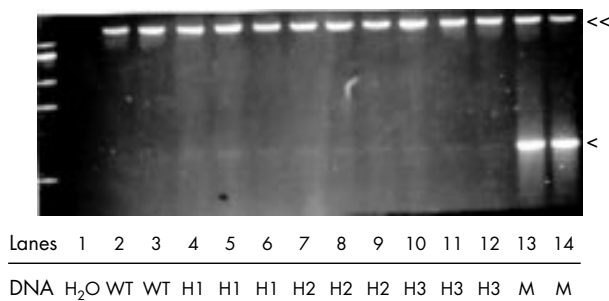


Figure 3 Amplification refractory mutation system (ARMS) screening of peripheral blood progenitor cell (PBPC) harvests in patient B11. Faint bands across all three PBPC harvests (H1, H2, and H3) in triplicate indicate the presence of minimal residual disease. The lane on the far left contains the ϕ X174/HaeIII molecular weight markers. The blank lane represents water control (H₂O) without DNA. <math><<</math> indicates the 140 bp ARMS product; \ll indicates the 360 bp internal PCR control product. M, mutant tumour DNA; WT, wild-type DNA.

mutant specific primer, this was followed by Ta and magnesium concentration as factors most likely to influence the specificity of the ARMS product.

To determine the sensitivity of ARMS to detect a minority population of tumour cells in normal cells, suspensions of T47D cells were mixed with peripheral blood leucocytes in 10 fold serial dilutions. Repeated experiments determined a sensitivity of detection in the order of one tumour cell in 1000 leucocytes (fig 2).

Study of clinical material

Clinical samples were studied initially by optimising an ARMS reaction for each of the 10 missense mutations (patients B3, B11, B16, B27, B28 (=B33), B30, B37, B45, B46, and B50). Of these 10 ARMS systems, three could not be optimised (B33, B46, and B50). Essentially, the amplification of WT and mutant DNA templates produced identical product yields. Of the remaining seven patients, only five could be optimised to detect minority mutant DNA species. Optimisation of the remaining two patients (B27 and B45) was difficult, such that when good specificity was achieved, the PCR product yield was too low to be of value in minimal residual disease detection. ARMS reactions for patients B3, B11, B16, B30, and B37 were optimised and could be used in minimal residual disease detection (table 3). For each patient, under optimal reaction conditions, the sensitivity of minimal residual disease detection was determined by preparing 10 fold serial dilutions of mutant DNA in WT DNA. The detection sensitivities achieved were limited to one part mutant DNA in 100–1000 parts WT DNA.

Five optimised ARMS were used to detect minimal residual disease in aliquots of PBPC harvests. Banding patterns on PAGE indicative of the presence of minimal residual disease were detected in three of five patients (B3, B11, and B37) and eight of 12 harvests. In two patients, evidence of minimal residual disease was seen in each PBPC harvest in all triplicate tests (fig 3), whereas in one patient (B3) one of three triplicate reactions detected minimal residual disease in each harvest.

DISCUSSION

The main purpose of our study was to assess the feasibility of using single base mutations, specifically in the p53 gene, as molecular markers for the detection of minimal residual disease. Rather than exclusively advocating p53 as a potential marker for this purpose, we used p53 simply as a model, and the findings of our study could be applied to other genes in which subtle alterations are present in malignant cells. p53 was chosen in this study for three main reasons, namely:

- (1) It is the single most frequently mutated gene in human cancers—one in five or more sporadic breast cancers have an alteration.^{11 18–21}
- (2) A large subgroup of the patient cohort selected for PBPC collection (patients with metastatic disease) had an adverse prognosis, or demonstrated aggressive or extensive disease, and historically this group has a higher incidence of p53 mutation.
- (3) The heterogeneous location and nature of p53 mutations, with most (79%) being single base mutations located in hot spot regions corresponding to exons 5–8, with both transversions and transitions represented,^{22 23} posed a major challenge but made this a good system to test the feasibility of such an approach.

The ras oncogene family has been used more often in the context of minimal residual disease detection, but differs from p53 by having mutations occurring largely within a small number of codons (codons 12, 13, and 61),¹⁰ hence reducing the complexity of ARMS.

“The main limitation to a more sensitive system is the inability of the amplification refractory mutation system to differentiate effectively between wild-type and mutant DNA”

The PCR primers for initial screening of p53 mutations ensured that approximately 90% of reported mutations in breast cancer were detected by DGGE.²⁴ The incidence of somatic p53 mutations in our patient cohort (approximately a quarter of the cases) was similar to other published series. Of 11 samples successfully sequenced, four were found in recognised hot spot codons (codons 175, 248 (two patients), and 273), consistent with other studies on sporadic breast cancer.²⁴ However, the number of patients amenable to the study of minimal residual disease was greatly reduced by (1) the failure to obtain reliable sequence information (two of 15); (2) the presence of constitutional polymorphisms (two of 15); and (3) a failure to optimise the ARMS stage fully (four of 15). Only a small number of studies describe methods to optimise ARMS, mostly based on models with one or a small number of single base changes.^{25–28} Our study attempted to optimise a heterogeneous system of mutant markers. Because of the subtle nature of the genetic alterations, only half of the 10 evaluable systems were sufficiently optimised. This experience is at variance with other studies. Whereas our results suggested that the important factors were the use of an additional mismatch in the ARMS primer, Ta, and the concentration of magnesium, others reported that the concentrations of magnesium and the primer were more crucial.²⁹ Ta and the dNTP concentration were reported to have little influence on optimising the reaction.^{25 30 31} Although pyrimidine–pyrimidine and purine–purine mismatches at the 3' end of a primer are most refractory to strand extension, this was not an important factor in determining ARMS optimisation.^{30 31}

Applying ARMS to the detection of minimal residual disease requires a high “discrimination sensitivity” between WT and mutant DNA. Reported sensitivities range from 1/100³¹ to 1/1000,⁸ 1/10,²⁸ and 1/10⁵,³² when radioisotopes are used, or glycerol added. Our range of sensitivities of 1/100 to 1/1000 falls within this range, and the main limitation to a more sensitive system is the inability of ARMS to differentiate effectively between WT and mutant DNA. Optimisation experiments for each ARMS system ensure that WT DNA is not amplified, but at the same time reduce the efficiency of amplifying the mutant species. DNA extracted from stored, paraffin wax embedded pathological specimens may be of inferior quality compared with that from fresh tumours, but we found no evidence that such DNA is not efficiently amplified by PCR. Moreover, tumour DNA quality would be more

Take home messages

- The detection of single base genetic changes to assess minimal residual disease in breast cancer is a complex process, which is labour intensive and therefore unlikely to play an important role in clinical practice
- In addition, it is relatively insensitive, lacks sensitivity, and is limited to a small number of patients and to certain mutations
- Reverse transcriptase polymerase chain reaction methods may be more suited to the detection of minimal residual disease

likely to influence the initial PCR, DGGE, and sequencing, but not the sensitivity of ARMS to detect minimal residual disease. Each ARMS reaction used 1 µg of genomic DNA, which contained approximately 3×10^5 copies of an autosomal gene.²⁸ p53 alterations occur commonly with loss of heterozygosity, resulting in only 1.5×10^5 copies/1 µg DNA. On the assumption that ARMS can amplify one single molecule of mutated p53 within 1 µg of template DNA, the maximum sensitivity would be in the order of 1/10⁵, and probably much lower in practice when non-radioisotopic methods are used to visualise the products, as our results suggest.

In conclusion, the detection of minimal residual disease using p53 missense mutations is a multistep procedure requiring mutation detection, sequencing, and ARMS optimisation. We found that this approach was labour intensive, lacked specificity, and was relatively insensitive. RT-PCR techniques, which use a much larger number of copies of nucleic acid, may be a more suitable vehicle to study minimal residual disease in these patients.³²⁻³⁴

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