

ORIGINAL ARTICLE

Detection of BRAF mutations in colorectal tumours and peritoneal washings using a mismatch ligation assay

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Aims: To detect cells bearing BRAF mutations in colorectal tumour samples and peritoneal washings, using a mismatch ligation assay (MLA).

Methods: DNA from 46 colorectal tumours was studied. Part of exon 15 of the BRAF gene was amplified using the polymerase chain reaction, and T→A mutations at codon 600 were detected using MLA. When a mutation was detected, the same mutation was sought in peritoneal washings from that patient.

Results: BRAF mutations were detected in five of the 45 analysable tumour samples. In four cases, this result was confirmed by sequencing analysis. More tumours with BRAF mutations were Dukes' stage C or D rather than A or B ($p < 0.02$). Dilution experiments revealed that one mutant cell could be detected in 1000 normal cells. Cells with the same BRAF mutation were present in the peritoneal washing taken at the start of the operation in four of the five patients.

Conclusions: MLA is a suitable technique for the detection of BRAF mutations, and allows the detection of small numbers of isolated tumour cells shed from the primary tumour.

BRAF belongs to a family of genes encoding serine/threonine kinases that are involved in mediating responses to growth factors, and are regulated by binding to K-ras. BRAF has recently been shown to undergo somatic missense mutation in about two thirds of melanomas and a smaller proportion of other human cancers, including around 10% of colorectal cancers.¹ Most (about 95%) of the mutations identified are in the form of a single base substitution (T→A) at position 1799 (NT_007914), which is in codon 600 of the protein²—for example, 60 of 63 BRAF mutations detected in one study.³ Colorectal cancers with BRAF mutations do not have K-ras mutations.⁴ BRAF mutations have been shown to occur at a similar stage in the adenoma–carcinoma sequence to K-ras mutations,⁵ but may be associated with an earlier tumour stage.

“The mismatch ligation assay is a technique used for the detection of malignant cells against a background of normal cells, by the identification of particular DNA mutations”

Previous descriptions have used direct sequencing techniques for the identification of BRAF mutations. These techniques are useful when a large proportion of cells in a sample has a particular mutation. However, for samples where a few mutant cells are present against a background of large numbers of normal cells, the excess wild-type base on the chromatogram obscures the presence of a mutant base. This may occur with unselected samples of whole tumour, or when attempting to detect isolated malignant cells shed from the primary tumour into lymph nodes, blood, or body cavities. Techniques that could detect disseminated cells with BRAF mutations would be particularly applicable for tumour types where a high proportion of cells have BRAF mutations, such as malignant melanomas.

The mismatch ligation assay (MLA) is a technique used for the detection of malignant cells against a background of normal cells, by the identification of particular DNA mutations. It involves the amplification of the gene under study by the polymerase chain reaction (PCR), and the

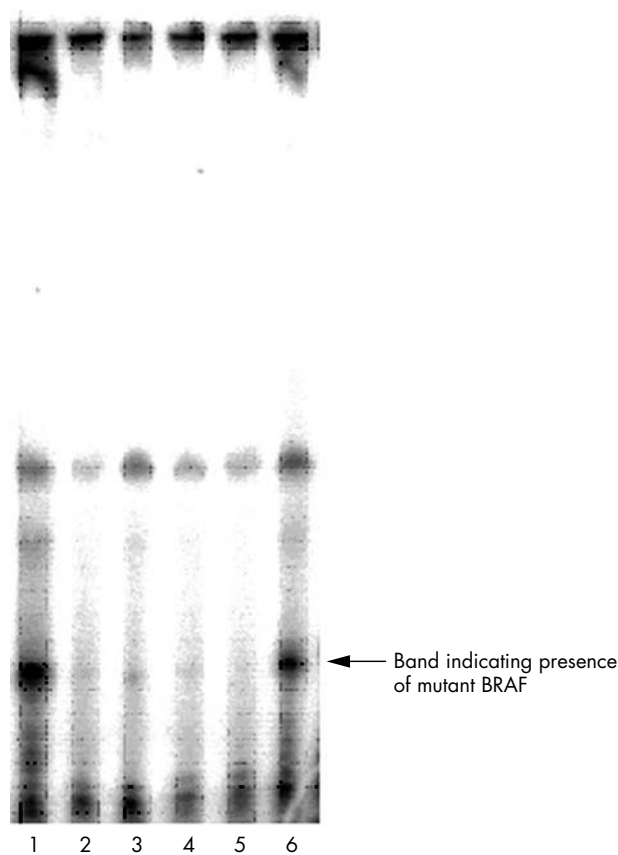


Figure 1 Example of a mismatch ligation assay (MLA) to detect mutant BRAF. Lane 1, positive control (COLO 205); lane 2, polymerase chain reaction product from DNA of healthy volunteer; lanes 3–6, tumours 30–33. Clear bands are seen in lanes 1 and 6.

Abbreviations: MACS, magnetic activated cell separation; MLA, mismatch ligation assay; PCR, polymerase chain reaction

detection of mutant DNA in the PCR product, by the ability of T4 ligase to join adjacent oligonucleotides (one of which is radiolabelled) only when they are matched accurately to the complementary strand.⁶ It has been used to detect K-ras and p53 mutations, in addition to alterations in mitochondrial DNA. DNA with these mutations has been detected in blood, faeces, aberrant crypt foci, and lymph nodes.⁷⁻¹¹

We describe the use of MLA to detect BRAF mutations in colorectal tumours and peritoneal washings from patients with these tumours.

METHODS

Ethical approval for our study was obtained from the South Warwickshire local research ethical committee, and all patients gave informed consent. Tumour samples (43 primary colorectal cancers, two severely dysplastic adenomas, and one recurrent colorectal cancer) were taken from surgical specimens and snap frozen in liquid nitrogen. A peritoneal washing with 0.9% saline was made at the start of the procedure, red blood cells were removed by density centrifugation, and epithelial cells in the sample were enriched by magnetic activated cell separation (MACS).¹² Epithelial cells were first labelled using mouse antihuman Ber EP4 antibodies (specific for epithelial cells; Dako A/S, Glostrup,

Denmark) and then with a secondary goat antimouse antibody conjugated to magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The epithelial enriched cells were stored at -80°C until use.

DNA was extracted from tumours (after grinding under liquid nitrogen) and epithelial enriched cells using a Qiagen DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Primers were designed using Primer Designer (Scientific and Educational Software, Durham, North Carolina, USA) software to amplify a short segment of exon 15 of BRAF (including the mutation hotspot at codon 600), and PCR was performed. Appropriate products could be generated from all but one tumour.

The MLA was performed using conditions similar to those described previously by Jen *et al.*⁸ Briefly, each reaction contained approximately 50 ng of PCR product combined with 25 ng of mutation specific oligonucleotide, 200 ng of blocking oligonucleotide (with a sequence complementary to that of wild-type BRAF), and 2 ng of a ³²P 5' end labelled oligonucleotide designed to anneal immediately 3' of the mutation site. The 20 µl reaction mixture also contained 1mM spermidine, 3 µg of T4 gene 32 protein (a single stranded DNA stabilising protein; Roche, Mannheim, Germany), and an appropriate volume of T4 DNA ligase buffer, with the volume being made up with 0.9% saline. The mixture was heated to 95°C for five minutes, and allowed to cool to room temperature for 15 minutes. One unit of T4 ligase (MBI Fermentas/Helena Biosciences, Tyne and Wear, UK) was added to each tube and incubated at 37°C for one hour, followed by heat inactivation at 68°C for 10 minutes. One unit of alkaline phosphatase (together with the dephosphorylation buffer supplied; Roche) was added to each tube, and incubated at 37°C for 30 minutes, to detach any unincorporated ³²P. The reaction products were separated on a 12% denaturing polyacrylamide gel, and visualised using a phosphoimager.

Primer and oligonucleotide sequences used were as follows: PCR sense primer, ACC CAC TCC ATC GAG ATT TC; antisense primer, GGT GAT TTT GGT CTA GCT AC; MLA mutation specific oligonucleotide, TAG CTA CAG A; blocking oligonucleotide, ACA GTG AAA TC; and end labelled oligonucleotide, GAA ATC TCG A.

Positive controls for MLA included PCR products from a cell line known to have the relevant BRAF mutation¹ (COLO 205; ATCC (American Type Culture Collection) number CCL-222). PCR products from the DNA of a healthy volunteer or a tumour shown to have wild-type BRAF on sequencing were used as negative controls. Each tumour sample was tested at least twice, using products from independent PCRs. When a BRAF mutation was detected in a primary tumour sample, the peritoneal washing from the same patient was tested for the presence of mutant BRAF. Sequencing analysis (Applied Biosystems Big Dye Terminator version 3.1, 3100 genetic

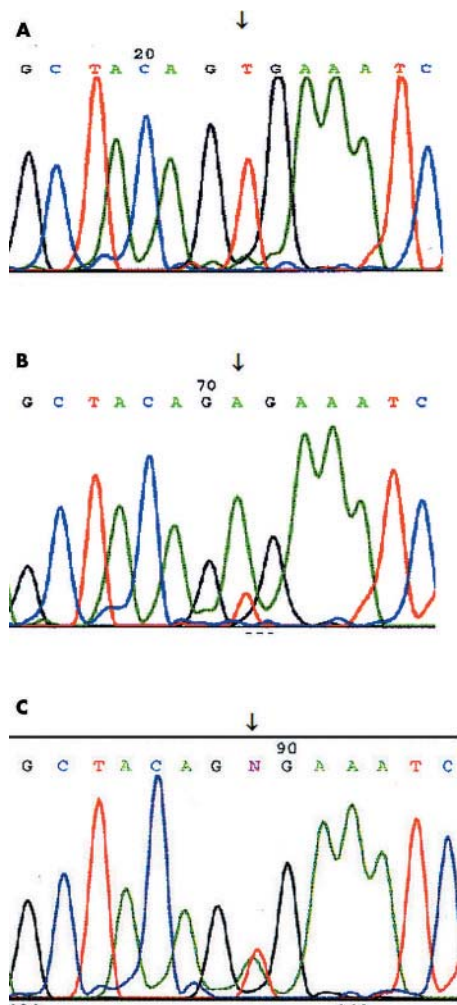


Figure 2 (A) Sequencing result for tumour 1. This shows wild type BRAF with T at position 1799 (arrow). (B) Sequencing result for the COLO205 cell line. This cell line is positive for the V600E mutation (arrow). (C) Sequencing result from tumour 48 with the V600E mutation shown by the arrow.

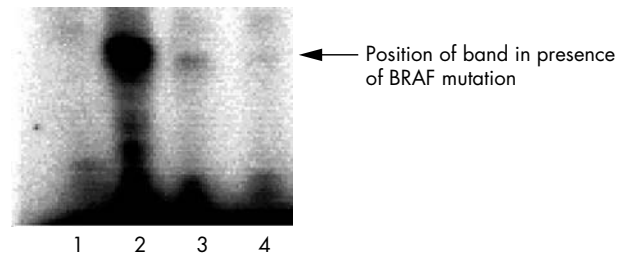


Figure 3 Results of spiked samples tested in the BRAF mismatch ligation assay (MLA). Lane 1, negative control; lane 2, positive control; lane 3, one mutant cell in 1000 normal cells; lane 4, one mutant cell in 3200 normal cells.

Table 1 Characteristics of tumours with BRAF mutations

No.	Dukes' stage	TNM stage	Age/Sex	Sequencing results	Site	Peritoneal sample
21	C1	T3N2MX	73/Male	T→A	Transverse	Positive
27	D	T4N2M1	63/Female	Wild type	Rectum	Negative
33	C1	T3N1MX	54/Male	T→A	Transverse	Positive
35	C1	T3N1MX	60/Female	T→A	Left	Positive
48	C2	T4N2MX	76/Female	T→A	Right	Positive

analyser; Applied Biosystems, Kiel, Germany) was performed to confirm BRAF mutations detected by MLA, using the primers described for exon 15 by Davies *et al.*¹

A series of samples, with increasing dilutions of COLO 205 cells in peripheral blood mononuclear cells from a healthy volunteer, was prepared to test the sensitivity of the MLA technique in detecting cells with BRAF mutations.

RESULTS AND DISCUSSION

Initial experiments confirmed the ability of the MLA to distinguish between DNA samples containing BRAF mutations and those with wild-type BRAF. Figure 1 is an example of a gel showing the results of an MLA with DNA from tumour containing a BRAF mutation.

BRAF mutations were detected by MLA in five of the 45 analysable tumour samples. Results were consistent on repeated testing. In four cases, the mutation was confirmed on direct sequencing analysis. Figure 2 shows examples of sequencing results from a tumour with wild-type BRAF, COLO 205, and one of the tumours with a BRAF mutation. In the tumour with a BRAF mutation found on MLA but not confirmed by sequencing, it may be that the mutant cells were in a minority, so that the presence of the mutation was masked by an excess of normal DNA. We performed sequencing analysis on 25 of the tumours giving negative results on MLA. All showed the wild-type sequence.

“This methodology has a wide applicability, especially in the context of malignant melanoma, where two thirds of tumours bear BRAF mutations”

These results are similar to those found in sequencing studies, with about 10% of colorectal tumours bearing the relevant mutation. Although some workers have suggested that tumours with BRAF mutations are associated with an earlier Dukes' stage,³ we did not confirm these findings. Four of the five BRAF mutant tumours were Dukes' stage C, with the other being Dukes' D, and the BRAF mutant tumours in our series were significantly more likely to be stage C or D, rather than A or B, than those without such a mutation (5/5 v 15/38 primary cancers). The data are significant ($p < 0.02$, Fisher's exact test), although the sample is small. The mismatch repair status of our tumours is unknown, and one explanation for the difference in stages could be a lower rate of microsatellite unstable tumours in our BRAF positive group than in other series. However, the patients' clinical data do not provide evidence to support this notion (table 1). These results indicate that the techniques we have described are able to distinguish between wild-type BRAF and that with a T→A mutation at position 1799, at least in those samples where a high proportion of cells would be expected to contain the mutation. An estimate of the sensitivity of the technique was obtained by “spiking” peripheral blood mononuclear cells of a healthy volunteer with known numbers of COLO 205 cells. We found that MLA could detect the BRAF mutation when there was one mutant cell for each 1000 normal cells (fig 3).

We applied the MLA technique to detect cells shed from these tumours into the peritoneal cavity. Any epithelial cells in the sample were first enriched using MACS. This process has the additional advantage of ensuring that only DNA from intact cells is studied, rather than DNA released from damaged cells. In four of five patients whose tumour had the relevant BRAF mutation, DNA with the same mutation could be found in cells from the peritoneal washing taken at the start of surgery. Table 1 shows the clinical details, sequencing result, and peritoneal washing status of the patients whose tumours showed BRAF mutations on MLA.

We hypothesised that the detection of malignant cells in the peritoneal cavity would act as a marker for likely local recurrence. We were surprised to find such a high proportion (four of five) with such cells and the clinical relevance of our results await further follow up. However, all these patients had either lymph node or distant metastases at the time of sample collection. MLA does not give information on the number of these mutant cells present or their viability, so that the methodology may be too sensitive to give clinically useful information. Perhaps the additional use of MACS, with at least a 20 fold enrichment of epithelial cells (data not shown), contributes to this.

We have shown that the MLA technique is useful for detecting BRAF mutations in colorectal tumours. Because of its sensitivity, it is also applicable to the detection of isolated cells shed from the primary tumour. This methodology has a wide applicability, especially in the context of malignant melanoma, where two thirds of tumours bear BRAF mutations. MLA could be used to look for cells that have disseminated from these tumours into other tissues, such as lymph nodes.

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Take home messages

- BRAF mutations were detected by the mismatch ligation assay (MLA) in five of 45 analysable colorectal tumours, and were confirmed by sequencing analysis in four cases
- Cells with the same BRAF mutation were present in peritoneal washings in four of the five patients
- More tumours with BRAF mutations were Dukes' stage C or D rather than A or B
- Dilution experiments revealed that one mutant cell could be detected in 1000 normal cells
- Thus, MLA is useful for detecting BRAF mutations in colorectal tumours, and because of its sensitivity can be used to detect isolated cells shed from the primary tumour

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