Frequency of allele loss of DCC, p53, RBI, WT1, NF1, NM23 and APC/MCC in colorectal cancer assayed by fluorescent multiplex polymerase chain reaction

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Summary We report here the use of multiplex fluorescent polymerase chain reaction (PCR) for quantitative allele loss detection using microsatellites with 2–5 base pair repeat motifs. Allele loss of APC, DCC, p53, and RBI in colorectal tumours has been reported previously using a variety of methods. However, not all workers used intragenic markers. We have used microsatellite polymorphisms which map within, or are closely linked to, these tumour-suppressor gene loci in order to determine whether these loci are indeed the targets for alteration in colorectal cancer. In addition, we have assayed two other tumour-suppressor genes, WT1 and NF1, to see whether they play a role in colorectal carcinogenesis. The putative metastasis-suppressor gene, NM23, was also investigated since there have been conflicting reports about its involvement in colorectal carcinogenesis. Allele loss was detected at the DCC (29%), p53 (66%), RBI (50%) and NF1 (14%) loci and in the APC/MCC region (50%), but not at the WT1 or NM23 loci. These rapid, and mostly gene-specific, fluorescent multiplex PCR assays for allele loss detection could be modified to devise a single molecular diagnostic test for the important lesions in colorectal cancer.

The progression of a colorectal tumour is a multistage process involving activation of oncogenes and inactivation of tumour-suppressor genes (Fearon & Vogelstein, 1990). If we are to establish which are the most important genes in this pathway we must be able to assay specific genes rapidly and easily.

The p53, adenomatous polyposis coli (APC), deleted in colorectal cancer (DCC) and retinoblastoma (RBI) genes are tumour-suppressor genes which are implicated in this pathway. To lose their suppressing effect, tumour-suppressor genes have to undergo allele loss to uncover recessive mutations in the remaining allele. Although loss of heterozygosity is accepted to be a later event than mutation of a tumour-suppressor gene, it is more widely studied because it is easier to assay. Most tumour-suppressor genes display a wide array of mutations and therefore require more time-consuming assays. Allele loss assays are used to determine which chromosome sites have undergone reduction to homozygosity. There have been several reports on APC (5q21), DCC (18q21) and p53 (17p13) allele loss in colorectal tumours, but not all used specific intragenic sequence polymorphisms to determine whether the tumour-suppressor gene itself had been lost. In almost all cases where intragenic restriction fragment length polymorphisms (RFLPs) have been used detection involved the use of a radiosotopic Southern blot analysis. This type of assay requires large amounts of good-quality DNA and is therefore not suitable for the study of paraffin-embedded specimens, is time consuming and is usually subjective rather than quantitative. Therefore rapid and reliable gene-specific quantitative PCR assays for allele loss need to be developed in order that the true significance of suppressor genes involved in colorectal cancer can be confirmed on large series.

Most previous workers assessed the p53 gene region for allele loss using Southern blot analysis. The frequencies of allele loss of the p53 region found using this method ranged from 48% (Lothe et al., 1992) to 68% (Meling et al., 1993). RFLP probes and Southern blot analysis of the APC/MCC region yielded allele loss frequencies of 31% (Neuman et al., 1991) to 48% (Ashton-Rickardt et al., 1989). Again, most previous work on allele loss of DCC was done using RFLP probes and Southern analysis, with frequencies ranging from 66% (Barletta et al., 1993) to 75% (Ookawa et al., 1993). However, a study using the same primers [DCC (1)] as our study, which amplify an intragenic region, revealed an allele loss frequency of only 33% (Huang et al., 1993).

Involvement of the RBI gene (13q14) in colorectal cancer has been shown by previous studies. Meling et al. (1991) showed that RBI was altered in 35% of cases, using an intragenic probe, but noted that both amplification and deletion occurred. Ookawa et al. (1993) used markers at the RBI locus and found allele loss in 46% of cases.

The importance of the NM23 gene (17q22) in colorectal carcinogenesis is controversial since both allele loss and protein overexpression have been found, and other workers have found no evidence of allele loss. Using RFLP probes allele loss of NM23 was shown in 52% of cases by Cohn et al. (1991), but in only 13% of cases by Wang et al. (1993). However, Whitelaw and Northover (1994) found no evidence of allele loss of NM23 in 34 informative cases. Further studies of NM23 expression have confounded the picture since increased expression of NM23 has been found in 80–88% of colorectal tumours (Haut et al., 1991; Myeroff & Markowitz, 1993). Evidence for mutational activation, as with p53, has not been found (Myeroff & Markowitz, 1993; Wang et al., 1993).

There are no reports to our knowledge on allele loss of the Wilms' tumour gene WT1 (11p13) or the neurofibromatosis type 1 gene NF1 (17q11), which are both tumour-suppressor genes, in colorectal cancer. The NF1 gene product is involved in the ras signal transduction pathway and could therefore be of importance in other cancers. The NF1 protein has GTPase-activating protein (GAP) activity, which can down-regulate ras. There has been one study of NF1 in colorectal cancer, which looked at mutations of the gene (Li et al., 1992), and in the one case of mutated NF1 found the mutant protein had 200–400 times reduced GAP activity.

The drawbacks to the use of RFLPs in allele loss studies include the amount and quality of DNA required and the length of time to produce results when using radiosotopic detection methods. Microsatellites are DNA sequence polymorphisms which exhibit length polymorphisms and are usually highly informative (Weber & May, 1989). Allele loss can be detected using microsatellites in a radioactive PCR assay (Jones & Nakamura, 1992; Gruis et al., 1993). We recently developed a fluorescence-based microsatellite PCR assay to detect and quantitate allele loss (Cawkwell et al., 1993) which made several improvements to the radiosotopic...
assay. Using this assay with intragenic markers where possible, we aimed to assess the possibility of multiplexing a number of markers to simplify such studies. We went on to determine the frequency of allele loss specific to DCC, p53, RB1, WTI, NFI, NM23 and the APC/MCC region. To our knowledge no microsatellite polymorphisms have as yet been identified at the APC locus and thus for this region, which also contains the MCC gene, we had to use markers which are closely linked to APC. Wherever possible two microsatellite markers for each locus were assayed. The use of a four-colour detection system enabled us to devise multiplex PCR assays in order to assess allele loss at several loci simultaneously.

We aimed to develop a panel of rapid gene-specific assays in order to assess the importance of the above genes in colorectal cancer.

**Materials and methods**

**Samples**

Freshly frozen samples of colorectal adenocarcinomas were obtained from 20 patients at Leeds General Infirmary between 1983 and 1987. Fresh normal colorectal tissue taken from as far away from the tumour as possible was also obtained from each patient. The range of age at operation was 36–82 years, and 11 of the patients were male. There were 15 left-side and 5 right-side tumours. There were one Dukes stage A, nine Dukes stage B and ten Dukes stage C tumours. Sections were stained with haematoxylin and eosin and assessed by an experienced gastrointestinal pathologist. All tumour sections used contained at least 50% malignant cells.

**DNA extraction**

DNA was extracted using a proteinase K digestion method as described by Bell et al. (1991).

**Primer sequences**

The APC (1) primers amplify a CA repeat motif at the D5S299 locus proximal to, and linked to, APC (van Leeuwen et al., 1991). The sequences were 5'-GTAAGCAGGACAAG-CA and 5'-GCTATTTCTCAGGATCTG (van Leeuwen et al., 1991) and give products of 156–182 bp.

The APC (2) primers amplify a CA repeat at the D5S82 locus, which is proximal to APC (Breukel et al., 1991). The sequences were 5'-CCCAAATGATTAGATTTAAGCT and 5'-ATCGAGATGATAGAAGT (Breukel et al., 1991) and give products of 169–179 bp. The p53 (1) primers amplify a CA repeat at the p53 locus (Jones & Nakamura, 1992). The sequences were 5'-AGGGATACTATTCAGCCC-GAGGTG and 5'-ACTGCCACTCTTGGCCCATT (Jones & Nakamura, 1992) and give products of 103–135 bp.

The p53 (2) primers amplify an AAAA 5 bp repeat in the first intron of the p53 gene (Futreal et al., 1991). The reverse primer sequence was 5'-AACAGCCTTCTTAAAATGGCGAC (Futreal et al., 1991), but the forward primer (5'-GAATCCGGAGGAGGTTG) was designed from the p53 genomic DNA sequence in order to develop a simple PCR assay. This primer gives products of 140–175 bp.

The DCC (1) primers amplify a TA repeat in an intron of DCC (Fearon et al., 1990; Risinger & Boyd, 1992). The sequences were 5'-TCCCTCTAGAAATTTGTG and 5'-TACCTTATTCTTATTG (Risinger & Boyd, 1992) and gives products of 106–160 bp. The DCC (2) primers are an alternative set of amplifiers which amplify the same TA repeat as the DCC (1) primers. The sequences were 5'-GATGACATTTTCTCTTAG and 5'-GTGGTTATGTGCC-TTGAAGAG (Huang et al., 1992) and give products of 150–210 bp.

The RB1 primers amplify a CCTT(4) 4–5 bp repeat in intron 20 of the RB1 gene (Yandell & Dryja, 1989). The sequences were 5'-CTCCTCCCTACTTACCTGT (Huang et al., 1992) and 5'-AATTTAACAGGGTGTGTTGAC (Onadim et al., 1992) and give products of 266–306 bp.

The WTI primers amplify a CA repeat in the 3' untranslated region of WTI (Haber et al., 1990). The sequences were 5'-AATGAGACTTCTGGGTAGGG and 5'-TTACAGATTATTTCAAGCAACGG (Haber et al., 1990) and give products around 144 bp.

The NM23-H1 primers amplify a CA repeat at the NM23-H1 locus (Hall et al., 1992). The sequences were 5'-TACTCCAGACTTTTCCCTCTAG and 5'-CTCCTACCCTTGTT and 5'-CTCCTACTTCTGT (Onadim et al., 1992) and give products of 94–104 bp.

The NFI primers amplify a CA repeat in intron 38 of the NFI gene (Lázaro et al., 1993). The sequences were 5'-CAGAAGAGCCCTGTCT and 5'-CTCCTACATTT-ATTAACCTTA (Lázaro et al., 1993) and give products of 171–187 bp.

**Primer synthesis, labelling and purification**

For each primer pair one primer only was fluorescently labelled. This was so that only one DNA strand was detected on the gel, which made interpretation easier. All primers were synthesised on a model 391 DNA synthesiser (Applied Biosystems, Foster City, CA, USA). Using red (rox) as the size standard colour we had three colours available (green, blue and yellow), with the choice of three fluorochromes (Applied Biosystems). Yellow primers were produced by using the tamra fluorochrome (dye-NHS ester), the blue primers were produced using either 5'fam (dye-NHS ester) or 6'fam amidite, and the green primers were produced using either eoe (dye-NHS ester) or hex amidite.

**Non-fluorescent primers**

Non-fluorescent primers required no purification before use in PCR and were stored in concentrated ammonia at –20°C. The ammonia was removed prior to each PCR by evaporation in a vacuum desiccator.

**Fluorescent primers**

Two different methods were used to fluorescently label the primers using either dye-NHS esters or dye-amidites.

1. Dye-NHS ester method. This method was as described in Cawkwell et al. (1993).

2. Dye-amidine method. Primers were synthesised by standard phosphoramidite chemistry on a model 391 DNA synthesiser (Applied Biosystems). The dye-amidine was incorporated into the 5' site via a fluorescent dye-amidine (Applied Biosystems). After elution and deprotection the fluorescent oligonucleotide was dried in a centrifugal evaporator, resuspended in 20 μl of distilled water, and applied to a thin-layer chromatography plate (Surepore oligonucleotide purification system, United States Biochemicals, Cleveland, OH, USA). The purified fluorescent primer was eluted from the plate in distilled water.

**Polymerase chain reaction**

The target DNA sequences were amplified by the PCR in 25 μl of 1 x Taq polymerase reaction buffer (Promega Corporation, Madison, WI, USA) containing 12.5 pmol of each primer (one fluorescent), 0.75 units of SuperTaq Taq polymerase (HT Biotechnology, Cambridge, UK), 1.5 mM magnesium chloride, 50 μM each of dATP, dCTP, dGTP, and dTTP and 25–50 ng of sample DNA. This was overlaid with mineral oil. The DNA was amplified in an MJ Research Thermal Controller (Genetic Research Instrumentation, Dunmow, Essex, UK) by one cycle at 95°C for 5 min, 55°C for 1 min followed by an average of 22 cycles consisting of 95°C for 30 s and 55°C for 1 min. The annealing temperature was reduced to 50°C for the DCC (1) primers and to 52°C for the NFI primers. The cycle number was optimised for each DNA sample to ensure that the PCR products were detectable but were not over amplified, as this caused the quantification of DNA.
peak area to be inaccurate and therefore unusable. A thermoprobe was included in a sample tube containing mineral oil alone to ensure that the samples reached the programmed cycle temperature before the timing of the cycle began.

**Multiplex PCR**

Multiplex PCR assays were designed on the basis that co-amplified products would be distinguished either by colour or by size range. The method for multiplex PCR was the same as above except that more than one set of primers were added to the same tube.

**Polyacrylamide gel electrophoresis**

PCR products were analysed on 6% polyacrylamide (Gelman-6, Gibco BRL, Uxbridge, Middlesex, UK) denaturing gels in 1 x TBE buffer in a model 373A (four filter wheel) automated fluorescent DNA sequencer (Applied Biosystems), which is a four-colour detection system. One microlitre of each PCR reaction was combined with 4 μl of formamide and 0.5 μl of a fluorescent size marker (GS2500P, Applied Biosystems). This mix was denatured for 3 min at 90°C, after which 5 μl was loaded into each well on the prewarmed gel. The tumour DNA samples were loaded 5 min after the normal samples so that any lane-to-lane spillage would not affect the subsequent quantitation. The internal size standard for each sample enables staggered loading to be carried out. The gel was run at 30 W and 40°C for 4 h except when using the Rβ1 primers, which required 6 h. While the samples were undergoing electrophoresis the fluorescence detected in the laser scanning region was collected and stored using the Genescan Collection software (Applied Biosystems).

**Data analysis**

The fluorescent gel data collected during the run were automatically analysed by the Genescan Analysis program (Applied Biosystems), using the appropriate dye matrix, at the end of the run. Each fluorescent peak was quantitated in terms of size (in base pairs), peak height and peak area.

**Calculation of allele ratios**

Allele ratios were calculated as described in Cawkwell et al. (1993). The sizes of the two alleles for heterozygous cases were assigned according to the two peaks of greatest height in the normal sample. The values for peak area of the two alleles in the paired normal and tumour samples were used to assign a figure for allele loss. The ratio of alleles was calculated for each paired normal and tumour sample and then tumour ratio was divided by the normal ratio, i.e. T1/ T2/N1/N2, where T1 and N1 are the area values of the shorter length allele peak and T2 and N2 are the area values of the longer length allele peaks for the tumour (T) and normal (N) sample. In cases where the allele ratio was above 1.00, a conversion was made using 1(T1/ T2/N1/N2) to give a result range of 0.00—1.00. A ratio of less than or equal to 0.50 was taken to be indicative of allele loss (Cawkwell et al., 1993) to allow for up to 50% contaminating normal cells in the tumour sample. All assays were performed at least twice, to ensure that consistent results were obtained, and then the mean value was taken. In the case of overamplified products, which would give unreliable area values, an aliquot of the PCR product was diluted in distilled water and rerun on a subsequent gel.

**Microsatellite instability**

Samples which consistently exhibited novel allele peaks in the tumour sample, as compared with the corresponding normal sample, for a particular marker were classed as being affected by microsatellite instability at that marker. Such markers were classed as uninformative for the allele loss study.

**Results**

Our assay is based on the alteration of allele ratio in the tumour when compared with the ratio in the corresponding normal sample, and as such will not distinguish between allele loss and amplification. Therefore it would be more accurate to describe the results of our assay in terms of allele imbalances, rather than loss, since the Rβ1 gene has been noted to undergo both loss and amplification.

The product size ranges we observed, as sized by the GS2500P standard (Applied Biosystems), were: APC (1), 158—192 bp; APC (2), 173—183 bp; p53 (1), 109—127 bp; p53 (2), 145—165 bp; DCC (1), 115—155 bp; DCC (2), 169—214 bp; Rβ1, 274—305 bp; WT1, 138—150 bp; NF1, 173—191 bp; NM23, 94—108 bp.

In the case of DCC we had two different sets of primers available to amplify the same TA repeat, but we found that the DCC (2) primers, which give longer products, were much easier to interpret than the DCC (1) primers, and so the DCC (2) primers will be used in the future.

Representative examples of the electropherograms are shown in Figures 1 and 2.

Both the dye-NHS ester- and the dye-amidite-labelled primers worked well, but the amidite dyes required less manipulation. The tamra dye was consistently found to be less intense than any of the blue or green dyes.

We observed preferential amplification of shorter products over larger products (Figure 1). This affected the success of some multiplex designs since markers which gave products of shorter length would be overamplified in relation to markers which gave longer length products. This was especially evident with the NM23 primers, but overamplification could usually be corrected by diluting an aliquot of the PCR product.

Some microsatellites produce 'stutter bands', which are PCR artefacts (Litt, 1991; Hauge & Litt, 1993) and can make interpretation of results difficult from autoradiographs. The fluorescence-based system overcame this problem in most cases. The p53 (1) and DCC (1) markers, which are 2 bp repeats, were the most problematic in terms of interpretation of results owing to excess stutter bands. The longer 4—5 bp repeat markers were not prone to this artefact and therefore the alleles were much easier to distinguish.

The results are shown in Table I.

The use of microsatellites for allele loss studies facilitates the identification of loci where microsatellite instability (Aaltonen et al., 1993; Itonov et al., 1993; Thibodeau et al., 1993) has occurred, and with the use of our fluorescent detection method these are easily recognised (L. Cawkwell et al., manuscript in preparation). The finding of microsatellite instability meant that such loci were designated uninformative and thus the total number of informative cases was reduced. The allele imbalance frequency was calculated as [A1/(A1 + N1)] × 100%, i.e. excluding all loci which were either homozygous or affected by microsatellite instability. Our microsatellite instability results will be further described in a separate paper (L. Cawkwell et al., manuscript in preparation).

Where two markers were used for a region we found no discordance between the two results where both markers were informative.

The frequencies of allele imbalance found were as follows: APC/MCC region, 50% (8/16); p53, 66% (10/15); DCC, 29% (5/17); Rβ1, 50% (7/14); WT1, 0% (0/10); NF1, 14% (2/14); NM23, 0% (0/14).

**Discussion**

We have used fluorescent multiplex PCRs in order to increase throughput for quantitating allele imbalance at seven suppressor loci in colorectal tumours. Our frequencies for allele imbalance of the p53 and Rβ1 loci are similar to the findings of others using RFLP probes and Southern blot analysis.
Our frequency of loss in the APC/MCC region is also similar to that reported by others, but an intragenic microsatellite in APC would still be preferred to ensure that no deletions specific to APC are missed. The markers used here forSq are linked to APC, but this means that we may miss small deletions. There is a CA repeat 30-70 kb downstream of APC (Spirito et al., 1991) which we are using in further studies, but no intragenic microsatellite polymorphisms within APC have been reported.

The frequency of allele imbalance of DCC that we found, however, is lower than that reported by RFLP probes but is similar to the frequency found by Huang et al. (1993) using the intragenic DCC (1) primers that we have also used. The reason for our low frequency is not clear. We would have expected all of our frequencies to be lower than expected if there was a common problem with our technique. Thus we have ruled out the possibilities that normal cell contamination of the tumour sample could have affected our results and that the stringency of our allele loss calculation could have produced our low frequency of DCC loss. Huang et al. (1993) also found a low frequency when using the same TA repeat markers, which could have indicated that there was a problem with this marker. We have noted that there is a very wide allele size range with this marker, and preferential amplification of the shorter allele was often seen. This could have affected the allele loss calculation if there was any inconsistency in the peak area quantitation. However, all of our results were repeated at least once and such inconsistencies did not appear to occur. Thus there could be a true difference in the frequency of DCC loss between our series and other published series.

WIT1 does not appear to be of importance in colorectal carcinogenesis, but the NF1 gene may be involved in a minority of cases. A further study of chromosome 17, especially between 17p13 (p53) and 17q11 (NF1), may be of interest in the cases with allele imbalance at NF1 to see whether the deletion involving p53 encompassed the NF1 locus or whether the NF1 region had been targeted separately. The finding of no allele imbalance at NM23 (17q22) in these cases indicates that loss or gain of a whole chromosome 17 had not occurred.

Our finding of no allele imbalance of NM23 supports the finding of Whitelaw & Northover (1994), and thus the involvement of NM23 in colorectal cancer remains unresolved.

Table 1 Results for allele imbalance in colorectal tumours

| Sample number | APC | APC | p53 | p53 | DCC | DCC | RB1 | WT1 | NF1 | NM23 |
|---------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| 1             | MI  | MI  | MI  | H   | N   | N   | MI  | MI  | MI  | H    |
| 2             | N   | N   | H   | N   | N   | N   | H   | N   | N   | N    |
| 3             | N   | H   | H   | Al  | Al  | Al  | N   | N   | N   | N    |
| 4             | Al  | H   | Al  | U   | Al  | Al  | N   | N   | Al  | N    |
| 5             | N   | N   | N   | MI  | N   | N   | Al  | N   | N   | N    |
| 6             | Al  | H   | Al  | H   | Al  | Al  | N   | N   | N   | N    |
| 7             | Al  | H   | Al  | H   | MI  | MI  | Al  | N   | N   | M    |
| 8             | Al  | Al  | Al  | H   | H   | H   | H   | N   | N   | N    |
| 9             | Al  | Al  | Al  | N   | N   | N   | N   | N   | Al  | N    |
| 10            | Al  | MI  | MI  | N   | MI  | MI  | N   | MI  | MI  | N    |
| 11            | MI  | H   | MI  | H   | N   | N   | MI  | MI  | MI  | M    |
| 12            | MI  | MI  | MI  | H   | N   | N   | MI  | MI  | MI  | M    |
| 13            | N   | N   | H   | Al  | N   | N   | N   | N   | N   | N    |
| 14            | N   | N   | H   | Al  | N   | N   | N   | N   | N   | N    |
| 15            | N   | N   | U   | N   | N   | N   | N   | N   | N   | N    |
| 16            | MI  | MI  | MI  | H   | N   | N   | MI  | MI  | MI  | H    |
| 17            | H   | Al  | U   | Al  | N   | N   | H   | N   | N   | N    |
| 18            | N   | Al  | Al  | Al  | Al  | Al  | H   | N   | Al  | N    |
| 19            | Al  | Al  | U   | Al  | Al  | Al  | N   | N   | H   | N    |
| 20            | H   | Al  | N   | N   | N   | N   | N   | N   | N   | N    |

H, homozygous; N, no allele imbalance; Al, allele imbalance; MI, microsatellite instability; U, uninterpretable due to excess stutter bands.
The use of our fluorescent microsatellite assay has enabled us to easily detect microsatellite instability. This caused problems with the allele imbalance study, since such loci had to be classed as uninformative. However, the assay does enable us to assess these two types of alteration simultaneously.

Multiplex design in future will take into account the preferential amplification of short products over long products. To counteract this, the shortest products could be labelled with the tamra dye since this is the weakest fluorescing dye. Alternatively, to counteract this effect, the separate amplification of short products and long products would enable the optimisation of both. The products could then be mixed and co-loaded into a single lane on the gel.

The use of fluorescent multiplex PCR for allele imbalance assays enabled us to assess several suppressor gene loci simultaneously. The most frequently occurring lesions in colorectal cancer will now be studied in an extended series with accompanying clinical and follow-up data to see whether any alteration, or specific combination of alterations, correlates with clinical features or prognosis. We will then attempt to devise a rapid and robust fluorescent multiplex PCR assay for allele imbalance, preferably in a single tube, to assess all of these important lesions simultaneously. Such a diagnostic test could be important in screening, prognosis and therapy in colorectal cancer, and in a modified form in other tumours.

In conclusion, in this preliminary study we have used rapid and quantitative fluorescent multiplex PCR assays to detect allele imbalance at several tumour-suppressor loci simultaneously, using microsatellite markers of 2–5 bp repeat motifs, in order to identify the specific genes which undergo alteration most frequently in sporadic colorectal tumours. These assays also identified microsatellite instability.

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