

Technical reports

Rapid microsatellite analysis of paraffin embedded tumour specimens from patients with hereditary non-polyposis colorectal cancer

Jochen Raedle, Angela Brieger, Jörg Trojan, Günter Herrmann, Stefan Zeuzem

Abstract

In screening for hereditary non-polyposis colorectal cancer (HNPCC)—an autosomal dominant disorder characterised by mutations in mismatch repair genes—detection of microsatellite instability is an important diagnostic criterion. The mono- or dinucleotide repeat DNA sequences are usually amplified from formalin fixed, paraffin embedded tissue by polymerase chain reaction after numerous time consuming steps including deparaffinisation, DNA extraction, and purification. A rapid single step method for direct DNA analysis is described, based on preincubation of paraffin embedded tissue with Triton X-100 followed by DNA amplification with fluorescence labelled primers and electrophoresis in an automated sequencer. This procedure allows precise allele sizing and analysis of genetic instability, is more efficient and time saving, reduces the risk of contamination, and is therefore of particular interest in screening for HNPCC.

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Keywords: hereditary non-polyposis colorectal cancer; rapid microsatellite analysis; paraffin embedded tissue

Microsatellites represent DNA loci with simple sequence repeats that are frequently and widely dispersed throughout the human genome. Owing to allelic polymorphisms they represent a valuable source for human genetic linkage studies with applications in forensic medicine, population genetics, paternity affiliation, and molecular cancer research. In hereditary non-polyposis colorectal cancer (HNPCC)—an autosomal dominant disorder accounting for two to six per cent of all colorectal cancers in the Western world—length alterations in mono- ($(A)_n$ or dinucleotide $(CA/GT)_n$ repeats, termed microsatellite instability (MIN) or replication error positive (RER⁺) phenotype, are used as a diagnostic criterion of mismatch repair deficiency.^{1,2} MIN, caused by various mutations in at least three mismatch repair genes (hMSH2, hMLH1, and hPMS2), can be detected by polymerase chain reaction (PCR) amplification of microsatellite loci in DNA

extracted from fresh frozen or, owing to standard archival procedures, mostly from paraffin embedded tumour specimens in comparison with normal colonic tissue. PCR analysis from paraffin embedded tissue is preceded by numerous steps—including deparaffinisation, proteinase K digestion, and DNA purification—which are necessary for sensitive and reproducible amplification.³ These time consuming and contamination-prone steps limit the clinical value of microsatellite analysis as a standard screening procedure for HNPCC.

Here we describe a modified protocol for convenient direct PCR amplification of microsatellite markers from paraffin embedded tissue. For HNPCC screening, representative 5 μ m sections of paraffin embedded tumour and peritumour tissue were mounted onto slides and dried for 60 minutes at 50°C. Areas of 2–3 mm² in size were microdissected and the tissue transferred into PCR tubes containing 20 μ l of 1% Triton X-100 (Sigma, Deisenhofen, Germany). After 10 minutes' incubation at 95°C, the PCR mixture was added and the amplification was started. We successfully amplified several mono- (BAT26, BAT40) and dinucleotide repeat loci (D2S119, D2S123, D2S136, D3S1266, D3S1298, D5S346, D15S120, D17S250, and D18S58) on chromosomes 2, 3, 5, 15, 17, and 18, respectively, using fluorescence labelled 5' primers. The products of the examined microsatellite loci ranged in size from 80 to 300 bp and were electrophoresed using an automated ABI 310 DNA sequencer.² Using primers labelled with HEX (4,7,2',4',5',7'-hexachloro-6-carboxy-fluorescein), TET (4,7,2',7'-tetrachloro-6-carboxy-fluorescein), and 6-FAM (6-carboxy-fluorescein) we simultaneously analysed up to seven amplified microsatellite loci in one electrophoresis lane. The accuracy and efficiency of MIN assessment of tumour DNA from HNPCC patients were compared with the conventional extraction of genomic DNA using proteinase K digestion and phenol-chloroform standard techniques. As shown in fig 1, no difference in the electrophoresis patterns between the two methods was observed. We were able to assess MIN and loss of

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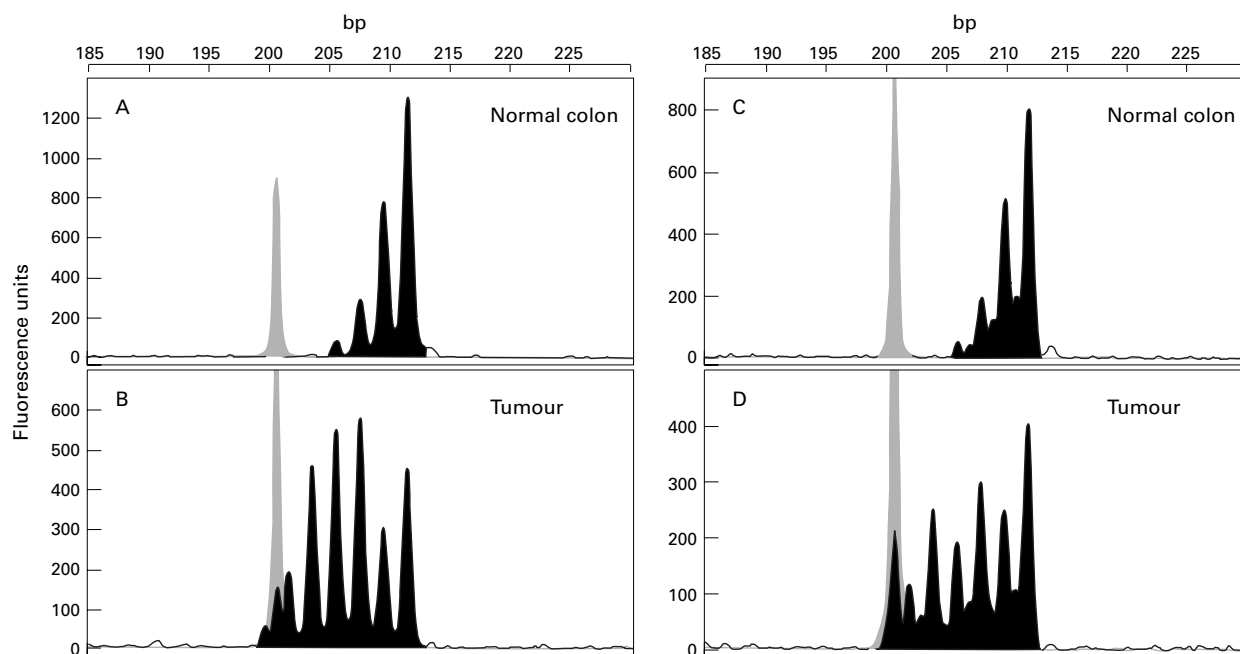


Figure 1 High resolution fluorescent microsatellite analysis of the D2S123 locus of a patient with hereditary non-polyposis colorectal cancer. Well characterised areas of 2–3 mm² in size were microdissected from slides containing paraffin embedded normal colonic epithelium (A) and tumour tissue (B) and directly transferred into polymerase chain reaction (PCR) tubes containing 20 µl 1% Triton X-100 (Sigma, Deisenhofen, Germany). After 10 minutes' preincubation at 95°C 30 µl PCR mixture, consisting of 3.5 mM MgCl₂, 15 mM ammonium sulphate, 60 mM Tris-HCl (pH 8.5), 250 µM of dATP, dTTP, dCTP and dGTP (Invitrogen, Leek, The Netherlands), 0.1 µM of HEX labelled, forward (5'-AAACAGGATGCCTGCCTTA-3') (Perkin Elmer, Weiterstadt, Germany) and 0.1 µM reverse (5'-GGACTTTCCACCTATGGGAC-3') (Biospring, Frankfurt, Germany) primers and 2.5 U AmpliTaq Gold DNA polymerase (Perkin Elmer, Weiterstadt, Germany) were added and the tubes were heated at 95°C for additional 10 minutes. To compare the accuracy of the proposed direct microsatellite analysis, slides containing paraffin embedded tissue adjacent to the previously used non-tumour (C) and tumour sections (D), respectively, were deparaffinised in xylene. DNA was subsequently released by digestion with proteinase K over three days at 40°C, extracted, and purified by standard phenol–chloroform techniques. The preincubation and amplification was carried out in a Perkin Elmer 9600 PCR system. The amplification conditions were 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s over 45 cycles, followed by a 10 minute extension at 72°C. For automated electrophoresis in an ABI 310 DNA sequencer with laser scanning and linear detection characteristics, 2 µl aliquots of a mixture of each amplified PCR product were mixed with 12.25 µl formamide and 0.75 µl TAMRA size standard (N,N,N',N'-tetramethyl-6-carboxyrhodamin) (Perkin Elmer, Weiterstadt, Germany) and finally analysed by the Gene Scan 2.01 software (Perkin Elmer, Weiterstadt, Germany). In all electrophoretic profiles of the PCR amplified microsatellite sequences (A–D) the 200 bp band of the TAMRA size standard was plotted in light grey. For final analysis, each quantitatively detected microsatellite profile derived from tumour and peritumour tissue was compared and demonstrated a characteristic cluster of additional bands in the tumour samples (B, D).

heterozygosity equally in all loci examined. Accuracy of allele sizing, which is important for linkage studies, was identical between the two techniques.

The amplification rate of paraffin embedded tissue specimens using the above described technique was significantly higher (130/140 tested loci) compared with the conventional extraction method (114/140 tested loci; $p < 0.01$). Using conventional protocols, MIN analysis of paraffin embedded tumour specimens requires three to four days. Application of the method described here reduces the duration for MIN analysis to 12 hours. Although lysis with Triton X-100 has recently been suggested for direct PCR from paraffin embedded tissue,^{4,5} no applications for microsatellite amplification with fluorescence labelled primers followed by automated DNA electrophore-

sis have been reported. Since the technique we describe is less time consuming, more efficient, and reduces the risk of contamination by limiting the number of steps required it might replace current DNA extraction and purification steps, especially for clinical screening purposes. In addition it is also applicable to genetic linkage studies.

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