Downloaded from mp.bmj.com on February 10, 2012 - Published by group.bmj.com

J Clin Pathol: Mol Pathol 1999;**52**:300–304

Technical reports

HLA-B*27 typing by sequence specific amplification without DNA extraction

D C Sayer, H S Cassell, F T Christiansen

Abstract

HLA-B27 appears to play a direct role in the pathogenesis of ankylosing spondylitis and almost all patients with this disease have HLA-B27. Therefore, a diagnosis of ankylosing spondylitis can virtually be excluded in the absence of HLA-B27. Many techniques have been used for HLA-B*27 typing. Of these, molecular methods are the most sensitive and specific but require extracted DNA as the testing material. A technique where HLA-B*27 is amplified directly from whole blood using sequence specific primers has been developed. This technique uses small sample volumes, is not restricted by choice of anticoagulant or sample age up to at least six weeks, and can be applied to other clinical polymerase chain reaction based procedures.

(*J Clin Pathol: Mol Pathol* 1999;**52**:300–301)

Keywords: ankylosing spondylitis; HLA-B*27; whole blood; sequence specific primers

Department of Clinical Immunology, Royal Perth Hospital, Wellington Street, Perth, Western Australia 6000, Australia
D C Sayer
H S Cassell
F T Christiansen

Correspondence to:
Mr Sayer.
email: david@dci.uwa.edu.au

Accepted for publication 15 June 1999

Testing for HLA-B27 is of clinical importance for the diagnosis of ankylosing spondylitis. Excluding HLA-B27 virtually excludes ankylosing spondylitis.[1] Serological techniques such as microcytotoxicity and flow cytometry for testing for HLA-B27 require viable cells that adequately express HLA-B27 and may give false negative results if HLA-B27 is downregulated or "masked".[2 3] Flow cytometry is rapid and relatively inexpensive, but has been reported to lack specificity, especially in the presence of antigens that crossreact with HLA-B27, such as HLA-B7.[4]

Molecular techniques are sensitive and specific. However, DNA extraction makes the procedure cumbersome, time consuming, and therefore unattractive to routine clinical laboratories.

We have developed a procedure for the detection of HLA-B*27 after amplification with sequence specific primers directly on whole blood (WBSSP).

Previous attempts to amplify directly from whole blood have been inconsistent, probably because of the physical entrapment of DNA by blood proteins that are denatured as a result of the high temperatures encountered during the polymerase chain reaction (PCR). However, incorporating formamide into the PCR mix reduces the melting temperature of DNA and allows the PCR cycles to be performed at much lower temperatures, resulting in successful amplification.[5]

Materials and methods

TEST SAMPLES

One hundred and forty two samples of blood collected into tubes containing acid citrate dextrose (Becton Dickinson, New Jersey, USA) were used in the initial WBSSP evaluation. All samples were selected randomly from routine HLA typing requests and were tested by microcytotoxicity and WBSSP. Ankylosing spondylitis was clinically indicated in 79 of these cases.

Microcytotoxicity was performed on cells after positive selection with magnetic beads (Dynabeads; Dynal, Oslo, Norway) using conventional techniques.

SAMPLE PREPARATION

Whole blood

Samples were mixed on a rotating mixer for one hour. An aliquot of 0.5 µl was removed and mixed with 6 µl of formamide (Sigma, St Louis, USA) and 3.5 µl of water and incubated at 95°C for five minutes. The final concentration of formamide in the PCR mix was 12%. This had been determined to be the optimum concentration by titration (data not shown) and may differ according to the application.

Buffy coat

The blood samples were centrifuged at 200 ×*g* for 15 minutes. An aliquot of 1 ml of buffy coat was removed and placed into a sterile tube. From this, 0.5 µl was treated in the same way as whole blood.

HLA-B*27 SPECIFIC AMPLIFICATION

The test primers are similar to those described by Olerup[6] (5' primer, B27ex294F: 5'-CTACGTGGACGACACGCT-3'; 3' primer, B27ex2199RC: 5'-AGTCTGTGCCTTGG CCTTGC-3') and amplify a region of 141 bp in exon 2 of HLA-B. However, in contrast to the primers described by Olerup, these primers do not contain the deliberately introduced mismatch in the 5' primer. The primers are specific for all subtypes of HLA-B*27 (HLA-B*2701–HLA-B*2713) except HLA-B*2712.[7] The control primers HGH I (5'-CAG

Downloaded from mp.bmj.com on February 10, 2012 - Published by group.bmj.com

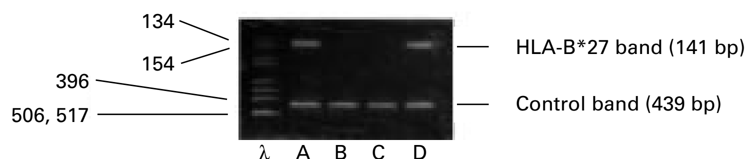

*Figure 1 Amplification with sequence specific primers using whole blood (WBSSP) detected by agarose electrophoresis. Products were resolved on a 2% ME agarose gel at 150 V for 25 minutes. HLA B*27 is reported as present when the 141 bp test band is amplified and negative when the test band is not amplified in the presence of a control product. λ is the molecular weight ladder. Patient A is HLA-A2,B27,B44; patient B is HLA-A19,A24,B13,B44; patient C is HLA-A2,A3,B49; patient D is HLA-A19,A23,B27,B35.*

TGCCTTCCCAACCATTCCCTTA-3') and HGH2 (5'-ATCCACTCACGGATTTCTG TTGTGTTTC-3') have been described previously,[8] and amplify a conserved region in the human growth hormone gene, yielding a product of 439 bp.

The PCR was performed in a final volume of 50 µl and contained 1 µM of each test primer, 0.4 µM of each control primer, 240 µM of each dNTP, 10 mM Tris HCl at pH8.3, 50 mM KCl, 1 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 2.5 U of Amplitaq polymerase (PE Biosystems, Foster City, USA), and 10 µl of the sample mix. Amplification was performed in a Perkin-Elmer 480 thermocycler as follows: seven cycles of 85°C for two minutes, 55°C for 45 seconds, and 60°C for one minute; 15 cycles of 85°C for one minute, 50°C for 45 seconds, and 60°C for 90 seconds; and 10 cycles of 85°C for one minute, 45°C for 45 seconds, and 60°C for two minutes. Products were resolved by electrophoresis in a 2% ME (SeaKam; FMC Products, Rockland, Maine, USA) agarose gel containing ethidium bromide at 150 V for 25 minutes.

SEQUENCING

The test product was confirmed as HLA-B*27 after sequencing with the HLA-B*27 specific primers using an ABI prism big dye terminator cycle sequencing ready reaction kit (PE Biosystems) on a PE Biosystems 373A automated sequencer containing the filter wheel for big dye sequencing.

Results and discussion

The presence or absence of HLA-B*27 was indicated by the presence or absence of the HLA-B*27 test product (fig 1).

There was adequate amplification in 132 of the 142 samples. Sixteen samples were positive for HLA-B*27 by WBSSP and microcytotoxicity. All of the remaining samples (116) were negative by both microcytotoxicity and WBSSP.

A result was not recorded for 10 samples because of PCR failure indicated by the lack of amplification with the control primers. Amplification failures may be explained by variations in cell numbers obtained in the small sample volume when samples are inadequately mixed. To improve the robustness of the assay, buffy coats were evaluated as the test material. Forty samples were tested in duplicate comparing buffy coat and whole blood. There was complete concordance for the typing result between the two groups. Six were positive for HLA-B*27 and 34 were negative. Of the subsequent 199 buffy coat samples, two were unreportable because of failed control band amplification. Both were successful on repeat. This failure rate of ~ 1% is consistent with previously reported SSP methods for HLA-B*27.[9]

Duplicate samples from a HLA-B*27 positive donor were stored at 4°C and room temperature. Each sample was tested at regular intervals for a six week period. The HLA-B*27 band and the control band were amplified for each sample for the entire testing period. This is consistent with Panaccio *et al*.[5]

The HLA-B*27 specific primers are not complimentary to HLA-B*2712. This allele was described by Balas *et al* as a Bw6 associated serological blank, which reacts with only some monoclonal antibodies with HLA-B40 reactivity.[10] Therefore, it is unlikely that this allele would be detected by microcytotoxicity and flow cytometry, particularly in the presence of HLA-B40. Importantly, however, Balas *et al* have shown the HLA-B2712 differs from all other HLA-B27 subtypes in the configuration of the B pocket of its peptide binding groove, and that it differs from the ankylosing spondylitis associated HLA-B2705 in its F pocket configuration. On this basis, they suggest that HLA-B2712 is unlikely to be associated with ankylosing spondylitis.[10] If this is correct, then our assay will maintain its sensitivity for ankylosing spondylitis.

HLA-B27 typing by WBSSP has the sensitivity and specificity of a DNA based test without the additional reagent cost and "hands on" time required for DNA extraction. In addition, testing can be performed on aged samples, long after the sample has become unacceptable for flow cytometry and microcytotoxicity.

We wish to thank M Corp for her help and patience in the preparation of the manuscript and the staff of the immunogenetics team of the department of clinical immunology at the Royal Perth Hospital.

1 Brewerton DA, Caffrey M, Hart FD, *et al.* Ankylosing spondylitis and HL-A 27. *Lancet* 1973;:904–7.
2 Amor B, Kahan A Georgiadis A. Transient loss of HLA-B27. *Lancet* 1978;**1**:167.
3 Neumuller J, Fischer M Eberl R. Failure of the serological determination of HLA-B27 due to antigen masking in patients with ankylosing spondylitis. *Rheumatol Int* 1993; **13**:163–7.
4 Neumuller J, Schwartz DWM, Dauber E, *et al.* Evaluation of four monoclonal antibodies against HLA-B27 for their reliability in HLA-B27 typing with flow cytometry (FC): comparison with the classic microlymphocytotoxic test (MLCT). *Cytometry* 1996;**26**:209–15.
5 Panaccio M, Georgesz M, Lew AM. FoLT PCR: a simple PCR protocol for amplifying DNA directly from whole blood. *Biotechniques* 1993;**14**:238–43.
6 Olerup O. HLA-B27 typing by group-specific PCR amplification. *Tissue Antigens* 1994;**43**:253–6.
7 Mason PM, Parham P. HLA class I region sequences, 1998. *Tissue Antigens* 1998;**51**:419–66
8 Bein G, Glaser R Kirchner H. Rapid HLA-DRB1 genotyping by nested PCR amplification. *Tissue Antigens* 1992:**39**: 68–73.
9 Steffens-Nakken HM, Zwart G van den Bergh FAJTM. Validation of allele-specific polymerase chain reaction for DNA typing of HLA-B27. *Clin Chem* 1995;**41**:687–92.
10 Balas A, Santos S, Garcia-Sanchez F, *et al.* Complete coding sequence of HLA-B*2712: a serologic B27-negative antigen associated to Bw6. *Tissue Antigens* 1998;**51**:394–7.

Downloaded from mp.bmj.com on February 10, 2012 - Published by group.bmj.com

Variability in the interpretation of microsatellite patterns with different electrophoretic conditions

A C Santos, Y Yamaoka, D Y Graham, A R Sepulveda

Abstract

Microsatellite markers permit the analysis of microsatellite instability and loss of heterozygosity. Frequently, the allelotypes of microsatellites are interpreted in the presence of numerous bands in gels. The importance of different gel electrophoresis conditions in the interpretation of microsatellite patterns was tested. Microsatellite markers were used to amplify DNA from gastric cancer samples and adjacent gastric mucosa. Polymerase chain reaction (PCR) products were separated by electrophoresis through 7% polyacrylamide gels containing either 5.6 M urea and 32% formamide or 7 M urea. PCR reactions separated on urea/formamide gels resulted consistently in clear allele definition (one or two bands), whereas 7 M urea gels resulted in allele patterns that comprised multiple bands. Analysis of microsatellite abnormalities using non-formamide gels gave false negative results in just under a third of cases (four of 13). In conclusion, the interpretation of microsatellite alterations in cancer DNA is improved by using electrophoresis conditions that result in complete DNA denaturation, such as urea/formamide/acrylamide gel electrophoresis.

(*J Clin Pathol: Mol Pathol* 1999;**52**:302–304)

Keywords: microsatellite instability; interpretation; electrophoresis

Microsatellite DNA sequence analysis has become an extremely valuable tool for the study of the human genome, and it has been used to characterise a number of human genetic disorders.[1–3] Microsatellite markers are DNA sequences that consist of short tandem nucleotide repeats. The tandem sequences consist of DNA repeats of less than six base pairs, and the total length of the repeated region is usually less than 100 base pairs. The most common repeat sequences are (A)n/(T)n and (CA)n/(GT)n and such repeats are usually located in intronic DNA regions. The number of core repeats is highly variable, determining a high frequency of heterozygosity in the population. This often results in distinct alleles in different individuals and in polymerase chain reaction (PCR) amplification of two products of different size, each corresponding to one of the two normal alleles. However, in some individuals both alleles have identical sizes, resulting in a single size PCR product. Microsatellite markers have been used for the analysis of microsatellite instability (MSI) and loss of heterozygosity (LOH), which characterise carcinogenesis in multiple organs.[1 4–7] The validity of data generated by PCR amplification of microsatellite markers rests upon the correct interpretation of the normal alleles of the proband. However, many studies have reported data collected using gel electrophoretic conditions that result in alleles constituted by numerous bands, when a maximum of two major bands should be visible from normal tissue DNA, because they result from normal diploid cells.

We compared different gel electrophoresis conditions to determine their effect on the analysis of MSI and LOH in tumour relative to non-tumour tissue from the same individual.

Department of Medicine, Veterans Affairs Medical Center and Baylor College of Medicine, Houston, Texas 77030, USA
A C Santos
Y Yamaoka
D Y Graham
A R Sepulveda

Correspondence to:
Dr A R Sepulveda, Veterans Affairs Medical Center (111D), 2002 Holcombe Blvd, Houston, TX 77030 USA.
email: asepulv@bcm.tmc.edu

Accepted for publication 20 April 1999

Materials and methods

TISSUE SAMPLES AND GENOMIC DNA EXTRACTION

Thirteen patients with gastric cancer were selected. Tissue was obtained from gastric carcinomas and from non-tumour, non-metaplasia, non-dysplastic containing gastric mucosa from the same patient. Serial 5 µm thick tissue sections were obtained on glass slides and the areas of interest were microdissected after matching with an adjacent section stained with haematoxylin and eosin. DNA extraction was performed with the QiAmp tissue kit (QIAGEN, Chatsworth, California, USA), according to the manufacturer's instructions. Cancer samples were included in the study when the tumour cells constituted greater than 70% of the tissue section.

PCR ANALYSES OF MICROSATELLITE SEQUENCES AND GEL ELECTROPHORESIS

Both tumour and non-tumour DNA were amplified by PCR using specific oligonucleotides for the microsatellite loci D2S123,[2] D13S170,[2] and TP53.[8] PCR reactions were performed using 2 mM $MgCl_2$, 1.25 U of Taq Gold DNA polymerase (Perkin Elmer Corporation, Branchburg, New Jersy, USA), 20 pmol of each dideoxynucleotide, and 50 pmol of both γ-^{32}P ATP labelled and unlabelled primer in a 50 µl reaction mixture. PCR was performed using a DNA engine (MJ Research, Watertown, Massachusetts, USA) with 45 cycles of one minute at 94°C, one minute at 50°C, and one minute at 72°C. PCR products were diluted in the same volume of formamide loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 2 mM EDTA) and denatured at 95°C for two minutes. The PCR products were then separated in formamide containing urea gels, essentially as described by Litt and colleagues[9] and in formamide free urea gels. "Formamide" gels contained 5.6 M urea, 32% formamide, and 7% polyacrylamide, whereas "formamide

Downloaded from mp.bmj.com on February 10, 2012 - Published by group.bmj.com

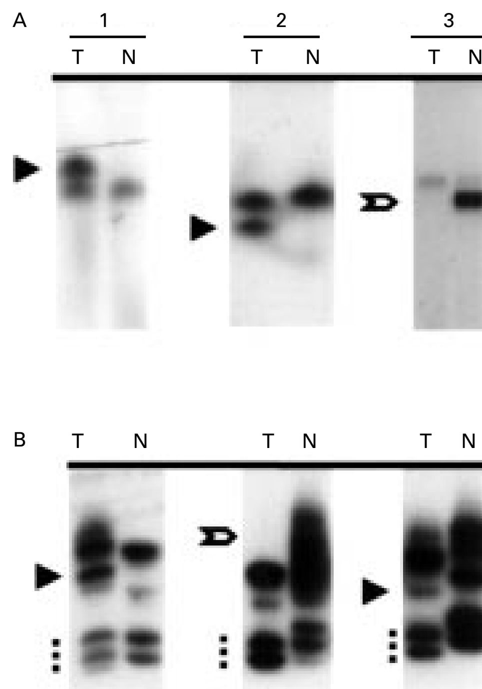

Figure 1 Variability in the interpretation of microsatellite patterns with different electrophoresis conditions. Gel electrophoresis of tumour DNA (T), and non-tumour DNA (N) samples (1, 2, and 3) separated through formamide containing acrylamide gels (A) and the same samples analysed using non-formamide containing gels (B). The solid arrows indicate a new size band in the tumour (microsatellite instability), whereas the empty arrow indicates loss of a band in tumour tissue relative to the normal counterpart DNA, indicating loss of heterozygosity. In (A) the markers show only one size allele in the normal tissue (samples 1 and 2) or alleles of two different sizes (sample 3). (B) Shows the same samples after electrophoresis in non-formamide containing gels, resulting in multiple bands. The lower group of two bands indicated by the interrupted line probably represents different conformations of the amplified alleles. A control sample of known size was included to locate the expected allele products.

free" gels contained 7 M urea and 7% polyacrylamide. The gels were prepared with the usual care required for the manipulation of acrylamide gels, on the open working bench. Electrophoresis was performed in 16 × 20 cm gel beds at 20 W for two hours, after an initial pre-run until the temperature of the gel plates reached 40°C. The gels were then exposed to *x* ray film. The interpretation of each band pattern was performed independently by two investigators (AS and ARS) and selected cases with discordant interpretations were confirmed by repeating the PCR and gel electrophoresis.

Table 1 Differences in the scoring of allele patterns with different gel electrophoresis conditions

Formamide gels	*Non-formamide gels*	*D13S170*	*TP53*	*D2S123*
MSI	Normal	1	1	2
MSI	LOH	3	1	–
MSI	NI	1	2	1
LOH	Normal	–	–	1
LOH	MSI	1	–	–
LOH	NI	–	–	1
Normal	LOH	–	1	–
NI	MSI	1	–	–
Number of discordant cases for each (total number: 13)		7	5	5

Different interpretation of microsatellite instability (MSI), loss of heterozygosity (LOH), and non-informativity (NI) for each microsatellite marker, when comparing the band pattern of PCR amplifications separated by the two different electrophoretic conditions are indicated.

Results

The examination of PCR amplification products of DNA extracted from gastric carcinoma and non-tumour gastric mucosa frequently revealed different patterns when run under the two different gel denaturing conditions. We selected a set of microsatellite markers (D13S170, D2S123, and TP53) that frequently renders informative allelotypes, to examine LOH and microsatellite instability in tumours. LOH was scored by comparing the relative intensity of the two separate alleles of an individual, and was identified when imbalance of the normal ratio of the two alleles was altered in the tumour DNA. Individuals with monomorphic alleles in non-tumour samples, resulting in a single size PCR product, were considered non-informative for LOH. MSI was detected by a size shift in one or both alleles, as a result of an increase or decrease in the number of core repeat sequences of the microsatellite region.

PCR reactions separated through formamide containing gels consistently resulted in unambiguous allele definition, whereas non-formamide gels produced allele patterns that frequently consisted of multiple bands of similar intensity, precluding a correct assessment of the allele status. Figure 1 shows examples of the same PCR product samples separated using the two different electrophoretic conditions. For example, sample 1 shows a similar interpretation with the two electrophoretic conditions (MSI), although the pattern of band shift is different; sample 2 shows MSI with formamide containing gel electrophoresis but LOH when non-formamide gels were used; and sample 3 shows LOH with formamide containing gel electrophoresis but MSI when analysed using non-formamide gels.

All possible differences in the interpretation of microsatellite instability, LOH, and non-informativity status of alleles were found when the two conditions were compared for each microsatellite marker (table 1). For example, with the D13S170 marker, three cases of MSI were scored as LOH and in five instances a case was read as normal when in fact it displayed MSI or LOH (table 1). Overall, the most frequent outcome was that MSI was missed under the non-formamide containing gel electrophoresis conditions (table 1).

Discordant results were found in over half of the cases when the D13S170 marker was used and in more than a third of the cases when the TP53 or D2S123 markers were used. Allelotyping using non-formamide containing gels produced false negative results in almost a third of the cases for LOH or MSI (four of 13 cases), and one of the 13 cases was a false positive that was read as LOH in non-formamide gels when the formamide containing gel showed a normal pattern.

Discussion

In our study, we used microsatellite markers to determine microsatellite instability and LOH

Downloaded from mp.bmj.com on February 10, 2012 - Published by group.bmj.com

in gastric tumours, and compared the effect of different gel electrophoresis conditions on the interpretation of allele patterns relative to that of DNA extracted from tumour free gastric mucosa. In studies on genetic disease, including the molecular characterisation of tumorigenesis pathways, comparative microsatellite analysis between non-tumour and tumour samples from the same individual can be performed to identify genomic alterations in tumours.[1] This analysis requires the correct identification of the alleles from both the tumour and non-tumour tissue. It has been shown previously that electrophoresis of PCR amplifications of microsatellite markers displaying more than two major bands in normal tissues can be the result of different migration rates of conformationally different forms of the same DNA molecule, and this phenomenon can result from incomplete DNA denaturation during gel electrophoresis.[9] In our study we found that gels lacking formamide did not provide reliable DNA denaturing capacity, frequently resulting in the presence of several bands in gels, when a maximum of two major bands is expected from normal somatic cells. Because allelic analysis with the markers used in our study resulted in clearly defined alleles in all cases when formamide containing gels were used, we selected these gels as the standard experimental conditions. We found that interpreting data with incompletely denatured DNA during electrophoresis (from urea gels lacking formamide) resulted in multiple types of data misinterpretation, with MSI frequently being missed in non-formamide containing gels. It is possible, however, that PCR amplification with other microsatellite markers could result in pronounced stutter bands, as a result of polymerase slippage, that would not be eliminated by separating the reactions in formamide containing gels. Nonetheless, the analysis of microsatellite data should be performed using technical conditions that allow the demonstration of no more than two major DNA bands, corresponding to the two expected alleles from normal tissue. Therefore, if microsatellite amplification results in multiple bands with no obvious major band within the expected allele size range, the use of formamide containing gels might help achieve a better DNA separation, with easier scoring of allele status.

Many publications are currently being generated by the use of microsatellite marker analyses with PCR to investigate both MSI and LOH at many loci in the genome of tumours and pre-neoplastic lesions.[1 4–7 10] It is therefore of great importance to assess such changes correctly, to avoid the accumulation of biased data in the literature. Furthermore, the future use of this technology in diagnostic applications might be hindered by lack of reproducibility among laboratories, indicating that strict conditions should be followed and guidelines should be available for laboratory reference. For example, MSI is a typical finding in colon tumours from individuals with hereditary nonpolyposis colorectal cancer, and a lack of identification of MSI could result in an index case being missed, potentially affecting the clinical follow up of the individual as well as other family members. LOH studies using multiple microsatellite markers are performed frequently in the research setting to search for putative tumour suppressor genes, and it is therefore important that scoring of allele changes should be accurate. In our study, we show that accurate analyses of MSI and LOH with some microsatellite markers can be achieved by using formamide containing gels run under denaturing conditions, in contrast to the more variable results obtained with typical urea/acrylamide gel electrophoresis.

This work was supported by the Department of Veterans Affairs and by general support from Hilda Schwartz.

1 Chung DC, Rustgi AK. DNA mismatch repair and cancer. *Gastroenterology* 1995;**190**:1685–99.
2 Weissenbach J, Gyapay G, Dib C, *et al.* Linkage map of the human genome. *Nature* 1992;**359**:794–801.
3 Martorell L, Johnson K, Boucher CA, *et al.* Somatic instability of the myotonic dystrophy (CTG)n repeat during human fetal development. *Hum Mol Genet* 1997;**6**: 877–80.
4 Shinmura K, Tani M, Isogaki J, *et al.* RER phenotype and its associated mutations in familial gastric cancer. *Carcinogenesis* 1998;**19**:247–51.
5 Lin JT, Wu MS, Shun CT, *et al.* Microsatellite instability in gastric carcinoma with special references to histopathology and cancer stages. *Eur J Cancer* 1995;**31A**:1879–82.
6 Liu B, Parsons RE, Hamilton SR, *et al.* hMSH2 mutations in hereditary nonpolyposis colorectal cancer kindreds. *Cancer Res* 1994;**54**:4590–4.
7 Meltzer SJ, Yin J, Manin B, *et al.* Microsatellite instability occurs frequently and in both diploid and aneuploid cell populations of Barrett's-associated esophageal adenocarcinomas. *Cancer Res* 1994;**54**:3379–82.
8 Hamamoto T, Yokozaki H, Semba S, *et al.* Altered microsatellites in incomplete-type intestinal metaplasia adjacent to primary gastric cancers. *J Clin Pathol* 1997;**50**:841–6.
9 Litt M, Hauge X, Sharma V. Shadow bands seen when typing polymorphic dinucleotide repeats: some causes and cures. *Biotechniques* 1993;**15**:280–4.
10 Hayden JD, Martin IG, Cawkwell L, *et al.* The role of microsatellite instability in gastric carcinoma. *Gut* 1998;**42**: 300–3.

Downloaded from mp.bmj.com on February 10, 2012 - Published by group.bmj.com

Variability in the interpretation of microsatellite patterns with different electrophoretic conditions.

A C Santos, Y Yamaoka, D Y Graham, et al.

Mol Path 1999 52: 302-304
doi: 10.1136/mp.52.5.302

Updated information and services can be found at:
http://mp.bmj.com/content/52/5/302

These include:

References Article cited in:
http://mp.bmj.com/content/52/5/302#related-urls

Email alerting service Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/