DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (DHPLC) IN DETECTION OF MICROSATELLITE INSTABILITY

DOLOČANJE MIKROSATELITNE NESTABILNOSTI Z DENATURACIJSKO VISOKO LOČLJIVOSTNO TEKOČINSKO KROMATOGRAFIJO (DHPLC)

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Abstract

Background *Microsatellite instability (MSI) is a phenomenon characterized by small deletions or insertions within short tandem repeats in tumour DNA compared to matching normal DNA. MSI analysis is becoming more and more important for detection of hereditary non-polyposis colorectal cancer as well as for sporadic primary colorectal tumours with MSI high phenotype. Use of five quasimonomorphic mononucleotide markers eliminates ultimate need for analysis of germline DNA corresponding to tumour DNA.*
Here we discuss our method for MSI analysis using denaturating high performance liquid chromatography (DHPLC) in combination with quasimonomorphic mononucleotide microsatellite markers in comparison with previously used methods. The method is high-throughput, accurate, quick and cost-effective and suitable for large-scale studies as well as for daily use with smaller numbers of samples.

Key words *microsatellite instability; mononucleotide microsatellite markers; multiplex PCR; DHPLC*

Izvleček

Izhodišča *Mikrosatelitno nestabilnost opredeljujejo manjše delecije in vstavitve na področjih tandemskih ponovitev tumorske DNA v primerjavi z DNA normalnega tkiva. Analiza mikrosatelitne nestabilnosti postaja vse bolj pomembno orodje pri odkrivanju dednega nepolipoznega kolorektalnega raka, kot tudi sporadičnih primarnih kolorektalnih tumorjev z visoko mikrosatelitno nestabilnim (MSI-H) fenotipom. Uporaba 5 skoraj monomorfnih mononukleotidnih mikrosatelitnih označevalcev odpravlja potrebo po analizi zarodne DNA v primerjavi s tumorsko DNA.*
Opisana je metoda za analizo mikrosatelitne nestabilnosti z uporabo denaturacijske visoko ločljivostne tekočinske kromatografije (DHPLC) v kombinaciji s skoraj monomorfnimi mononukleotidnimi mikrosatelitnimi označevalci in primerjava s predhodno uporabljanimi metodami. Metoda je visoko zmogljiva, zanesljiva, hitra in cenovno ugodna. Primerna je tako za študije na večjem številu vzorcev, kot tudi za dnevno uporabo z manjšim številom vzorcev.

Ključne besede *mikrosatelitna nestabilnost; mononukleotidni mikrosatelitni označevalci; multipla PCR; DHPLC*

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Introduction

Microsatellites (MS) are polymorphic regions present in DNA that consist of repeating units of 1–4 base pairs in length. An example of microsatellite DNA with 2 base pairs repeating units is CACACACA or $(CA)_4$. The number of repeating units (in this case 4) in the same DNA region may vary between individuals. Microsatellite DNA can be therefore used as molecular marker in wide-range of applications including clinical. A phenomenon in which the number of repeating units differs between normal in tumorous DNA of the same individual is called microsatellite instability (MSI). MSI was first described in colorectal cancer (CRC). Approximately 10 to 15 percent of sporadic CRC and majority of hereditary non-polyposis colorectal cancer show MSI.[1–4] MSI is also seen in a significant proportion of other extracolonic cancers.[5] Since determination of MSI could be diagnostically and therapeutically important the use of standardized panel of MS markers is necessary. So-called Bethesda panel of two mononucleotide (BAT-25, BAT-26) and three dinucleotide (D5S346, D2S123, D17S250) microsatellite markers was therefore proposed by National Cancer Institute Workshop in 1997. With regard to MSI status tumours can be classified in three groups: MSI-high (MSI-H, showing instability in ≥ 40 % of tested markers); MSI-low (MSI-L, showing instability in < 40 % of tested markers); and MSS (microsatellite stable, showing no microsatellite instability). MSI-H is a distinct group of tumours associated with specific clinicopathological features and favourable prognosis.[6] Distinction between MSI-L and MSS group is still a mater of debate due to contradictory data.[7–11] Determination of MSI status has become very important tool in hereditary non-polyposis colorectal cancer (HNPCC) screening and prediction of the responsiveness to chemotherapy.[12, 13]

Improvements in determination of MSI in colorectal cancer

In recent years a lot has been done in MSI analysis concerning accuracy and rapidness. Developments in PCR technology enabled co-amplification of several microsatellite markers in a single multiplex reaction.[14–16] To determine the best set of microsatellite markers for identification of MSI-H tumours and to simplify MSI analysis extensive studies have been done concerning sensitivity and specificity. The Bethesda panel provides a uniform set of markers and criteria in MSI analysis, however it has some limitations because of three dinucleotide markers used in the panel.[17] Each of dinucleotide repeats in the aforementioned panel generally shows instability in only 60–80 % of MSI-H tumours,[14] their highly polymorphic nature requires the analysis of tumour and corresponding germline DNA (not always available), and results in misclassification of MSI if samples from two individuals are mixed.[18] Misinterpretation of allelic profiles using dinucleotide repeats can also be the consequence of mutations in *hMSH6* gene which cause alterations primary in mononucleotide repeats,[19] and the presence of stutters, polymerase chain reaction (PCR) artefacts produced by DNA polymerase slippage.[20, 21] To overcome these problems several authors suggest the use of BAT-26 alone for evaluation of MSI without the need for corresponding germline DNA analysis due to its quasimonomorphic nature.[22–24] Population-based studies showed that most of the Caucasian populations have less than 1 % of polymorphic alleles on BAT-26 locus but African-American population is polymorphic in 7–13 % of alleles.[25, 26] These data suggest that misclassification of MSI can occur in different populations if only BAT-26 is analysed without matched normal tissue DNA. In effort to improve existing panel of MSI markers proposed by Bethesda guidelines more mononucleotide markers were tested for germline polymorphisms and several of them showed quasimonomorphic nature.[20, 27] Suraweera et al. [2002] proposed set of five quasimonomorphic mononucleotide microsatellite markers (BAT-25, BAT-26, NR-21, NR-22, and NR-24) with

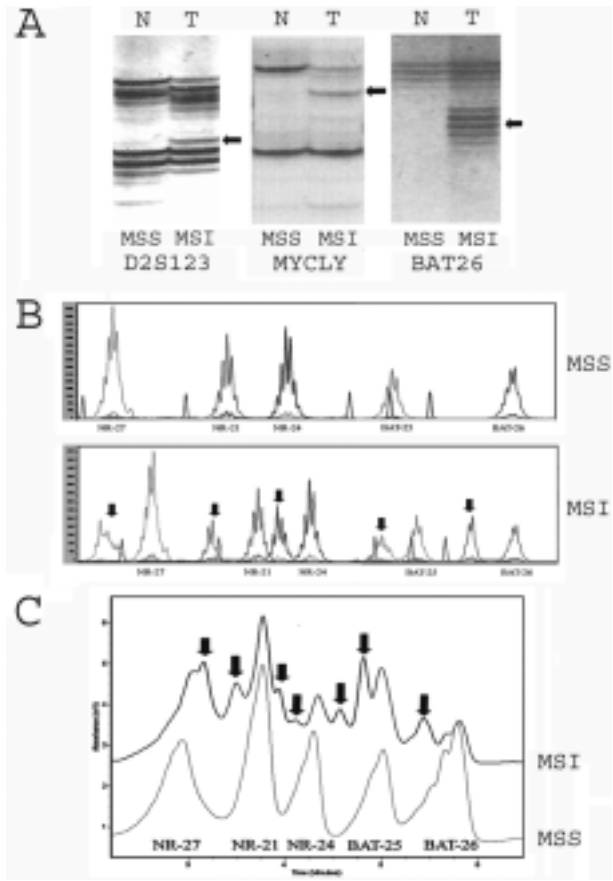

Figure 1. *MSI analysis using SSCP (A), capillary electrophoresis (B) and DHPLC (C). Arrows indicate presence of new sized alleles. MSI- microsatellite instability, MSS – microsatellite stable, T – tumour sample, N – normal sample.*

Sl. 1. *MSI analiza z uporabo SSCP (A), kapilarna elektroforeza (B) in DHPLC (C). Puščice označujejo nove alele. MSI – mikrosatelitna nestabilnost, MSS – mikrosatelitna stabilnost, T – tumorski vzorec, N – normalen vzorec.*

nearly 100 % sensitivity and specificity eliminating the need for corresponding germline DNA analysis. Tumour is defined as MSI-H when at least three out of five mononucleotides show instability.[14] With polymorphisms occurring in 1 % of the Caucasian population and 10 % of the African-American the probability of having 3 polymorphic markers would be 10^{-6} and 10^{-3} respectively.[15]

Introduction of DHPLC in analysis of MSI

Conventional methods used for MSI analysis are non-automated single-stranded conformation analysis (SSCA) and semi-automated fluorescence-based electrophoresis. Potočnik et al. [2001] identified 10 % of MSI-H tumours in 345 tested samples using SSCA. Other studies identified 12 % of MSI-H tumours (509 samples tested),[28] 6.8 % of MSI-H tumours (1222 samples tested),[29] and 12 % of MSI-H tumours (535 samples tested)[30] with fluorescence-based electrophoresis. Several weaknesses were observed using these methods. The first method is labour-intensive, time consuming, and needs strict electrophoretic conditions; the second needs fluorescently labelled primers and additional software.[31] Aforementioned drawbacks cause lower throughput and higher costs of MSI detection, which is not convenient for large scale and everyday screening.

We recently introduced denaturating high performance liquid chromatography (DHPLC) for determination of MSI-H tumours (Berginc et al. submitted). Main advantages of DHPLC are: no need for modified PCR primers, customized specific reagent arrays, detection labels, and any sample treatment other than PCR.[32] Using multiplex system with a set of five quasimonomorphic mononucleotide markers [14, 15, 20] and DHPLC we managed to eliminate the need for analysis of corresponding germline DNA and minimize the time for analysis. Using this method we screened Slovenian colorectal cancer patients, identified 43 (of 595 tested) new MSI-H patients, and checked for polymorphisms in mononucleotide microsatellite markers.

All 5 microsatellite markers showed quasimonomorphic nature with percent of polymorphisms not exceeding 2,1 (BAT-26 0.07 %, BAT-25 1.4 %, NR-24 2.1 %, NR-21 1.4 %, and NR-27 1.4 %).

Advantages of DHPLC MSI analysis

Large-scale studies as well as procedures in daily diagnostic practice need fast, accurate, and cost-effective methods. Methods conventionally used for MSI analysis (SSCA, fluorescence-based electrophoresis) do not fulfil all of the proposed criteria.[31, 33] Gel-based SSCA analysis is widely used because it is inexpensive and does not need additional equipment. However, SSCA does not meet criteria of high-throughput method due to its several labour-intensive steps: making of the gel, sample loading, long staining procedures, and difficulties in data interpretation.[31] Capillary electrophoresis solved several previously mentioned problems; nevertheless, there are still several drawbacks. The need for fluorescently labelled primers and additional analysis software affect cost-effectiveness of this method.[33] Alternative methods using DHPLC were proposed for MSI analysis.[31, 33] Two different approaches were described; the first used nondenaturing conditions[31] and the second used fully denaturing conditions.[33] Simple elution profiles with one peak per marker under nondenaturing conditions allowed us to perform multiplex analysis, which would be very difficult under conditions described by Pan et al.[33]

In order to shorten the time needed for MSI analysis, we have chosen mononucleotide repeats multiplex assay (MRP) proposed by Buhard et al.,[15] containing BAT-25, BAT-26, NR-24, NR-21, and NR-27 quasimonomorphic mononucleotide microsatellite markers. This multiplex assay was particularly suitable for DHPLC analysis because the size difference between two PCR products is at least 22 base pairs. Due to average deletion of 5–12 base pairs for these markers in MSI tumours, shortened alleles do not overlap with normal allele of neighbouring MSI marker,[14, 15] therefore they can be seen on DHPLC chromatogram. One of the main advantages of MRP assay is elimination of the need for corresponding germline DNA analysis due to its nearly 100 % sensitivity and specificity for detection of MSI-H tumours. These facts enabled us to radically reduce the time needed for analysis of one sample without decreasing accuracy. Our method enabled us to identify MSI status of one sample in 9 minutes which is one third of the time needed for MRP assay analysis using capillary electrophoresis (Berginc et al. submitted).

Conclusion

Determination of microsatellite instability (MSI) in colorectal cancers (CRC) is important since highly MSI tumours represent first step in screening of hereditary non-polyposis colorectal cancer (HNPCC). Furthermore MSI is connected with better prognosis in all CRC (hereditary and sporadic), however patients with MSI tumours do not benefit from chemotherapy with fluorouracil. MSI determination is therefore important in diagnostic and clinical applications. Development of high-throughput, robust, accurate and cost-effective method for detection of MSI-H tumours using quasimonomorphic mononucleotide markers and DHPLC is therefore very beneficial. Method is suitable for large-scale population studies as well as for daily use with smaller numbers of samples and performs well even in populations with higher percent of polymorphic microsatellite markers.

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