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ORIGINAL ARTICLE

Detection of microsatellite instability in Czech HNPCC patients

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Summary
The detection of microsatellite instability (MSI) is a standard part of mutational analysis in hereditary nonpolyposis colorectal cancers (HNPCC). A characteristic phenotypic feature of MSI indicates loss of mismatch repair (MMR) in tumour cells.

We studied MSI in 205 tumours from 152 patients with HNPCC. Of these, 37 patients fulfilled the Amsterdam criteria, 72 patients were familial and 43 were sporadic cases. We used the method of fragmentation analysis (ABI Prism 310 Genetic Analyzer) with fluorescent labelled primers; three mononucleotide (BAT-RII, BAT-25, BAT-26) and five dinucleotide (D2S123, D3S1029, D5S346, D17S250, D18S58) repeat loci were analysed. We detected 75 tumours with a high degree of MSI (MSI-H), 12 tumours with a low degree of MSI (MSI-L) and 118 tumours with stable microsatellites (MSS). We found a loss of heterozygozity (LOH) in 44 MSS tumours. In 30 patients with MSI-H tumours mutation in one of mismatch repair genes was detected.

Keywords: microsatellite instability – loss of heterozygozity – HNPCC – fragment analysis

INTRODUCTION

Microsatellite regions of DNA are composed of short tandem repeats up to six nucleotide bases. They are ubiquitously scattered throughout the human genome. The most common types of repeats are $(A)_n$ and $(CA)_n$. Microsatellites are stably inherited and are the same in every tissue (Marra and Boland 1996).

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The observation of microsatellite instability (MSI) was first associated with colorectal tumours from families with hereditary nonpolyposis colorectal cancer (HNPCC), and is also found in sporadic colorectal cancer and endometrial and gastric cancer. Identification of families with HNPCC relied on a detailed family history because of the lack of a phenotypic marker for HNPCC that could be used in clinical screening (Cawkwell et al. 1995). Minimal criteria for the definition of HNPCC have been proposed by the International Collaborative Group on HNPCC (Amsterdam criteria):
(i) at least three relatives should have histologically verified colorectal cancer, one of them should be a first-degree relative of other two; (ii) at least two successive generations should be affected; (iii) one

of the relatives should have been diagnosed before the age of 50; (iv) familial adenomatous polyposis must have been excluded (Vasen et al. 1991).

Microsatellite instability (MSI) is caused by germline or somatic mutations affecting different DNA mismatch repair genes (*hMLH1*, *hMSH2*, *hMSH6*, *hPMS1*, *hPMS2* and *hMSH3*) and leading to loss of mismatch repair functions (Liu et al. 1996). Failure to repair DNA mismatches and DNA loops caused by slipped strand mispairing during the replication, would lead to a generation of incorrectly replicated DNA strands containing inserted or deleted bases that would appear as novel alleles and is detected as a change in allele size between tumour and nontumour DNA sample.

For good quality MSI testing and definition a reference panel of markers were recommended; at least five (mono and dinucleotide) of them have to be analysed (Boland et al. 1998), and also to be included as part of the explanation of the results obtained. Tumours with at least two microsatellite unstable markers (or with instability in 30–40% of markers) are termed as MSI-H (high degree of MSI), tumours with one unstable marker (or less than 30–40% of markers) termed as MSI-L (low degree of MSI) and tumours with any unstable marker as MSS (microsatellite stable tumours).

MATERIAL AND METHODS

Tissue and DNA Isolation

A total of 205 neoplastic tissue (202 colorectal cancers, 1 breast, 2 endometrial cancers) from 152 patients were studied. There were 30 frozen tissues, 175 paraffin embedded tissues. A paired control sample (peripheral blood lymphocytes) was not available in six cases. DNA from peripheral blood cells was extracted by using the standard method, DNA from neoplastic tissues by using a Nucleospin C+T kit (Macherey-Nagel, Düren, Germany).

Polymerase chain reaction (PCR)

The following microsatellite markers were used: BAT-RII (3p22) F: 5´-ctg ctt ctc caa agt gca tta, R: 5´-gca ctc atc aga gct aca gga; BAT-25 (4q12) F: 5´-tcg cct cca aga atg taa, R: 5´-tct gca ttt taa cta tgg ctc; BAT-26 (2p16) F: 5´-tga cta ctt ttg act tca gcc, R: 5´-aac caa tca aca ttt tta acc c; D2S123 (2p16) F: 5´-aca ttc ctg gaa gtt ctg gc, R: 5´-cct ttc tga ctt gca tac ca; D3S1029 (3p21.3) F: 5´-ata ctg tgg acc cag att gat tac, R: 5´-taa ttc cca aat ggt tta ggg gag; D5S346 (5q21-21) F: 5´-act cac tct agt gat aaa tcg gg, R: 5´-agc aga taa gac agt att act agt t; D17S250 (17q11.2-q12) F: 5´-gga aga atc aaa tag aca at, R: 5´-gct ggc cat ata tat att taa acc; D18S58 (18q21) F: 5´-gct ccc ggc tgg ttt t, R: 5´-gca gga aat cgc agg aac tt (Applera, Prague, Czech Republic).

PCR conditons for D2S123 were 94°C 1´, $(94°C\ 40´´, 58°C\ 40´´, 72°C\ 1´)_{40}$, 72°C 7´, for the other markers 94°C 1´, $(94°C\ 30´´, 50°C\ 30´´, 72°C\ 30´´)_{40}$, 72°C 7´; 1x PCR buffer with $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.2mM dNTP, 1 μM of each primer, 2u/100 μl Taq DNA polymerase (Fermentas Inc., Hannover, USA). Fluorescent primers (upstream strand) were labeled on 5´end with a fluorescent mark.

Analysis

The fluorescent labeled fragments were analysed by an ABI Prism 310 Genetic Analyzer. In most cases multiplex analysis was used. Labeled fluorescent fragments from the same tissue sample were mixed together and analysed in one run. Both tumour and nontumour fragments were compared and analysed for MSI and/or loss of heterozygosity (LOH).

RESULTS

We analysed 205 samples from 152 patients. Only paired (tumour and nontumour) samples with five informative analysed markers were added into the study. We studied also six patients without available nontumour DNA with microsatellite instability in at least two mononucleotide markers.

Patients were divided into three groups: Group "A+" fulfilled the Amsterdam criteria, group "A-" were familial cases and group "S" were sporadic cases (Table 1).

37 patients were from group "A+": 31 patients (83.7%) had a high degree of MSI (MSI-H phenotype), 2 patients (5.4%) with a low degree of MSI (MSI-L) and 4 patients (10.8%) with stable microsatellites (MSS phenotype). 72 patients were from group "A-": 19 patients (26.4%) were with MSI-H phenotype, 5 patients with MSI-L (6.9%) and 48 patients (66.7%) with the MSS phenotype. 43 patients were from Group "S": 5 patients (11.6%) with MSI-H phenotype, none with MSI-L phenotype and microsatellite stability was observed in 38 patients (88.4%).

We had two or more tissue samples from 43 patients. A discrepancy in the degree of MSI in tumour samples was detected in five patients. Patients 837 and M390 had MSI-L phenotype in the first sample and MSI-H in the second one. Both patients were classified as having MSI-H phenotype. MSS was detected in one sample of patient 628 and MSI-H in other three samples. Samples of breast carcinoma and colon were analysed in woman 478. MSI-H was detected in a colon cancer sample and MSS with LOH on D3S1029 (*MLH1*) in breast sample tissue. Two samples with MSI-H and another with MSS were detected in patient 431.

37.2% MSS samples showed LOH in at least one marker, most frequently in the D5S346 (*APC* gene) and the D18S58 (*DCC* gene). Twelve samples from seven patients had an MSI-L phenotype, with LOH of some other marker in five cases.

Mutational analysis of mismatch repair genes *MLH1*, *MSH2* and *MSH6* was performed in 119 of 152 patients (Plevova et al. 2004). Mutations were detected in 33 patients: in 30 MSI-H patients, two MSI-L patients and in one MSS patient. 81% MSI-H patients had a mutation in the *MLH1* or *MSH2* gene. Patient number 386 with MSI-L phenotype had a mutation in the *MSH6* gene.

Table 1: **Results of microsatellite analysis**. A+ pacients fulfilling Amsterodam criteria, A- familiar cases, S sporadic cases, LOH loss of heterozygozity

	MSI-H	MSI-L	MSS(LOH)	Total
A+	31	2	4(3)	37
A-	19	5	48(14)	72
S	5	0	38(15)	43
Total	55	7	90(32)	152

In a parallel study, 69 samples from 66 patients were analysed by immunohistochemistry analysis for detection of the expression of mismatch repair proteins MSH2 and MLH1 (Plevová et al. 2004).

92.4% (61 patients) were positively correlated. Twenty three MSI-H patients lost expression of MLH1 protein, and three MSI-H patients lost expression of MSH2 protein. No changes in expression of mismatch repair genes were detected in MSS patients.

DISCUSSION

Detection of microsatellite instability is a standard part of the mutational analysis of hereditary nonpolyposis colorectal cancers. The characteristic phenotypic feature of microsatellite instability indicates a loss of mismatch repair in tumour cells. MSI is detected and analysed by the standard protocol defined by the International Collaborative Group on HNPCC (Boland et al. 1998).

The frequency of microsatellite instability in sporadic colorectal cancers is 10–15%; in patients who fulfilled the Amsterdam criteria HNPCC up to 93%. This frequency depends on the group of patients analysed. A high frequency is shown in large pedigrees of cancer families. Our group of patients involved no relatives from the one family except one pair (brother/sister).

We analysed 152 patients: 31of the 37 patients fulfilling the Amsterdam criteria (83.7%) showed the MSI-H phenotype, 2/37 patients (5.4%) MSI-L and 4/37 patients (10.8%) MSS phenotype. 19 of the 72 patients with a positive family history (26.4%) were with MSI-H phenotype, 5/72 patients with MSI-L (6.9%) and 48 patients (66.7%) with MSS phenotype. 5 of the 43 sporadic cases (11.6%) were with MSI-H phenotype, none with MSI-L phenotype and microsatellite stability was present in 38/43 patients (88.4%). This is in agreement with the frequency of MSI in other studies (Vasen et al. 2007).

37% of samples lost heterozygosity in at least one marker. Markers D5S346 and D18S58, located in the noncoding region of the APC and DCC genes, respectively, were the most sensitive. The same findings were shown in other studies (Thibodeau et al. 1998, Dietmaier et al. 1999, Gebert et al. 2000). In these cases, tumours may result from an accumulation of somatic mutations in tumour-suppressor genes, that may influence clinical prognosis (Gebert et al. 2000).

81% of MSI-H patients had germline mutation in the mismatch repair gene hMLH1 or hMSH2. Two mutations were found in patients with the MSI-L phenotype: patient M145 had a germline mutation of the MSH2 gene, and the degree of instability was limited to MSI-H. In two tissue samples from this patient one, respectively two markers with MSI were detected and two, respectively three LOHs in other markers, too. Patient 385 with a clear MSI-L phenotype (BAT-26) showed a mutation in the MSH6 gene. This is in agreement with the study of Wu, who showed that patients with HNPCC with a low degree of microsatellite instability in the mononucleotide markers are frequently mutated in the MSH6 gene (Wu et al. 1999).

Samples from 66 patients were studied by immunohistochemical analysis of mismatch repair proteins MLH1 and MSH2 (Plevova et al. 2004).

92.4% of MSI-H patients had a reduced expression of MLH1 or MSH2.

Microsatellite analysis is a verified method for the preselection of patients suspected of HNPCC.

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