SUMMARY THESIS

MICROSATELLITE INSTABILITY IN HEREDITARY NONPOLYPOSIS COLORECTAL CANCER PATIENTS

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SUMMARY

INTRODUCTION

The observation of microsatellite instability (MSI) was first associated with colorectal tumors from families with hereditary nonpolyposis colorectal cancer (HNPCC), also is found in sporadic colorectal cancer and endometrial and gastric cancer. Identification of families with HNPCC relied on a detailed family history because of any phenotypic marker for HNPCC that can 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): - at least three relatives should have histologically verified colorectal cancer, one of them should be a first-degree relative of other two; - at least two successive generations should be affected; - one of the relatives should have the diagnosis before ther age of 50 years; -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, occuring by slipped strand mispairing during the replication, would lead to the generation of incorrectly replicated DNA strands containing inserted or deleted bases that would appear as novel allels and is detect as a change in allele size between tumor and nontumorDNA sample.

For good quality of MSI testing and definition were recommended the reference panel of markers: at least five (mono and dinucleotide) of them have to be analyzed (Boland et al. 1998). Also were united in the explanation of obtain results. Tumors with at least two microsatellite unstable markers (or with instability in 30-40 % of markers) are termed as MSI-H (high degree of MSI), tumors with one unstable marker (or less than 30-40 % of markers) termed asMSI-L (low degree of MSI) and tumors with any unstable marker as MSS (microsatellite stable tumors).

RESEARCH OBJECTIVES

Main research objectives are:

- Instalation of fragment analysis with fluorescent-labeled PCR method as a part of diagnosis of HNPCC
- Attestation of new method effectivity in comparision to polyacrylamid gel analysis

- To analyse group of patients suspected to HNPCC
- To corelate results of MSI testing with mutational analysis and immunohistochemistry of expression MMR genes

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. Paired control sample (peripheral blood lymphocytes) was not available in six cases. DNA from peripheral blood cells was extracting by using standard methods, DNA from neoplastic tissues by using standard purifying kits (Macherey-Nagel, Düren, Germany; QIAamp DNA Mini Kit, Qiagen, Hilden, Germany).

Polymerase chain reaction (PCR) analysis

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, 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, 1μM of each primer, 2u/100μl Taq DNA polymerase (Fermentas Inc., Hannover, USA). Fluorescent primers (upstream strand) were labeled on 5´end with fluorescent mark.

Analysis

Amplified fragments were separated on denaturing polyacrylamid gel with ethidium bromid staining, fluorescent labeled fragments were analysed by ABI Prism 310 Genetic Analyzer. In the second method multiplex analysis was used; labeled fluorescent fragments from the same tissue sample were mixed together and analysed in one run. Both tumor and

nontumor fragments were compared and analysed for MSI and/or loss of heterozygosity (LOH).

RESULTS

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

Patients were divided into three groups: Group „A+“ fulfilled Amsterodam criteria, group „A-“ were familial cases and group „S“ were sporadic cases.

37 patients were from group „A+“: 31 patients (83,7 %) were with high degree of MSI (MSI-H phenotype), 2 patients (5,4 %) with 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 MSS phenotype. 43 patients were from Group „S“: 5 patients (11,6 %) with MSI-H phenotype, none

with MSI-L phenotype and microsatellite stability were in 38 patients (88,4 %).

From 205 tissue samples 75 were MSI-H, 12 MSI-L and 118 MSS. In 44 MSS cases loss of heterozygosity (LOH) was detected.

We had two or more tissue samples from 43 patients. Discrepancy in the degree of MSI in tumor 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. In Patient 628 MSS was detected in one sample and MSI-H in other three samples. Samples of breast carcinoma and colon were analysed in woman 478. MSI-H was detected in colon cancer sample and MSS with LOH on D3S1029 (MLH1) in breast sample tissue. Two samples with MSI-H and other one with MSS were detected in patient 431.

37,2 % MSS samples showed LOH in at least one marker, most frequently in D5S346 (APC gene) and D18S58 (DCC gene).

Twelve samples from seven patients had MSI-L phenotype, with LOH of some other marker in five cases. Mutation in one of mismatch repair gene were detected in two

of those patients (Plevová et al., 2004, Křepelová unpublished).

MSI present only in dinucleotide markers was detected in thirteen patients (8,6 %). Samples from patients O3, 336,834,M145, M266 and M390 were classified MSI-L, from patients 338, 431, 460, 544, 628, 832 and 965 MSI-H.

Marker BAT-25 was added to reference panel of analysed markers on september 2002 to improve analysis and informativity of results in samples without nontumor control tissue. Retrospective analysis of this marker in informative patients to date was not performed.

Marker BAT-RII is less sensitive (23,3 %) for MSI than others (about 30 %). 22 samples from 16 patients were classified as MSI-H in all markers except BAT-RII.

Fifty two patients were analysed for detection of effectivity of both methods by using markers BAT-RII, BAT-26, D2S123, D3S1029, D5S346 and D18S58. Of those, twenty five patients showed the same results by both methods, fluorescent and nonfluorescent fragment analysis. Thirteen noninformative patients by polyacrylamide gel analysis were

informative by fragment analysis with fluorescent PCR. In ten patients loss of heterozygosity was found and four showed MSS and MSI discrepancy.

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

In paralel study, 69 samples from 66 patients were analysed by immunohistochemistry analysis for detection of 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, three MSI-H patients lost expression of MSH2 protein. No changes in expression of mismatch repair genes were detected in MSS patients. Details of this study are in Apendix 1.

DISCUSSION

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

Frequency of microsatellite instability in sporadic colorectal cancers is 10-15 %, in patients fulfilled Amsterodam criteria HNPCC up 93 %. This frequency depends on analysed group of patients. High frequency is shown in large pedigrees of cancer families. Our group of patiens involved any relatives one family except one pair (brother/sister).

We analysed 152 patients, 31/37 patients fulfilling Amsterodam criteria (83,7 %) showed MSI-H phenotype, 2/37 patients (5,4 %) MSI-L and 4/37 patients (10,8 %) MSS phenotype. 19/72 patients with 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. 5/43 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 frequency of MSI in other studies (Vasen et al., 2007)

81 % MSI-H patients had germline mutation in mismatch repair gene hMLH1 or hMSH2. Two mutations were found in patients with MSI-L phenotype: patient M145 had germline mutation of MSH2 gene, 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 clear MSI-L phenotype (BAT-26) showed mutation in MSH6 gene. This is in agreement with the study of Wu, who showed that patients with HNPCC with low degree of microsatellite instability in mononucleotide markers are frequently mutated in MSH6 gene (Wu et al., 1999).

Samples from 66 patients were studied by immunohistochemical analysis of mismatch repair proteins MLH1 and MSH2 (Plevová et al., 2004). 92,4 % of MSI-H patients had reduced expression of MLH1 or MSH2. Details see Appendix 1.

Up to 2001 MSI was detected in our laboratory by fragment analysis in denaturing polyacrylamid gel and staining with ehtidium bromide. When the Genetic analyser ABI Prism 310 became available, the method of fragment analysis with fluorescent labeled PCR was introduced. 52 patients were analysed by both methods. Discordant results were detected in 14 patients. Method of fragment analysis of fluorescent labeled PCR was more effective in detection of LOHs and new fragments with insertion/deletion of one repetitive unit. The same results were published by Christensen study (Christensen et al., 1999).

Sometimes we had troubles with amplification of DNA from formalin-fixed paraffin embedded tumor tissue. 85 % of analysed tissue were from parffin embedded samples. DNA from these samples had lower quality, was degraded into shorter fragments. In comparision to 22 tissue samples with all amplified markers in 175 fixed samples, all analysed markers from frozen tissue were amplified in every patient. Later, new marker (D17S250) was added from reference panel to get more informative results (Boland et al., 1998).

We also analysed nine patients without non-tumor DNA as a control. It is recomended to analyse at least three markers from reference panel in these cases (Boland et al.,

1998). We added one more mononucleotide marker, BAT-25, for this situation. Samples with two or more unstable mononucleotide markers were included to the study. Samples with one or no unstable successfully analyzed mononucleotide marker were classified as noninformative and were excluded.

Two quasimonomorphic markers are recommended in the reference panel, BAT-25 and BAT-26. Both are monomorphic in Caucasian population, where only one allele is detected. Some research groups proposed to analyse only tumor DNA with BAT-26 for rapid and effective analysis of microstellite instability (Iacopetta and Hamelin, 1998; Iacopetta and Grieu, 2000; Samowitz et al., 1999). Iacopetta, Fridrichová (Fridrichová et al., 2000), Cunningham (Cunningham et al., 2001) and Lamberti (Lamberti et al., 1999) detected instability in BAT-26 marker in almost 100 % of MSI-H samples. Since in our series of patients we found thirteen samples (seven MSI-H) with instability only in dinucleotide markers, we always compared tumor and non-tumor DNA whenever possible.

Lower sensitivity of marker BAT-RII (22 MSI-H tumors without any change in BAT-RII marker) observed by us is simmilar to results published by others (Iacopetta et

Hamelin 1998; Parsons et al., 1995; Akiyama et al., 1997, Gebert et al., 2000). Instability in marker BATRII is caused by somatic frameshift mutation in tumor cells, this marker is in coding region of the TGF-β-RII gene. Due to this fact, 60-90% of MSI-H tumors have one or two basepair insertion/deletion in this gene (**62 %** Johannsdottir et al., 2000; **72,7 %** Markowitz et al., 1995; **82 %** Gebert et al., 2000; **85 %** Akiyama et al., 1997; **85 %** Fujiwara et al., 1998; 86 % Iacopetta et al., 1998; **90 %** Parsons et al., 1995), in correspondence with our result of 70,6 %.

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

Some difference from the observation of Dietmaier (Dietmaier et al., 1999) were found in our study, where we observed cases with both MSI and LOH, in one tissue sample. While no sample from 148 demonstrated both LOH and MSI in Dietmaier's study, 15/152 (10%) samples demonstrated both LOH and MSI in our study. This difference may be caused by

different methods of tissue sample processing. Dietmaier's group used microdissection of tumor cells from paraffin-embedded tissues. This method is more effective and sensitive.

The second possible explanation is an incorrect interpretation of MSI. We can detect only gained or missed fragments in tumor sample by analysis of MSI. We can not detect loss or deletion/insertion of the first allele comigrating with the second allele of the same lenght. It is necessary to quantify both DNA fragment samples for verifying this situation. It may be impossible because of the poor quality of DNA extracted from formalin-fixed, paraffin-embedded tissue.

SUMMARY

Detection of microsatellite instability (MSI) is the standard part of mutational analysis in hereditary nonpolyposis colorectal cancers (HNPCC). Characteristic phenotypic feature of MSI indicates loss of mismatch repair (MMR) in tumor cells.

We studied MSI in 205 tumors from 152 patients with HNPCC. Of these, 37 patients fulfilled Amsterdam criteria, 72 patients were familial and 43 were sporadic cases. We used methods of fragmentation analysis on polyacrylamide gel

and/or with fluorescent labelled primers (ABI Prism 310 Genetic Analyzer). Three mononucleotide (BAT-RII, BAT-25, BAT-26) and five dinucleotide (D2S123, D3S1029, D5S346, D17S250, D18S58) repeat loci were analysed. We detected 75 tumors with high degree of MSI (MSI-H), 12 tumors with low degree of MSI (MSI-L) and 118 tumors with stable microsatellites (MSS). In 44 of these, loss of heterozygozity (LOH) was found.

In 30 patients with MSI-H tumors a mutation in one of mismatch repair genes was detected. Microsatellite analysis was positively correlated with immunohistochemical detection of MLH1 and MSH2 proteins.

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