Letters to the Editor

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Incidence of germline *hMLH1* and *hMSH2* mutations (HNPCC patients) among newly diagnosed colorectal cancers in a Slovenian population

EDITOR—Hereditary non-polyposis colorectal cancer (HNPCC) syndrome is a common autosomal dominant predisposition to colorectal cancer. Clinical diagnostic features of sporadic and HNPCC associated colorectal cancer do not differ significantly and until recently the identification of HNPCC patients was based mainly on their family history. Because of the importance for relatives of HNPCC patients to be clinically examined frequently in order to detect the disease at an early, curable stage, the International Collaborative Group (ICG) on HNPCC proposed criteria for identification of HNPCC families. According to the guidelines agreed by the ICG in Amsterdam in 1990, an HNPCC family has to fulfil the following criteria: there should be at least three relatives with colorectal cancer (one of whom is a first degree relative to the other two), at least two successive generations should be affected, and one relative should be diagnosed under the age of 50.[1] CRC patients with HNPCC syndrome can also develop cancer of the endometrium, stomach, ovary, and urinary and hepatobiliary tracts.[2–5] In several epidemiological studies, the incidence of HNPCC has been estimated to be between 0.5%[6] and 15%[3] of all colorectal cancers. The identification of mismatch repair genes (MMR), of which at least five (*hMLH1*,[7 8] *hMSH2*,[9 10] *PMS1*,[9 10] *PMS2*,[7] and *hMSH6* (*GTBP*)[11]) are associated with HNPCC, has enabled mutational analysis in families fulfilling complete or partial Amsterdam criteria. Carriers of germline MMR mutations have a higher than 80% risk for cancer by the age of 75.[2] The great majority of germline mutations were found in approximately equal proportions in *hMLH1* and *hMSH2*, while mutations in the other three MMR genes have been reported only in a limited number of cases.[12] Germline *hMLH1* and *hMSH2* mutations were also found in a considerable proportion of colorectal cancer patients from families not fulfilling the Amsterdam criteria,[13] especially if they were young,[14 15] indicating that some HNPCC families might be missed if they are preselected before mutational analysis.

Mutations in MMR genes result in microsatellite instability (MSI),[16] which is characteristic of more than 90% of tumours in HNPCC patients but was also found in 12-15% of sporadic colorectal tumours.[17–19] In the absence of other diagnostic criteria, MSI analysis of tumours could be valuable markers in HNPCC identification.

Here we report an effective MSI analysis of CRC and subsequent mutational analysis of the *hMLH1* and *hMSH2* genes in tumours with considerable MSI for identification of HNPCC among randomly collected, newly diagnosed colorectal cancers. This approach allowed us to identify HNPCC families and to estimate the minimal incidence of HNPCC in a Slovenian population based solely on molecular genetic analysis.

Primary colorectal adenocarcinomas and corresponding normal tissue samples were collected from patients who gave consent for testing of their DNA. Between 1996 and 1998, 300 newly diagnosed CRC patients from clinics all over Slovenia participated in this study. The sample of CRC is thus representative for the Slovenian population. All samples were gathered in a central institution where two physicians histologically evaluated each resection for a high proportion of tumour tissue. We also confirmed a high proportion of cancer cells versus normal cells in tumour tissue in many samples that exhibited LOH during MSI analysis. Normal colorectal mucosa taken from a site distant from the tumour was used as a normal control in the study.

We isolated DNA after tissue digestion using standard phenol/chloroform extraction and ethanol precipitation from frozen colorectal tumours and corresponding normal tissue samples. For MSI analysis of tumour and control

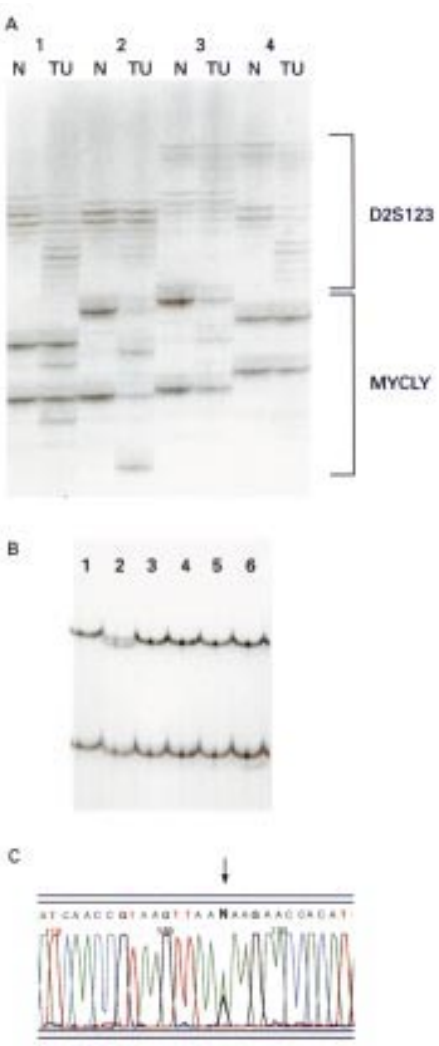

Figure 1 Strategy of molecular genetic based identification of HNPCC patients. (A) Microsatellite instability (MSI) analysis; D2S123 and MYCLY microsatellite markers were amplified in a multiplex PCR reaction and products were resolved on a 6% denaturing gel and silver stained. Tumour samples 1-3 exhibit MSI at both markers while sample 4 exhibits MSI only at D2S123. N=normal, TU=tumour. (B) SSCP analysis of hMLH1 exon and exon/intron boundary 9; aberrant bands in sample 2 indicates sequence alteration. (C) Sequence analysis of exon/intron boundary 9 of hMLH1 gene of sample 2. Arrow indicates heterozygous A→G substitution.

Table 1 Pathogenic germline hMLH1 and hMSH2 mutations identified in high MSI tumours

Gene and patient	*Exon*	*Nucleotide change*	*Consequence*	*Reference*
hMLH1				
OG/98-2989	2	199 G→A	G67R	31
OG/97-4266	15	1684 C→T	Q562X	This study
OG/97-3941	17	1964 T→C	I655T	32
hMSH2				
OG/97-2426	3	561 del TGAGGCTCT	In frame del of codons 188-190	33

tissue pairs, we tested the majority of samples with the following markers: mononucleotides BAT26,[11] BAT25,[11] and BAT40,[20] dinucleotides D2S123,[21] D5S346,[22] TP53,[23] D11S1294,[24] D112179,[24] D17S250,[25] D18S58,[26] and D18S69,[21] and tetranucleotide MYCLY.[27] Tumours were scored as high MSI if more than 40% of tested markers were positive and were scored as low MSI if less than 20% of tested markers were positive. During the analysis, it turned out that for determination of high MSI status four selected markers were sufficient, BAT26, D2123, BAT25, and D5S346. Therefore, we analysed additional samples in multiplex PCR reactions for microsatellite markers BAT26 and D2123, and BAT25 and D5S346, respectively. In this case, tumour tissue was scored as high MSI if at least two of four markers were altered.

MSI analysis was performed as previously described.[28] Briefly, after PCR amplification products were run on a thin (0.4 mm) denaturing polyacrylamide gel matrix fixed on one of the glass plates followed by an optimised silver staining protocol that markedly improved the resolution. MSI was detected as some additional bands in tumour DNA compared to control DNA (fig 1A).

For mutational analysis of *hMLH1* and *hMSH2* mismatch repair genes (MMR), we used PCR/non-isotopic conformation analysis (article in preparation). The basic principle of this method is a combination of three analyses which are all based on changes in three dimensional DNA structures, that is, single strand conformation analysis (SSCA), heteroduplex analysis (HA), and double strand conformation analysis (DSCA). We conducted them simultaneously on the same thin polyacrylamide gel.

Altogether, of 300 randomly collected primary colorectal tumours, 29 (9.7%) were classified as high MSI tumours and 23 (7.7%) as low MSI tumours. The use of BAT26 only has been previously proposed for determination of high MSI status.[29 30] Our results show that using only this marker we would have missed one of 29 high MSI tumours. On the other hand, no low MSI tumour was positive for BAT26 or BAT25.

Genomic DNA from 29 subjects with high microsatellite instability tumours were further analysed for the presence of germline mutations in two MMR genes. The whole coding regions as well as all exon/intron boundaries of *hMLH1* and *hMSH2* were tested with PCR/conformational analysis. We observed 17 different aberrant gel migrations in 29 DNA samples from patients with high MSI tumours. All 17 alterations were detected as single stranded conformational polymorphisms, one sample also showed altered double stranded conformation, and in two samples heteroduplexes were also formed. Eleven alterations were in *hMLH1* and six in *hMSH2*. Sequencing showed four presumably pathogenic mutations, three in *hMLH1* and one in *hMSH2* (table 1). A new polymorphism IVS9+10A>G in *hMLH1* (allele frequency 0.02) has also been detected (fig 1B, C).

Mutations G67R and I655T in *hMLH1* have been previously reported[31 32] in connection with hereditary bowel cancer, while Q562X has been detected for the first time among Slovenian patients with high MSI CRC tumours. We also detected a germline deletion of three consecutive amino acids from 188-190 in *hMSH2* in a person with a high MSI tumour (OG/97-2426-T). The same mutation was previously found in a Slovenian patient (HNPCC-8-2B) with a family history of CRC.[33] Although these two patients live in different parts of Slovenia and have different family names, a retrospective pedigree search showed common roots two generations back and resulted in the identification of an extensive Slovenian HNPCC pedigree (fig 2). Similarly, other patients with germline mutations were contacted for their family history. All patients with germline mutations accessed via MSI analysis had at least one first degree relative with HNPCC associated cancer.

In this study, we used a molecular genetic approach for identification of HNPCC families. Our approach was based firstly on microsatellite instability (MSI) analysis of newly diagnosed randomly collected CRC and subsequently on mutational analysis of mismatch repair genes (MMR) in MSI positive tumours. We used a solely molecular genetic approach for detecting HNPCC families because (1) a considerable proportion of germline MMR mutations were found in patients from families not fulfilling the Amsterdam criteria,[13–15] and (2) access to well organised cancer registries is not available in many countries. In Slovenia, a cancer registry exists but it does not contain family data. No specialised registries for hereditary forms of cancer are available. In our first attempt to identify Slovenian HNPCC families, 600 colorectal cancer patients were questioned for their family history data. A total of 44 patients reported at least one first degree relative with CRC and 16 families agreed to contribute blood samples for DNA analysis. Germline mutation was detected in one family only.[33] This approach

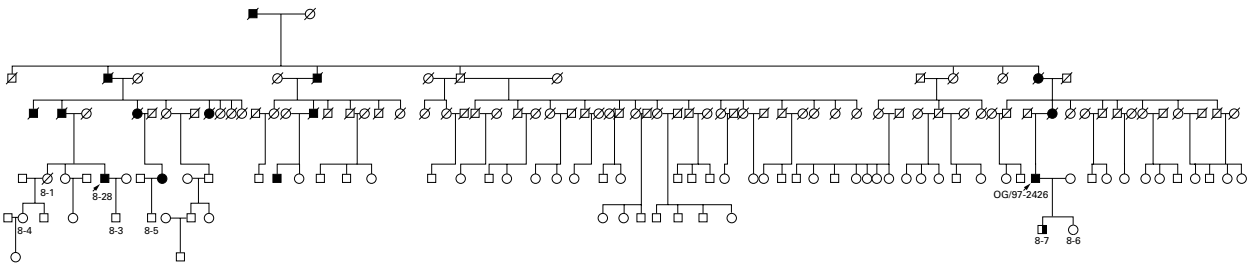

Figure 2 Extended Slovenian HNPCC family with deletion of three amino acids (188-190) in hMSH2. We found this germline deletion in a subject with high MSI tumour (OG/97-2426). We also previously detected the same mutation in a Slovenian patient (HNPCC-8-2B) with a family history of CRC.[32] A retrospective pedigree search showed common roots of both patients' families two generations back. The resulting extended HNPCC family spans six generations and has 162 family members. Affected members are shaded. Presymptomatic DNA testing identified five non-carriers and one carrier of the mutation (8-7).

showed some inaccuracy of data obtained directly from patients and suggested that epidemiological studies of HNPCC incidence based on patients' answers should be treated with caution.

With a molecular genetic approach, we found germline mutations in four out of 300 newly diagnosed colorectal cancers. These four alterations are unambiguously pathogenic mutations; one is a deletion, one is nonsense, and two are missense mutations. Two mutations are so far specific for Slovenian families: a deletion of codons 188-190 in exon 3 of *hMSH2* (fig 2) and a C to T substitution in exon 15 of *hMLH1* resulting in a stop codon at 562. A substitution of Ile to Thr at codon 655 detected in a patient OG/97-3941 was previously reported in a patient with gastric carcinoma.[32] Interestingly, also, Slovenian patient OG/97-3941 had a metachronic gastric cancer two years after colon cancer. The Gln to Arg substitution at codon 67 was previously described in a Swedish HNPCC family[31] and its pathogenicity was proved in a functional assay in yeast.[34]

It is possible that we missed some tumours with MMR gene mutation, because we analysed only MSI positive tumours. Cases with MMR mutations without MSI were also reported.[15] However, 1.3% (4/300) tumours with germline MMR gene mutations in the Slovenian population is lower than the 2% observed in a similar study in a Finnish population. If the common ancestral Finnish specific mutation, which accounts for a half of all Finnish HNPCC families,[35] is excluded the percentage in our country is even higher than in Finland. No comparison with other populations using a similar approach is currently available. Finnish and Slovenian molecular genetic based estimations of HNPCC incidence are considerably lower than the 5-10% estimated in the majority of epidemiological studies.[36] However, Evans *et al*[37] also reported the incidence of HNPCC to be 1.4%. Their population based study of 1137 consecutive cases of colorectal cancer showed a lower frequency of familial bowel cancer than previous studies and may reflect a lower incidence of inherited mutations in the HNPCC MMR genes than is currently accepted.[37] Since germline mutation in MMR genes is a reliable indicator of HNPCC syndrome, the estimation of incidence of this hereditary disorder in a Slovenian population could be calculated. If we consider 19 000 newborns annually and 850 newly diagnosed colorectal cancer cases annually[38] (of which 1.3% have germline MMR gene mutation), the HNPCC incidence in the Slovenian population is approximately 1 in 1700. However, this estimate represents the absolute minimum. It is possible that we missed some mutations because of technical limitations and because we did not analyse three minor genes (*hPMS1*, *hPMS2*, and *hMSH6*), which are also predisposing factors for HNPCC. Another reason for underestimation of the disease frequency might be that we only searched for mutations in patients with colorectal cancer, but this cancer accounts for only approximately two thirds of cancers in HNPCC affected families.[39]

The proportion of patients younger than 50 years in our study was the same as it is for colorectal cancer patients in the complete National Cancer Registry indicating that there was no bias in overestimation according to age.

With molecular genetic analysis of colorectal cancers, we were able to identify Slovenian families with a hereditary form of CRC. Only patients with germline MMR mutations who wished to be acquainted with the results of DNA testing were further contacted by both a gastroenterologist and a geneticist for future medical surveillance and family history data. Some extended HNPCC pedigrees have thus been detected. Through this study, we succeeded in providing more information about the role of molecular genetic analysis to general physicians and HNPCC family members. Presymptomatic DNA testing was offered to well informed and consenting non-symptomatic relatives. A prevention programme in the sense of periodic clinical examination of relatives with a constitutional mutation has been initiated. In particular, records of young CRC patients have been re-examined for family and clinical data and additional potential HNPCC families have been identified. This enabled us to initiate building of a national HNPCC registry.

We conclude that a molecular genetic approach with evaluated genetic markers for efficient MSI analysis and subsequent MMR gene analysis of patients with MSI positive tumours is valuable for identification and surveillance of HNPCC families and may serve also as a model for detection of familial cases of CRC in other populations.

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Does the survival motor neuron protein (SMN) interact with Bcl-2?

EDITOR—Spinal muscular atrophy (SMA) is an autosomal recessive disease resulting from mutations in the telomeric copy of the survival motor neuron gene (*SMN1*),[1–7] which results in reduced expression of the survival of motor neuron protein (SMN).[4 7] The SMN protein is ubiquitously expressed but is found at high levels in motor neurons.[4 7 8] The SMN protein associates with Sm proteins,[9 10] SIP-1 protein,[9 10] and itself.[8–11] SMN is found in structures termed gems[8] that are associated with coiled bodies in the nucleus. The SMN protein is involved in RNA biogenesis[10] and is important for the maturation of a functional snRNP complex that performs splicing.[12] The complete loss of SMN is lethal[13] whereas the low levels of SMN found in SMA cause loss of the motor neurons.[4 7] The mechanism by which the reduction of SMN protein results in the loss of motor neurones is unknown. Some groups have suggested it occurs by apoptosis.[14 15] Apoptosis is a conserved, highly regulated mechanism of non-chronic cell death for the removal of surplus, aged, or damaged cells.[15] Apoptosis is regulated by the interactions of apoptosis agonists and antagonist with the Bcl-2 protein being one of the key inhibitors of apoptosis.[16]

Recently Iwahashi *et al*[17] have suggested a direct interaction between SMN and Bcl-2 using transfected constructs. In an effort to confirm and extend their results, we have attempted to coimmunoprecipitate SMN and Bcl-2 both in a native environment and using transfected cells. The SMN protein and Bcl-2 are expressed in Jurkat cells and in spinal cord. Jurkat cells are a human lymphoblast T cell line that has been previously shown to express Bcl-2 and can be induced to undergo apoptosis.[18 19]

Immunoprecipitation experiments were performed using two different methods.[17 20] Fig 1A shows a western blot of the immunoprecipitation reactions using the method of Iwahashi *et al*.[17] Jurkat cell lysate was precipitated with anti-SMN (MANSMA2), anti-Bcl-2 (Bcl-2(100)), and anti-dystrophin (MANDYS-1) monoclonal antibodies. The precipitated proteins were western blotted and probed with anti-SMN or anti-Bcl-2 polyclonal antibodies. As shown in fig 1A-1, anti-SMN polyclonal antibody SMN-C3 detects SMN only in the immunoprecipitation using anti-SMN monoclonal antibody MANSMA2 and not in the immunoprecipitation reaction using anti-Bcl-2 monoclonal Bcl-2(100). The reciprocal experiment is shown in fig 1A-2 in which the blot is probed with anti-Bcl-2 polyclonal antibody Bcl-2 (ΔC21). Bcl-2 is detected only in the immunoprecipitation reaction using Bcl-2(100) but not in the sample immunoprecipitated with MANSMA2. The blots were stripped and reprobed with the anti-Bag-1 (fig 1A-3) and anti-Sm antibody Y12 (fig 1A-4). Sm proteins and Bag-1 have been previously shown to interact with SMN and Bcl-2 respectively and act as controls for the immunoprecipitation procedure.[8 21] The expected results were obtained with Bcl-2 antibodies immunoprecipitating Bag-1 and SMN antibodies precipitating Sm proteins. Similar results were obtained using Jurkat cells undergoing apoptosis after induction with phytohaemagglutinin (data not shown).

In normal spinal cord (fig 1B), SMN-C3 detects a 38 kDa band in the immunoprecipitation reaction using MANSMA2 but not in the immunoprecipitation reactions using Bcl-2(100) or MANDYS-1. Similarly, in the reciprocal experiment, the anti-Bcl-2 rabbit polyclonal antibody Bcl-2 (ΔC21) detects a 29 kDa band only in the sample immunoprecipitated with Bcl-2(100) and not in the other immunoprecipitation reactions.

As immunoprecipitation experiments failed to show a direct interaction of native SMN and Bcl-2 in proliferating and apoptotic Jurkat cells and in spinal cord, we investigated cells transfected with SMN/Bcl-2 expression constructs. We have prepared various SMN expression constructs with or without the HA epitope tag at the amino-terminus. Immunoprecipitations were performed