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Short Communication

Microsatellite Instability in Adenomas as a Marker for Hereditary Nonpolyposis Colorectal Cancer

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Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common of the well-defined colorectal cancer syndromes, accounting for at least 2% of the total colorectal cancer burden and carrying a greater than 80% lifetime risk of cancer. Significant reduction in cancer morbidity and mortality can be accomplished by appropriate clinical cancer screening of HNPCC patients with mutations in mismatch repair (MMR) genes. Thus, it is desirable to identify individuals who are mutation-positive. In individuals with cancer, mutation detection can be accomplished relatively efficiently by germline mutation analysis of individuals whose cancers show microsatellite instability (MSI). This study was designed to assess the feasibility of screening colorectal adenoma patients for HNPCC in the same manner. Among 378 adenoma patients, six (1.6%) had at least one MSI adenoma. Five out of the six patients (83%) had a germline MMR gene mutation. We conclude that MSI analysis is a useful method of prescreening colorectal adenoma patients for HNPCC. ***(Am J Pathol 1999, 155:1849–1853)***

Germline mutations in DNA mismatch repair genes (*MLH1*, *MSH2, PMS1*, *PMS2*, and *MSH6*)[1] underlie HNPCC. Deficient DNA mismatch repair results in reduced replication fidelity. Cells deficient for both alleles of a mismatch repair gene acquire a mutator phenotype, leading to accumulation of somatic mutations, which can be demonstrated by analyzing microsatellite sequences in the tumor DNA. These sequences display frequent somatic deletions and insertions, often referred to as microsatellite instability (MSI). HNPCC patients form adenomas at a slightly but not strikingly increased rate as compared with the general population[2] (Järvinen HJ, Aarnio M, Mustonen H, Aktan-Collan LA, Peltomäki P, de la Chapelle A, Mecklin J-P, submitted). Adenomas in HNPCC tend to be large and show a villous architecture and high-grade dysplasia.[3] It is possible that the mutator or MSI phenotype characteristic of HNPCC tumors drives the promotion of adenoma to carcinoma.

MSI can be seen at an early stage in HNPCC tumors, but usually at later stages in sporadic colorectal tumorigenesis.[4] Adenomas from patients with HNPCC frequently show MSI as opposed to 0 to 3% of apparently sporadic colorectal adenomas.[5,6] The frequency of the MSI is 80 to 95% in HNPCC cancers[6–8] and 10 to 15% in sporadic colorectal cancers.[7–10] The fact that a considerable proportion of sporadic colorectal cancers displays MSI makes microsatellite analysis a relatively unspecific marker for HNPCC when applied to malignant tumors.[11]

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Most colorectal cancers arise within a pre-existing epithelial neoplasm, an adenoma, though only a small proportion of adenomas progresses to malignancy.[12] Adenoma is the precursor lesion in familial adenomatous polyposis (FAP), a rare inherited cancer syndrome. By contrast, in the more common hereditary nonpolyposis colon cancer (HNPCC), typically very few if any adenomas are seen, and their role in the process leading to cancer is less clear.[13–15] However, it has been hypothesized that adenomas in HNPCC patients are more likely to progress to carcinoma than are adenomas in the general population.[13] Nevertheless, colonoscopic screening in individuals with HNPCC gene mutations provides an efficient method of cancer prevention.[16] In a recently completed 15-year screening program aimed at HNPCC mutation-positive individuals, both early detection of cancer and removal of premalignant adenomas resulted in reduction in cancer morbidity and mortality (Järvinen HJ, Aarnio M, Mustonen H, Aktan-Collan K, Aaltonen LA, Peltomäki P, de la Chapelle A, Mecklin J-P, submitted). Similar but less dramatic benefits of colonoscopic cancer surveillance in the general population have been observed before.[17] Given the high (>80%) risk of colorectal cancer in individuals who have germline HNPCC mutations[18,19] and the common occurrence of metachronous tumors in HNPCC patients, it is highly desirable to determine which patients with colorectal adenoma have HNPCC. This study was undertaken to test the hypothesis that the determination of microsatellite instability (MSI) in adenomas, a relatively simple procedure, can serve as a screening method for this purpose.

Materials and Methods

Patients and Population Controls

We screened 402 adenoma specimens obtained from 378 patients for MSI. The number of adenomas obtained from each individual varied from one to seven, but was typically one. Of the adenomas, 71% were distal, 17.5% were proximal, and in 11.5% of cases the site of the adenoma was not reported. The individuals ranged in age from 23 to 90 years with a mean age of 66 years. Fresh-frozen samples were collected between June 1994 and June 1998 at nine large regional hospitals in eastern Finland. Informed consent was obtained from the patients. The design of the study called for accrual of every adenoma whenever this was technically possible. No selection in favor of adenomas from young patients, patients with a family history of cancer, or clinical parameters was allowed. However, in view of the large number of participating hospitals, physicians, and other personnel, some such unintentional selection may nevertheless have occurred. Clearly, the main reason for not including adenomas was small tumor size. It is not feasible to divide very small adenomas, and procuring a good specimen for histological analysis had precedence over procuring a specimen for this study. Most (338, 84%) of the samples were obtained during colonoscopy and some (45, 11%) during surgery; information was not available in 19 cases. Pathologists at the respective hospitals histologically evaluated the lesions. The patient data were cross-linked with the Finnish HNPCC registry data and Finnish Cancer Registry data. The series was found to contain 11 patients from known HNPCC families. Seven families segregate characterized *MLH1* mutations. DNA from 2497 cancer-free anonymous blood donors (age range, 18–65) from the eastern part of Finland were collected at local Finnish Red Cross Blood Transfusion Centers proportional to the geographic distribution of tumors collected from the participating hospitals and were used as population controls in mutation studies.

Analysis of MSI

DNA extracted from the adenoma tissue was studied for MSI using BAT26 and TGF-βRII mononucleotide (polyA) markers by fluorescence-based polymerase chain reaction (PCR). The forward and reverse primers used were, for BAT26: forward, TGA CTA CTT TTG ACT TCA GCC; reverse, AAC CAT TCA ACA TTT TTA ACC; for TGF-βRII: forward, CTT TAT TCT GGA AGA TGC TG; reverse, GAA GAA AGT CTC ACC AGG C. PCR reactions were carried out in a 10-μl reaction volume containing 100 ng genomic DNA, 1× PCR buffer (Perkin Elmer Applied Biosystems Division, Foster City, CA), 200 μmol/L of each dNTP (Finnzymes, Espoo, Finland), 300 μmol/L (TGF-βRII) or 600 μmol/L (BAT26) of each primer and 1.5 units of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 1.5 mmol/L. The following PCR cycles were used for amplification: BAT26: 95° for 10 minutes, 30 cycles of 95° for 45 seconds, 55° for 1 minute, 72° for 30 seconds and TGF-βRII: 94° for 10 minutes, 28 cycles of 94° for 30 seconds, 55° for 75 seconds, 72° for 20 seconds. Final extension was 72° for 10 minutes. PCR products were loaded on a 6% polyacrylamide 8-mol/L urea gel and run in an ABI PRISM 377DNA Sequencer (Perkin Elmer) according to manufacturers instructions. The data were collected automatically and analyzed by the GeneScan 3.1 software (Perkin Elmer). From all MSI patients normal tissue (normal mucosa or venous blood) DNA was available and the MSI analysis was repeated by comparing paired normal/adenoma DNA pairs to confirm somatic origin of the aberrant alleles.

Detection of Mutations

All 378 patients were scrutinized for the three most common mismatch repair gene mutations in Finland. In approximately half of all cases normal tissue was not available for mutation analysis, and adenoma DNA was used. Founder mutation 1 is a 3.5-kb genomic deletion of *MLH1* comprising exon 16. Founder mutation 2 is *MLH1* exon 6 splice site mutation G→A at 454–1. Founder mutation 3 is *MLH1* exon 4 missense type of change T→G at 320. Together these three mutations account for 79% (65/82) of mutation-positive HNPCC families diagnosed in Finland[20] (unpublished data). Mutation 1 was detected by a PCR-based method that has been described previously.[21] Mutations 2[21] and 3 were detected by allele-specific

Table 1. Results of MSI and Mutation Analyses

Patient	Genealogical connection to a HNPCC family	MSI	Mutation detected in patient	Mutation segregating in the family
A38	+	+	mut 1	mut 1
A83	+	+	mut 3	mut 3
A190	+	−	—*	?‡
A262	+	+	*MLH1* ex 17	*MLH1* ex 17
A263	+	−	—*	?‡
A367	+	−	—*	?‡
A369	+	+	mut 2	mut 2
A380	+	+	mut 1	mut 1
A387	−	+	—†	—
A390	+	−	—*	mut 1
A396	+	−	—*	?‡
A507	+	−	—*	mut 2
All others	−	−	—*	—
Totals 376	11	6	5	7

*Sample was analyzed for Finnish founder mutations 1, 2, and 3.
†Sample was analyzed for mutations 1, 2, and 3, and genomic sequencing of *MLH1* and *MSH2* was performed.
‡No mutation has been found in the family despite germline mutation analysis of *MLH1* and *MSH2* by genomic sequencing in individuals affected by cancer.

oligonucleotide hybridization. The protocol for mutation 3 detection is identical with that of mutation 2, except that the hybridization temperature is 62°C. The forward and reverse primers used for mutation 3 detection were: forward, CAG ATA ACC TTT CCC TTT GGT G; reverse, TAT GCA CAC TTT CCA TCA GC and the PCR reactions were carried out in 20-μl reaction volume containing 100 ng genomic DNA, 1× PCR buffer (Perkin Elmer), 500 μmol/L of each dNTP (Finnzymes), 700 μmol/L of each primer and 1 unit of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 2.75 mmol/L. The following PCR cycles were used for amplification: 95° for 10 minutes, 40 cycles of 95° for 1 minute, 57° for 1 minute, 72° for 1 minute. Final extension was 72° for 10 minutes. PCR products were run in 2% agarose (NuSieve) gel to verify the amplification, thus avoiding the need for hybridization with a wild-type probe. PCR products from three individuals were pooled together into the filter. Filters were hybridized with a probe containing the mutant sequence (mutation 2: 5′ CTT CTG TTC AAG TGG AGG AC 3′, mutation 3: 5′ CTT TGG CCA GCA TAA GCC AT 3′). If a positive signal was obtained, the respective samples were rehybridized separately in a new filter.

If none of the founder mutations was detected in the MSI samples, full mutation analysis of *MLH1* and *MSH2* was performed by direct genomic sequencing of the coding exons, including the flanking intron region and promoter region, as previously described.[11] Normal tissue DNA from members of known HNPCC families was studied for the mutation known to segregate in the family even if the adenoma of the patient was microsatellite-stable (MSS).

To estimate roughly the population frequency of the founder mutations studied, the two most frequent defects, mutations 1 and 2, were analyzed from DNA derived from 2497 healthy anonymous blood donors. A rapid method to lyse white cells from 0.5 ml of whole blood was used.[22] Mutation 1 was screened for as previously described[21] with the exception of a reverse primer (5′-GAACACATCATCAAGATGGAGAGCC-3′) located in intron 16, downstream from the deletion breakpoint, instead of the previously published reverse primer located in exon 17. Mutation 2 was screened for by a solid phase minisequencing method[23] suitable for semi-automated screening of single nucleotide changes.

Results

Among 378 tested patients, six (1.6%) had MSI adenomas. In each MSI case BAT26 deletions of at least 6 bp were observed. Of these six individuals, one (A38) was represented by two adenomas (both displaying MSI), whereas one adenoma was derived from the other five individuals. All seven tumors displayed instability at the BAT26 locus, and three displayed TGF-βRII polyA deletions (three adenomas were negative and one failed in analysis). Of the 12 adenomas derived from HNPCC families, six (50%), derived from five different individuals, were among the MSI samples, whereas the incidence of MSI in sporadic adenomas was one out of 367 (0.3%). A germline mutation in *MLH1* or *MSH2* was detected in five of the six MSI patients. All were members of previously identified HNPCC families (mutation 1 in two patients, mutation 2 in one patient, mutation 3 in one patient and *MLH1* exon 17 non-sense mutation C→T at 1975 [20] in one patient). No mutation was detected in the sixth MSI patient, who did not belong to a known HNPCC family (Table 1).

Six (50%) adenomas derived from known HNPCC family members were MSS. In four of these families *MLH1* and *MSH2* mutations had been previously excluded by germline mutation analysis. Two families were known to harbor *MLH1* mutations, but the MSS adenoma patients did not have the germline mutation segregating in the family.

None of the three founder mutations was detected in the 372 MSS patients. Eight MSS samples failed in mu-

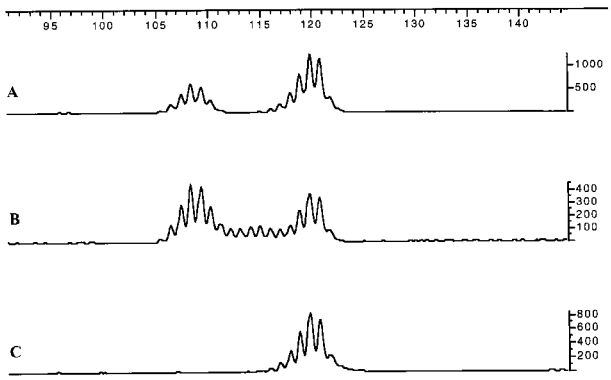

Figure 1. MSI analysis using BAT26 mononucleotide (polyA) marker by fluorescence-based PCR. Graphs **A** and **B** represent two separate adenomas and graph **C** represents the normal DNA of the same individual (A38). Tumor DNA displays novel BAT26 alleles as compared with the normal tissue DNA. Allele sizes in basepairs can be determined using the scale at the top of the figure.

tation 1 analysis, one sample failed in mutation 2 analysis, and four samples failed in mutation 3 analysis. No cases with mutation 1 and no cases with mutation 2 were detected among the 2424 cancer-free anonymous blood donors (73 samples failed) with the screening methods used. The carrier frequency of these mutations in cancer-free population appears to be low, perhaps less than 1/2500 or 0.0004% in the geographic area of highest HNPCC incidence in Finland.

Discussion

In a series of 378 adenoma patients we found six (1.6%) cases that were MSI. This frequency is in accordance with previous data.[5,6,24] MSI detected in adenomas predicted germline mutations in *MLH1* or *MSH2* with a high specificity, as five out of six (83%) MSI adenoma patients were mutation-positive. The one patient with MSI adenoma who had no association to an HNPCC kindred, and in whom no *MLH1* and *MSH2* mutation detected, was a 66-year-old woman. The lesion was removed during colonoscopy that revealed approximately 10 polyps, four of which were biopsied. Two of the lesions were adenomatous, one hyperplastic, and in one biopsy specimen no histological changes were reported. The pedigree of the patient was extended, and data on the relatives were linked to the Finnish Cancer Registry. No cancer had been reported for the patient's brother (69 years of age) or four children (37 to 40 years of age). Thus, strong features of HNPCC were not revealed.

Considering the proportion of HNPCC (patients with *MLH1* or *MSH2* mutations[11]) of all colorectal carcinomas (approximately 2%), the proportion of HNPCC in the current series of adenoma patients, 1.6%, may appear unexpectedly high, as adenomas frequently occur in the general population. In addition to possible bias in patient selection, the high frequency of mutation-positive individuals is explained by the fact that the Finnish HNPCC screening program is actively conducted in the participating hospitals. Hundreds of at-risk individuals undergo colonoscopic tumor screening in these centers.

Because of the quasimonomorphic nature of the BAT26 polyA tract (size variation is uncommon between germline alleles), this marker can be used to screen initially for MSI without matching normal DNA.[25] However, possible MSI cases should be reanalyzed with a matching normal DNA to confirm the somatic origin of the aberrant alleles.[24]

Three out of seven (43%) MSI lesions carried somatic truncating mutations of the TGF-βRII gene. These mutations have also previously been found in adenomas from individuals with HNPCC,[26] suggesting that TGF-βRII inactivation is an early event in HNPCC colon neoplasia. The sporadic MSI adenoma also displayed a somatic TGF-βRII mutation. These findings do not support the notion that TGF-βRII mutations are late events in MSI tumors.[27] Obviously, larger numbers of samples need to be analyzed to clarify this issue.

MSI in adenomas appears to be a relatively specific pointer for HNPCC. As MSI is very rare in sporadic adenomas, screening such lesions routinely for MSI may not be a high priority. However, MSI analysis in adenomas is likely to be useful in cases where clinical features or family history suggest hereditary predisposition. For example, MSI analysis in an adenoma patient from a colon cancer family can give important clues to the true risk status of such an individual. This aspect is illustrated in the two cases where the adenoma patients were members of known HNPCC families, but had MSI-negative adenomas and were demonstrated not to carry the mutation segregating in the family. Also, in a prospective, long-term colonoscopic screening organized in 22 Finnish families, adenoma was removed in 12 at-risk family members who subsequently proved to be mutation-negative in predictive tests (Järvinen HJ, Aarnio M, Mustonen H, Aktan-Collan K, Aaltonen LA, Peltomäki P, de la Chapelle A, Mecklin J-P, submitted). The case for using MSI analysis in adenomas is even stronger in colon cancer families with MSI tumors and unidentified mutations. Adenomas are common in the general population, but the detection of an adenoma in colonoscopic screening for hereditary colon cancer frequently causes uncertainty and confusion. MSI analysis adds an important parameter to be considered in the interpretation of the patient's cancer risk.

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