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INCIDENCE OF HEREDITARY NONPOLYPOSIS COLORECTAL CANCER AND THE FEASIBILITY OF MOLECULAR SCREENING FOR THE DISEASE

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ABSTRACT

Background Genetic disorders that predispose people to colorectal cancer include the polyposis syndromes and hereditary nonpolyposis colorectal cancer. In contrast to the polyposis syndromes, hereditary nonpolyposis colorectal cancer lacks distinctive clinical features. However, a germ-line mutation of DNA mismatch-repair genes is a characteristic molecular feature of the disease. Since clinical screening of carriers of such mutations can help prevent cancer, it is important to devise strategies applicable to molecular screening for this disease.

Methods We prospectively screened tumor specimens obtained from 509 consecutive patients with colorectal adenocarcinomas for DNA replication errors, which are characteristic of hereditary colorectal cancers. These replication errors were detected through microsatellite-marker analyses of tumor DNA. DNA from normal tissue from the patients with replication errors was screened for germ-line mutations of the mismatch-repair genes *MLH1* and *MSH2*.

Results Among the 509 patients, 63 (12 percent) had replication errors. Specimens of normal tissue from 10 of these 63 patients had a germ-line mutation of *MLH1* or *MSH2*. Of these 10 patients (2 percent of the 509 patients), 9 had a first-degree relative with endometrial or colorectal cancer, 7 were under 50 years of age, and 4 had had colorectal or endometrial cancer previously.

Conclusions In this series of patients with colorectal cancer in Finland, at least 2 percent had hereditary nonpolyposis colorectal cancer. We recommend testing for replication errors in all patients with colorectal cancer who meet one or more of the following criteria: a family history of colorectal or endometrial cancer, an age of less than 50 years, and a history of multiple colorectal or endometrial cancers. Patients found to have replication errors should undergo further analysis for germ-line mutations in DNA mismatch-repair genes. (N Engl J Med 1998; 338:1481-7.)

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SEVERAL hereditary disorders predispose people to colorectal cancer. Among these the polyposis syndromes have distinctive clinical features,¹ whereas the diagnosis of hereditary nonpolyposis colorectal cancer is based mainly on family history. The lack of characteristic diagnostic features has prompted the use of the so-called Amsterdam criteria to establish the diagnosis: the presence of histologically verified colorectal cancer in at least three relatives (one of whom is a first-degree relative of the other two), the presence of the disease in at least two successive generations, and an age at onset of colorectal cancer of less than 50 years in one of the relatives. In addition, the various polyposis syndromes must be ruled out.²

Typical features of hereditary nonpolyposis colorectal cancer include a family history of colorectal cancer at a relatively young age, predominance of proximal tumors, and a tendency to have multiple primary tumors. Certain types of extracolonic tu-

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tumors are associated with the disease, especially tumors of the endometrium.³⁻⁶ Germ-line mutations of five genes involved in DNA nucleotide mismatch repair, *MSH2*, *MLH1*, *PMS1*, *PMS2*, and *MSH6* (also known as *GTBP*), have been identified in patients with the disease, but only occasional examples of mutations of *PMS1*, *PMS2*, and *MSH6* have been reported. In the great majority of families with hereditary nonpolyposis colorectal cancer the mutations affect *MSH2* or *MLH1*.⁷⁻⁹ One additional mismatch-repair gene, *MSH3*, has not been associated with hereditary nonpolyposis colorectal cancer.¹⁰ These genes encode enzymes that survey newly replicated DNA for errors and repair mismatched bases in the molecule. Defective DNA mismatch-repair genes result in replication errors and genetic instability.¹¹

Recent evidence suggests that members of families with hereditary nonpolyposis colorectal cancer benefit from clinical screening by colonoscopy.¹² For such screening to be effective and efficient, it should only be offered to family members with a mutation. Programs to identify carriers of mutations in mismatch-repair genes have been implemented in some centers in which affected families have been identified.^{3,13} However, only a fraction of such families have been identified. A further complication is that the incidence of hereditary nonpolyposis colorectal cancer is not known. Epidemiologic and genetic studies have estimated that 0.5 to 13 percent of cases of colorectal cancer are due to hereditary nonpolyposis colorectal cancer,^{14,15} leaving a high level of uncertainty about the actual incidence. Given that carriers of mismatch-repair gene mutations have a greater than 80 percent risk of cancer by the age of 75,³ it is important to determine the frequency of the disease in the population and to devise efficient and cost-effective ways of detecting this condition.

Benign and malignant colorectal tumors in patients with hereditary nonpolyposis colorectal cancer are characterized by a high frequency of DNA replication errors.^{16,17} These errors can be identified by detecting microsatellite marker alleles in tumor DNA that are not present in DNA from normal tissue. The microsatellites are short, repetitive nucleotide sequences containing, for example, multiple consecutive cytosine (C) and adenine (A) bases. These novel alleles presumably reflect the genetic instability caused by mutations in mismatch-repair genes. The length of these sequences can be determined by designing primers that will flank the repeated sequence and performing a polymerase chain reaction (PCR) for microsatellite marker analysis. By analyzing DNA from normal tissue and tumor tissue from the same patient, one can evaluate the fidelity of the replication of these sequences in the tumor. A finding of alleles in the tumor DNA that are not present in the normal tissue may indicate that the fidelity of DNA

replication in the tumor cells is poor. About 10 to 15 percent of all colorectal carcinomas have replication errors,¹⁶⁻²⁰ and it has been suggested that the presence of such errors can be a useful marker for hereditary nonpolyposis colorectal cancer.¹⁶ There is a need for such a marker because in the absence of characteristic clinical features, the diagnosis rests largely on family history²; moreover, screening for germ-line mutations in mismatch-repair genes is likely to be inefficient.²¹

The present study was conducted to determine the frequency of hereditary nonpolyposis colorectal cancer among patients with colorectal cancer and to test a screening strategy for the disease in patients with newly diagnosed colorectal cancer.

METHODS

Patients and Tissue Preparation

We collected fresh-frozen specimens of colorectal adenocarcinomas from 509 consecutive patients between May 1994 and April 1996 at nine large regional hospitals in southeastern Finland. Informed consent was obtained from each patient before any molecular analyses were carried out. Specimens were examined histologically so that the sections used for DNA extraction would have as high a proportion of tumor cells as possible.²² In 96 percent of the patients this proportion was over 50 percent. Normal mucosa (from a separate site, not from the margins of the tumor) or blood was used as a source of normal tissue for DNA extraction.

We documented the patients' family histories by identifying all first-degree relatives (parents, siblings, and children) in the official population registries and verifying the diagnoses of cancer in the relatives through the Finnish Cancer Registry (Table 1). Both the population registries and the Cancer Registry have almost complete coverage,^{23,24} and this method of documenting family history has been successfully used in the past.¹⁴ The data on previous cancers in the patients were also obtained from the Cancer Registry. The Finnish Cancer Registry is nationwide, is population based, and has legal status. The great majority of the cases of cancer reported to the registry are histologically or cytologically documented; in 1994, 94 percent of cases were so documented.²⁵

Analysis for Replication Errors

In the first 236 carcinomas of the series, analysis for replication errors was performed with radioactive-labeling techniques, whereas the last 273 carcinomas were analyzed by fluorescence-based PCR methods that became available during the study and that have been described previously.²⁶ The newer method allowed efficient and precise determination of the lengths of the alleles and was safe to work with since it did not involve the use of radioactive isotopes. In a separate study, the comparability of the results of the two methods was confirmed.²⁶ The presence or absence of replication errors was determined by a reviewer with no prior knowledge of the clinical features of the patients.

With the radioactive technique the tumors were first analyzed with a set of seven microsatellite markers (D5S404, D17S787, D5S346, D1S216, D11S904, D10S197, and TP53). If tumor DNA (as compared with the respective normal-tissue DNA) unambiguously displayed novel microsatellite alleles, reflecting the presence of replication errors, at two or more loci as evaluated by two reviewers, the tumor was scored as positive for replication errors. If the two reviewers disagreed (as occurred in three cases), a third reviewer evaluated the results. If none of the markers displayed novel alleles, the tumor was scored as negative for replication errors, provided that at least five of the seven loci had been amplified successfully. In the few cases in which only

TABLE 1. CHARACTERISTICS OF THE 509 PATIENTS WITH COLORECTAL CARCINOMAS.*

CHARACTERISTIC	NO REPLICATION ERRORS (N=446)	REPLICATION ERRORS (N=63)	GERM-LINE MUTATION (N=10)	ALL PATIENTS (N=509)
Site of tumor — no. (%)				
Proximal to splenic flexure	118 (26)	49 (78)	7 (70)	167 (33)
Distal to splenic flexure	325 (73)	14 (22)	3 (30)	339 (67)
Dukes' stage — no. (%)				
A	97 (22)	7 (11)	1 (10)	104 (21)
B	160 (36)	37 (59)	6 (60)	197 (39)
C	120 (27)	13 (21)	2 (20)	133 (26)
D	63 (14)	6 (10)	1 (10)	69 (14)
Adenocarcinoma — no. (%)	407 (91)	54 (86)	10 (100)	461 (91)
Cell type — no. (%)				
25–50% mucinous	20 (4)	5 (8)	0	25 (5)
>50% mucinous	18 (4)	4 (7)	0	22 (4)
Signet-ring-cell tumor — no. (%)	1 (0.2)	0	0	1 (0.2)
Average age at onset — yr	68	69	49	68
First-degree relative with colorectal or endometrial cancer — no. (%)	64 (14)	16 (25)	9 (90)	80 (16)
History of colorectal or endometrial cancer — no. (%)	5 (1)	11 (17)	4 (40)	16 (3)
Age at diagnosis <50 yr — no. (%)	31 (7)	9 (14)	7 (70)	40 (8)
Any of the 3 preceding characteristics — no. (%)	102 (23)	20 (32)	10 (100)	122 (24)
Average no. of first-degree relatives with verified cancer	8.1	7.8	7.1	8.1

*Because data were missing in some cases, the numbers shown do not equal the totals in the groups.

I locus showed novel alleles, we used additional markers (DCC, D13S175, D7S519, D20S100, D15S120, D2S136, and D14S79) to study a minimum of 10 loci. If no additional evidence of replication errors was obtained, the tumor was scored as negative. If at least one additional marker displayed instability, the tumor was designated as positive for replication errors. Thus, in practice the number of loci successfully analyzed with the radioactive method ranged from 5 to 12 per pair of samples, but was typically 5 to 7.

With fluorescent labeling and subsequent fragment analysis by an automated sequencer, all pairs of samples were directly analyzed with 16 markers (D8S254, MYC, NM23, D5S346, TP53, D1S228, D8S261, D7S496, D8S137, DCC, D7S501, MCC, D5S318, D1S507, D19S394, and RB1). The number of successful amplifications ranged from 7 to 16, but was typically more than 12. Because of the larger number of markers analyzed, at least 30 percent of the studied marker loci had to have novel alleles to meet the minimal requirement for positivity for replication errors. When the technique was introduced, amplifications were extensively repeated. Since the results were consistent, routine repetition of the analyses was considered unnecessary. Finally, to verify uniformly the replication-error status of the patients, the whole series of tumors was analyzed by a PCR-based method using radioactively labeled BAT-26, a recently introduced mononucleotide marker that is particularly sensitive to replication errors.^{27,28} Tumors displaying BAT-26 alleles at least 7 bp apart were considered unstable. Patients identified as having replication errors were contacted and offered genetic counseling.

Detection of Mutations

In patients whose tumors had replication errors, a search for germ-line mutations in *MLH1* and *MSH2* was performed by two-dimensional denaturing gradient gel electrophoresis in the first 198 patients.²⁹ The sensitivity of this method in detecting point mutations is 90 to 100 percent.^{30–33} For the 311 subsequent pa-

tients, tumors with replication errors were analyzed for mutations by direct sequencing. The promoter regions and each exon of the *MLH1* and *MSH2* genes from genomic DNA were individually amplified in a Perkin-Elmer cyler (model 9600, Perkin-Elmer, Norwalk, Conn.)³⁴ and subsequently sequenced directly with fluorescence-labeled M13 forward and reverse primers (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.) with a Prism dye primer cycle-sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Chromatograms of the sequences were analyzed with Factura and Sequence Navigator software (Applied Biosystems).³⁴

In addition to the above, samples of normal tissue from all 509 patients were analyzed with a PCR-based method³⁵ regardless of replication-error status for a common founder mutation, referred to as mutation 1, that cannot be detected by the analytic approaches described above (Fig. 1). It consists of a 3.5-kb genomic deletion comprising exon 16 of *MLH1* and has so far been identified in more than 30 families in Finland and Sweden.^{35–37}

RESULTS

On the basis of data from the Finnish Cancer Registry, we estimate that we examined approximately two thirds of all patients with newly diagnosed colorectal carcinoma in the participating centers. Of the 509 samples of colorectal cancer, 63 (12 percent) had replication errors (Table 1). The proportion of tumors with replication errors was very similar with both methods used to detect such errors: 13 percent (31 tumors) in the 236 samples analyzed with radioactive reagents and 12 percent (32 tumors) in the 273 samples analyzed by fluorescence labeling.

To study the whole series of 509 tumors uniform-

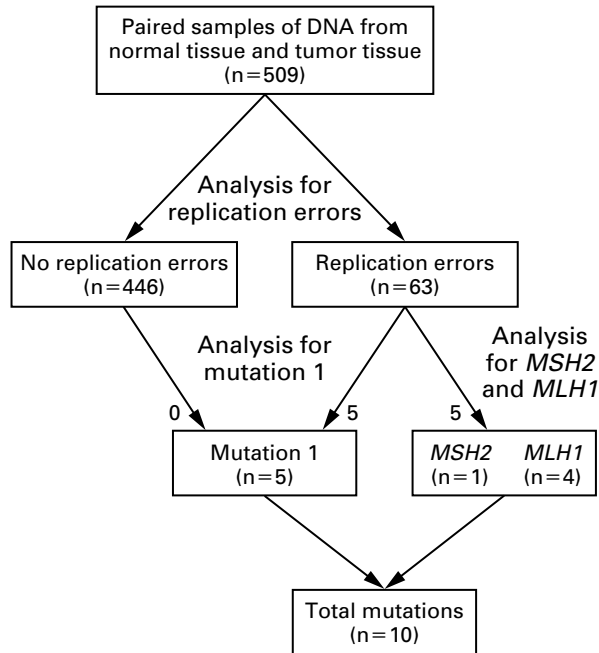


Figure 1. The Approach Used to Identify Hereditary Nonpolyposis Colorectal Cancer in Patients with Newly Diagnosed Colorectal Cancer.

Mutation 1 is a founder mutation that cannot be detected by the methods used to find mutations in *MLH1* and *MSH2*.

ly we used BAT-26, a highly sensitive microsatellite marker for DNA replication errors.^{27,28} Altogether, 64 tumors showed evidence of genetic instability with the BAT-26 marker. Of these, 58 were among the 63 samples that were found to be positive for replication errors with multiple markers. Six samples judged to be negative with the other types of tests were positive on BAT-26 testing: 2 were among the 236 samples tested with the radioactive method, and 4 were among the 273 samples tested by fluorescence labeling. Of 31 samples deemed positive with the radioactive method, 3 were negative on BAT-26 testing, and 2 of the 32 positive samples detected by fluorescence were negative on BAT-26 testing.

Mutation analysis of normal-tissue DNA (the source being either normal colonic mucosa distant from the neoplasm or blood lymphocytes) identified 10 unambiguous germ-line mutations. Two of these mutations were revealed by two-dimensional DNA electrophoresis and sequencing,²⁹ two by direct automated sequencing, and five by the test for mutation 1. The sample from one patient (Patient 115), who had had a prior colon carcinoma, was analyzed more thoroughly and was shown to have a deletion of *MLH1* exons 3, 4, and 5 in complementary DNA.³⁸ Thus, the total number of germ-line mutations was 10 (2.0 percent) (Table 2). DNA from six patients whose tumors were negative for replication errors on

initial testing but were positive on BAT-26 testing was also sequenced, and none of the sequences had a mutation of *MSH2* or *MLH1*.

A number of previously described polymorphisms in *MLH1* and *MSH2* and three apparently neutral intronic sequence variants in *MLH1* were also detected in the normal-tissue samples (453+79A→G, 885-24T→A, and 2104-8A→T). In samples from 20 patients, changes in the promoter regions were observed. Eleven of these patients were heterozygous for the substitution of glycine for alanine 93 bp upstream from the initiation codon of exon 1 of *MLH1*, and four patients were homozygous for this change. Seven patients were heterozygous for a change from threonine to cysteine 118 bp upstream from the beginning of exon 1 of *MSH2*. This change was also present in 11 of 32 healthy controls. Both of these changes are likely to be neutral variants.

Data were obtained on all patients through population registries and the Finnish Cancer Registry (Table 1). All but 1 of the 10 patients with germ-line mutations had a first-degree relative with colorectal or endometrial cancer (Table 2). Four of the 10 families fulfilled the Amsterdam criteria for the syndrome,² as evaluated by a registry search, and 3 additional families fulfilled these criteria on the basis of existing information on the extended pedigrees.² None of the 53 patients with replication errors but without mutations had a family history of cancer that was compatible with the Amsterdam criteria, nor did any of the 446 patients without replication errors.

DISCUSSION

We used replication-error analysis to screen samples from 509 consecutive patients with colorectal cancer for evidence of genetic instability consistent with the presence of hereditary nonpolyposis colorectal cancer. We found 63 samples with replication errors and were able to detect germ-line mutations of *MLH1* and *MSH2* (genes that encode DNA repair enzymes) in 10 of these specimens. We can therefore say that hereditary nonpolyposis colorectal cancer was diagnosed by molecular methods in 10 (2 percent) of the specimens. In many of the remaining 53 samples with replication errors, the cancer may be the result of somatic inactivation of a mismatch-repair gene, rather than a germ-line mutation. Five of the 10 germ-line mutations of the *MLH1* and *MSH2* genes involved the founder mutation 1 of *MLH1*, and all 5 had replication errors. This founder mutation, a deletion of exon 16 of *MLH1*, has been detected in more than 30 families with hereditary colon cancer in Finland and Sweden.³⁵⁻³⁷ This mutation was not found in any of the 446 patients without replication errors, which supports the potential value of replication-error analysis as an initial screen for hereditary nonpolyposis colo-

TABLE 2. CHARACTERISTICS OF 10 PATIENTS WITH GERM-LINE MUTATIONS.

PATIENT No.	AGE AT ONSET (YR)	No. OF 1ST-DEGREE RELATIVES IDENTIFIED	No. OF 1ST-DEGREE RELATIVES WITH COLORECTAL OR ENDOMETRIAL CANCER	No. OF 1ST-DEGREE RELATIVES WITH OTHER CANCERS	MUTATION DETECTED*
34	46	6	2	2	Mutation 1
52	35	4	0	0	<i>MSH2</i> , 1388–1G→T at splicing acceptor site of exon 9†
62	43	5	1	0	<i>MLH1</i> , 454–1G→T at splicing acceptor site of exon 6
115‡	63	9	1	0	Genomic deletion of <i>MLH1</i> exons 3, 4, and 5§
125‡	42	10	3	0	Mutation 1
179	49	7	1	1	<i>MLH1</i> , nonsense mutation R659X(C→T)†
279‡	66	10	2	1	Mutation 1
360	36	6	1	0	Mutation 1
477‡	65	8	3	0	<i>MLH1</i> , 1107R(T→G)
483	41	6	1	0	Mutation 1

*Mutation 1 is a 3.5-kb genomic deletion comprising exon 16 of *MLH1*.

†This mutation has been described previously.²⁵

‡This patient had multiple primary cancers in the colorectum, endometrium, or both.

§This mutation has been described previously.³⁰

rectal cancer. If the effect of the relatively prevalent founder mutation 1 is excluded, the proportion of patients with hereditary nonpolyposis colorectal cancer is 1 percent. These figures are based on the identification of germ-line mutations and are somewhat lower than the results obtained in epidemiologic studies. We can use these figures to estimate the frequency of hereditary nonpolyposis colorectal cancer in the general population as follows. According to the data base of the Finnish Cancer Registry, the risk of colorectal cancer in Finland is 5 percent by the age of 85. If, as we found, 2 percent of all colorectal cancers are hereditary nonpolyposis colorectal cancer, then the risk of this type of cancer is 2 percent of 5 percent, or 1 in 1000. These estimates need to be confirmed and expanded by extensive population studies.

Our finding that 2 percent of the samples had hereditary nonpolyposis colorectal cancer represents the absolute minimum in our series. Mutations can be missed because of technical limitations, and we did not analyze three minor genes, *PMS1*, *PMS2*, and *MSH6*, that are predisposing factors for this disease. Liu et al. used several methods to detect mutations in four DNA mismatch-repair genes and found a mutation in 70 percent of families with hereditary nonpolyposis colorectal cancer that met the Amsterdam criteria for the disease.³⁹ These authors also concluded that some mutations had escaped detection. Another factor that may have led us to underestimate the population frequency of heredi-

tary nonpolyposis colorectal cancer is that we only screened patients with colorectal cancer, although colorectal cancer accounts for no more than two thirds of the cancers in affected families.⁴⁰ Screening patients with endometrial cancer for hereditary nonpolyposis colorectal cancer would increase the estimates of the population frequency. None of the patients without replication errors fulfilled the Amsterdam criteria, but it is likely that screening for replication errors does not identify patients with other types of hereditary colorectal cancer and that there are as yet unidentified syndromes of hereditary colorectal cancer.

In each participating unit one physician was responsible for organizing the collection of samples. We obtained samples from two thirds of all patients with colorectal cancer who were treated in the participating units during the study. In the case of the remaining third, the patients did not consent to participate, emergency laparotomies were performed, or the samples were collected improperly.

The possibility that samples from patients with a family history of colorectal cancer were overrepresented cannot be ruled out. Such a bias could cause overestimates of the frequency of hereditary nonpolyposis colorectal cancer. However, the proportion of young patients (those less than 50 years of age) in our series (8 percent) was the same as in the complete Cancer Registry for this geographic area, indicating that the sampling was unbiased with respect to age.

Only 3 of 10 mutations (in Patients 52, 115, and

179) have not been previously identified in multiple, apparently unrelated Finnish families.^{35,38} Studies of Finnish families with hereditary nonpolyposis colorectal cancer have been extensive, and the most prevalent mutations in the population have probably been identified.^{16,17,35,37,38} Five of the 10 patients with germ-line mutations in our study were members of previously identified families. Although the Finnish Hereditary Nonpolyposis Colorectal Cancer Registry has promoted clinical screening in these families for more than a decade,¹² in only two (Patients 115 and 483) were the tumors diagnosed by a screening endoscopy. This suggests that even more intensive screening efforts are needed.

Analysis of all colorectal cancers for replication errors followed by direct sequencing of *MLH1* and *MSH2* in patients with replication errors as a strategy for detecting hereditary nonpolyposis colorectal cancer has not been tested on a large scale or in a prospective manner. The results of our study support the feasibility of this approach, but also suggest that screening could be made more efficient by certain modifications. Importantly, the disease phenotype in our patients appeared to be very similar to that described in previous studies of high-risk families. All the patients in whom germ-line mutations were detected had at least one of the three major hallmarks of hereditary nonpolyposis colorectal cancer: a family history of colorectal or endometrial cancer, a young age at onset (less than 50 years), or multiple primary cancers. Three of the 10 patients with germ-line mutations were more than 60 years old (Table 2), and 1 of the 10 did not have a family history of hereditary nonpolyposis colorectal cancer. Perhaps, then, the efficiency of screening for hereditary nonpolyposis colorectal cancer in patients with colorectal cancer could be improved if all young patients, all patients with a first-degree relative with colorectal or endometrial cancer, and all patients with multiple primary cancers in the colorectum, endometrium, or both were analyzed for replication errors. In our study, 8 percent, 16 percent, and 3 percent of the 509 patients would have fallen into these respective categories, but because of overlap among the groups, only 24 percent would have been analyzed for replication errors. Among these patients, there would have been 20 with replication errors (4 percent of patients), and mutation analysis would have led to the identification of the same 10 patients who were identified with the other approach.

We conclude that analysis for replication errors is a useful way of detecting possible cases of hereditary nonpolyposis colorectal cancer in clinical practice. However, since only 10 of 63 patients (16 percent) with replication errors had a detectable germ-line mutation, and since all patients with these mutations were young, had a family history of the disease, or had had a previous cancer, the number of patients in

whom molecular analyses are indicated can be reduced through the selection of appropriate patients. In this way, molecular screening for hereditary nonpolyposis colorectal cancer would be feasible for large populations of patients with newly diagnosed cancer.

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