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Identification and Survival of Carriers of Mutations in DNA Mismatch-Repair Genes in Colon Cancer

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ABSTRACT

BACKGROUND

The identification of mutations in germ-line DNA mismatch-repair genes at the time of diagnosis of colorectal cancer is important in the management of the disease.

METHODS

Without preselection and regardless of family history, we recruited 870 patients under the age of 55 years soon after they received a diagnosis of colorectal cancer. We studied these patients for germ-line mutations in the DNA mismatch-repair genes *MLH1*, *MSH2*, and *MSH6* and developed a two-stage model by multivariate logistic regression for the prediction of the presence of mutations in these genes. Stage 1 of the model incorporated only clinical variables; stage 2 comprised analysis of the tumor by immunohistochemical staining and tests for microsatellite instability. The model was validated in an independent population of patients. We analyzed 2938 patient-years of follow-up to determine whether genotype influenced survival.

RESULTS

There were 38 mutations among the 870 participants (4 percent): 15 mutations in *MLH1*, 16 in *MSH2*, and 7 in *MSH6*. Carrier frequencies in men (6 percent) and women (3 percent) differed significantly ($P < 0.04$). The addition of immunohistochemical analysis in stage 2 of the model had a sensitivity of 62 percent and a positive predictive value of 80 percent. There were 35 mutations in the validation series of 155 patients (23 percent): 19 mutations in *MLH1*, 13 in *MSH2*, and 3 in *MSH6*. The performance of the model was robust among a wide range of cutoff probabilities and was superior to that of the Bethesda and Amsterdam criteria for hereditary nonpolyposis colorectal cancer. Survival among carriers was not significantly different from that among noncarriers.

CONCLUSIONS

We devised and validated a method of identifying patients with colorectal cancer who are carriers of mutations in DNA repair genes. Survival was similar among carriers and noncarriers.

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GENES RESPONSIBLE FOR SEVERAL AUTOSOMAL dominant and recessive colorectal-cancer-susceptibility syndromes have been mapped and causative mutations characterized.^{1,2} Most autosomal dominant syndromes are defined empirically on the basis of family history and clinical and pathological criteria, such as the criteria for hereditary nonpolyposis colorectal cancer (also called the Lynch syndrome).^{3,4} In clinical practice, however, the use of these approaches creates a bias against low-penetrance alleles, small families, nonpaternity or adoption, and newly arisen mutations. The Lynch syndrome is caused by inactivating mutations of DNA mismatch-repair genes (mostly *MSH2*, *MLH1*, and *MSH6*),⁵ but many patients with colorectal cancer who have such mutations do not fulfill the empirical criteria for the Lynch syndrome.⁶⁻⁸ Moreover, about 1 in 3100 people between the ages of 15 and 74 years carries a defective DNA mismatch-repair gene.⁹ This finding implies that patients with incident colorectal cancer who fulfill the criteria for the Lynch syndrome do not account for all mutation carriers. The fact that asymptomatic carriers have a substantial risk of colorectal and other cancers¹⁰⁻¹² underscores the importance of identifying these carriers early enough to allow for counseling and surveillance.

Practical and financial constraints often require initial testing (prescreening) of tumor DNA for microsatellite instability or immunohistochemical assessment of tumor sections for DNA mismatch-repair proteins, or both, before the genotyping of incident cases of cancer. Prescreening assumes that microsatellite instability is a consistent feature of tumors from carriers, but some tumors do not have microsatellite instability.⁸ Moreover, prescreening misses an appreciable number of mutations in DNA repair genes^{6,8} and has a relatively poor positive predictive value.^{6,8,13} In addition, analysis of patients with incident colorectal cancers for microsatellite instability and DNA mismatch-repair proteins¹⁴ without previous genetic counseling and explicit consent raises ethical concerns, because patients with tumors that do not have DNA mismatch-repair proteins and do have microsatellite instability are likely to carry heritable germ-line mutations.

There is inconclusive evidence that the prognosis of colorectal cancer among patients with the Lynch syndrome is better than that among patients with sporadic cases.¹⁵⁻¹⁷ Sporadic colorectal cancers with microsatellite instability have a

better prognosis than do microsatellite-stable sporadic tumors,¹⁸⁻²⁰ but this difference does not necessarily apply to tumors with germ-line defects in DNA mismatch-repair genes.

Mutational analysis of germ-line DNA mismatch-repair genes without previous testing of the tumor has not been undertaken in a prospective, population-based series.²¹ In this study, we looked for such mutations without considering the family history or the results of tumor testing in a prospective, population-based series of 870 cases of early-onset colorectal cancer, a group enriched for genetically determined disease.^{6-8,13,22,23} This approach allowed us to construct a clinically driven predictive model that aids in the prediction of carriers of germ-line mutations of DNA mismatch-repair genes.

METHODS

Beginning January 2, 1999, we identified all patients in Scotland (population, 5.06 million) who had received a diagnosis of colorectal cancer before the age of 55 years. All such patients were identified soon after the diagnosis and regardless of family history. After the exclusion of 15 patients with dominant polyposis syndromes, 1259 patients agreed to undergo genetic counseling and gave informed written consent for germ-line and tumor analysis before the end of 2005. The study population comprised 870 consecutive patients who received a diagnosis of colorectal cancer between February 1999 and July 2003 and in whom mutational analysis and follow-up were complete. The study population also included 72 percent of 1206 patients with incident cases identified independently by the Scottish Cancer Registry. Of these patients, 80 did not respond to our request, 156 had psychiatric or physical illnesses that precluded their enrollment in the study, and only 100 patients declined to participate, which left us with 90 percent compliance overall. The study was approved by the research ethics committees and National Health Service (NHS) management of every participating hospital.

An invitation to participate in the study was extended to patients within a few weeks after diagnosis to minimize survival bias. Family history was obtained at the time of the interview, a blood sample was taken for DNA analysis, and tumor samples were acquired from NHS pathology departments. The date of disease onset was defined as the date of the first histologic diagnosis of

colorectal adenocarcinoma. The cancer history of patients was obtained by personal interview and cross-reference of personal identifiers with the Scottish Cancer Registry. Tumors were staged according to Dukes' classification and tumor-node-metastasis (TNM) criteria in accordance with recommendations of the American Joint Committee on Cancer.

MUTATIONAL ANALYSIS

Germ-line DNA obtained from blood leukocytes was analyzed for *MLH1*, *MSH2*, and *MSH6* mutations. Denaturing high-performance liquid chromatography analysis (Transgenomic) was used for 11 exons of *MSH2* and 16 exons of *MLH1*. Variants noted on chromatography were sequenced, as were *MSH2* exons 1, 4, 5, 10, and 13 and *MLH1* exons 8, 12, and 15 in every sample (ABI 3730 DNA Analyzer, Applied Biosystems). All 10 *MSH6* exons were sequenced. Sequencing chromatographs were assessed visually and with the use of Sequencher software. Mutations were confirmed by reamplification of an independent sample of DNA and resequencing in both directions. *MLH1* and *MSH2* were assessed for deletions by multiplex ligation-dependent probe amplification (MLPA, MRC-Holland), with products separated on an ABI 3100 *xl* Genetic Analyzer (Applied Biosystems) and analyzed with the use of custom software (available on the Web site of the United Kingdom National Genetics Reference Laboratory at www.ngrl.org.uk/Manchester/Publications.htm#MLPA).

ANALYSIS OF MICROSATELLITE INSTABILITY

Tumor DNA that had been purified from microdissected 10- μ m tumor sections (QIAamp DNA minikit, Qiagen) was subjected to multiplex polymerase-chain-reaction assay with the use of BAT25, BAT26, D2S123, D5S346, and D17S250 markers²⁴ and compared with control DNA from blood or normal tissue in the section. Products were analyzed with the use of ABI PRISM 3100 and Genescan software (Applied Biosystems). Tumors with more than one shifted marker were categorized as having a high degree of microsatellite instability, those with one unstable marker were categorized as having a low degree of microsatellite instability, and those with no instability were categorized as being microsatellite stable.

IMMUNOHISTOCHEMICAL ANALYSIS

Paraffin-embedded sections that had been stained with antibodies against *MLH1*, *MSH2*, and *MSH6*

were assessed independently by two microscopists. If discrepancies could not be resolved, a third pathologist reviewed all slides.

STATISTICAL ANALYSIS

Two-Stage Predictive Model

Univariate analysis of clinical variables by logistic regression identified independently significant predictors of mutational status. These predictors were included in multivariate logistic regression to construct a two-stage model for the prediction of whether patients carry germ-line mutations of DNA repair genes on the basis of clinical variables (see the Supplementary Appendix, available with the full text of this article at www.nejm.org). The least significant variables were removed one by one until all variables remained significant. All variables (except the age at onset) were included as categorical variables. The use of automatic stepwise backward and forward selection criteria, on the basis of the likelihood ratio and the Wald statistic, yielded similar results on logistic regression with SPSS software, so we selected the simplest model. The final multivariable analysis that estimates carrier probabilities for stage 1 of the model can be described by the following equation, which can be solved electronically at <http://www1.hgu.mrc.au.uk/Softdata/MMRpredict.php>²⁵: $Pr/(1-Pr) = 1.39 \times 0.89^{\text{AGE}} \times 2.57^{\text{SEX}} \times 4.45^{\text{LOCATION}} \times 9.53^{\text{SYN/MET}} \times 46.26^{\text{CRCFH} < 50} \times 7.04^{\text{CRCFH} \geq 50} \times 59.36^{\text{ECFH}}$

In this equation, Pr is the carrier probability, and age refers to the age at diagnosis. Male sex is assigned a value of 1, and female sex a value of 0. A proximal tumor location is assigned a value of 1, and a distal location a value of 0. The presence of a synchronous (SYN) metachronous (MET) tumor is assigned a value of 1, and the absence of a SYN/MET tumor is assigned a value of 0. Colorectal-cancer family history (CRCFH) is assigned a value according to the age of the youngest relative with the disease, so CRCFH < 50 and CRCFH \geq 50 are 1 and 0, respectively, if the youngest relative is less than 50 years of age and 0 and 1, respectively, if the youngest relative is 50 years of age or older; the assigned values are 0 and 0 if there are no affected relatives. The endometrial cancer family history (ECFH) is assigned a value of 1 if any first-degree relative has endometrial cancer.

Stage 1 of the model used exclusively clinical variables to represent the situation at diagnosis and identify subgroups enriched for carriers. These groups then proceeded to stage 2 (analysis of the tumor for microsatellite instability and immuno-

Table 1. Clinical and Molecular Data on Patients under the Age of 55 Years Who Had Colorectal Cancer with Pathogenic Mutations.*

Patient No. and Mutation	Sequence	Protein Change	MSI Status†	Immunohistochemical Analysis‡	Sex	Age at Diagnosis	Amsterdam Modified Criteria	HNPCC Criteria‡	Tumor Location
MLH1									
2399	EX1del	Unknown	MSI-H	Negative	M	27	Positive	Positive	Ascending colon or hepatic flexure
2667	c.3G>A	Unknown	MSI-H	Negative	M	39	Negative	Positive	Ascending colon or hepatic flexure
1828§	c.116G>T	Splice site	MSI-H	Negative	M	34	Positive	Positive	Cecum
1812§	c.116G>T	Splice site	NA	Negative	M	37	Negative	Positive	Cecum
2425	c.116 +1G>A	Splice site	MSI-L	NA	F	19	Negative	Positive	Ascending colon or hepatic flexure
2159	c.200G>A	p.G67E	ND	ND	M	48	Positive	Positive	Ascending colon or hepatic flexure
1946¶	c.428_429insC	p.A143fsX171	MSI-H	Negative	F	39	Positive	Positive	Transverse colon
1092¶	c.428_429insC	p.A143fsX171	MSI-H	Negative	M	43	Positive	Positive	Rectum
2977	c.588+1G>T	Splice site	ND	ND	M	43	Negative	Positive	Descending colon
2332	c.1017_1018delC	p.S339fsX366	MSI-H	Negative	F	50	Negative	Positive	Rectum
1867	c.1190_1191delT	p.L397fsX400	MSI-L	Negative	F	48	Positive	Positive	Ascending colon or hepatic flexure
2116	c.1347_1368del22insTAAAA	p.G449fsX472	MSI-H	Negative	M	36	Positive	Positive	Cecum
2869	c.2041G>A	p.A681T	MSI-H	Negative	M	35	Negative	Positive	Cecum
2293	c.2041G>A	p.A681T	MSI-H	Negative	F	45	Negative	Positive	Ascending colon or hepatic flexure
2353	c.2099-2102delIAGCA	p.Q700fs[X722]	MSI-H	Negative	M	36	Negative	Positive	Ascending colon or hepatic flexure
MSH2									
2400	EX1_6del	Unknown	MSI-L	NA	M	37	Negative	Positive	Cecum
2187	c.1A>C	Unknown	MSI-L	Positive	M	51	Negative	Negative	Sigmoid colon
1974	c.388_389delCA	p.Q130fsX131	MSI-H	Positive	M	49	Positive	Positive	Cecum
2794**	c.754C>T	p.Q252X	MSI-H	NA	M	31	Positive	Positive	Descending colon
7356**	c.754C>T	p.Q252X	N/D	ND	F	46	Negative	Positive	Sigmoid colon
2355	c.754C>T	p.Q252X	MSI-H	Positive	M	51	Positive	Positive	Ascending colon or hepatic flexure

2643	c.942+3A>T	Skip exon 5	MSI-H	NA	Negative	Negative	M	40	Negative	Positive	Rectum
2394	c.942+3A>T	Skip exon 5	MSI-H	Negative	Negative	Negative	M	41	Positive	Positive	Cecum
1815	c.942+3A>T	Skip exon 5	MSI-H	Positive	Negative	Negative	M	52	Positive	Positive	Rectum
1839	c.1320_1321insA	p.L440fsX442	MSI-H	NA	NA	NA	M	40	Negative	Positive	Rectosigmoid colon
2252	c.1786_1788delAAAT	p.N596del	MSI-H	NA	Negative	Negative	F	35	Positive	Positive	Rectum
4976	c.1786_1788delAAAT	p.N596del	ND	ND	ND	ND	M	43	Negative	Positive	Transverse colon
1800	c.1786_1788delAAAT	p.N596del	ND	ND	ND	ND	M	45	Positive	Positive	Cecum
2416	c.1786_1788delAAAT	p.N596del	MSI-L	Positive	Negative	Negative	M	51	Negative	Positive	Ascending colon or hepatic flexure
2573	c.2038C>T	p.R680X	ND	ND	ND	ND	F	43	Positive	Positive	Sigmoid colon
2938	c.2634+5G>C	Skip exon 15	MSI-H	Positive	Negative	Negative	M	43	Negative	Positive	Ascending colon or hepatic flexure
MSH6											
1846	c.3261_3262insC	p.F1088fsX1092	ND	ND	ND	ND	F	53	Negative	Negative	Sigmoid colon
2670	c.3516_3527del12	p.V1173_R1176del	MSI-L	Positive	Negative	Negative	M	51	Negative	Positive	Cecum
2844	c.3519_3520insA	p.V1173fsX1175	MSI-L	Positive	NA	NA	M	44	Negative	Positive	Rectosigmoid colon
2685	c.3840_3846delGGAGACT	p.Q1280fsX1324	MSI-L	NA	NA	NA	F	44	Negative	Positive	Transverse colon
2324	c.3958_3959ins19	p.A1320fsX1324	MSI-H	Positive	NA	Negative	M	50	Negative	Positive	Sigmoid colon
2656	c.3958_3959ins19	p.A1320fsX1324	MSS	Positive	Negative	Negative	F	52	Negative	Positive	Rectum
2728	c.3984_3985insATCA	p.Q1328fsX1339	MSS	Positive	Positive	Positive	M	45	Positive	Positive	Rectum

* Mutations were categorized as pathogenic as follows: frameshifts, previously defined pathogenic in-frame deletions, nonsense mutations, and splice-site variants. Amino acid changes required substantial evidence of pathogenicity through independent genetic linkage, association, or tumor analysis of microsatellite instability. Mutation detection with the use of the combination of direct sequencing and denaturing high-performance liquid chromatography analysis had 95 percent sensitivity for mutations. Any mutations that were missed with the use of techniques employed here would have minimal effect, because the "noncarrier" group was large. In addition to categorized pathogenic mutations, a further 22 missense variants of uncertain pathogenicity were identified (see the Supplementary Appendix). We did not undertake functional assays because they have not necessarily correlated well with clinical observations. For all analyses, we included these in the noncarrier group because we found no significant differences in any variables studied in the 22 patients and the noncarrier group as a whole. We retained the sample identification numbering of patients in order to facilitate further analysis and potential exchange of specific samples.

† For analysis of microsatellite instability (MSI) and immunohistochemical analysis, NA denotes not assessable (the assay was performed, but the technical quality was insufficient to provide an unambiguous result, despite repeated assay), and ND not done (no tumor material available). For immunostaining, representative 3- μ m sections of paraffin-embedded tumor and normal tissue was assessed for MLH1, MSH2, and MSH6 protein expression by immunohistochemical analysis. Mouse monoclonal antihuman antibodies against MLH1 (Pharmingen), MSH2 (Oncogene Research Products), and MSH6 (Transduction Laboratories) were used at dilutions of 1:50, 1:100, and 1:100, respectively. Sections were treated with citric acid buffer at 95°C for 10 minutes for epitope enhancement, and immunohistochemical analysis was performed with the use of an LSAB immunoperoxidase staining kit (Dako). Sections were counterstained with Harris hematoxylin, dehydrated with xylene, and mounted under coverslips. Negative denotes a loss of normal staining, indicating a loss of the normal pattern of expression, and positive a normal staining pattern and retained protein expression.

‡ For patients with hereditary nonpolyposis colorectal cancer (HNPCC) evaluated by the Amsterdam and Bethesda criteria, positive refers to the fulfillment of criteria and negative means a lack of fulfillment of the criteria.

§ Patients 1828 and 1812 were related.

¶ Patients 1946 and 1092 were related.

|| Patients 2869 and 2293 were related.

*** Patients 2794 and 7356 were related.

histochemical analysis). Combining stage 1 with stage 2 allowed for the assessment of the model's performance and usefulness in predicting the carrier state at various cutoff probabilities, as compared with the use of modified Amsterdam and Bethesda criteria, which were similarly combined with data on microsatellite instability and immunohistochemical analysis.

Replication Set

We assessed the validity of the model in an independent retrospective series of 155 Scottish patients under the age of 45 years (mean age, 34 years) who had received a diagnosis of colorectal cancer between February 1973 and June 1998. The mutational analysis was undertaken without consideration of the family history, the microsatellite instability of tumors, or immunohistochemical analysis.

Survival Analysis

Patients were followed by contacts with hospital teams, general practitioners, and systematic flagging with the Registrar General for Scotland. We also cross-referenced our data with records from the Scottish Health Service Information and Statistics Division. Data were censored on April 30, 2005, and no patients were lost to follow-up. Patients were followed until they died or the study ended. Kaplan–Meier analysis was used to compare survival according to genotype, first for all patients and then in an analysis that excluded patients who had undergone surveillance screening or had had previous cancers. Data were excluded for patients who had undergone previous surveillance (4 carriers) or had a previous potentially lethal cancer (26 noncarriers and 1 carrier); 1 carrier fulfilled both criteria.

RESULTS

Pathogenic mutations were identified in 38 of 870 patients (4 percent) (Table 1). Carriers were younger than noncarriers (mean age, 42.7 and 48.2 years, respectively; $P < 0.001$) (Table 2), although 37 percent of all carriers were between the ages of 45 and 54 years. The mean age was 38.5 years for carriers of *MLH1* mutations, 43.8 years for those with *MSH2* mutations, and 49.0 years for those with *MSH6* mutations ($P = 0.005$), suggesting gene-specific penetrance effects. Carrier frequencies in men (6 percent) and women (3 percent) were

significantly different ($P < 0.04$). Most carriers fulfilled Bethesda criteria (95 percent), but only 42 percent fulfilled Amsterdam criteria.

Sites of tumors in carriers were approximately evenly distributed (Table 2 of the Supplementary Appendix), but most sites were distal in noncarriers ($P < 0.001$); 87 percent of carriers of *MLH1* mutations had proximal tumors, similar to the percentage of patients with tumors bearing somatic *MLH1* hypermethylation.²⁶ Seven of 38 carriers (18 percent) and 14 of 832 noncarriers (2 percent) had synchronous or previous colorectal cancers. Three of 38 carriers (8 percent) and 37 of 832 noncarriers (4 percent) had synchronous or previous extracolonic tumors. A greater proportion of carriers (13 of 37 patients, or 35 percent) had mucinous tumors than did noncarriers (121 of 798, or 15 percent) ($P < 0.003$). Genotype did not influence the tumor stage or differentiation; 12 of 38 carriers presented with metastases (32 percent).

MICROSATELLITE INSTABILITY AND IMMUNOHISTOCHEMICAL ANALYSIS

The presence of a high degree of microsatellite instability had a sensitivity of 67 percent for germline mutations, as compared with a sensitivity of 27 percent for the presence of a low degree of microsatellite instability and a sensitivity of 93 percent for the presence of any degree of microsatellite instability. The positive predictive values were 45 percent, 24 percent, and 36 percent, respectively (Table 3). The presence of a high degree of microsatellite instability had a sensitivity of 83 percent for the detection of *MLH1* mutations, of 75 percent for the detection of *MSH2* mutations, and of 17 percent for the detection of *MSH6* mutations. The presence of a low degree of microsatellite instability had sensitivities of 17 percent, 25 percent, and 50 percent, respectively, and the presence of any degree of microsatellite instability had sensitivities of 100 percent, 100 percent, and 67 percent, respectively. Table 3 lists the results of immunostaining for mismatch-repair proteins *MSH2*, *MHL1*, and *MSH6*, along with sensitivities, positive predictive values, and 95 percent confidence intervals. The absence of *MSH6* protein predicted mutations in *MSH2* or *MSH6* (positive predictive value, 56 percent), as did the absence of *MSH2* for mutations in *MSH2* or *MSH6* (positive predictive value, 52 percent), reflecting the biologic interaction between these proteins.

Table 2. Clinical Features and DNA Mismatch-Repair Genotype.*

Variable	No. of Patients (%)	Mean Age yr	
Age at onset of colorectal cancer†			
Noncarrier	832	48.2±6.0	
Carrier	38 (4)	42.7±7.7	
<i>MLH1</i>	15 (39)	38.5±8.4	
<i>MSH2</i>	16 (42)	43.8±6.1	
<i>MSH6</i>	7 (18)	49.0±3.9	
Frequency of Mutation			
<i>no. of carriers/no. of noncarriers (%)</i>			
Sex			
Male	27/435 (6)		
Female	11/397 (3)		
	Carriers (N = 38)	Noncarriers (N = 832)	Total (N = 870)
	<i>no. (%)</i>		
Fulfillment of established HNPCC criteria‡			
Bethesda criteria			
Positive	36 (95)	519 (62)	555 (64)
Negative	2 (5)	313 (38)	315 (36)
Modified Amsterdam criteria			
Positive	16 (42)	18 (2)	34 (4)
Negative	22 (58)	814 (98)	836 (96)

* Plus-minus values are means ±SD. Further clinical details on tumor characteristics are provided in Table 2 of the Supplementary Appendix.

† A younger age at onset was significantly associated with the mutational status of the patient ($P < 0.001$; odds ratio, 0.9; 95 percent confidence interval, 0.85 to 0.94). There were significant differences in the age at onset between *MLH1*, *MSH2*, and *MSH6* mutation carriers ($P = 0.005$, for the analysis of variance).

‡ Bethesda criteria refers to the 2004 revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (HNPCC) (also called the Lynch syndrome),⁵ and the modified Amsterdam criteria refers to the 1999 revised family-history criteria.⁶

TWO-STAGE PREDICTIVE MODEL

Tumor microsatellite instability and immunohistochemical data were assessed in stage 2 to refine the carrier prediction derived from stage 1. Clinical variables that were significant on univariate analysis (Table 4, and Table 3 of the Supplementary Appendix) were used to construct stage 1 of the model. The factors and P values in univariate analysis were age ($P < 0.001$), sex ($P = 0.03$), the location of the tumor ($P < 0.001$), the presence of synchronous or metachronous tumors ($P = 0.001$), having a first-degree relative with colorectal cancer ($P < 0.001$), and having a first-degree relative with endometrial cancer ($P = 0.006$). (The equation for the calculation of carrier probability is listed and described in the Methods section.²⁵)

The number of affected relatives was not a significant predictor of mutational status. Instead, the model gave priority to the youngest affected first-degree relative, resulting in three possible categories of family history (as described in the Methods section). Performance of the model can be assessed at various cutoffs, allowing tailoring of the proportion of patients proceeding to stage 2.²⁶ The results can be compared with Amsterdam or Bethesda criteria combined with microsatellite instability and immunohistochemical data (Table 4, and Table 3 of the Supplementary Appendix). The use of Amsterdam criteria yielded a sensitivity and specificity similar to those obtained with the use of stage 1 of the model at the 0.45 probability cutoff, whereas Bethesda criteria perform

Table 3. Microsatellite Instability of Tumor and Immunohistochemical Analysis.*

Variable	Carriers		Noncarriers	
	No. of Patients/ Total No.	Sensitivity % (95% CI)	No. of Patients/ Total No. (%)	Positive Predictive Value % (95% CI)
Microsatellite-instability analysis				
High degree of instability	20/30	67 (47–83)	24/322 (7)	45 (38–61)
Low degree of instability	8/30	27 (12–46)	26/322 (8)	24 (11–41)
Any degree of instability	28/30	93 (78–99)	50/322 (15)	36 (25–48)
Stable	2/30	7 (1–22)	272/322 (84)	NA
Immunohistochemical analysis				
Loss of protein expression†				
MSH2	9/11	82 (48–98)	12/317 (4)	43 (22–66)
MLH1	12/12	100 (78–100)	12/300 (4)	50 (29–71)
MSH6	3/4	75 (19–99)	15/324 (5)	17 (4–41)

* CI denotes confidence interval, and NA not applicable.

† Among carriers 31 tumors were assessable, and 359 tumors were assessable among noncarriers.

similarly to a model cutoff approaching 0.005. In stage 2, immunostaining identified two thirds of all carriers (95 percent confidence interval, 0.46 to 0.77) and had a positive predictive value of 80 percent (95 percent confidence interval, 0.66 to 0.95; probability cutoff, 0.05) (Table 3 of the Supplementary Appendix). The addition of immunostaining can refine carrier prediction because stage 1 of the model (at a cutoff of 0.05) identified the 17 percent of the population of patients who are enriched for mutation carriers. Immunostaining of biopsy specimens from these patients provides good overall sensitivity and positive predictive values but indicates a requirement for mutational analysis in only 1 in 29 (3 percent) of all cases (Table 3 of the Supplementary Appendix).

As compared with stage 1 of the model combined with microsatellite-instability analysis at stage 2 (at the optimal 0.05 probability cutoff), the Amsterdam criteria combined with microsatellite instability had a low sensitivity (39 percent, as compared with 65 percent in our model) but a positive predictive value of 100 percent. A combination of the Bethesda criteria and microsatellite instability was sensitive (88 percent) but had a positive predictive value of only 32 percent, as compared with 80 percent in our model. Although the inclusion of microsatellite-instability analysis enhanced the ability to predict the carrier state for all cutoff points in stage 1 of our model and for the Amsterdam and Bethesda criteria, to be

clinically useful it would necessitate the genotyping of DNA from biopsy specimens within a very short time after diagnostic biopsy.

REPLICATION SET

Mutation analysis of germ-line DNA from the replication series identified mutations in 35 of 155 samples (23 percent): 19 mutations in *MLH1*, 13 in *MSH2*, and 3 in *MSH6*. Clinical variables for all 155 patients were entered into the prediction algorithm,²⁵ and carrier probabilities were generated for each subject. The discriminatory power of the model across cutoff values was similar in both the prospective and retrospective series (receiver-operating-characteristic [ROC] curves are shown in Fig. 1 of the Supplementary Appendix). Furthermore, there was no significant difference ($P=0.3$) between the area under the ROC curve for these independent series (0.85 for the prospective series; 95 percent confidence interval, 0.77 to 0.93; and 0.82 for the replication series; 95 percent confidence interval, 0.72 to 0.91).

SURVIVAL ANALYSIS

Follow-up for a maximum of six years three months generated a total of 2938 patient-years for survival analysis (134 carriers and 2804 noncarriers). There was no significant difference in survival between carriers and noncarriers (five-year survival rate of 74 percent for carriers and 63 percent for noncarriers, $P=0.18$) (Fig. 1A). The censoring of data from

Table 4. Comparison of the Effectiveness of a Two-Stage Predictive Model at Various Cutoff Values with Bethesda and Amsterdam Criteria.*

Variable	After Stage 1			Patients in Stage 2 no. (%)	After Stage 2 (MSI or IHC)			Positive Predictive Value			Number Identified by Stage 2 (% Identified in Stage 1)	
	Sensitivity	Specificity	Positive Predictive Value		Sensitivity	IHC	MSI	MSI	IHC	MSI		IHC
Model cutoff value												
0.005	0.95	0.14	0.05	728 (86)	0.88	0.81	0.87	0.94	0.27	0.38	138 (16)	81 (9)
0.01	0.89	0.42	0.07	506 (60)	0.83	0.76	0.91	0.95	0.30	0.42	104 (12)	68 (8)
0.05	0.68	0.86	0.19	140 (17)	0.65	0.62	0.97	0.99	0.53	0.80	46 (5)	30 (3)
0.15	0.55	0.96	0.40	53 (6)	0.52	0.49	1.00	1.00	0.87	1.00	23 (3)	19 (2)
0.2	0.47	0.97	0.45	40 (5)	0.44	0.44	1.00	1.00	0.86	1.00	20 (2)	17 (2)
0.25	0.39	0.98	0.45	33 (4)	0.36	0.36	1.00	1.00	0.84	1.00	16 (2)	14 (2)
0.35	0.39	0.99	0.71	21 (2)	0.36	0.36	1.00	1.00	0.87	1.00	16 (2)	14 (2)
0.45	0.34	1.00	1.00	13 (2)	NA	NA	NA	NA	NA	NA	NA	NA
Criteria†												
Bethesda	0.95	0.38	0.06	555 (64)	0.88	0.84	0.92	0.95	0.32	0.44	103 (12)	72 (8)
Modified Amsterdam	0.42	0.98	0.47	34 (4)	0.39	0.39	1.00	1.00	1.00	1.00	15 (2)	15 (2)

* Further detail on the replication set and 95 percent confidence intervals is provided in Table 3 of the Supplementary Appendix. Sensitivity, specificity, and positive predictive value are shown for stage 1 of the model²⁵ and stage 2 (tumor microsatellite-instability status or immunohistochemical analysis), as compared with the performance of Bethesda and Amsterdam criteria in this series. Negative predictive values were never less than 97 percent in stage 2 and so are not shown. Mutation analysis is taken as the gold standard. In the two-stage model, a cutoff probability of “x” means that patients are categorized as mutation carriers if their predicted probability of being a mutation carrier is $\geq x$ (noncarrier, <x). The model cutoff relates to the stringency of the prediction, whereas Bethesda and Amsterdam criteria are dichotomized. The 0.05 cutoff indicates the optimal combination of clinical utility and efficiency. We pooled data from patients with a low degree of microsatellite instability and those with a high degree of microsatellite instability, so that microsatellite instability means any degree of instability because both low-degree and high-degree status had similar predictive values in the univariate analysis at all model cutoff levels and for both Bethesda and Amsterdam criteria. (Indeed, any degree of microsatellite instability performed better than a high degree of microsatellite instability alone. Data are available on request.) Immunohistochemical status refers to a loss of expression of any DNA mismatch-repair protein, not necessarily that relevant to the germ-line mutation. Although losses of *MLH1*, *MSH2*, and *MSH6* were individually significant predictors of mutation status in the univariate analysis, there was a strong relationship between a loss of expression of *MSH2* and *MSH6*, which meant that adding *MSH6* data into the model did not provide a substantially better explanation of the data. Furthermore, the category “any protein loss” is useful in practice because it accounts for poor-quality immunohistochemical analyses that might be obtained for one antibody but good-quality slides for another showing a clear loss of expression. Stage 2 of the model (0.45 cutoff) identifies a subgroup of patients who all had mutations, and so no further refinement was possible with microsatellite instability or immunohistochemical analysis, as indicated by “not applicable” (NA). MSI denotes microsatellite instability, and IHC immunohistochemical analysis.

† Bethesda criteria refers to the 2004 revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (HNPCC) (also called the Lynch syndrome),⁵ and the modified Amsterdam criteria refers to the 1999 revised family-history criteria.⁶

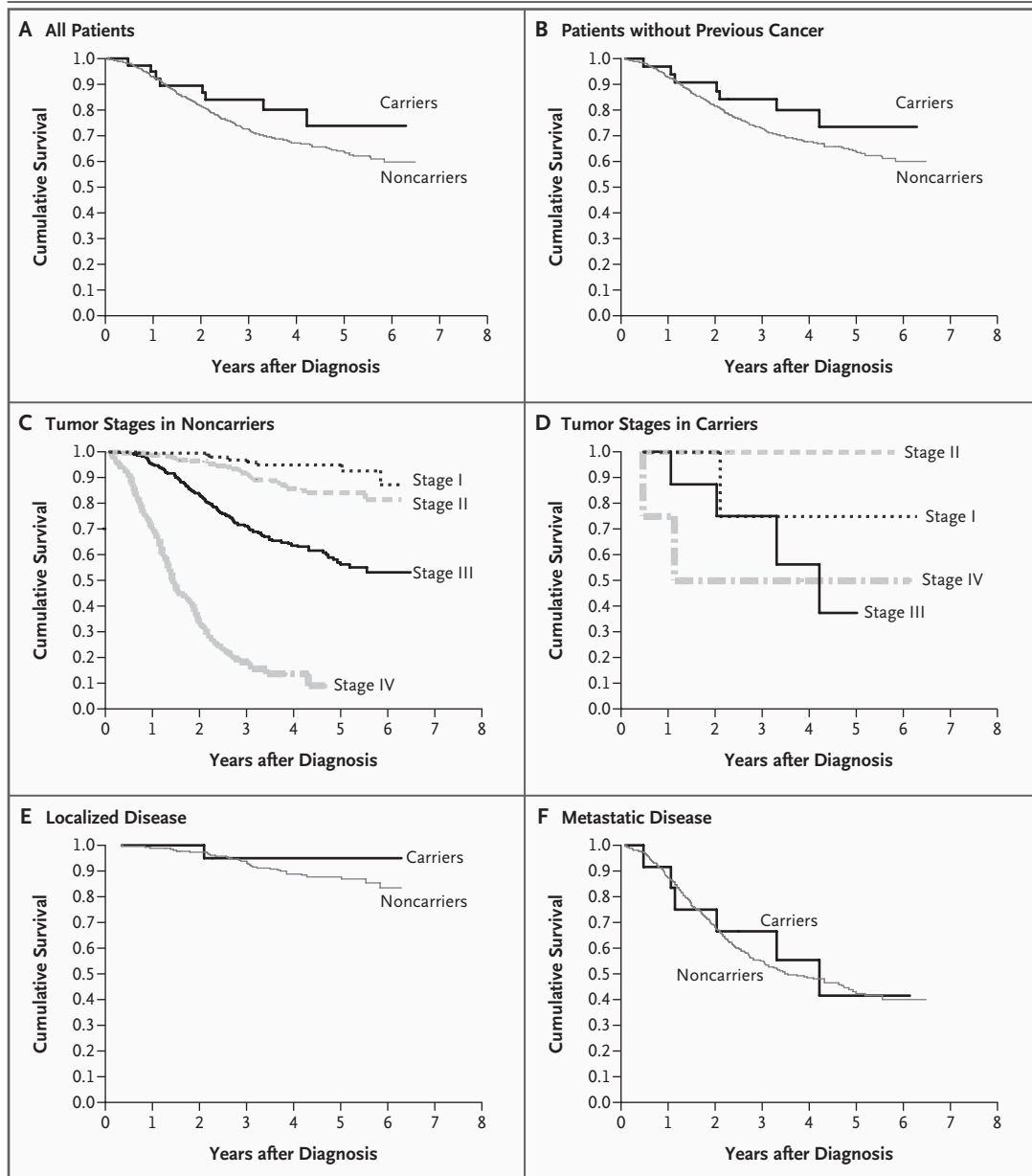


Figure 1. Survival Analysis of 870 Patients, According to Mutational Status and Tumor Staging.

The graphs show 2938 patient-years of prospective follow-up from the time of diagnosis — 134 patient-years for patients with pathogenic mutations (carriers) and 2804 patient-years for those without such mutations (noncarriers). No significant difference in overall survival was observed between carriers and noncarriers ($P=0.18$ by the log-rank test) (Panel A). To minimize any potential survival bias, in all other analyses (Panels B through F), we excluded patients (5 carriers and 25 noncarriers) who had undergone any form of colonic surveillance or screening or who had previously had a potentially fatal cancer. Panel B shows the overall survival in this group, and again there was no significant difference in survival ($P=0.24$ by the log-rank test). Panels C and D, which show tumor stages I through IV in noncarriers and carriers, respectively, demonstrate, as expected, that there were significant differences in survival according to stage, even when the analysis was adjusted according to mutational status ($P<0.001$ by the log-rank test). Panel E shows survival curves for carriers and noncarriers with localized disease (stages I and II), and Panel F survival curves for those with metastatic disease (stages III and IV). There was no significant difference in survival between mutation carriers and noncarriers after adjustment for the extent of the disease ($P=0.6$ by the log-rank test).

patients who had previous cancers or had undergone previous screening or surveillance did not change the result significantly ($P=0.24$) (Fig. 1B). Figure 1C and 1D show survival according to tumor stage; carriers with stage II tumors survived longer (five-year survival rate of 100 percent) than those with stage I tumors (five-year survival rate of 75 percent). There was no significant difference in survival between carrier groups according to the extent of tumor spread at diagnosis: the five-year survival rate among patients with localized disease was 95 percent for carriers and 87 percent for noncarriers (Fig. 1E); for those with metastatic disease, the five-year survival rate was 42 percent for both carriers and noncarriers (Fig. 1F).

DISCUSSION

This large prospective, population-based study provides robust estimates of the prevalence of mutations in DNA mismatch-repair genes in incident cases of colorectal cancer. We did not prejudice the analysis by using the family history or by first testing the tumor for microsatellite instability or expression of DNA mismatch-repair proteins. This strategy allowed us to identify predictors of mutational status using univariate analysis and to develop a two-stage, clinically driven predictive model using a multivariate analysis that estimates carrier probabilities. As was previously stated, the factors involved in the model have been fitted into the equation that appears in the Methods section. A clinician-friendly electronic version to estimate the likelihood that a given patient with colon cancer has a mutation is available at www1.hgu.mrc.au.uk/Softdata/MMRpredict.php.²⁵

The model was replicated in an independent series, even though the replication set consisted of younger patients and recruitment was retrospective. Among the 870 patients with cancer, the prevalence of the mutation was 4 percent, which causes some imprecision in the values in Table 3. For instance, testing for microsatellite instability combined with the use of Bethesda or Amsterdam criteria or stage 1 of our model (probability cutoff set at 0.05) gave sensitivities of 88 percent (95 percent confidence interval, 77 to 98 percent), 39 percent (95 percent confidence interval, 24 to 55 percent), and 65 percent (95 percent confidence interval, 50 to 80 percent), respectively, whereas positive predictive values were 32 percent (95 percent confidence interval, 23 to 41 percent), 100 percent (95 percent confidence interval,

82 to 100 percent), and 53 percent (95 percent confidence interval, 39 to 68 percent), respectively. All the confidence intervals are shown in Table 3 of the Supplementary Appendix.

Immunostaining of biopsy specimens obtained during colonoscopy (stage 2 of the model) is a feasible means of preoperative prediction of the carrier state. Of patients who underwent surgical resection, 80 percent underwent a preoperative endoscopic diagnostic biopsy. At the 0.05 cutoff, the model identifies a subgroup of 17 percent of patients for whom tumor immunostaining could inform a decision concerning the surgical procedure. The use of a combination of clinical measures and immunohistochemical staining of the tumor gives a positive predictive value of 80 percent and a sensitivity of 62 percent for mutation carriers. This information could be used in preoperative counseling about options for surgical prophylaxis, including total colectomy, rather than segmental resection, or combining colectomy with hysterectomy for postmenopausal women or those who do not want more children. Carrier identification could also be used to inform decisions about adjuvant therapy, since there may be differences between carriers and noncarriers in their responsiveness to chemotherapy.^{27,28} In all, combining the model with immunohistochemical data identifies only 1 in 29 of all patients (3.4 percent) as being likely to carry a mutation (positive predictive value, 80 percent) and so represents a highly efficient means of identifying patients for mutation testing.

By varying the cutoff values, the model allows resource-constrained health systems to optimize the efficiency of carrier detection by matching available financial and sample resources with the capacity for immunohistochemical analysis, microsatellite-instability analysis, and an evaluation of mutational factors.

The model requires cautious application, because its performance has been assessed in only 73 patients with mutations in the primary set of 870 patients and the replication set of 155 subjects. The prevalence of mutations reported by Aaltonen et al.⁶ and Hampel et al.⁸ was half that in our primary set (4 percent) and 1/10th that in the replication set (23 percent), which probably is a reflection of the age groups studied (mean ages, 68 years, 62.9 years, and 34 years, respectively), and reinforces our rationale for studying early-onset cases. The sex-specific difference in the frequency of mutations (a ratio of 1:17 for men and 1:37 for women)

is noteworthy and consistent with sex-dependent penetrance effects^{10,12} and environmental factors, such as hormonal protective effects and sex-linked modifier genes. Such modifiers may explain sex-based differences in the population risk of colorectal cancer.

A few patients had the same mutations; some of these patients were related to each other, whereas others may have carried founder mutations. The proportion of *MSH6* mutations (18.4 percent) is higher than that previously reported,^{29,30} although it is in accord with findings in families with the Lynch syndrome with predominantly extracolonic or late-onset colorectal cancers.³¹ A minority of tumors from *MSH6* carriers had a high degree of microsatellite instability, as noted previously.^{29,30,32} The presence of other DNA mismatch-repair defects may increase the risk of cancer that does not have a microsatellite-instability phenotype — the *MLH1* D132H variant, for example.³³ The absence of microsatellite instability in these carriers emphasizes the importance of systematic approaches, without prescreening, to the identification of carriers among patients with incident cases.

Our study population was not categorized according to family history or patterns of referral, reducing the likelihood of bias. In contrast, previous reports^{23,34,35} studied selected populations referred to genetic centers. Amsterdam criteria seem to be insufficiently sensitive (42 percent) for routine use in incident cases, supporting our previous observations.⁷ Furthermore, 18 patients who fulfilled the Amsterdam criteria had no identifiable mutation. Some of these patients may have had genomic rearrangements of *MSH6*, intronic or promoter mutations, or *PMS2* mutations, but use of the criteria for identifying hereditary nonpolyposis colorectal cancer fails to detect a clinically significant number of carriers. Although the Bethesda criteria are highly sensitive for the identification of carriers (95 percent), they require the analysis of microsatellite instability of 64 percent of tumors and three times as many analyses of germ-line genes as does our stage 1 model combined with immunostaining.

We found no significant difference in survival

between genotypes, perhaps because of the relatively small number of carriers, but any possible trend is small and clinically irrelevant. This result concurs with results of population-based studies of family history³⁶⁻³⁸ but conflicts with results of retrospective analyses of families with the Lynch syndrome.^{15-17,39} Lead-time and selection bias probably explain these discrepancies. Thus, families with the Lynch syndrome with several surviving affected relatives are more likely to be included in retrospective studies. Effects on reproductive fitness also mean that fewer patients with alleles associated with a poor prognosis would be recruited. In a similar way, highly penetrant alleles impart excess rates of death with each incident tumor, as well as an increased likelihood of having multiple fatal synchronous or metachronous tumors. This contrasts with cases involving families with the Lynch syndrome who have a survival benefit as a result of surveillance.⁴⁰ In any case, our finding emphasizes the importance of early cancer detection and prevention in patients with germ-line mutations in DNA mismatch-repair genes, whether or not such patients fulfill the criteria of having the Lynch syndrome.

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