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Cancer risk associated with germline DNA mismatch repair gene mutations

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The autosomal dominant syndrome of Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is due to germline DNA mismatch repair gene mutations in most cases. However, the penetrance of such mutations outwith classical HNPCC kindreds is unknown because families studied to date have been specifically selected for research purposes. Using a population-based strategy, we have calculated the lifetime cancer risk associated with germline DNA mismatch repair gene mutations, irrespective of their family history. We identified 67 gene carriers whose risk to age 70 for all cancers was 91% for males and 69% for females. The risk of developing colorectal cancer was significantly greater for males than for females (74% versus 30%, *P* = 0.006). The risk of uterine cancer (42%) exceeded that for colorectal cancer in females, emphasising the need for uterine screening. Our findings give further insight into the biological effect of defective DNA mismatch repair. We have demonstrated a systematic approach to identifying individuals at high risk of cancer but who may not be part of classical HNPCC families. The risk estimates derived from these analyses provide a rational basis on which to guide genetic counselling and to tailor clinical surveillance.

INTRODUCTION

The increased familial incidence of colorectal, uterine and other cancers that characterises Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (1,2) is due, in most families, to germline alterations in any one of four human DNA mismatch repair (MMR) genes (3). Almost all reported germline MMR gene mutations have been identified in HNPCC kindreds that were specifically selected for study because of a striking number of cancer cases (3–6). Hence, ascertainment bias is an inherent problem with cancer risk estimates derived empirically from such families (2,7,8). A recent study assessed the cancer risk for individuals with germline hMSH2 or hMLH1 mutations (9). However, all 19 families studied were already registered in the Dutch HNPCC Registry and fulfilled criteria for HNPCC. Clearly, such studies are highly relevant to genetic counselling in classical HNPCC kindreds, albeit that such families are relatively small in number, but the cancer risk has not been delineated for individuals with germline MMR gene mutations who are not from known HNPCC families. Knowledge of the cancer risk for these individuals is of substantial importance, since gene carriers identified as part of systematic genetic screening programs seem likely to outnumber probands from known HNPCC families.

Tumours from HNPCC patients exhibit genetic instability at simple DNA repeat sequences (3,10–12) due to DNA mismatch repair deficiency (13–15), that we refer to here as the RER phenotype (for Replication ERror). Some studies suggest that identification of RER tumours alone does not predict well for familial cases (16,17). In this study we evaluated targeting genetic analysis to patients with both early-onset colorectal cancer and an RER tumour as a means of identifying kindreds with germline MMR gene mutations. We then assessed the lifetime cancer risk associated with such mutations in relatives of the index cases. Our findings in this cohort of relatives, in whom ascertainment bias has been minimised, provide a rational basis for tailoring clinical surveillance programs on a population-wide basis.

RESULTS

Tumour RER analysis

We assessed tumour RER status in 27 patients who fulfilled the inclusion criteria. RER analysis was equivocal in four tumours due to technical problems with DNA degradation in paraffin-embedded material. Thirteen of the remainder were RER (56%), a fraction significantly less than that previously reported by our laboratories for tumours from known HNPCC patients (3), where identical RER analyses were employed (13 of 23, 58% versus 68 of 74, 92%; $P < 0.0003$).

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Table 1. Germline mismatch repair gene mutations identified in index patients with early onset colorectal cancer

Patient	Age (years)	Sex	Gene involved	DNA change	cDNA change	Protein alteration
528	28	F	hMSH2	C→T	CAG→TAG	Truncation at codon 406
666	35	M	hMSH2	del AAT	del AAT	del Asn at codon 596
814	20	F	hMSH2	C→T	CAG→TAG	Truncation at codon 601
817	29	F	hMLH1	N/I	del exon 13	del codons 470–520
818	23	M	hMSH2	C→T	CAG→TAG	Truncation at codon 252
825	28	F	hMSH2	delCTGT	del exon 5	del codons 265–314

Further details of analyses employed to facilitate screening relatives for the respective mutations found in patients 666, 814 and 817 are available on request.

Table 2. Cancer experience of the 67 relatives who carry germline mismatch repair gene mutations

Tumour type	Sex (32M,35F)	No. of relatives with tumour	Mean age (range) at first occurrence
Large bowel[a]	Male	17	47 years (31–79)
	Female	7	50 years (41–67)
Uterine	Female	7	54 years (45–68)
Oesophagus/gastric	Male	3	59 years (37–88)
	Female	–	–
Pancreatic	Male	1	42 years
	Female	1	76 years
Lung (adenoca.)	Male	–	–
	Female	1	54 years

[a]One of the tumours included in the large bowel category was an adenocarcinoma of the appendix.

MMR gene mutations in index cases

hMSH2 and hMLH1 mutation analysis for the 13 patients with RER tumours identified germline mutations in six cases (46%), a proportion consistent with previous studies (3). Five of these mutations have been reported previously (3,18). There were two males and four females with a mean age at diagnosis of 27 years (range 20–35 years). With the exception of patient 666, all mutations are predicted to have a dramatic effect on gene function (Table 1). The mutation carried by patient 666 involves deletion of a residue which does not exhibit marked inter-species conservation (19). Nonetheless, this alteration is highly likely to be pathogenic because all affected family members carried that same mutation, while an identical mutation has been reported in an unrelated HNPCC family (3) and in a sporadic colorectal tumour as a somatic alteration (20).

We devised tests to facilitate screening relatives for the respective MMR gene mutation. Alterations conducive with such screening were: that found in patient 666, resulting in the creation of a *Taq*I restriction endonuclease site; that in patient 814, detectable by PCR modification of adjacent sequences resulting in mutation-specific ablation of an *Msl*I restriction endonuclease site; that in patient 817 which induced the ablation of a *Pst*I restriction endonuclease site. Other mutations were screened by direct sequencing or IVSP analysis of cDNA. The mutation in patient 528 was not present in either parent or in siblings. Analysis with a battery of CA repeat markers indicated non-paternity and hence no further attempts were made to trace relatives. Multiple repeat analyses were performed to verify a positive or a negative result in all families.

Pedigree ascertainment and tracing of relatives of index cases

Pedigree tracing for the six probands with germline MMR gene mutations resulted in the ascertainment of 156 individuals over 18 years old related to the index case by common ancestry. Only two families fulfilled criteria for HNPCC (1), even when considering extended pedigrees. An average of 30 at-risk relatives were identified for each index case with a characterised mutation in whom non-paternity did not confound family tracing. Blood samples were obtained from family members regardless of their previous cancer history and nucleic acids screened for the mutation identified in the proband. Excluding index cases, 67 relatives (35 females and 32 males) carried a germline MMR gene mutation identified directly by mutation analysis or by inference from analysis of multiple descendants. The proportion of relatives that were gene carriers (67 of 156, 43%) did not differ significantly from that expected (50%) for an autosomal dominant genetic disorder ($P = 0.09$).

Summary data on cancer occurrence in family members are shown in Table 2. Including metachronous tumours, there were 27 separate colorectal cancers, seven uterine cancers, and seven other malignancies in a total of 35 relatives.

Assessment of lifetime cancer risk for relatives carrying germline MMR gene mutation

Cancer risk estimations for male and female relatives with gene mutations are presented in Figure 1A–D. Cancer incidence increased rapidly from age 40 but many patients destined to develop cancer did not do so until a relatively elderly age. This is

intriguing, given the very early onset disease in the proband. The risk of colorectal cancer is significantly higher at all ages in males than in females (Fig. 1A). By 70 years of age, the male risk was 74% while the female risk was only 30% (P = 0.0066). The relatively low lifetime risk of colorectal cancer for females is a striking feature and the risk of uterine cancer actually exceeds it by age 58 years, giving an estimate of 42% by age 70 years. The results of these estimates indicate that >90% of males who carry a MMR gene mutation will develop at least one type of cancer by 70 years of age.

Figure1B–D shows the results in a form readily applicable to counselling and to tailoring clinical screening. For clarity, only analyses that include direct ancestors of the proband are shown. For a family member of a given age (Y), these curves give an estimate of the risk of developing cancer over the ensuing 10–40 years. Figure 1B and C shows the projected risk of colorectal cancer for males and females, respectively, while Figure 1D shows the uterine cancer risk. When assessing cancer risk generated from these data, it is important to consider possible cohort effects that may result in true penetrance differences between generations. Such influences could be the result of dietary differences as well as the phenomenon of anticipation. Figure 1B and C does take these possible variations into account, but it is a formal possibility that future generations could have different age dependent cancer risk curves.

Although only analyses that included ancestors are presented, exclusion resulted in only a small reduction of the estimated penetrance at all ages. This was as predicted, since these ancestors survived to reproduce and so may be healthier and longer-lived than collateral relatives. The statistical methodology appeared satisfactorily robust as the results were highly insensitive to alterations in the assumed general population cancer incidence. Changes of ±25% had a negligible effect on the estimated penetrance for gene carriers. Standard errors associated with penetrance estimates were ~10%.

DISCUSSION

The results of this study allow assessment of cancer risk for individuals who carry mismatch repair gene mutations. These risk estimates are less liable to the ascertainment bias that inevitably arises when analysing cancer risk in previously identified HNPCC families. Clearly, the least biased of all possible samples would involve a population screening approach regardless of disease state, but at present this is not practical. However, our findings represent a reasonable balance between an unbiased sample and the practical constraints of wholesale population screening. Thus, the risk estimates allow tailoring of invasive screening procedures such as colonoscopy or endometrial biopsy for individuals identified as part of similar systematic population-based approaches.

One important finding from the present study is the substantially lower colorectal cancer risk for female gene carriers than for males. This conflicts with empirically derived estimates in HNPCC families (2,8) which suggest approximately sex-equal colorectal cancer risk. One recent study of HNPCC patients with proven MMR gene mutations suggested a lower colorectal cancer risk in females, although the difference was not statistically significant (9). A recent report has suggested an improved survival rate for female hMLH1 mutation carriers (21) which may have a confounding effect on pedigree data gleaned from

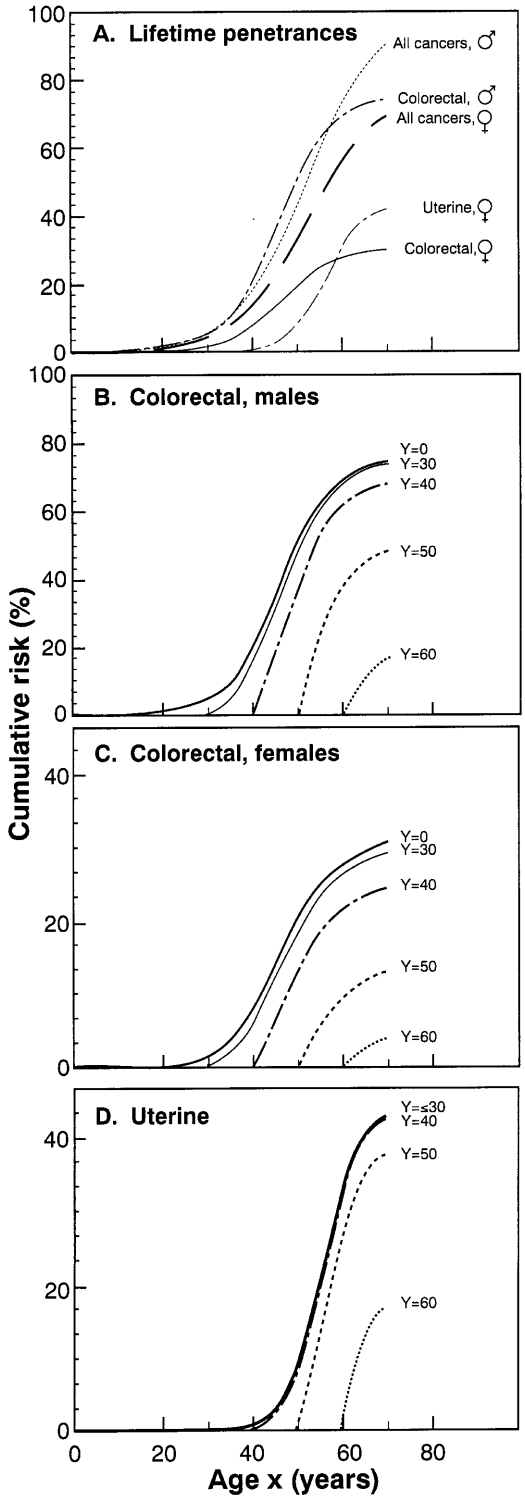

Figure 1. (**A**) Estimated lifetime penetrance in mismatch repair gene carriers for colorectal cancer (M and F), uterine cancer (F) and for all cancers (M and F). The latter included colorectal, uterine, gastric, pancreatic and lung (adenocarcinoma). Population estimates for adenocarcinoma of the lung were taken as 15% of all cancers of trachea, bronchus and lung for males and 25% for females. (**B**) Estimated risk of colorectal cancer as a function of advancing age (X years) for male gene carriers, given disease-free at Y years. (**C**) Colorectal cancer risk as in (B) for female gene carriers. (**D**) As (B) for uterine cancers in female gene carriers.

mortality statistics and from family members recollection of cancer as a cause of death in classical HNPCC families. Thus, females with MLH gene mutations could have an equivalent risk to males for developing cancer but a lower risk of cancer-related death and hence be less likely to be identified. However, such bias cannot explain our observations, since we cross-referenced the database to incidence data from Cancer Registration and did not rely exclusively on colorectal cancer mortality. It seems likely that the lower female incidence is real and that females are protected in some way, perhaps due to environmental factors or even a sex-linked modifier gene. Further studies may shed light on this important influence on cancer risk.

Risk estimates from the present study indicate that females are at greatest risk of uterine cancer, the risk being almost 50% higher than that for colorectal cancer (42% versus 30%). Hence, it is vital that integrated clinical screening protocols address this substantial uterine cancer risk and that counselling should include discussion of the option of prophylactic hysterectomy. Since colorectal cancer risk for men is so high and interval cancers are well described on surveillance programs (22), it is clear that non-directive counselling should include the option of prophylactic colectomy.

The statistical model that we employed is not a literal representation of the disease process but has certain notable advantages over other methods, including the intuitively reasonable implication that the cancer risk for carriers is never less than that for non-carriers. The model assumes that the existence of a test result conveys no information regarding the carrier status for an individual family member. This is unlikely to be strictly true, since a carrier has an increased chance of dying of cancer and thus being unavailable for testing. However, the effect is likely to be small for relatively late onset disease.

Our findings provide evidence that the underlying molecular pathogenesis in an appreciable proportion of patients with early onset colorectal cancer does not involve the genetic instability that manifests as the tumour RER phenotype. Significantly fewer patients who developed colorectal cancer aged ≤35 years had RER tumours than we have reported for HNPCC patients (P <0.0003) (3). Since the majority of sporadic tumours are non-RER (10–12,23), further studies are required to investigate whether such early-onset non-RER tumours are also genetically determined. Indeed four of 12 HNPCC kindreds previously studied from New Zealand were non-RER (3), further supporting the notion that genes other than known MMR genes are involved (3). Attenuated familial adenomatous polyposis, which is due to truncating mutations in the first four exons of the APC gene (24), may also account for a proportion of patients with non-RER tumours and a search for such mutations is underway.

It is clear that the cancer risk to MMR gene carriers is substantial and that timely institution of clinical surveillance is essential. Outside known HNPCC families, a proactive approach such as described here will allow identification of gene carriers from small families, those with few affected family members due to low penetrance, and those cases that arise as new mutations. Although RER analysis of tumours without stratifying age groups does not appear to be sufficiently discriminatory to identify those with a family history of HNPCC (16,17), the efficiency of the strategy that we employed here was acceptable given the extensive molecular analysis required for each patient. Extrapolation of these and previous data from our laboratories (18,23,25) indicates that 58% of patients <35 years of age with colorectal cancer have RER tumours and 24% of the total will have a germline MMR gene mutation. This compares with findings in the non-age stratified group where 15% have RER tumours and ~1% of the total have germline mutations. Thus the prevalence of RER tumours is substantially higher in the patients <35 years of age at diagnosis compared with the older age groups while the germline MMR gene mutation prevalence in the younger age groups with RER tumours is also far higher. In conclusion, this study demonstrates a practical and efficient approach to identification of MMR gene mutation carriers and provides a rational basis on which to build accurate risk assessments for such individuals.

MATERIALS AND METHODS

Proband and family history ascertainment

Index patients were identified from the Scottish National Cancer Registry and were diagnosed with colorectal cancer aged 35 years or less between 1970 and 1993. Patients referred specifically because of a family history fulfilling HNPCC criteria (1) were excluded. Despite extensive investigation, only two of the probands evaluated in the current study were found to have a family history that met these criteria.

Index cases chosen for subsequent analysis had to meet the following criteria: histological confirmation of colorectal cancer from pathology records; survival of the index case to allow blood sampling and agreement to participate by informed written consent; availability of paraffin-embedded or fresh tumour material for assessment of tumour RER phenotype. Family history was obtained from each patient by interview or questionaire and subsequently extended and verified through central Scottish records of births, deaths and marriages. All surviving relatives in the extended kinships were traced and interviewed. Personal and family history of cancer was ascertained from these relatives and again verified from clinical, pathology and death certificate records as appropriate. Family trees are not presented for reasons of confidentiality.

Tumour RER analysis

Methodology for tumour RER analysis is described in detail elsewhere (3,18,26). In brief, we compared the allele patterns generated by PCR amplification across simple repetitive elements of (A)n or (CA)n using matched tumour and normal DNA purified from paraffin-embedded or from fresh material. Tumour-specific variations had to be present in >50% of at least four markers analysed for the tumour to be classified as RER.

MMR gene analysis

RNA and DNA purified from peripheral blood lymphocytes or from lymphoblastoid cell lines from patients with RER tumours was screened for MMR gene mutations using an *in vitro* synthesised protein truncation assay (IVSP) in combination with single stranded conformational polymorphism analysis (PCR-SSCP). IVSP analysis was performed on PCR products amplified from cDNA templates reverse-transcribed from peripheral blood lymphocyte or cell line RNA as described (3,18). IVSP reliably detects truncating or splice mutations (3,18,26–28) but will not detect missense mutations. Hence, patients with no detectable IVSP alterations were assessed by PCR-SSCP for hMSH2 and

hMLH1 mutations, employing previously described methodology (28). Samples showing variants on IVSP or SSCP assays were re-amplified and purified PCR products sequenced to identify the specific mutation (28). All relatives of index patients with germline MMR gene mutations were then tested for that mutation.

Statistical methodology and cancer risk estimations for gene carriers

Cancer history including age-of-onset, tumour site and type, along with date of birth and, where relevant, date and cause of death was ascertained for all descendants of the earliest traceable ancestors of index patients with MMR gene mutations. Pedigree data and cancer incidence were recorded on a computerised database. In practice, the earliest traceable relatives were those alive around 1850 when Parish Registers were replaced in Scotland with a national registration system of births, deaths and marriages. Live relatives were classified as disease-free if alive and well when data collection terminated (June 1995) and deceased relatives classified as disease-free if death certification recorded only non-malignant disease. Only the onset of the first malignancy was included in the assessment of cancer risk, after which that relative did not contribute years at risk. After determining carrier status for each relative with regard to the MMR gene mutation identified in the respective proband, each family member was then classified as proband (P); direct ancestor of proband (DA); tested carrier (TC); tested non-carrier (NC); inferred carrier (IC) (untested but with one or more direct descendants carrying the mutation); other untested individuals (UT). We assumed that all gene carriers in the same family inherited the mutation from a common ancestor and that no mutant alleles entered the family independently.

Index cases were excluded from the analysis of disease-free survival of gene carriers since they were selected to have early onset disease. Direct ancestors could also be subject to bias since survival to reproductive age was a prerequisite. Hence, analyses were carried out both including and excluding direct ancestors. The probability (P_c) of being a gene carrier was computed for all family members, conditional on their own carrier status if tested, or on that of their relatives if untested. Thus P_c was 1.0 for categories DA, TC and IC, and 0.0 for category NC. If all relatives had been available for testing, we could have calculated disease-free survival curves by standard life-table analyses for gene carriers. However, we had to allow for (mainly deceased) individuals of unknown carrier status (UT), where $1 > P_c > 0$.

For untested individuals where P_c did not depend on the carrier status of another UT individual, the likelihood was expressed in the form:

$$P(x = X) = \{P_c \times [F(X + 1) - F(X)]\} + \{(1 - P_c) \times [G(X + 1) - G(X)]\}$$

for affected relatives and,

$$P(x > X) = \{P_c \times [1 - F(X)]\} + \{(1 - P_c) \times [1 - G(X)]\}$$

for unaffected individuals (X denotes age at onset or when last known disease-free: F(X), G(X) denote the penetrance functions for carriers and non-carriers, respectively).

For parent-child pairs (UT) where P_c for each individual was dependent on the probability that the other was a carrier, the joint likelihood for unaffected pairs was expressible as:

$$\{P_{cc} \times [1 - F(X_1)] \times [1 - F(X_2)]\} + \{P_{c\bar{c}} \times [1 - F(X_1)] \times [1 - G(X_2)]\} + \{P_{\bar{c}\bar{c}} \times \{1 - G(X_2)] \times [1 - G(X_2)]\}$$

where P_{cc} = Prob(both parent and child are carriers); $P_{c\bar{c}}$ = Prob(parent is a carrier and the child a non-carrier); $P_{\bar{c}\bar{c}}$ = Prob(both are non-carriers); X_1 and X_2 are the X-values of parent and child, respectively. Analogous expressions were also derived where either or both members of the pair were affected. The assumed penetrance function, G(X), for non-carriers was modeled by the Gompertz-type form:

$$G(X) = b_0/(1 + e^{-s(X)}), \text{ where}$$

$$s(X) = b_1 \times (X - b_2).$$

b_0 can be interpreted as the cancer risk at 'infinite age', b_1 as related to rate of increase of risk with age and b_2 as the age by which half the total risk is experienced. We used fixed values for b_0, b_1 and b_2 derived from cancer registration data for the Scottish population (29). F(X) was modelled by postulating an additional competing risk attributable to the carrier status:

$$F(X) = 1 - \{[1 - G(X)] \times [(1 - a_0)/(1 + e^{-r(X)})]\}, \text{ where}$$

$$r(X) = a_1 \times (X - a_2).$$

Maximum likelihood estimates of the parameters, and of their asymptotic variances and co-variances, were derived by maximizing the overall likelihood using the SEARCH suite of programs (30).

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