Short Report

Family cancer histories predictive of a high risk of hereditary non-polyposis colorectal cancer associate significantly with a genomic rearrangement in hMSH2 or hMLH1

Ainsworth PJ, Koscinski D, Fraser BP, Stuart JA. Family cancer histories predictive of a high risk of hereditary non-polyposis colorectal cancer associate significantly with a genomic rearrangement in hMSH2 or hMLH1.


Hereditary non-polyposis colorectal cancer (HNPCC) results from inactivating germline mutations in a set of DNA-mismatch-repair genes, of which the most clinically relevant are hMSH2 and hMLH1. Computer-assisted pedigree risk assessment tools are available to assist in the calculation of an individual’s likelihood of bearing such a deleterious mutation. One such tool, CANCERGENE version 3.4 (http://www3.utsouthwestern.edu/cancergene) was used to assess the risk of a deleterious mutation in the genes hMSH2 and/or hMLH1 in a series of probands selected from a panel of 67 South-western Ontario kindreds previously identified as likely candidates for HNPCC by established clinical criteria. A DNA sample isolated from peripheral blood leukocytes obtained from each of these probands was examined for genomic rearrangement using the multiplex ligation-dependent probe amplification (MLPA) method. Of the individuals calculated to have a risk of >50% of a hMSH2 or hMLH1 gene mutation by the CANCERGENE risk assessment tool, 69% (9/13) were shown to have a genomic rearrangement resulting in the deletion of one or more exons of one of these two genes. Family cancer histories predictive of a high risk of HNPCC significantly associate with a genomic rearrangement in hMSH2 or hMLH1.

Hereditary non-polyposis colorectal cancer (HNPCC) (MIM 114500) is the most common form of hereditary colon cancer, accounting for 1–5% of this disease (1, 2). A critical role in HNPCC predisposition is played by the DNA-mismatch-repair genes, which include hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6. Inactivating mutations of two of these genes, hMSH2 and hMLH1, have been considered to be the major cause of DNA-mismatch-repair deficiency in humans (3, 4), and many of these mutations are reported in an internet-accessible database (http://www.nfdht.nl/). Initially, the majority of deleterious mutations reported were nonsense, missense, or frameshift mutations as well as mutations affecting splice junctions. This may well have been due at least in part to an ascertainment bias, as more recently utilization of elegant newer detection techniques [e.g. multiplex ligation-dependent probe amplification (MLPA)] (5, 6) have shown that a surprisingly large proportion of deleterious mutations can result from genomic rearrangements commonly consisting of single or multi-exonic deletions affecting the hMSH2 and/or hMLH1 genes (2).

The first generally accepted diagnostic criteria for HNPCC were the Amsterdam criteria (I), where at least three close relatives had to be affected with colon cancer in two successive generations where the age at diagnosis in at least one individual was <50 years (7). These initial criteria proved to be
too stringent and a more relaxed version; the Amsterdam criteria (II) were developed, which included in addition to colon cancer, extra-colonic cancers, notably endometrial cancer, and to a lesser extent other cancers, including cancers of the small bowel, ureter, and renal pelvis (8). Clinical guidelines, derived using as a starting point both the Amsterdam and Bethesda (3) criteria, have been established in the Province of Ontario to provide eligibility criteria for HNPCC molecular genetic screening (Materials and methods section below).

More recently, computer-assisted risk analysis programs, which can assess the likelihood of a deleterious mutation in the genes predisposing to HNPCC, have become available. One such program is available in CANCERGENE version 3.4 (9). Available within this program is a colon cancer risk assessment tool which analyzes family cancer history information using a logistic regression model (10) to specifically estimate the likelihood of a mutation in the DNA-mismatch-repair genes hMSH2 and/or hMLH1.

Materials and methods

A series of families with a history of colon and/or endometrial cancer who had been referred in to the Cancer Genetics Unit at the London Regional Cancer Center were assessed for risk of HNPCC using the clinical criteria outlined below.

Clinical risk criteria to select families at risk of HNPCC

Affected and unaffected individuals from families with a known HNPCC mutation: affected individual from modified Amsterdam families

Three affected relatives, one with colorectal cancer and the other two with any combination of colorectal, endometrial, small bowel, ureter, transitional cell kidney cancer, and/or sebaceous adenoma/carcinoma.

Family must meet all of the following criteria:
1. One should be a first-degree relative of the other two.
2. At least two successive generations should be affected.
3. At least one diagnosis of CRC must be before age 50.
4. Tumor type should be confirmed by review of pathology or other medical records.

Affected individuals from families as defined below

Three affected individuals, one with colorectal cancer and the other two with any combination of colorectal, endometrial, small bowel, ureter, sebaceous adenoma/carcinoma, ovarian, pancreatic, kidney (transitional cell cancer only), gastric, and primary brain or primary hepatobiliary cancer.

1. Two of the three family members must be in a first-degree relationship.
2. At least one diagnosis must be under the age of 50.
3. Tumors should be verified by pathological examination.

An individual affected with CRC and a second primary HNPCC-associated cancer

This includes synchronous and metachronous colorectal cancers. At least one primary cancer must be diagnosed under age 55. Families are eligible with or without family history of HNPCC-associated cancer, and tumors should be verified by pathological examination.

An individual diagnosed with CRC under the age of 35

Families are eligible with or without family history of HNPCC-associated cancer, and tumors should be verified by pathological examination.

DNA sampling and calculation of HNPCC risk

After genetic counseling and confirmation of family history together with review of relevant pathology records, 67 families were identified to be eligible for molecular genetic screening of the hMSH2 and hMLH1 genes, and the highest risk individual from each of these families (the proband) provided a blood and, where possible, a tumor (colon) sample for analysis. Genomic DNA isolated from peripheral blood leukocytes was utilized for molecular genetic screening (MLPA). In addition, the risk of mutation in the hMSH2 or hMLH1 gene was estimated using the established family cancer history as calculated by CANCERGENE version 3.4 (9), as noted above. One mathematical model included in this program assesses the relevant family HNPCC-related cancer history and utilizes a logistic regression model (10) to establish the likelihood of a mutation in the DNA-mismatch-repair genes hMSH2 and/or hMLH1.

Multiplex ligation-dependent probe amplification

MLPA represents a simple and elegant method (5) to detect unusual copy numbers of genomic sequences such as the deletion or duplication of single or multiple exons and is available in kit form (6). In MLPA, pairs of oligonucleotides are brought into close proximity by hybridization to specific target (genomic) DNA sequences. Following a
ligation reaction, these copy sequences are then amplified in a quantitative, multiplex PCR reaction with the use of a common pair of primers, one of which is labeled with a fluorophore. Upwards of 40 sets of such oligonucleotide pairs can be employed in a single hybridization reaction, the sizes of the PCR amplicons derived from these being varied by the incorporation of stuffer sequences of increasing size into one of each of the primer pairs to create a size gradient and allow for their electrophoretic separation. The PCR products, the amount of which is directly proportional to the amount of template DNA, can then be quantified on a standard automated DNA sequencer.

For the purposes of this study, the hybridization procedure was performed using as a template a DNA sample derived from the proband from each of the 67 distinct HNPCC families noted above, according to manufacturer’s protocol for the P003 MLPA kit (6). The probe mixture included in this kit contains probes for each of the 19 exons of the hMLH1 gene as well as for each of the 16 exons of the hMSH2 gene. PCR of the hybridized and ligated probes was carried out using an Applied Biosystems 9700 thermal cycler using half the recommended volumes. PCR products were diluted with water, mixed with an equal portion of denaturing solution, heated to 94°C for 1 min, and then cooled in an ice block prior to gel electrophoresis. Samples were separated using 6% denaturing PAGE on an automated DNA sequencing system (Visible Genetics Clipper, Bayer Health Care, Berkely, CA) at 50°C, 50% power and 1400 V for 40 min. Areas under the peaks were calculated using the OpenGene software available with the Visible Genetics system and were exported to a Microsoft Excel spreadsheet for copy number analysis. The proportion of each peak relative to the area for all peaks was calculated for each sample and then compared to proportions for the corresponding peak averaged for a set of 11 normal DNA samples. Ratios of 1.0 (±20%) were treated as normal copy number. Ratios of 0.5 were considered as deletions and ratios of 1.5 as duplications.

Discussion

Of the 67 selected patients considered to be at high risk of HNPCC by clinical criteria, 10 were shown to have a genomic rearrangement by MLPA, a number in close agreement with a recently published study (2). Because of the possibility of single nucleotide polymorphisms potentially interfering with probe hybridization and causing a false-positive result, especially in the detection of single exon deletion events, these latter were confirmed by either long-range PCR (LR-PCR) or by corresponding cDNA analysis where possible (results not shown). A false-positive MLPA result was felt to be far less likely in the detection of contiguous multi-exonic deletions. The multi-exon deletions (hMLH1 EX12_16 and hMSH2 EX1_8del) seen in four distinct families were not confirmed by alternate methodology.

The logistic regression model which was derived by Wijnen (10) and which is used in the CANCERGene program was used to calculate the risk of a mutation in hMSH2 or hMLH1 in the 67 proband DNA samples analyzed. Of these 36% (24/67) were calculated to have a risk of >0.2 of a deleterious mutation in either of these two genes (Table 1). All 10 probands identified by MLPA to have an inactivating hMSH2/hMLH1 genomic rearrangement had a calculated risk of mutation of >0.2 and represented 40% (10/24) of this arbitrarily defined subgroup. More strikingly, of the individuals calculated by CANCERGene to have a risk of >0.5 of an hMSH2 or hMLH1 gene mutation, 69% (9/13) were demonstrated by MLPA to have a genomic rearrangement in one of these two genes.

A clearly significant association of the degree of calculated risk of a mutation in hMSH2 or hMLH1 with the presence of a deletion in either of these two genes was confirmed with a two-tailed Fisher’s exact test (p < 0.001).

The CANCERGene program was used to calculate the risk of a mutation in hMSH2 or hMLH1 in the 67 proband DNA samples examined. These comprised five distinct mutations: hMSH2 EX1_8del (2), hMSH2 EX12_16del (2), hMSH2 EX8del (2), hMLH1 EX2_6del (1), and hMLH1 EX12del (3) typical examples of which are shown in Fig. 1.
The likelihood of finding a family cancer history highly predictive of HNPCC in a kindred where the DNA mismatch repair function is disrupted by a deletion event in either \( \text{hMSH2} \) or \( \text{hMLH1} \) is consistent with the likelihood that these types of gene mutations eliminate the expression of a viable protein by the affected allele. This predicted loss of viable protein correlates with our own immuno-histochemical (IHC) findings showing a corresponding loss of either \( \text{hMSH2} \) or \( \text{hMLH1} \) in tumors from patients where these were available for analysis. A similar disruption of expression of a functional \( \text{hMSH2} \) or \( \text{hMLH1} \) protein would be expected to result from nonsense or smaller I/D frameshift mutations, and these types of mutations might similarly be expected to show a relatively high calculated risk of a deleterious mutation in \( \text{hMLH1} \) or \( \text{hMSH2} \) as well as the loss of viable protein. Indeed, this appeared to be the case; of four additional HNPCC kindred where an \( \text{hMSH2} \) or \( \text{hMLH1} \) protein terminating mutation (nonsense or frameshift) had previously been confirmed, three of these (where tissue was available for IHC analysis) showed loss of immunohistochemically detectable \( \text{hMSH2} \) or \( \text{hMLH1} \) protein (results not shown). These four families demonstrated a calculated risk of \( \text{hMSH2} \) or \( \text{hMLH1} \) mutation ranging from 0.18 to 0.82.

The other individuals shown in Table 1 to have a relatively high calculated risk of mutation, but where the MLPA for \( \text{hMSH2} \) and \( \text{hMLH1} \) was normal, could be harboring genomic rearrangements in any of the other mismatch repair genes, e.g. \( \text{hMSH6} \) and or \( \text{hPMS2} \), for which an MLPA kit has recently become available (6). Interestingly, the individual with the highest calculated risk (Table 1) was shown to have a \( \text{hMSH2} \) missense mutation, and somewhat surprisingly colon tumor tissue in this individual was shown to be deficient in \( \text{hMSH2} \) protein by IHC.
It seems likely that families with a lower calculated risk of mutation in *hMSH2* and *hMLH1* will have a preponderance of potentially less severe missense mutations in these DNA-mismatch-repair genes.

It is recognized that this is a relatively small study, but our results do suggest that an economic first step in screening for HNPCC would involve initial assessment of gross genomic integrity by MLPA analysis of leukocyte-derived genomic DNA. This would be especially valuable when individuals undergoing testing are identified by clinical history to be at high risk, as this approach would bypass the logistical difficulties of tumor analysis (assessment of microsatellite instability and immunohistochemistry).

**Conclusions**

The presence of a genomic rearrangement, resulting in the deletion of one or more exons of the DNA-mismatch-repair genes *hMSH2* and/or *hMLH1*, shows a highly significant association (p < 0.001 by Fisher’s exact test) with the likelihood of a mutation in one of these two genes, as calculated by an HNPCC-related familial cancer history assessment using the logistic regression model available in CANCERGENE program

A preliminary screen utilizing MLPA to identify genomic rearrangements in the proband from families at risk of HNPCC would represent a cost-effective first step in any algorithm for the detection of inactivating mutations in the DNA-mismatch-repair genes *hMSH2* and/or *hMLH1*.

We suggest that the MLPA assay be utilized as an economical primary screen in families calculated to be at an elevated risk of HNPCC.

**References**


