MOLECULAR GENETICS IMPROVES THE MANAGEMENT OF HEREDITARY NON-POLYPOSIS COLORECTAL CANCER

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Background. The syndrome of hereditary non-polyposis colorectal cancer (HNPCC) can be diagnosed fairly accurately using clinical criteria and a family history. Identifying HNPCC helps to prevent large-bowel cancer, or allows cancer to be treated at an early stage. Once the syndrome has been diagnosed a family member's risk can be judged approximately from a family tree, or it can now be predicted accurately if the causative mutation is known.

Objective. This study involved attempts to improve the management of a family with HNPCC over a period of 10 years. Clinical diagnostic criteria, colonoscopic surveillance, surgical treatment, genetic counselling, molecular genetic research, and finally predictive genetic testing were applied as they evolved during this time.

Subjects and methods. A rural general practitioner first noted inherited large-bowel cancer in the family and began screening subjects as they presented, using rigid sigmoidoscopy at the local hospital. At the time that the disorder was recognised as being HNPCC (1987), screening by means of colonoscopy at our university hospital was aimed primarily at first-degree relatives of affected individuals. After realising how many were at risk, screening was brought closer to the family. A team of clinicians and researchers visited the local hospital to identify and counsel those at risk and to perform screening colonoscopy. Family members were recruited for research to find the gene and its mutation that causes the disease, to develop an accurate predictive test and to reduce the number of subjects undergoing surveillance colonoscopies.

Results. There are approximately 500 individuals in this family. In the 10 years of this study the number of subjects who have been counselled for increased genetic risk or who have requested colonoscopic surveillance for HNPCC in this kindred has increased from 20 to 140. After the causative mutation was found in the *hMLH1* gene on chromosome 3, a test for it has reduced the number of subjects who need screening colonoscopy by over 70%. A protocol has been devised to inform family members, to acquire material for research in order to provide genetic counselling for (pre-test and post-test) risk, and to test for the mutation. Eventually, identifying those with the mutation should focus surveillance accurately.

Conclusions. The benefits of restricting screening to subjects with the mutation that causes colorectal cancer and of performing operations to prevent cancer are hard to measure accurately. However, it is likely that at least half the family members will be able to avoid colonoscopic screening, some deaths from cancer should be prevented, and the cost of preventing and treating cancer in the family should fall substantially.

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Although hereditary non-polyposis colorectal cancer (HNPCC) was described in 1913,[1] it has only been widely recognised as a clinical entity in the last few decades. Once the syndrome is recognised colorectal cancer can be identified early by screening family members. In addition, when the causative mutation is found appropriate surgery may prevent cancer in family members. Estimates of HNPCC prevalence vary widely from 0.3% to 10% of all colorectal cancers,[2-9] probably because of differences between populations as well as in the methods of ascertainment. There are no data available from South Africa. HNPCC has different clinical features from sporadic large-bowel cancers. It typically causes large-bowel cancer between the ages of 40 and 50 years. Unlike sporadic colorectal cancer, of which three-quarters occur in the rectum and sigmoid colon, most HNPCCs occur proximal to the splenic flexure. Approximately one-quarter of subjects ultimately develop more than one colorectal cancer.[10,11] Some families only develop cancer in the large bowel, while others have an increased risk of other cancers, such as those of the endometrium, stomach, biliary tract, urinary tract and ovary.[12] Unlike familial adenomatous polyposis (FAP), HNPCC is usually only diagnosed when cancer is confirmed because polyps are no

more common in those at risk than in the population at large.

HNPCC has a dominant pattern of inheritance. It is caused by a mutation in the germ cells, affecting any one of five known genes that normally repair mismatches between single base pairs or within short loops in newly synthesised DNA. Over 80% of affected families have mutations in the *hMSH2*[13,14] or *hMLH1* genes,[15,16] while mutations in *hPMS1*, *hPMS2*[17] and *hMSH6*[18] occasionally cause the disease.

This paper outlines the advances that have occurred in the management of HNPCC in the last 10 years. It illustrates their application and their limitations in a large South African family.

SUBJECTS AND METHODS

Study subjects and geographical setting

Most members of this family live in a remote village in the Northern Cape province of South Africa, some 50 km from the nearest tarred road and over 500 km from Cape Town, the nearest city. A nearby diamond mine is the largest source of employment. It has a small hospital. Subsistence livestock farming in the semi-desert countryside provides an alternative livelihood. Other family members live in coastal villages between Port Nolloth in the north and Cape Town in the south, or in isolated inland settlements.

Our first contact with the family was in 1987. A doctor working at the mine hospital referred a 30-year-old man to Groote Schuur Hospital in Cape Town for possible surveillance because he had had a cancer of the sigmoid colon removed at the age of 19 years. The patient's brother had survived the same disease at the age of 23 years, while their father as well as 3 of his 5 siblings had died of abdominal cancer. The doctor at the mine hospital had assembled a detailed family tree, which was recognised by a clinical geneticist as being typical of HNPCC.[19]

Anecdotal information from family members indicated that 17 men and 2 women in three generations had had bowel cancer, although histological proof was only available for 3 subjects. Individuals who were considered at risk because they were related to an affected individual were offered colonoscopic screening. At the same time they were given information on the disorder, on their risk of developing the disease, and on the need for colonoscopy. Colonoscopic screening began in 1988. At that time the technology for establishing linkage between the gene causing this disease and a particular chromosome was relatively primitive in our laboratory, but it was anticipated that technical advances would make stored leucocyte DNA or paraffin-embedded formalin-fixed tissue valuable provided that it came from individuals known to have HNPCC, or known to be elderly and unaffected.

Genetic studies

Finding the gene

During their clinical consultation family members were told their relative risk of developing colorectal cancer and asked for details of relatives who might be at risk. They were also asked to take part in research to find the responsible genetic defect. They were told that this might not benefit them, but that it might help their offspring. Formal recruitment with detailed informed consent began in 1991, when the family was told that finding the mutation that caused cancer would make it possible to identify those who had inherited it by means of a blood test. Recruitment involved visits to towns and villages such as Kommagas, Nababiep, O'Kiep, Port Nolloth, Springbok and Steinkopf in the Northern Cape province.

The DNA we studied was obtained either from lymphocytes from venous blood, or from archived histopathology specimens. The latter DNA was isolated from paraffin blocks of tissue obtained from surgical specimens examined at Groote Schuur Hospital. Specimens of colorectal adenocarcinomas from 6 members of the family were used. Sections of normal colon and of carcinoma tissue were cut from the paraffin blocks. The first and last sections were stained with haematoxylin and eosin (H&E). The intervening sections were used unstained for microdissection. Using the H&E slides as a guide, the carcinoma tissue was dissected off each slide while attempting to exclude normal tissue. Although the DNA from preserved biological material was primarily used to detect microsatellite instability, there was enough material for linkage analysis and for the direct detection of mutations. Microsatellite instability was studied because of its known association with mutations in DNA repair genes, which cause HNPCC.[13-16,20] In order to detect microsatellite instability each carcinoma extract was tested with a set of 7 microsatellite markers. Linkage analysis was then performed to localise the disease gene to a region of the genome.

For purposes of linkage analysis the microsatellite markers D3S1298, D3S1277 and D3S1611 on chromosome 3p were used because they lie near or in the *hMLH1* gene (Genome Data Base; http//gdbwww.gdb.org), which was considered a candidate for causing the disease. In order to find the mutation, each of the 19 exons (coding parts) of the candidate gene, *hMLH1*, was amplified. Single-stranded conformational polymorphism (SSCP) analysis[21] was then used to investigate each exon to identify any variation that segregated with the disease in the family. Once such a variation was found, the precise sequence of this fragment of DNA was determined. DNA from 50 unrelated individuals of mixed racial ancestry from the same geographical area were investigated in the same way to confirm that the variation was not an innocent polymorphism and that it was only found in subjects with the disease.

RESULTS

Pedigree

After 10 years of study the pedigree contains approximately 500 individuals. Of these, more than 350 have an increased risk of HNPCC because they are related to an affected subject. Of this cohort, 160 either have had cancer or are at 50% risk because they have an affected first-degree relative. This makes them eligible for surveillance colonoscopy, together with their offspring who are at 25% risk, and the progeny of such offspring who are at 12.5% risk (a nuclear section of the NPC1 kindred is shown in Fig. 1 to illustrate the relative risk of developing disease). By the end of 1998, blood had been obtained from 175 individuals, of whom 81 are at 50% risk and 50 are at 12.5 - 25% risk.

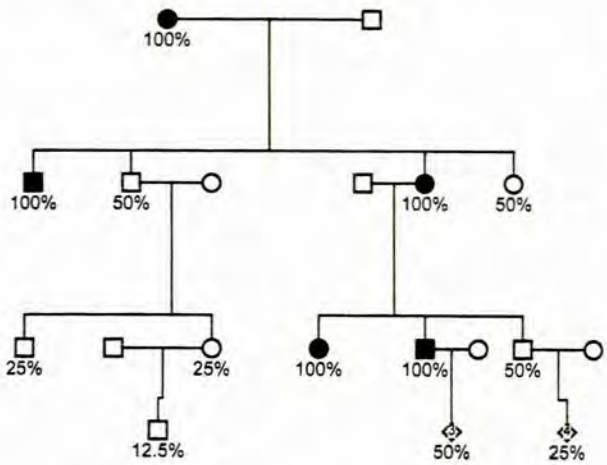

Fig. 1. Nuclear section of the larger NPC1 pedigree illustrating risk for HNPCC. Key: ■ = affected male; ● = affected female; □ = unaffected male; ○ = unaffected female.

Initial screening for cancer

We started colonoscopic assessment in 1988 in 20 first-degree relatives of affected family members using the local hospital's facilities and our own colonoscope. Of 18 asymptomatic subjects, 1 had isolated tubular adenomas in the caecum and the descending colon, as well as severe dysplasia in a tubulovillous polyp in the transverse colon. Of the 2 brothers who had had sigmoid cancer previously, 1 had two small tubular adenomas, in the ascending and in the transverse colon.

The initial assessment of the family tree suggested that scores of family members were at risk of HNPCC. They were referred for colonoscopic screening in Cape Town when possible, or screened during subsequent visits to the mine. Seven visits were made by colonoscopy teams to the local hospital during 10 years. Fortunately the university hospital administration agreed that allowing a team to visit the mine hospital once a year would be more cost-effective than screening family members as inpatients in our own institution. This allowed for biennial and then annual visits.

The travelling team included a geneticist for genealogical tracing, risk assessment and counselling of family members, a colorectal surgeon to perform colonoscopy and make clinical decisions, and an endoscopy nurse. Doctors and nurses working at the mine hospital helped to recruit the relatives of affected individuals, both for colonoscopic screening and to obtain DNA.

Pathology of cancers

Surgical specimens from 9 family members aged from 31 to 49 years (mean 39 years) were examined thoroughly. Only 1 subject was female. Seventeen carcinomas were present in the 9 subjects (3 cancers in 2 subjects, 2 cancers in 4 subjects, 1 cancer in the remaining 3 individuals). Only 3 were distal to the splenic flexure. Two subjects who had had carcinomas diagnosed previously presented with metachronous cancer. Mucinous carcinomas were present in all but 2 subjects. Breast carcinoma has been proven in 1 woman in the family who had previously undergone a total colectomy for two Dukes C carcinomas in the caecum and the ascending colon.

Molecular genetics

Microsatellite instability was detected in at least 2 of the 7 markers for each of the 5 carcinoma specimens investigated. This suggested that a DNA mismatch repair gene was involved in the pathogenesis of the family's disorder. Linkage was then sought to the genes known to cause HNPCC, the *hMSH2* gene on chromosome 2[13,14] and the *hLMH1* gene on chromosome 3.[15,16] Markers close to *hMSH2* showed that the disease locus in the kindred under investigation did not lie close to this gene (within 30 centimorgans). Early linkage analysis in the family, using markers known to be linked to the *hMLH1* gene, suggested that this might be the causative gene (log of odds score of 2.98 at theta = 0.01 with marker D3S1277).

Screening the *hMLH1* gene using SSCP analysis revealed a variation within exon 13. The DNA showing this variation was amplified using the polymerase chain reaction (PCR), and the base sequences of the variant and non-variant fragments of DNA were compared. The transversion of a single base, C to T at nucleotide 1 528 in exon 13, occurred in all family members with colorectal cancer and in a proportion of asymptomatic first-degree relatives of affected individuals, but not in 100 chromosomes from population-matched controls. This alteration is easily assayable using PCR-based restriction enzyme analysis; it destroys the highly specific *Mva*I restriction enzyme recognition site within the mutated allele of exon 13 in mutation carriers (Fig. 2). The C1528T alteration results in a premature stop codon (CAG-TAG) being introduced at codon 509 of the transcribed message. The transcription process is

therefore prematurely truncated at this point, producing a potentially ineffective peptide. Similar premature truncations within the *hMLH1* gene have previously been shown to cause HNPCC (hMLH1 mutation database: http://www.nfdht.nl/database/mlh1.htm).

Recognition of a biologically significant genetic change has meant that it is now possible to test blood samples for this change, in this way separating family members into those at high risk and those at negligible risk for HNPCC. We had to consider carefully how to use this information. It raised well-known but difficult ethical issues about providing the results of genetic tests. In addition, we had to explain the implications of testing to a large group of people in a remote village, and to their widely scattered relatives. An experienced genetics nurse (CH) with a special interest in colorectal cancer was recruited to help design the screening programme. Together with a geneticist, she travelled to village clinics and to individuals' houses to tell family members about the test for the mutation that caused the disease, its significance, and the possible advantages and disadvantages of being provided with the 'test result', whether positive or negative. Pamphlets containing this information were given to each person identified as being at risk. Those who wanted to be tested were asked for fresh samples of blood. These second samples served to confirm the findings from the original samples collected for research earlier in the project.

All individuals who have been told of the test have been eager to learn their results, except for 1 woman aged 23 years who was hesitant. However, when visited again she understood the implication of learning whether she harboured the mutation or not, and wanted to know the outcome. To date test results, together with pre- and post-test counselling, have been provided to 86 individuals, most of whom were previously calculated to be at 25% risk, or higher, of developing HNPCC.

Assay for the mutation on stored DNA from the 141 individuals deemed to be at 12.5% risk or higher, showed that 40 (28%) have the mutation and require colonoscopic follow-up. This reduced the number of subjects who would normally have been offered biennial colonoscopic screening by 72%.

DISCUSSION

This family shows that the diagnosis of HNPCC can be initiated by a general practitioner who takes a careful family history, and that the implications of diagnosing the syndrome are considerable.

This kindred's clinical features are typical of HNPCC. They have large-bowel cancer inherited in a dominant pattern (Fig. 2), often with multiple cancers, usually affecting the proximal colon and presenting at a young age, with very few adenomas. The family has the site-specific form of the disease, which does

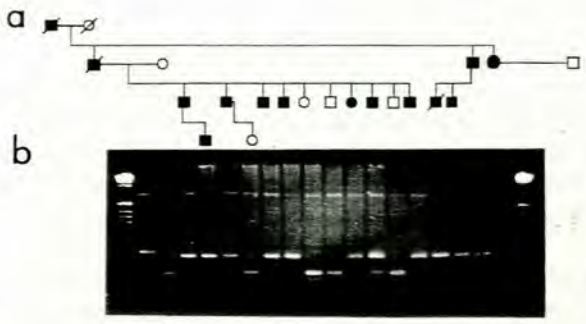

Fig. 2 (a). A section of the NPC1 pedigree showing affected and unaffected individuals. (b) Restriction enzyme digested DNA from the subjects in (a), illustrating the MvaI *restriction enzyme resistant alteration in mutation carriers (symbols in 2a are as for Fig. 1).*

not increase the frequency of other malignancies. Other families with HNPCC have an increased frequency of cancer of the endometrium, stomach, biliary tract, urinary tract and ovary,[12,22] and require more comprehensive organ-based screening.

Diagnosing HNPCC — clinical features

In smaller families HNPCC is difficult to diagnose because a dominant pattern of inheritance may not be obvious. At first sight it seems that two other clinical features should lead the clinician to suspect HNPCC, namely a young patient with colorectal cancer, a right-sided cancer, or both characteristics in one patient. However, objective evidence suggests that neither finding predicts the syndrome. A population-based study[23] of 305 cases of colorectal carcinoma from Northern Ireland showed that young age at diagnosis, cancer in the proximal large bowel, or the combination of both features was not associated with a family history of large-bowel cancer. It appears that age and cancer site are unimportant. Only a family history of bowel cancer will warn the clinician that his patient may not have sporadic bowel cancer.

A positive family history is less likely in small families. A study by Mecklin *et al.*[24] involving 122 patients from 22 affected Finnish kindreds found that at the time of diagnosis only 40% of subjects had at least two first-degree relatives already diagnosed. It is, therefore, not surprising that the ability of a family to meet the rigid 'Amsterdam criteria', formulated to standardise the diagnosis of HNPCC for research purposes, increases by 24% with each additional first-degree relative diagnosed in the family.[25] On the other hand, the Amsterdam criteria, conventionally used to confirm HNPCC in a family, are deliberately rigid in order to minimise the risk of a family entering a research project in error. The criteria are: *(i)* that at

least three relatives have histologically proven colorectal cancer, one being a first-degree relative of the other two; *(ii)* that at least two successive generations are affected; and *(iii)* that one subject should have had colorectal cancer diagnosed under the age of 50 years.[26] The syndrome may exist without these rigid criteria;[27] it should also be considered in families that approach but do not meet these standards.

Laboratory evidence

Histological features usually found in HNPCC are mucinous carcinoma, poor differentiation, an expanding or circumscribed growth pattern and lymphocytes infiltrating the tumour or around it.[28] Another biological peculiarity is the presence of DNA microsatellite instability in more than 40% of loci tested, provided that this is shown in cancers taken from at least two members of the family. To prove the diagnosis absolutely, a mutation in a DNA repair gene must be shown.

The role of molecular genetics

At the time of writing five genes have been found in which a mutation can cause HNPCC. Within a family the mutation will generally be in one gene and will be specific to that family or to families with a common ancestor. The defective gene must be identified to allow a reliable test for the family's specific mutation to be developed. Unless a mutation is identical to previously published disease-causing changes, or obviously introduces a biologically significant change, e.g. a stop codon, as identified in this study, tissue or blood from several affected and unaffected individuals is needed to prove that a mutation is associated with HNPCC and is not an innocent DNA variation. In addition, a few score unaffected individuals with no family history of HNPCC and a similar ethnic background to the affected family are needed to confirm that the mutation found in the affected family is not widespread and unimportant, but definitely causes the disease. Because few families can provide this number of tissue samples and because of the cost involved, in our country testing of blood samples for the mutation will probably only be possible in a few families, and then only by research laboratories, for some time to come. However, when a family's mutation is known this should reduce the number of people needing colonoscopic screening by more than half. In the family reported here it reduced the number by more than 70%. In a large family this may possibly make a search for the mutation cost-effective.

Guidelines for genetic testing and screening

Several ethical and practical implications affect the use of a test for a mutation such as this one.[29,30] The test may do harm as well as good. A positive test may cause anger and resentment. It may also have to be disclosed in applying for a job, medical insurance, life insurance or a housing loan.[31] There is obviously a conflict between the interests of an individual who does not disclose a mutation that causes a disease, and the interests of that individual's insurer, whose other customers and profits may suffer as a result. The obvious benefit for those who test positive is that screening will probably allow cancer to be detected early so that it may be cured by means of appropriate surgery. Moreover, and importantly, subjects with a negative test can be discharged from screening but should know that their risk of sporadic large-bowel cancer is not zero; it is similar to that of the general population.

Although implications of testing for the mutation are overwhelmingly positive, they are complex. Subjects should understand the implications before they accept testing.[32,33] Our current recommendations for the Lynch I HNPCC kindred described here involve offering an initial colonoscopy at 16 years of age, since we have encountered colorectal cancer in a mutation-positive individual who was under 18 years old. When subjects reach 18 a genetic counsellor explains what testing for the mutation implies. Those who do not want mutation analysis are offered surveillance colonoscopy every 2 years until 30 years of age, and every year thereafter. Those with a negative test for the mutation will not undergo colonoscopic surveillance. In future, subjects with a positive test may be offered a choice between the colonoscopic surveillance programme outlined above, or a prophylactic colectomy with ileo-rectal anastomosis. Because of the absence of the Lynch II spectrum of cancers in members of NPC1, we have not recommended additional screening, e.g. a pelvic examination, although mutation-positive women are advised of the symptoms of the Lynch II cancers and the need for timeous clinical consultation.[34]

Treating an individual with HNPCC

Cancer that occurs as part of the HNPCC spectrum involves different operations from those used in management of sporadic large-bowel cancer. Unless systemic metastases are present, patients with colon cancer due to HNPCC should undergo a total colectomy with an ileo-rectal anastomosis.[35] This reduces their risk of a later metachronous carcinoma, and makes it easy to monitor the rectal remnant endoscopically. On the other hand, rectal cancer in a patient with HNPCC may necessitate an ileo-anal pouch procedure to avoid a permanent stoma.

Should asymptomatic carriers of the mutation be offered a colectomy to prevent colorectal cancer? For 50 years this has been performed in FAP patients to reduce the risk of subjects dying from large-bowel cancer. However, it is hard to make the conceptual leap between operating on patients with visible polyposis, and operating on subjects with a mutation shown by a laboratory test, although the aim in both situations is identical. We expect prophylactic surgery to be performed increasingly for HNPCC now that genetic testing is feasible. However, it is important to consider the penetrance of the

mutation analysis to include a consecutive series of 28 FAP families (including the 11 kindreds from the initial study), using the protein truncation test (PTT) and indirect ophthalmoscopy to determine the presence of CHRPE lesions. The 6.5 kb exon 15 of the APC gene was divided into four overlapping segments that were individually amplified by the polymerase chain reaction (PCR) and subjected to the protein truncation test[16,17] using a coupled transcription-translation system.

MATERIAL AND METHODS

Patients

Blood samples were obtained from 28 apparently unrelated FAP families from private clinics and the gastro-intestinal tract clinics at Tygerberg and Groote Schuur hospitals. The families originated from six different South African ethnic groups, viz. black, coloured (mixed ancestry), German immigrant, English, Indian and Afrikaner. The families included in the study were selected on the basis of multiple colorectal polyps detected, supported by histopathological examination of mucosal specimens in affected individuals.[13] Blood was drawn after obtaining informed consent and ethical approval from the appropriate institutions.

Methods

Genomic DNA was extracted from whole blood.[13] It was PCR amplified according to the method of Miyoshi *et al.*[18] and was screened for mutations in exons 1 - 14 (codons 1 - 653) of the APC gene using combined HEX-SSCP analysis.[19] In addition, the PTT was performed in index cases as described by Powell *et al.*[16] Templates for the PTT analysis of exon 15 of the APC gene were generated by PCR on 100 - 200 ng DNA using the T7-modified primers described by Prosser *et al.*[20] These primers consist of a T7 consensus promoter sequence-spacer (Kozak consensus sequence – initiation sequence (ATG)) and unique APC sequence in-frame with the ATG in order to generate overlapping fragments of exon 15 when suitable reverse primers from the published sequences[5] were used. Unpurified PCR products were used in a coupled transcription/translation reaction (Promega UK), incorporating 40 μCi of [^{35}S] methionine according to manufacturer's instructions. The *in vitro* transcription and translation reactions were performed in a 12.5 μl reaction volume. The resultant products were diluted in buffer, heat-denatured and resolved using 10% and 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Gels were washed in fixation solution (25 minutes in 10:20:70 :: acetic acid/methanol/water) and autoradiographed overnight at room temperature. To confirm a variant in an index individual, segregation analysis was performed using DNA from relatives. Direct DNA sequence analysis was performed using fluorescent dideoxynucleotides on an Applied Biosystems (AB373) DNA sequencer and/or a sequenase PCR product kit and Sequenase Version 2.0 DNA Polymerase (Amersham Life Science, United States Biochemicals).

Indirect ophthalmoscopy following pupil dilation was performed in the index cases and in several of their family members. The criteria used to differentiate CHRPE-positive and CHRPE-negative status were as previously described.[10]

RESULTS

Mutations were identified in 20 of the 28 FAP patients screened for mutations in the coding area of the APC gene (Table I). In 8 cases no molecular change was found although three different mutation detection methods were applied. Single mutations were detected in exons 8, 10, 13 and a further six mutations in exon 15. Three mutations constituted deletions (of 1bp[12] and 5 bp), and two mutations were caused by insertions (of 1bp), thereby creating downstream stop codons. The truncated proteins resulting from an *in vitro* transcription/translation reaction of three APC mutations identified are shown in Fig. 1. Both the 1bp insertions were novel mutations, one at codon 1095[15] and the other at codon 1426 (Fig. 1 (Lane 2) and Fig. 2). The mutations in exons 8 and 13 were C-T transitions at CpG sites,[21] in each case changing an arginine codon to a stop codon.

Table I. Mutations identified in the coding region of the APC gene in South African FAP patients

Exon	Codon	Nucleotide change	No. of families	Reference
8	302	C ->T at 906	1	Miyoshi *et al.*[18]
10	441	Del T after 1323	1	Grobbelaar *et al.*[12]
13	564	C->T at 1692	5	Fodde *et al.*[31]
15	862 - 1022	?	1	This study
15	901	C->T at 2071	1	This study
15	1061	Del AGAAA after 3182	2	Miyoshi *et al.*[18]
15	1095	Ins A at 3285	1	Kotze *et al.*[15]
15	1309	Del AGAAA after 3926	7	Miyoshi *et al.*,[18] Grobbelaar *et al.*[14]
15	1426	Ins A at 4276	1	This study

The mutation in exon 15 was a C-T transition changing a glutamine codon to a stop codon. Of the six mutations found in exon 15 (representing two-thirds of all mutations detected), three were in the mutation cluster region (MCR) encompassing codons 1286 - 1513.[7] Segregation analysis was performed in families to confirm the sequence variants detected in the index individuals. In one family the mutation had not been characterised by DNA sequencing, relatives were screened by means of an *in vitro* transcription/translation reaction, and co-segregation of the truncated protein product with the disease phenotype was demonstrated. No double mutations were detected despite analysis of the entire coding region in each patient.

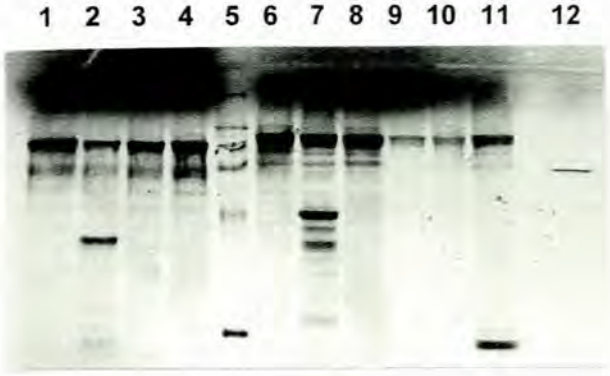

Fig. 1. Autoradiogram demonstrating protein products of in vitro *transcription/translation reaction. The PCR products of segments 2 (codons 686 - 1283) lanes 6 - 11 and 3 (codons 1099 - 1701) lanes 1 - 4 of exon 15 of the APC gene of 10 FAP-affected patients were resolved by electrophoresis in a 10% SDS PAGE gel. Lane 2 illustrates an insertion A at codon 1426 (nucleotide 4276) of exon 15 of the APC gene. Lane 7 represents a five-base pair deletion at codon 1061 of exon 15. Lane 11 shows a protein truncation with a mutation at codon 901 of exon 15. Size marker $^{14}/_{C}$ is shown in lane 5. Lanes 1, 3, 4, 6, 8, 9 and 10 represent full-length APC protein. A luciferase control (61 kDalton) is shown in lane 12.*

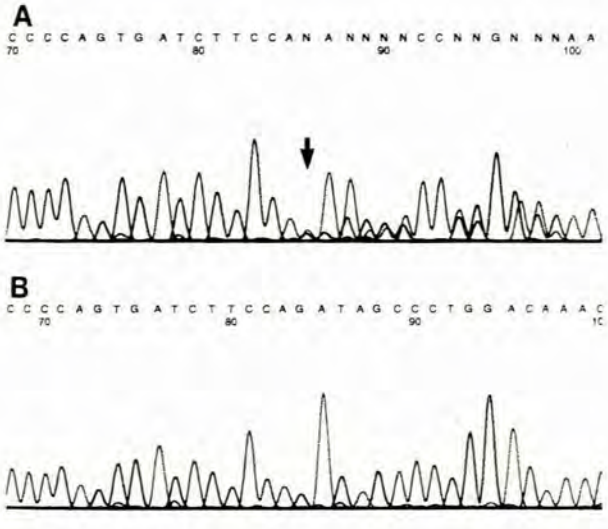

Fig. 2. Direct DNA sequencing of exon 15 of the APC gene. The PCR amplification was performed using nested primers derived from segment 3 (codons 1099 - 1701). The sequencing of the double-strand DNA template was performed on an Applied Biosystems (AB373) DNA sequencer using fluorescent dideoxynucleotides. A: Identification of an insertion A at codon 1426 (nucleotide 4276) in PCR-amplified DNA of an FAP-affected individual. This insertion results in a frameshift of the reading frame and creates a stop codon at residue 4280. B: Partial DNA sequence of exon 15 in a normal control individual.

Genotype and haplotype studies performed previously in five families[13,22] carrying the exon 13 mutation demonstrated that all affected subjects shared the same disease-related haplotype. Following indirect ophthalmoscopy, blood sampling and counselling, it became apparent that these five families belonged to a common genealogical tree. Indirect ophthalmoscopy was performed on the index cases and some of their family members (Table II). The presence or absence of CHRPE lesions correlated fully with the presence or absence of a mutation located in the region between codons 312 - 438 and 441 - 1444, respectively (Table II).

DISCUSSION

In this study 15 mutations were detected in the coding region of the APC gene in 28 apparently unrelated FAP patients. Pedigree analysis, HEX-SCCP analysis in polyacrylamide gels and direct sequencing facilitated the detection of 15 mutations. Subsequently 5 additional mutations were identified using the PTT. The relatively common mutation at codon 1309 of the APC gene was found in 7 unrelated families (25%) representing six different South African ethnic groups.[14] This mutation has been reported previously in the American, English, American black, Japanese,[18,23] Indian[24] and Italian[25] populations at similar frequencies. We[13,22] and others[26] have detected the deletion mutation on different haplotype backgrounds for closely linked markers associated with the disease locus. This phenomenon largely excludes the possibility that the common 5bp deletion represents a founder-type mutation. Evidence that the 1309 mutation leads to early development of colonic polyps in 4- and 5-year-old children[27,28] emphasises the importance of pre-symptomatic molecular diagnosis. In a study by the Genetic Interest Group in the UK,[29] the following principle regarding predictive testing in children has been accepted: 'After suitable counselling parents have the right to make an informed choice about whether or not to have their children tested for carrier status. Ideally, children should only be tested when of age to be involved in the decision.' We and Goldberg *et al.* (this issue)[30] support this statement by the Genetic Interest Group, but propose a more liberal view, especially with regard to this particular subset of FAP individuals, since the risk of progression to malignancy before adolescence is currently unknown.

The second most common 5bp deletion at codon 1061 was

Table II. Dilated fundal examination using indirect ophthalmoscopy. (The presence or absence of mutations and CHRPE are designated by '+' and '–' respectively. Large lesions were bigger than half optic disc diameter (DD), small lesions were smaller than one half DD.)

Patient	APC codon	Mutation	CHRPE	CHRPE size
65	302	+	–	
485,* 491	441	+	+	Small
499,* 497, 487, 496	441	–	–	
498,* 388, 421	441	+	+	Large
314, 210, 213	564	+	+	Large
212, 222	564	–	–	
285	901	+	+	Large
286, 287	901	–	–	
513, 521	862 - 1022	+	+	Large
522, 523	862 - 1022	–	–	
460, 457, 458	1061	–	–	
416, 515	1061	–	–	
459, 455, 514	1061	+	+	Large
493	1095	+	+	Large
425	1309	+	+	Large
520	1426	+	+	Small

* Grobbelaar *et al.*[12]

found in two of our unrelated families (7%), one of English origin and the other of mixed ancestry.

Five families displaying the same mutation in exon 13, and presumed to be unrelated, were subsequently traced to the same genealogical tree after further family history was disclosed. In one family an 83-year-old individual carried a predisposing germline mutation with non-penetrance of FAP (J J Grobbelaar *et al.*, in preparation). The genotype of all the members of this family, with the exception of the 83-year-old individual, correlated with the number of colonic polyps. This family illustrates the difficulty that can be encountered in genetic counselling when dealing with an hereditary predisposition that presents with such variable clinical manifestation.

The novel 1bp deletion identified in exon 10 (codon 441) has contributed to a better definition of FAP-related mutations.[12] In cases examined ophthalmoscopically, the screening of the entire coding region of the APC gene could be narrowed down to specific gene regions (codons 312 - 438 and 441 - 1444).[12]

Despite the application of two different mutation detection methods in the study population, eight FAP families could not be characterised at the molecular level. Failure to detect mutations in these families could be ascribed to several factors. The location of several oligonucleotides used in this study[5,18] was such that the detection of variation at splice junctions was limited. In addition, mutations located within promoter or enhancer regions of the APC gene may also have accounted for the failure to identify mutations in all the study participants. At present there is no single completely reliable mutation detection technique, and therefore haplotype association in family studies remains useful. Future research will include the analysis of the APC promoter in mutation-negative patients once this region of the gene has been fully characterised. DNA-based molecular examination of at-risk family members relieves the anxiety that they might develop the disease as well as the necessity of regular expensive and cumbersome medical examinations. Therefore, tracing a disease-causing mutation within a family provides an accurate test, which reduces cost for the family and health authorities.

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