Rapid detection of rare variants and common polymorphisms in the APC gene by PCR-SSCP for presymptomatic diagnosis and showing allele loss

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Abstract

During the course of screening the 5' half of exon 15 of the APC gene for germline and somatic mutations in two groups of patients, those with the inherited cancer prone syndrome adenomatous polyposis coli (APC) or with sporadic colorectal cancer, we have identified a number of intragenic changes that are not associated with the disease phenotype. Four of these changes are rare variants, each confined to one or two families and not detected in 50 additional unrelated people. Two common polymorphisms, at codon 1493 (exon 15I) and codon 1678 (exon 15J), were extensively investigated and found to be in almost complete linkage disequilibrium not only with each other but with a previously described polymorphism at codon 1960 (exon 15N). The rapid and sensitive single strand conformation assay used provides an efficient method for pre-symptomatic diagnosis using intragenic variants and was additionally used to show allele loss at the APC locus in sporadic colorectal carcinomas.

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The inherited premalignant condition adenomatous polyposis coli (APC) is characterised by the presence of multiple benign adenomas, usually more than 100, which develop throughout the colon and rectum of affected subjects during adolescence.¹ These precancerous lesions will progress to carcinoma unless surgically removed by prophylactic colectomy. APC, a dominantly inherited syndrome, results from mutation in the recently isolated gene (APC) localised to chromosome 5q21-22.²⁻⁵ APC is large, consisting of approximately 8500 bp of coding sequence and as a consequence screening of the entire gene for germline and somatic mutations has been performed by a small number of groups only. Such investigations have used a variety of mutation detection techniques which have, in general, shown deleterious germline or somatic mutations in about two thirds of affected patients and tumour material analysed.⁶ Thus, at present, predictive testing requires linked or intragenic polymorphisms in a large proportion of families. We have screened the 5' half of exon 15 of the APC gene for mutations in a

series of families segregating for the disease and, in addition to several germline mutations that have been reported,⁷ a number of non-disease variants were also discovered. Here we report the characterisation and frequency analysis of four rare variants and two common polymorphisms in APC detected by means of a rapid, non-radioactive single strand conformational polymorphism (SSCP) assay.

Materials and methods

The method used for analysis in this investigation was a non-radioactive SSCP assay using the Pharmacia PhastSystemTM that involves the electrophoresis of polymerase chain reaction (PCR) generated fragments in pre-prepared non-denaturing polyacrylamide gels followed by automated silver staining.

SAMPLES

Mutation screening was carried out in a total of 45 families with histologically confirmed APC from St Mark's and the Northern Region Polyposis Registries and the Wessex Regional Genetic Services Unit as previously described.⁷ Additionally paired normal and tumour tissue DNA samples from 46 sporadic colorectal cancer patients from St Mark's Hospital, London, were similarly screened for evidence of germline and somatic mutation in APC. To determine the frequency of variant alleles, lymphocyte DNA samples from random normal people and spouses from polyposis families were also tested.

POLYMERASE CHAIN REACTION AMPLIFICATION
PCR amplification was performed using a Hybaid DNA thermal cycler with oligonucleotide sequences specific for exon 15, amplicons A to J, of the APC gene as described by Groden *et al.*⁵ Each reaction contained 0.2 mmol/l dNTPs, one unit *Taq* polymerase (HT Biotechnology), 50 pmol of each primer, 50 mmol/l Tris-HCl, pH 9.0, 50 mmol/l KCl, 7 mmol/l MgCl₂, 16 mmol/l (NH₄)₂SO₄ in a 100 µl volume. Following initial denaturation at 94°C for four minutes, amplification involved 35 cycles of 94°C for 30 seconds, 48-62°C for 45 seconds, and 72°C for 45 seconds followed by a final elongation step at 72°C for 10 minutes. A total of 10 µl of

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Table 1 Rare variants detected in the 5' half of APC exon 15

APC family	Amplicon	Codon/nucleotide	Sequence change	Amino acid change	Allele frequency*
391	C	870 2610	CCA to TCA	Proline to Serine	<0.01
552	E	1083/ 3249	GAT to GAG	Aspartic acid to Glutamine	<0.01
564 II	E	1129/ 3386	TTG to TCG	Leucine to Serine	<0.01
564 I, III, and IV 5998	G	1317/ 3949	GAA to CAA	Glutamine to Glycine	0.0125

* A minimum of 100 chromosomes tested.

amplification product was analysed on 2% agarose (Sigma) gels before SSCP analysis to estimate DNA concentration.

SSCP ANALYSIS

Aliquots (1 µl) of the PCR generated DNA product were mixed with an equal volume of 95% formamide and reduced to a single strand state by heating at 99°C for 10 minutes followed by snap cooling on ice. SSCP analysis was an automated procedure performed on the PhastSystem™ (Pharmacia, LKB) using appropriate media supplied by the manufacturers. Single stranded DNA fragments were then separated on Homogenous 20 PhastGels with PhastGel native buffer strips. PhastGels were pre-run at 400 V, 20 mA, 2 W, and at 4–15°C for 10 volt hours (Vh) and electrophoresis performed at 400 V, 20 mA, 2 W, and 4–15°C for 300 Vh. Gels were silver stained, again an automated procedure using the PhastSystem™ according to the manufacturer's instructions.

DNA SEQUENCING

DNA templates for sequencing were prepared by excising the PCR product previously electrophoresed through 2% Nusieve agarose gels and followed by purification with Wizard™

PCR purification preps (Promega) according to the manufacturer's instructions. Direct sequencing was performed by the chain termination method⁸ modified with the use of thermostable DNA polymerase for cycle sequencing using the Circum Vent™ cycle sequencing kit (NEB).

Results

RARE VARIANTS SEGREGATING IN APC FAMILIES

As table 1 illustrates, four rare single base pair substitutions, all resulting in a change of the predicted amino acid, were characterised from the series of APC families. None of these alterations was associated with the disease phenotype.

FAMILY 564

Analysis of four members of family 564 which included one affected subject (III) resulting from fresh mutation, showed two non-disease variants by SSCP analysis (fig 1). The first was detected in amplicon 15G of APC in the affected subject and her sister (IV) and had been inherited from their father (I). Sequence analysis of DNA from all three subjects with the variant SSCP pattern showed the alteration to be the result of a previously reported G to C substitution at codon 1317 causing an amino acid change from glutamine to glycine (table 1).⁹ An identical alteration was also observed in two unaffected people from another APC family (5998) in which the germline mutation has been characterised as a 5 bp deletion occurring at codon 1309 of the APC gene.⁷

The second variant was detected in amplicon E of exon 15 and was observed in the affected subject's mother (II) only. Sequence analysis characterised this alteration as a T to C substitution at codon 1129 resulting in a predicted amino acid change from leucine to serine. This rare non-disease variant has also been detected in an APC kindred by Allan *et al.*⁹

The affected member of family 564 exhibits an additional SSCP variant in amplicon 15G of the gene which is the result of a single base pair insertion at codon 1323; this is the disease causing mutation.⁷

FAMILY 552

An SSCP variant was also observed in amplicon 15E of the APC gene in both affected and unaffected members of family 552. Subsequent

Figure 1 SSCP analysis of amplicons E and G of exon 15 of the APC gene in four members of family 564. Three subjects (I, III, and IV) show electrophoretic variants in the single strand DNA pattern on analysis of amplicon G, characterised as a single base pair substitution at codon 1317 of APC. Subject III shows additional SSCP variation which results from a new mutation (a 1 bp insertion at codon 1323) and which is predicted to truncate APC. Analysis of amplicon E of exon 15 in the same four subjects shows that one (II) exhibits a SSCP variant characterised as a base pair substitution at codon 1129.

Figure 2 Characterisation of nine subjects in three generations from a large APC pedigree for the frequent polymorphism at 1493 of APC detected in amplicon I of exon 15. Previously derived haplotypes segregating within the family, including the disease associated haplotype, are also illustrated.

sequence analysis showed this SSCP variant to be the result of a T to G substitution at codon 1083 creating a glutamine recognition codon from aspartic acid. That this change was observed in unaffected people indicated that it was likely not to be the disease causing mutation in this family and it was subsequently shown to segregate with the unaffected haplotype. However, as the disease causing mutation in this family has not been found, the variant described here allowed the gene status of a person at 50% risk of inheriting the disease to be determined.

FAMILY 391

In family 391, a rare variant SSCP pattern was observed to segregate with the disease. It was subsequently shown to result from a single base pair substitution of a T for a C at codon 870 (exon 15 C) causing an amino acid change from proline to serine. This variant has been reported by Powell *et al.*¹¹ The disease associated mutation in this family was shown to be the common 5 bp deletion at codon 1061 (amplicon 15E).⁷

Amplicons C, E, and G of exon 15 were analysed by SSCP in at least 50 unrelated

people from a variety of geographical and ethnic origins. None of the rare variants described was detected in these samples.

COMMON POLYMORPHISMS

The most frequent alteration initially observed through analysis of exon 15 of APC was detected in amplicon I, the result of a single base pair substitution of G for A at codon 1493. This is a silent alteration resulting in no amino acid change. Initially SSCP analysis to detect this change was performed at 15°C at which temperature the variant single strand pattern differed between unrelated subjects appearing as either two poorly separated bands or one of two single bands with slightly different mobilities. Electrophoresis performed at 10°C, however, resulted in clear separation of all single strand bands, unrelated subjects being classified as either slow migrating homozygotes, fast migrating homozygotes, or slow/fast migrating heterozygotes (fig 2).

The nature of this polymorphism was characterised in families segregating for APC, a panel of normal subjects, and a series of sporadic colorectal cancer (CR) patients with corresponding carcinoma tissue. In total, 155 unrelated people were tested (38 patients with APC, 46 sporadic CR patients, and 71 people unaffected with either disorder).

As table 2 illustrates, a little over half of all unrelated subjects (51%) were heterozygous for the common polymorphism at codon 1493 (exon 15 I), with approximately one third (32.9%) slow migrating homozygotes and the remainder (16.1%) fast migrating homozygotes. The resultant allele frequencies were 0.58 for the slow migrating allele and 0.42 for the fast migrating allele. There appears to be little variation between the allele frequencies of the three different subsets although among the group of APC patients there was an increased homozygote frequency (table 2) (for separate analysis, this subset was increased by the inclusion of seven fresh mutation cases).

Extending the region of the APC gene analysed in sporadic colorectal cancer patients showed a second frequent alteration detected as an SSCP variant and characterised as a single base pair substitution of an A with a G at codon 1678 (exon 15, region J) also without a resultant amino acid change. In total, DNA from 87 unrelated people was analysed for both of these polymorphisms and none was discordant, indicating complete linkage disequilibrium between them. For both polymorphisms the two alleles of heterozygotes representative of both polymorphisms were clearly distinguishable as variant conformers in the single strand DNA pattern and as such both are potentially valuable for analysis of allelic loss in colorectal tumours. Subsequently analysis of tumour and normal tissue for each of the 46 sporadic colorectal cancer patients showed loss of heterozygosity in eight tumours from the 25 patients (32%) who were heterozygous for the common polymorphisms and therefore informative (fig 3). Additionally, DNA from 50 unrelated people that had been analysed for

Table 2 Analysis of allele frequency for the common polymorphism at codon 1493

Individual subset (total number)	Slow allele homozygous		Slow/fast allele heterozygous		Fast allele homozygous	
	No	%	No	%	No	%
Unrelated subjects (155)	51	32.9	79	51.0	25	16.1
Normal subjects (73)	23	31.5	39	53.4	11	15.1
APC patients (45)	16	35.6	19	42.2	10	22.2
Sporadic colorectal cancer cases (46)	16	34.8	25	54.3	5	10.9

Figure 3 Allele loss detected in colorectal carcinomas with matching constitutional DNA analysis from four sporadic colorectal cancer patients. Detection of allele loss as shown (arrows) involves the analysis of two frequent polymorphisms at codons 1493 and 1678 in amplicons 151 and 15J of the APC gene respectively.

the exon 15 I and J variants were also typed by PCR for the previously described *MspI* polymorphism, FB54D,¹² that occurs in the APC exon 15 region N (codon 1960). With the exception of two CR cancer patients linkage disequilibrium was complete in all samples with the slower migrating alleles corresponding.

Discussion

Despite determination of the causative mutation in a high proportion of families there remains a need to determine the gene status of subjects within APC kindreds by linkage analysis with polymorphic markers around the APC locus. Recently a highly polymorphic (CA) repeat locus adjacent to the APC gene has been reported and its potential use in pre-symptomatic diagnosis highlighted.¹³ Although potentially more informative than the intragenic polymorphisms described in the current study, analysis using such markers is difficult and time consuming and frequently involves the use of radioisotopes. As an initial determinant of gene status in at risk subjects, therefore, the non-radioactive SSCP analysis described here is ideal.

We have also shown the use of such a system for rapid and efficient characterisation of allele loss in tumours. The clarity of the results that we have obtained compares favourably with those derived from the use of dinucleotide repeat markers.

The frequent polymorphism at codon 1493 (exon 15 I) characterised in detail here has previously been reported by others.^{11,14} The single base pair substitution itself creates a *BsaI* restriction enzyme site within the APC gene and this has recently been used to determine the gene status of 20 subjects based on digestion of PCR generated fragments en-

compassing the polymorphic site before agarose gel electrophoresis.¹⁵ Similarly, the codon 1678 (exon 15 J) polymorphism was described earlier¹⁴ and analysis by *HgiAI* digestion of PCR products has been reported.¹⁶ The SSCP analysis performed here to characterise these polymorphisms is preferable to the digestion assays in that it eliminates the intermediary digestion step which in itself presents a possible source of error (that is, incomplete or failed digestion), and is also a more rapid procedure taking approximately two and a half hours to perform. Furthermore, the SSCP analysis described does not appear to suffer from the imprecision produced by similar radioisotopic assays. From our data linkage disequilibrium between these two polymorphisms and the one at codon 1960¹² appears almost complete; for routine diagnostic use it is therefore only necessary to type one of the three. The implication of this finding is that intragenic recombination within exon 15 of APC is rare.

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