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CpG island clones from a deletion encompassing the gene for adenomatous polyposis coli

(hereditary disease/somatic cell genetics/fragment end cloning)

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ABSTRACT **Adenomatous polyposis coli (APC), a dominantly inherited disorder, has been mapped to chromosome 5q15-q21 by family linkage studies. Cells from patients with deletions in this region, in one case associated with polyposis in a family, have been used to construct human hamster hybrid cell lines that retain either the normal or deleted chromosome 5. These lines have been used to identify markers from the region of the polyposis gene obtained by cloning the ends of 0.5- to 2-megabase *Bss*HII fragments purified by pulsed-field gel electrophoresis. Three markers are described that map within the deletions and must therefore be close to the *APC* gene.**

Adenomatous polyposis coli (APC) is a dominantly inherited disorder in humans in which, by the second or third decade of life, the colon becomes coated with a large number of benign adenomas. There is an extremely high probability that some of these will progress to adenocarcinomas. Understanding the mechanism underlying the formation of the adenomas and the steps in the progression to carcinomas will depend on isolating the *APC* gene and identifying its function. The gene responsible for APC has been localized to chromosome 5q15-q21 by family linkage studies (1, 2). A more extensive genetic map of the region has subsequently been constructed by using additional polymorphic markers in APC and reference families (3).

The same region of chromosome 5 is also implicated in sporadic colon cancer. This has been shown by allele loss of chromosome 5 markers in tumor as compared to normal tissue (4–10). Although the *APC* gene is not the only somatic mutation involved in the development of colon carcinoma as shown by the involvement of p53 on chromosome 17 (ref. 11 and unpublished data) and allele loss on 18q (7, 12–14), it clearly plays an important role in many colorectal carcinomas, possibly at an early stage of carcinogenesis.

The next step toward the functional analysis of the defect in APC, through the molecular identification of the gene, is to superimpose the genetic map surrounding the gene onto a DNA restriction map. The relevant DNA can then be cloned and analyzed further for the presence of the gene. From the experimental point of view this requires a large number of DNA markers within a small region of a chromosome. A fast method for mapping probes to a particular region and a strategy for isolating only the most informative markers are essential for such a project. We have solved the mapping problem by constructing hybrid cell lines containing either only human chromosome 5 or a chromosome 5 deleted for the *APC* region on a hamster background. This allows localization of a probe to the deleted region by a single Southern blot. The deletion chromosomes we have used are similar to the one described by Herrera *et al.* (15) that provided the original clue to the location of the *APC* gene. One of the patients with such a 5q deletion comes from a family with polyposis in which two affected sibs, with an affected mother, carry the same deletion (16). This confirms the dominant inheritance of susceptibility by deletion of gene function and assures that the *APC* gene is within the deleted region, so that markers localized there must be close to the *APC* gene.

To minimize the number of clones needed to construct a long-range restriction map, we chose to isolate clones from CpG-rich, undermethylated islands (17) containing recognition sites for enzymes cutting rarely in mammalian DNA. Such clones can be isolated as linking clones (18), flanking the restriction sites on both sides or as fragment end clones containing the sequence from one side (19). Clones crossing or ending in rare-cutter restriction sites provide a set of well-spaced markers and often identify conserved or transcribed sequences, thus offering immediate access to some of the genes in a region. We report here the isolation of three markers for the region of the polyposis gene using this combination of mapping and cloning strategies.

METHODS

Cell Culture. Lymphoid lines were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. TsH1, a temperature-sensitive Chinese hamster ovary cell line (20), was grown at 33°C in E4 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. After fusion the temperature was raised to 39°C to select for hybrid cells and 10 μm ouabain was added to select against human cells.

Fusion. Epstein–Barr virus-transformed lymphoid lines were made (21) from two patients with constitutional deletions in the region of the *APC* gene (see *Results*). For each fusion, 5×10^6 tsH1 cells were harvested by trypsinization and resuspended with 5×10^6 lymphoid cells in 20 ml of serum-free medium. The cells were fused using polyethylene glycol (22). After fusion the cells were plated in ten 90-mm dishes and grown at 39°C with ouabain. Colonies were picked at 3–4 weeks and expanded for analysis.

Polymerase Chain Reaction Analysis of Hybrids. Approximately 1000 cells from each hybrid clone were harvested and lysed (24), and a polymerase chain reaction (23) was performed using oligonucleotides from human complement factor 9 (25).

Southern Blot Analysis. DNA from the hybrids and the parental lines was digested with the appropriate restriction enzyme, size separated by electrophoresis through a 0.7%

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Abbreviation: APC, adenomatous polyposis coli.

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agarose gel, and transferred to nylon membranes (Hybond-N; Amersham). Filters were hybridized with random-primed ^{32}P-labeled probes (26). C11P11 (D5S71) was hybridized to *Taq* I-cut DNA, and λMS8 (D5S43) (27) was hybridized to *Hin*fI-cut DNA. Filters were exposed to XAR-5 film (Kodak).

Chromosomal Analysis. Chromosome preparations were made from the hybrids by standard techniques. Slides were prebanded by incubation in 0.3 M NaCl/30 mM sodium citrate at 60°C followed by staining with Wright's stain. After photographs had been taken, the slides were destained and hybridized *in situ* with biotinylated total human DNA (28). Extended banding following BrdUrd incorporation was used to confirm which chromosome 5 was present in each hybrid.

Construction of a *Bss*HII End Clone Library. Agarose blocks containing high molecular weight DNA from the hybrid cell line H64 (29) were digested with *Bss*HII. The digested DNA was then run on an inverted field gel at 1.5 V/cm, 1:3 pulses from 20 sec to 999 sec for 86 hr and then with 999 sec unramped for 48 hr. One lane of the gel containing this digest and one lane containing yeast size markers were cut off, stained with ethidium bromide, photographed, and blotted according to standard procedures. The remainder of the gel was cut horizontally into 3-mm slices. One lane equivalent of each slice was melted at 68°C in 1.1 ml of 10 mM Tris/50 mM EDTA/0.5 M NaOH and applied to a dot blot. The rest of the slice was stored in 10 mM Tris, pH 7.6/50 mM EDTA. The dot blot and the strip from the side of the gel were then hybridized to C11P11, and the correct matching slice was identified. DNA was electroeluted from a slice hybridizing to the C11P11 probe, treated with agarase, ethanol precipitated, taken up in 10 mM Tris, pH 7.6/1 mM EDTA, and partially digested with *Mbo* I as for genomic library construction (e.g., ref. 30). Approximately 30 ng of *Mbo* I-digested DNA was ligated with gel-purified vector arms, the left arm of EMBL3 was digested with *Bam*HI and *Hin*dIII and the right arm of EMBL7 (31) was digested with *Mlu* I and *Pvu* II. The ligation mixture was packaged (Gigapack Gold) and plated onto bacterial strain NM539 (31). Human clones were identified by hybridization to labeled total human DNA in the presence of excess unlabeled hamster DNA.

Library Characterization. Plaques hybridizing to human DNA were picked into microtiter wells. Replicas were made and hybridized to human DNA, hamster DNA, and an oligonucleotide (5′-GCTAGAGTCGACGCGC-3′) complementary to the polylinker of the insertion site of the right vector arm (EMBL7). This allowed us to distinguish true *Bss*HII ends from *Mbo* I–*Mbo* I insertions ligated onto small amounts of contaminating *Bam*HI-cut EMBL3 right arm. After an initial sample of 60 random clones, only unambiguous end clones were mapped. In addition, unique fragments from 13 random end clones were hybridized back to the microtiter plate replicas to determine the complexity of the library.

Mapping of Phage Clones. DNA was prepared from individual 2-ml phage cultures (32), labeled by the random priming method (26), and hybridized to mapping panel filters using an excess of unlabeled human DNA (33).

Pulsed-Field Gel Analysis. Blocks were prepared as described by Herrmann *et al.* (34). Analytical pulsed field gels were run in a Bio-Rad CHEF-DRII system at 1.6 V/cm (55 V) for 130 hr with a 3- to 60-min pulse ramp, blotted, and hybridized as described (34).

RESULTS

Derivation of Hybrid Cell Lines. In 1986 Herrera *et al.* (15) described a man with a constitutional interstitial deletion in chromosome 5 [del(5)(q13-q15) or del(5)(q15-q21)] who had polyposis coli along with mental retardation and developmental abnormalities. Following linkage studies and *in situ* hybridization with C11P11 that localized the *APC* gene to the 5q21 region (1, 2) we obtained material from individuals with similar constitutional interstitial deletions. Patient M is a 12-year-old girl who was investigated for developmental delay and was found to have a 5q15-q21 deletion. Her chromosomes 5 are shown in Fig. 1. She has mild dysmorphic features and is, as yet, too young to screen for APC. Patient P is one of two brothers who are intellectually handicapped and have APC in addition to dysmorphic features. Their mother was also mentally retarded and died in her forties of an inoperable carcinoma of the colon with APC. Both brothers have a constitutional interstitial deletion of chromosome 5q13-q15 or 5q15-q21 (16).

Epstein–Barr virus-transformed lymphoid lines from these patients were fused with a temperature-sensitive hamster cell line, tsH1 (20). This cell line has a temperature-sensitive leucyl tRNA synthetase and will grow at 33°C but dies at 39°C. The human leucyl tRNA synthetase gene is located on chromosome 5 cent-q11 (35), and so hybrids containing human chromosome 5 will grow at 39°C.

To identify colonies containing human chromosome 5, and to exclude hamster revertants, colonies were initially characterized by a polymerase chain reaction using oligonucleotides from human complement factor 9 that has been mapped to chromosome 5 (25). DNA from positive hybrids was analyzed on Southern blots for several polymorphic markers. In this way haplotypes for each chromosome were established.

According to this protocol the following hybrid cell lines were isolated: MD/TS-1, which contains the deleted chromosome 5 from patient M and no other detectable human material; PD/TS-1, which contains the deleted chromosome 5 from patient P and some further human chromosomes but not the normal chromosome 5; and PN/TS-1, which contains the normal chromosome 5 of patient P and no other human material. These three cell lines were used as the basic mapping panel to assign markers to the neighborhood of the polyposis gene by Southern blot hybridization. The line containing only the normal chromosome 5 is being used for the isolation of the marker clones.

The *APC*-linked marker D5S71 (C11P11) was hybridized to the mapping panel and shown not to be within either deletion (Fig. 2*A*). A further linked marker on the proximal side, D5S37 (π227) (37), is also present in both deleted chromosomes and so not within the deleted region, which is consistent with its *in situ* localization in band 5q14 (data not shown).

Strategy for the Isolation of Markers. Many rare-cutting restriction enzymes typically cut within undermethylated CpG-rich islands, and analysis of mammalian DNA with these enzymes indicates that large regions with few undermethylated CpG islands alternate with long stretches of DNA in which these islands occur quite frequently (38, 39). Analysis of the region of chromosome 5 containing D5S71 (C11P11) with rare-cutting enzymes showed that most restriction fragments generated by these enzymes and recognized by D5S71 were very large (>1 megabase). This indi-

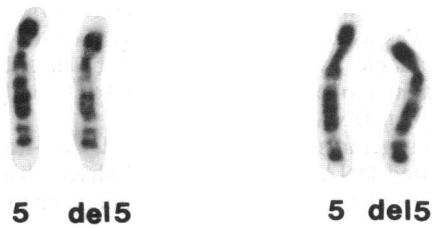

FIG. 1. Partial karyotype of patient M showing chromosomes 5. The deleted chromosome is on the right.

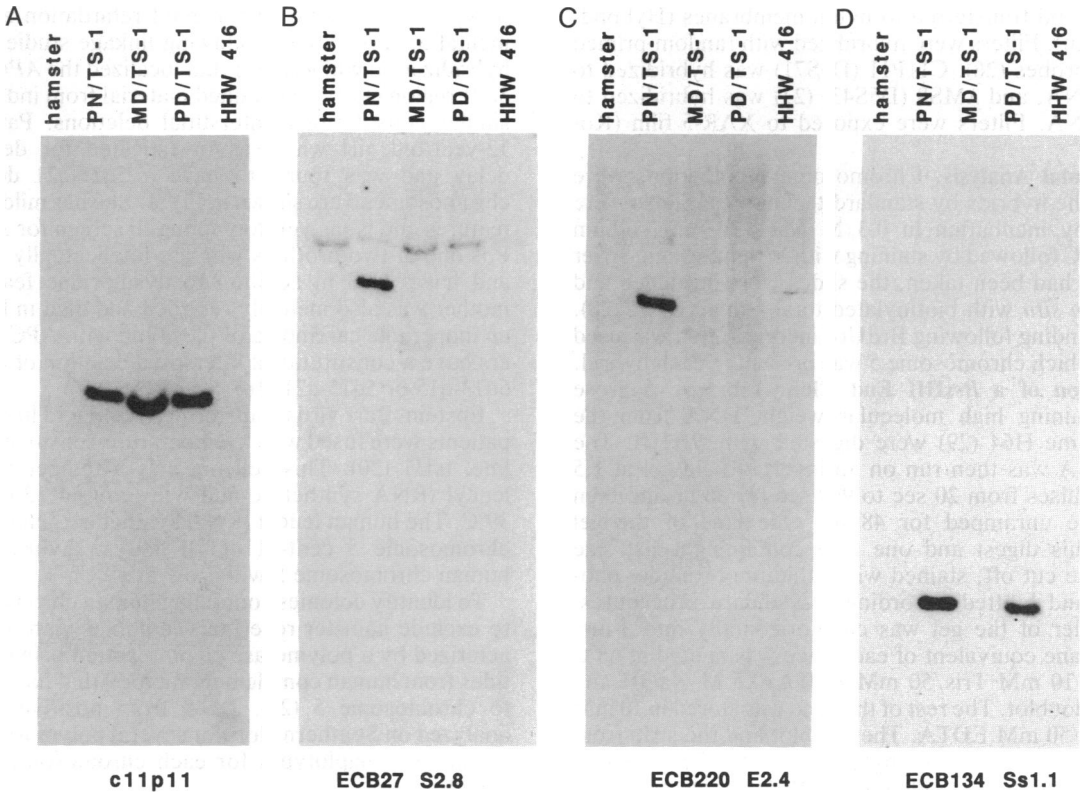

FIG. 2. Mapping of end clones by hybridization to a hybrid cell panel. Genomic DNA of hamster, PN/TS-1 (normal chromosome 5), MD/TS-1 [del(5)(q15-q21), status unknown], PD/TS-1 [del(5)(q15-q21), polyposis], and HHW 416 (chromosome 4 only) (36) was digested with *Eco*RI. Single-copy fragments of C11P11 (D5S71) present in both deleted chromosomes (*A*) as well as ECB27 (D5S98) (*B*), ECB220 (*C*), and ECB134 (D5S97) (*D*) were used as probes in these hybridizations.

cates that in this region undermethylated CpG-rich islands are rare, so that the *APC* gene might well lie within or close to the highly methylated region that contains C11P11. To obtain probes from this region we isolated sequences specifically from the small number of CpG-rich islands, by cloning the ends of the long *Bss*HII restriction fragments (19). These clones should provide markers at distances of a few megabases—that is, the distance between islands. Thus, a small number of markers should uniformly cover a large region, a very advantageous situation for genetic and physical mapping.

Isolation of *Bss*HII End Clones. C11P11 hybridizes to a 1.5-megabase *Bss*HII fragment in DNA from several different sources. We therefore cloned the ends of *Bss*HII fragments of approximately that size. To enrich for sequences on chromosome 5 we isolated these fragments from the DNA of the interspecies hybrid H64 (29) that contains human chromosomes 4 and 5 on a hamster background. (The hybrid PN/TS-1 was not available at the time.)

H64 DNA was digested with *Bss*HII and separated on inverted field gels; the desired size class was eluted from the gel slices, partially digested with *Mbo* I to an average size of 10–20 kilobases, and ligated into an EMBL vector with *Bam*HI ends on the left and *Mlu* I ends on the right arm. The *Mlu* I site was used because there is no λ vector for *Bss*HII cloning available and *Bss*HII ends can be ligated to *Mlu* I ends. Human clones were identified by hybridization to labeled human DNA.

Thirty nanograms of *Bss*HII-digested, size-fractionated, and gel-eluted DNA yielded 2.5×10^4 phage plaques. Nine percent of the clones hybridized with human repetitive DNA. Of these, 367 clones were picked into microtiter wells. About 20% of these clones were not true *Bss*HII end clones but had resulted from the ligation of internal *Mbo* I fragments to small amounts of contaminating *Bam*HI-cut right arm of EMBL3. This was determined by hybridization with an oligonucleotide complementary to the polylinker next to the *Bss*HII insertion site (see *Methods*). In addition, there is usually a number of clones that arise from blunt end ligation of damaged ends. These are difficult to detect since all *Bss*HII sites are destroyed by ligation into the *Mlu* I site of the vector.

To estimate the representation of *Bss*HII sites in the library, unique fragments from characterized clones were hybridized back to filters containing all of the clones: one was represented five times, five twice, and seven once. This indicates that 70–80% of the *Bss*HII fragments in the chosen size class should be present among the 367 picked clones.

The total number of human *Bss*HII fragments in the 0.5- to 2-megabase size class should be quite small. For example, if chromosomes 4 plus 5 constitute about 10% of the human genome and 20% (by weight) of the *Bss*HII fragments are in this size class, this corresponds to 60 1-megabase fragments or 120 different clonable *Bss*HII ends.

Mapping of Clones. One hundred and forty-seven clones were analyzed by labeling total phage DNA and hybridizing in the presence of unlabeled human DNA to a panel of DNAs from H64 (chromosomes 4 plus 5) (29), HHW213 (5pter-q11) (40), HHW416 (4 only) (36), hamster and human, or the panel shown in Fig. 2. Fifty-nine mapped on chromosome 4, and 88 mapped on chromosome 5. Clones assigned to chromosome 5 were further mapped on the following panel: PN/TS-1 (normal chromosome 5), PD/TS-1 [del(5)(q15-q21), polyposis], MD/TS-1 [del(5)(q15-q21), status unknown], HHW416 (chromosome 4 only), and hamster. An example is shown in Fig. 2*A*. The probe C11P11 is present in both deleted chromosomes and serves as a control. Two end clones, ECB27 (D5S98) (Fig. 2*B*) and ECB220 (Fig. 2*C*), were absent from both MD/TS-1 and PD/TS-1. In contrast, ECB134 (D5S97)

(Fig. 2*D*), a fortuitously cloned internal fragment, was present in PD/TS-1 but absent in MD/TS-1. This shows that the two cytologically similar deletions, though they may be largely overlapping, are not identical.

To check whether the end cloning had produced clones hybridizing to the expected size range, a unique subclone of ECB27 was hybridized to the same filter previously hybridized to C11P11 (Fig. 3). The *Bss*HII fragment recognized by this probe is clearly smaller than the corresponding C11P11 band but consistent with the separation on the field-inversion electrophoretic gel. A band in this size class was also observed with ECB220 and ECB134. None of these clones hybridizes to a *Bss*HII fragment of exactly the same mobility as the one recognized by C11P11. The end of that fragment was not found among the sample we analyzed, possibly because it is not within the region deleted in the two chromosomes 5 used for mapping. All probes are derived from a region in which CpG-rich islands occur at large distances, as expected from the size of the *Bss*HII fragments. The *Mlu* I fragments, though larger than the *Bss*HII fragments, do not provide any indication of physical connection of the probes.

The probe ECB27 (D5S98) recognizes a restriction fragment length polymorphism with the enzyme *Bgl* II, giving allele frequencies in our population of about 0.62 and 0.38. It is therefore possible to place it on the genetic map. Table 1 shows the results of linkage analysis of the ECB27 probe in 22 families with familial adenomatous polyposis (186 typed individuals). Forty-three meioses were fully informative, and 15 were partially informative. The peak logarithm of odds score for linkage of D5S98 to *APC* was 6.94 at a recombination fraction (θ) of 0.03. These results indicate that ECB27 should be a useful diagnostic marker.

The probe ECB27 contains sequences that are conserved in different species, pointing to a possible coding sequence. This is not surprising in view of the fact that it has been derived from a CpG-rich island. However, since recombination is found between the disease gene and the probe (see Table 1), it is unlikely that the conserved sequence should correspond to the *APC* gene.

Table 1. Logarithm of odds score from two-point analysis for linkage between D5S98 (ECB27) and *APC*

Recombination fraction										
0	0.001	0.01	0.05	0.10	0.15	0.20	0.30	0.40	$Z(\theta)$	θ
−INF	5.88	6.76	6.84	6.27	5.48	4.59	2.64	0.85	6.94	0.03

CONCLUSION

We have used cells from individuals with constitutional deletions of the q15-q21 region of chromosome 5 as a basis for identifying markers near the *APC* gene. In one case the deletion is associated with APC in a family, and in the other the APC status of the child is unknown. The *APC* gene has an estimated mutation rate between 2.5×10^{-4} and 10^{-5}, about an order of magnitude higher than most dominantly inherited disorders. This estimate is based on a population incidence of 1 in 5000 to 1 in 10,000 and mutation selection balance assuming 30–40% of cases are new mutations (41). With a mutation rate as high as this it might be expected that a broad range of mutational types would exist for APC, including submicroscopic deletions and rearrangements, as well as the cytologically visible deletions described by us and others. Just as mutational variation has greatly aided the isolation and identification of genes involved in other genetic disorders, most notably retinoblastoma (42) and Duchenne muscular dystrophy (43), the same may apply to APC. If the genetic situation for APC is analogous to these other diseases, it would be expected that a sufficient number of probes physically close to the gene, isolated by pursuing approaches similar to those we have used so far, would soon identify submicroscopic changes and so further restrict the APC gene's localization. These probes should readily point to one gene, or a small number of candidate genes, that can be assessed by functional studies and by consistent presence of mutations in different APC individuals.

Since the initial description of the linkage of the *APC* gene to 5q21 by the relatively uninformative D5S71 (C11P11) marker, we and others (3) have now produced probes that, collectively, should make most APC families informative. This immediately poses the question of the appropriate use of the genetic information for counseling APC families and for planning optimal prophylactic treatment. The eventual identification of the functional *APC* gene will enhance enormously the power of the genetic analysis for counseling families and for the analysis of sporadic tumors, with associated prospects of using knowledge of the gene's function to

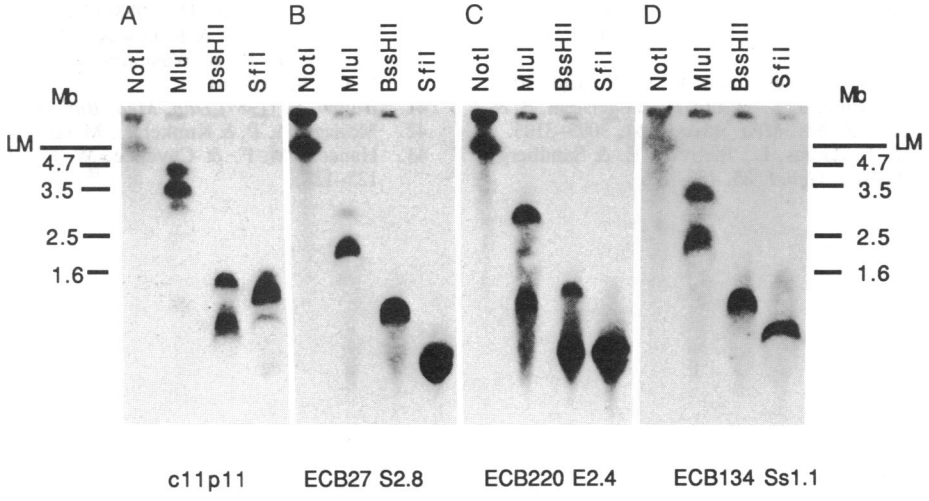

FIG. 3. Pulsed-field gel blot of GM1416B DNA (human lymphoblastoid cell line, NIGMS Human Genetic Cell Repository, Camden, NJ) digested with *Not* I, *Mlu* I, *Bss*HII, and *Sfi* I. The same filter was hybridized with single-copy fragments of C11P11 (*A*), ECB27 (*B*), ECB220 (*C*), and ECB134 (*D*). In this cell line C11P11 recognizes two *Bss*HII fragments. The 1.5-megabase (Mb) fragment corresponds to the band that is present in the hybrid H64 that was used as donor DNA in the cloning experiment. LM (limiting mobility), contains all unresolved DNA fragments.

design approaches for prevention, early detection, and treatment of many colorectal carcinomas.

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