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#27

A Test of the Role of Two Oncogenes in Inherited Predisposition to Colon Cancer

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Summary Inheritance of mutationally altered oncogenes could predispose individuals to the development of specific tumors and account for familial tumor phenotypes. Using adjacent DNA sequence polymorphisms as genetic markers, we have examined two oncogenes, the Kirsten ras2, isolated from a human colon cancer cell line, and the Harvey ras1, isolated from a human bladder cancer cell line, for their role in the genetic etiology of inherited colon cancer in Gardner syndrome. Both oncogene loci have been shown to be unlinked to the Gardner syndrome locus and are, therefore, eliminated as candidates for the Gardner syndrome gene.

Introduction

The recent isolation of activated oncogenes from human tumor cell lines (Murray *et al.*, 1981; Goldfarb *et al.*, 1982; Pulciani *et al.*, 1982; Shih & Weinberg, 1982), together with the observation that such genes are normal components of the human genome, raises the possibility that mutant alleles of oncogenes that predispose their carriers to

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specific tumors may exist in the human population. Knowledge of such alleles would be important as it might permit the identification of predisposed individuals as well as indicate a role for the oncogene in the early steps of initiation of tumorigenesis. Families showing inherited predisposition to tumors, although rare, constitute a unique opportunity to define the genes that are involved in the initial events leading to tumor development.

A marked predisposition to colon cancer has been characterized in families carrying an allele for Gardner syndrome (Wennstrom *et al.*, 1974). This syndrome is characterized by autosomal dominant inheritance of multiple adenomatous polyps of the colon accompanied by any of several benign extra-intestinal growths including epidermoid cysts, fibromas, osteomas and specific dental abnormalities (Gardner *et al.*, 1980). Since workers in several laboratories have been able to isolate activated oncogenes from human colon cancer cell lines, it seemed possible that, in the case of Gardner syndrome, inheritance of an activated oncogene might predispose to multiple adenomatous polyps, believed to be precursors of colon carcinoma (Morson, 1978), and thereby predispose to carcinoma of the colon.

Materials and methods

One of the most appealing aspects of this hypothesis is that it can be rigorously tested by the classical methods of genetics for each candidate oncogene. Cloned DNA segments define the oncogene loci by the method of Southern (1975) and permit the identification, using restriction enzymes, of adjacent polymorphic DNA sequences. These serve as genetic markers to track the segregation of putative oncogene alleles within pedigrees (Botstein *et al.*, 1980). Specifically, if an allele at the oncogene locus is an allele that causes Gardner syndrome, then the two loci should map to the same place in the human genome. Operationally, within a single pedigree segregating the Gardner syndrome allele, there should be extremely tight cosegregation of a specific allele of the oncogene with the Gardner allele, whose presence is defined by the characteristic syndrome.

Two oncogene probes have been selected for this initial study, one isolated from human colon carcinoma cell lines (Murray *et al.*, 1981) and found to be synonymous with the Kirsten ras2 gene (c-Ki-ras2) (Der *et al.*, 1982; McCoy *et al.*, 1983; Chang *et al.*, 1982; Shimizu *et al.*, 1983), and the other isolated from a human bladder carcinoma line (Pulciani *et al.*, 1982; Shih & Weinberg, 1982) and found to be synonymous with the Harvey ras1 gene (c-Ha-ras1) (Chang *et al.*, 1982; Shimizu *et al.*, 1983; Parada *et al.*, 1982).

Results and discussion

Segregation of alleles at the oncogene loci has been examined in the original family from which Gardner syndrome was described (Gardner *et al.*, 1980; Gardner, 1951; Gardner & Richards, 1953). Figure 1 is an abbreviated pedigree chart of the family and indicates which individuals were sampled for DNA in this study.

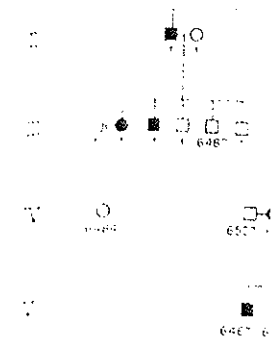

Figure 1 ... abbreviated form ... Gardner, 1951; Gardne ... pedigree drawing. Spouses ... Unaffected male and ... syndrome in living kin ... somatous polyposis and tw ... children, however, (6459, ... special extracolonic manifesta ... syndrome in expired kindred ... polyposis coli and the expect ... standard ... as well as a detaile ... symb... has been published (C ... present study are indicated on ... the illustrated pedigree are det...

The probes used to det... cloned segments contain... p640, derived from a ... activated" human c-Ki-... in pBR322 (McCoy *et al.* ... also cloned in pBR322, w... (Goldfarb *et al.*, 1982). I... The c-Ki-ras2 probe h... polymorphisms in the vic... with each of several diffe... *Hin*dIII, *Kpn*I, *Pvu*II, *Bg*... ...ages ... monomorphic b... were ... bed with p640. T... single 5·7 kb† segment ... polymorphism was obser... ...zyme-recognition sequ... ...rangements.

† Abbreviations used: kb, 10^3

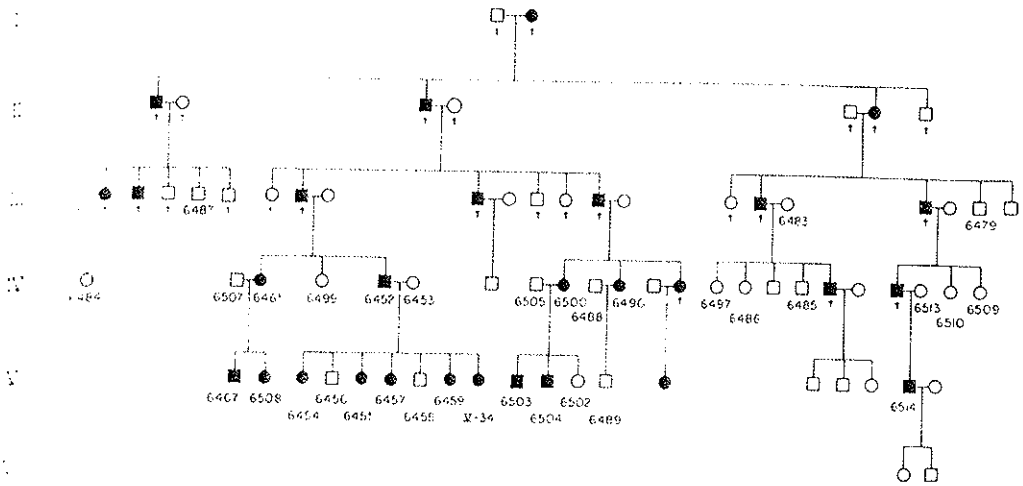

Figure [illegible] abbreviated form and updated pedigree of a previously described Gardner syndrome kindred, [illegible] (Gardner, 1951; Gardner & Richards, 1953). All affected members and their offspring are included in [illegible] pedigree drawing. Spouses of affected family members are represented wherever children are present. (□) [illegible] Unaffected male and female, respectively; (■) and (●) the affected counterparts; †, dead. Presence [illegible] syndrome in living kindred members has been determined in each case by the finding of colonic [illegible] matous polyposis and two or more of the expected benign extra-intestinal growths (see the text). Three [illegible] children, however, (6459, 109-V-34, 6489) are considered to have the syndrome on the basis of multiple [illegible] extracolonic manifestations, although proctoscopy has not yet been performed. Presence of the [illegible] drome in expired kindred members has been determined from medical records indicating colon cancer [illegible] polyposis coli and the expected extra-intestinal growths. A complete pedigree (i.e. including unaffected [illegible] as well as a detailed summary of the extra-intestinal growths present in each involved family [illegible] been published (Gardner *et al*, 1980). Family members and spouses who were sampled for the [illegible] study are indicated on the pedigree chart by a 4-digit laboratory number. Informative segments of the illustrated pedigree are detailed in the text and other Figures.

The probes used to define the oncogene loci in the present study were derived from cloned segments containing all or part of an oncogene coding sequence. The probe p640, derived from a phage bearing a segment of DNA containing part of an "activated" human c-Ki-ras2 gene, is a 640 base-pair *Eco*RI-*Hin*dIII fragment cloned in pBR322 (McCoy *et al.*, 1983). The probe pTBB-2 is an 800 base-pair *Pst*I fragment, also cloned in pBR322, which contains part of the human c-Ha-ras1 coding sequence (Goldfarb *et al.*, 1982). Each probe uniquely identifies its respective oncogene locus. The c-Ki-ras2 probe has been used to screen for restriction fragment length polymorphisms in the vicinity of this gene. Nine different human DNAs were digested with each of several different enzymes including *Mbo*I, *Msp*I, *Taq*I, *Eco*RI, *Hin*dIII, *Bam*HI, *Kpn*I, *Pvu*II, *Bgl*II, *Pst*I, *Hin*cII and *Bcl*I. With the exception of the *Taq*I digests, a monomorphic band or set of bands was seen when transfers of these digests were [illegible]bed with p640. The random human DNAs digested with *Taq*I revealed either a single 5·7 kb† segment or both a 5·7 kb and a 3·3 kb segment. The fact that polymorphism was observed only with the restriction enzyme *Taq*I suggests that the enzyme-recognition sequence is affected and that there are no detectable DNA rearrangements.

† Abbreviations used: kb, 10^3 base-pairs.

The c-Ha-ras1 probe has previously been demonstrated to reveal ge... polymorphism in human placental and tumor cell line DNAs digested with *Bgl*II ... *Bam*HI (Goldfarb *et al.*, 1982). We have confirmed the observation with lympho... DNAs isolated from individuals and have also probed DNAs digested with *Ta*... revealing at least eight different allelic fragments ranging in length from 2·3 kb ... 4·4 kb. The *Taq*I polymorphism is related to that seen with *Bam*HI, since individu... with larger *Taq*I fragment alleles also show the larger *Bam*HI fragment alleles. Each ... the polymorphisms revealed in *Taq*I digests by the pTBB-2 and p640 probes has be... examined for Mendelian inheritance in several nuclear family units from unaffect... families (data not shown). The *Taq*I alleles revealed by the probe p640 were examin...

TABLE 1

Genotypes observed for the Taq*I alleles of the c-Ki-ras2 locus and C-Ha-ras1 locus am... individuals from Kindred 109*

	p640 alleles	pTBB-2 alleles
6487	1, 2	6, 6
6483	1, 2	2, 6
6484	1, 1	6, 8
6507	1, 2	6, 8
6465	1, 2	3, 6
6499	1, 2	6, 6
6452	1, 2	3, 6
6453	1, 1	1, 6
6512	1, 2	3, 6
6500	1, 1	6, 6
6505	1, 2	6, 6
6490	1, 1	3, 6
6488	1, 1	6, 6
6497	1, 2	6, 6
6486	1, 1	2, 6
6485	1, 2	6, 6
6510	1, 1	6, 8
6509	1, 1	6, 6
6467	1, 1	6, 8
6508	1, 2	3, 6
6454	1, 2	1, 6
6456	1, 2	1, 6
6455	1, 2	1, 6
6457	1, 2	6, 6
6458	1, 2	1, 6
6459	1, 1	1, 3
109-V-34	1, 2	1, 6
6503	1, 2	6, 6
6504	1, 2	6, 6
6502	1, 2	6, 6
6489	1, 1	6, 6
6514	1, 1	6, 6

in 11 such units with 29 offspring. The allelic patterns seen in all 29 offspring were consistent with codominant Mendelian inheritance of alleles at the polymorphic locus. A similar examination of the inheritance of the *Taq*I fragments revealed by the probe pTBB-2, within 14 nuclear family units with a total of 39 offspring, again showed the fragment patterns of offspring to be consistent with codominant Mendelian inheritance of alleles.

The genotypes obtained for the *Taq*I alleles of the c-Ki-ras2 locus among individuals from Kindred 109 are summarized in Table 1. Individuals affected with Gardner syndrome are seen to be either heterozygous for the *Taq*I alleles or homozygous for allele 1, suggesting that if the Gardner and c-Ki-ras2 loci are tightly linked, the Gardner mutation must be associated with allele 1 at the c-Ki-ras2 locus. Two affected individuals with offspring, 6465 and 6452, are heterozygous at the c-Ki-ras2 locus and therefore their children are potentially informative for linkage. Since the Gardner mutation is rare and has been shown to be inherited as an autosomal dominant, affected individuals are assumed to be heterozygous for the mutation. Figure 2 shows the informative portion of the pedigree and the corresponding portion of the Southern transfer from which allelic assignments were determined.

If the Gardner mutation affects the c-Ki-ras2 gene, then it must be very tightly linked to the polymorphism revealed by p640, since the polymorphic *Taq*I site is at most 20 to 30 kb distant from any site in the gene. We would expect recombination disrupting the association to occur at a frequency less than 2×10^{-4} to 3×10^{-4} per meiosis, aassuming a correspondence of one centimorgan per million base-pairs. In principle, two different haplotypes could exist, one in which the Gardner mutation is associated with allele 1 and one in which the mutation is associated with allele 2. However, since affected individuals homozygous for allele 1 have been demonstrated within this kindred (e.g. 6500 and 6490), allele 2 is not likely to be associated with the Gardner mutation in this kindred and only one haplotype is possible. Inspection of the allelic pattern shown in Figure 2 reveals that the assumption of tight linkage between the two loci is contradicted by the occurrence of four recombinant offspring. For example, individual 6454 must be a recombinant since this individual received both allele 2 at the c-Ki-ras2 locus and the Gardner mutation from the affected parent. Calculation of the logarithm of the relative likelihood of linkage at a specific recombination fraction (r) to that of no linkage (LOD score), given this data set, gives values of $-13{\cdot}6$ at $r=0{\cdot}0001$, $-9{\cdot}6$ at 0·001 and $-5{\cdot}6$ at $r=0{\cdot}01$; tight linkage between the Gardner locus and the c-Ki-ras2 locus is therefore extremely unlikely. Including the possibility that the parental haplotypes might also represent association of the Gardner mutation with allele 2 changes these values only slightly. These data do not rule out the possibility of loose linkage of the c-Ki-ras2 locus to the disease but do strongly indicate that the locus is not the site of the mutation that causes the disease.

The genotypes obtained at the c-Ha-ras1 locus by examining *Taq*I digests of K109 family DNA samples with the pTBB-2 probe are presented in Table 1. Figure 3 shows the informative portion of the pedigree and the corresponding section of the Southern transfer from which allelic assignments were determined. Again the affected parents 6465 and 6452 are heterozygous, and their families are informative with respect to the possible linkage of the c-Ha-ras1 locus to the Gardner mutation. Although there are five different *Taq*I alleles present in the K109 pedigree, affected individuals carry in

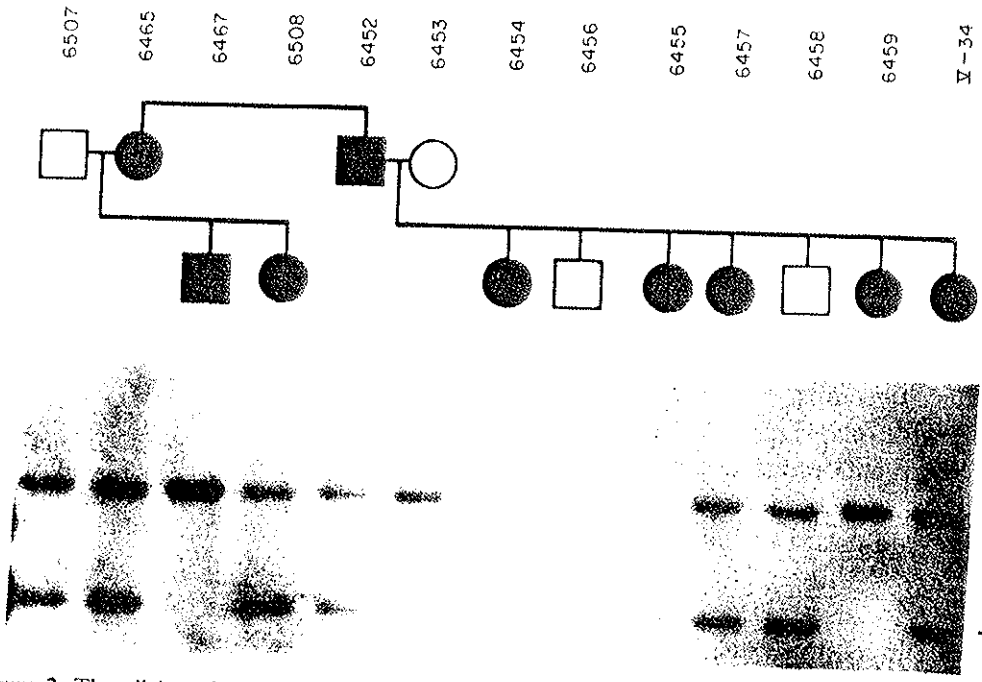

Figure 2. The alleles of the *Taq*I polymorphism at the c-Ki-ras2 locus in individuals from an informative portion of Kindred 109.

High molecular weight DNA obtained from lymphocytes (except for individual V-34 where cultured fibroblasts were the DNA source) was digested with *Taq*I, electrophoresed in a 0·8% agarose gel and transferred to Genatran™ (D&L Filter Corporation). The plasmid, p640, was labeled by nick-translation and hybridized to the filter for 24 h at 42°C in 50% formamide, 5 × SSC, 1 × Denhardt's solution; 20 mM sodium phosphate buffer (pH 6·6), 100 μg denatured salmon DNA/ml and 10% dextran sulfate. The filter was then washed briefly in 2 × SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate), 0·1% sodium dodecyl sulfate (SDS) at room temperature and twice for 30 min each at 65°C in 0·1 × SSC, 0·1% SDS, and then exposed to Kodak XAR-5 film backed by a DuPont Lightning-Plus intensifier screen for 9 days. The 5·7 kb and 3·3 kb fragment alleles are referred to in the text as allele 1 and allele 2, respectively. Fragment sizes were determined by loading 5 pg of standard DNA fragments, which include homology to the vector portion of the p640 probe, onto one lane of the gel.

common only the allele 6 (with one exception, individual 6459). Thus, if the Gardner mutation is tightly linked to the c-Ha-ras1 locus, it is likely to be associated with allele 6 in this pedigree. Examination of the allelic patterns of the progeny again contradicts the hypothesis of close linkage. If association of the Gardner mutation with the allele 6 is assumed, then individuals 6508 and 6459 must be recombinants since they can inherit the disease but not allele 6. Individuals 6456 and 6458 must be recombinants since they inherit the allele 6 but not the disease, from the affected parent. On the alternative hypothesis that allele 3 is associated with the Gardner mutation in the parents, individuals 6467, 6454, 6455, 6457 and 109-V-34 must likewise represent recombination events. This high frequency of recombination is inconsistent with the occurrence of the Gardner mutation at the c-Ha-ras1 locus. Calculations of LOD scores give negative values comparable to those obtained for the c-Ki-ras2 locus.

The linkage test has established unequivocably that mutant alleles of neither the

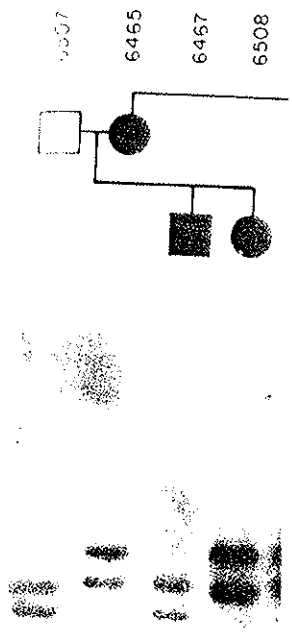

Figure 3. The alleles of the c-Ha-ras
...tion of Kindred 109. Eight diffe
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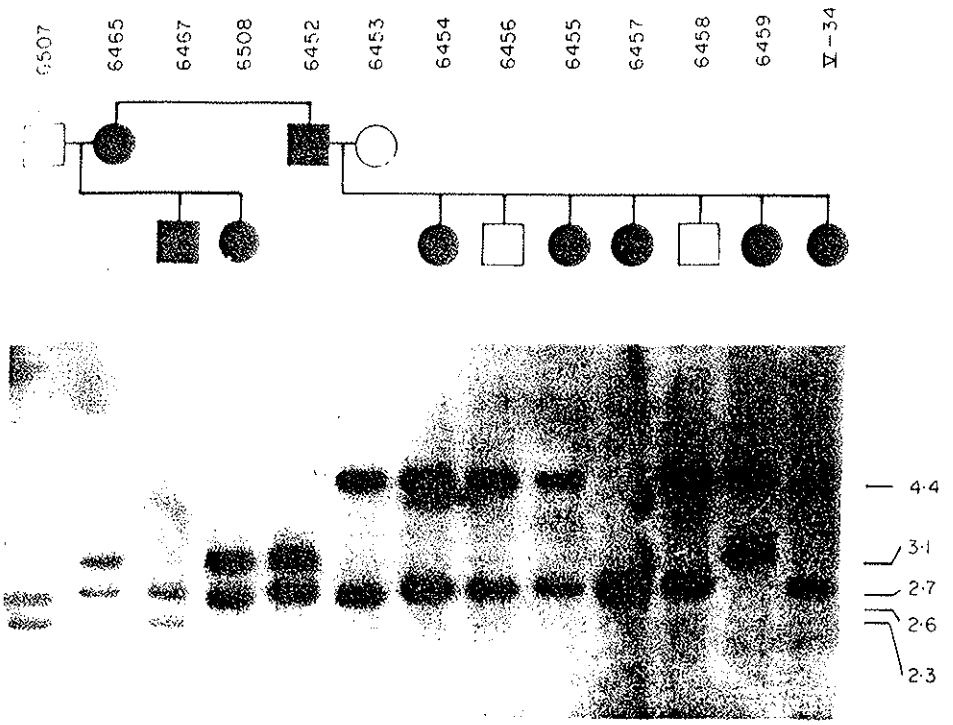

Figure 3. The alleles of the c-Ha-ras1 polymorphism revealed by *Taq*I in individuals from an informative portion of Kindred 109. Eight different allelic *Taq*I fragments are revealed at the Ha-ras1 locus with the probe pTBB-2. The 4·4, 3·7 and 3·1 kb alleles are referred to as alleles 1, 2 and 3, respectively. Alleles 4 and 5 are 2·9 and 2·85 kb, respectively. These two alleles are rare and have not been observed in Kindred 109. Two different fragments of approximate length 2·7 and 2·6 kb are distinguishable when run in adjacent lanes; as the individuals 6508 and 6452 shown here, however, owing to the difficulty of scoring these alleles accurately on different transfers, these 2 fragment sizes were each scored as allele 6. Allele 8 is a 2·3 kb fragment.

A duplicate filter of that described in the legend to Fig. 2 was prepared and hybridized with nick-translated pTBB-2 plasmid, washed and exposed identically. To ensure that allele assignments in this critical portion of the pedigree were correct, these 2 filters were subsequently stripped of radioactive DNA by treatment with 0.4 M-NaOH at 42°C for 30 min and re-hybridized with the alternative probe. Every individual showed the same allelic pattern with each probe on both filters.

c-Ki-ras2 nor c-Ha-ras1 genes are responsible for Gardner syndrome in Kindred 109. It is reasonable then to propose that if colon carcinoma cells from individuals with Gardner syndrome carry an activated oncogene that is the same as one of those tested in this study, then the activation event is likely to be a somatic mutation occurring in the progression to colon carcinoma.

Acknowledgments

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