

ORGANIZATION OF ACTIN-CONTAINING CABLES IN CULTURED SKIN FIBROBLASTS FROM INDIVIDUALS AT HIGH RISK OF COLON CANCER

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Actin-containing cables were examined by immunofluorescence in cultured skin fibroblasts from individuals genetically prone to colon cancer. The study confirmed our earlier finding of an altered distribution of actin-containing cables in skin fibroblasts of patients with hereditary adenomatosis of the colon and rectum (ACR) (Kopelovich *et al.*, 1977). Abnormalities were also found in about one-half of the asymptomatic offspring at risk for ACR, while a polyposis-free branch of one ACR family showed a normal pattern of actin-containing cables. Persons from colon cancer-prone (CCP) families without polyposis, and normal controls, showed no disturbance in the actin patterns. The results suggest that this phenotypic marker may be useful in identifying ACR gene carriers and in probing cellular controls of carcinogenesis.

The delineation of precursor states in colorectal cancer may help to clarify susceptibility mechanisms and identify high-risk individuals most likely to benefit from screening programs (Anderson and Romsdahl, 1977; Fraumeni, 1977; Knudson, 1977; Kopelovich, 1979; Lipkin *et al.*, 1979; Lynch, 1976; Swift, 1976). Adenomatosis of the colon and rectum (ACR) occurs in about 1 of 8,000 live births and is inherited in an autosomal dominant pattern. Carcinomas of the large bowel arise in virtually all untreated cases (Almy and Licznerski, 1973; Morson and Bussey, 1970). A genetic variant called Gardner's syndrome combines ACR with bone tumors, especially osteomas, and lesions of the skin and soft tissue, such as sebaceous cysts and fibromas (Gardner and Richards, 1973). It has been previously shown that, while cutaneous biopsies of ACR patients and their progeny are histologically normal, the cultured fibroblasts are abnormal in several aspects of *in vitro* growth control (Kopelovich, 1977a; Kopelovich *et al.*, 1977, 1979b; Kopelovich and Sirin, 1980; Pfeffer and Kopelovich, 1977; Pfeffer *et al.*, 1976). These findings suggest a systemic disorder of stromal cells in ACR patients that might provide clues to carcinogenic mechanisms involving the large bowel and possibly other sites (Kopelovich, 1977, 1978, 1979).

To further evaluate these findings, we examined individuals from colon cancer-prone (CCP) families in which the pattern of cases suggested autosomal dominant inheritance (Lynch *et al.*, 1977a, b). Despite the absence of ACR, occasional adenomas were seen in the colonic mucosa of many of these individuals. In line with other studies on CCP families, tumors developed at a younger than usual

age, tended to arise at multiple foci, and often involved the proximal part of the colon (Fraumeni, 1977; Lipkin *et al.*, 1979; Lynch *et al.*, 1977a, b).

The present report shows that, in contrast to the disorganization of the actin-containing cables (ACC) seen in ACR cells, there was no evidence of aberrant ACC pattern in skin fibroblasts from CCP individuals.

MATERIAL AND METHODS

Determination of actin distribution in skin fibroblasts

Processing of skin biopsies and culture conditions were as previously described (Kopelovich *et al.*, 1979b). Passage number was kept at a range of 5-12, since human cell strains beyond this stage are likely to undergo crisis (Hayflick and Moorehead, 1961), which might affect the distribution pattern of ACC in the skin fibroblasts (SF) in an unreproducible fashion.

The SF were seeded at an initial plating density of 4×10^3 cells per coverslip (about 25% confluency) per well, 2 cm², (Linbro; 24-well Dispo-tray, Cat. No. FB 16'24TC) in 1.0 ml of EMEM/15% FCS, and were examined for evenness of distribution, homogeneity of morphology, and sterility. After 48 hours incubation in EMEM/15% FCS, the medium was changed to EMEM/1% FCS for 48 h and the cells were then fixed with 3.8% formaldehyde in Ca²⁺- and Mg²⁺-free phosphate-buffered saline, pH 7.0, at room temperature. Under these conditions, all cell types appeared to spread to a similar extent at final densities of about 6×10^3 and 9×10^3 for the normal and ACR cells respectively.

Immunofluorescent localization of actin in skin fibroblasts

Cover-slips were generally kept in formalin for 4-8 days at 4°C, then acetone post-fixed, and stained sequentially with rabbit antiactin (1:80 in the same phosphate-buffered saline) and fluorescein isothiocyanate-conjugated goat antiserum to rabbit IgG (Cappel) (1:20 in phosphate-buffered saline), and mounted cell-side down in Aquamount (Kopelovich *et al.*, 1977; Pollack and Rifkin, 1975). When multi-

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ple coverslips prepared from the same culture were fixed in formaldehyde for 5 minutes, 20 minutes, 3 hours, 25 hours, 48 hours, 4 days and 8 days before staining, no significant differences were observed in the subsequent appearance of the actin cytoskeletons. The mean and standard deviation of coverslips scored for cables in these fixation tests (see below) were 70.6 ± 11 for cells from a control individual, and 44.4 ± 13 for cells from a symptomatic ACR individual. Stained cells were scanned by epi-illumination through an X63 objective with a Zeiss photomicroscope.

Method of scoring ACC

In normal pre-crisis fibroblasts from certain species, including man, the adherent cables are very thick and extensive. Many run the length of the cell, passing under the nucleus. In transformed cells, cables in this subset are diminished in width and length so that, in the limiting case, they are not resolvable at all (Rifkin and Pollack, 1979; Tucker *et al.*, 1978; Weber *et al.*, 1974). Quantitation of the fraction of cells with adherent cables has shown the difference between normal and transformed cells to be statistically significant (Goldman *et al.*, 1976; Kopelovich *et al.*, 1977; Pollack and Rifkin, 1979). SF with less than total loss of adherent-plane cables can also be quantitatively distinguished from normal by scoring cells for the presence or absence of some arbitrary number of adherent cables per cell.

Scoring of ACC in cells was carried out by three independent investigators and patterns were judged by the degree to which cables extended throughout the cytoplasm and by their orientation to the adherent plane of the cell. The success of this assay depends upon a high-titer, mono-specific, antibody to actin (Burrige, 1976), kindly provided by Dr. K. Burrige, Cold Spring Harbor Labs., and upon a careful choice of culture conditions (Kopelovich *et al.*, 1977). Cells were scored as positive for actin cables if fluorescent bands were seen to run the length of the cell when the edge of the cell was in focus. We have found that the threshold of two or more cables per cell under the nucleus clearly distinguishes normal from apparently transformed ACR cell cultures (Kopelovich *et al.*, 1977). More than 100 cells were scored on each cover-slip, and all experiments were scored by an observer «blind» to the clinical status of the cells examined.

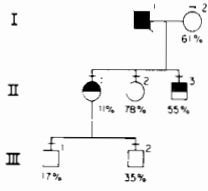
Study subjects

Forty-nine members of 11 families (see Fig. 1) were tested for actin cable in SF. Families A-H are affected by ACR, with two examples of Gardner's syndrome (B+D), while I, J, and K are CCP families. In total, seven groups of individuals were tested: affected ACR patients; family members at risk of ACR; family members in an unaffected branch of an ACR family; affected CCP patients; family members at risk of CCP; spouse controls of CCP and ACR patients; and normal individuals without a family history of cancer. Several of the subjects in the first two groups had been studied previously (Kopelovich *et al.*, 1977), and they were re-assayed for the present report concomitantly with CCP and control individuals.

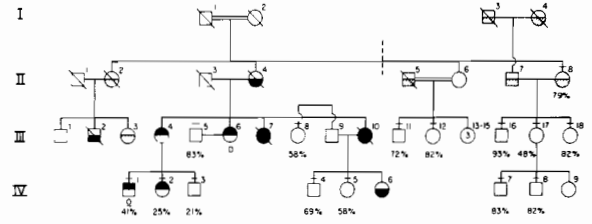
TABLE I
DISTRIBUTION OF ACTIN CABLES IN SF OF INDIVIDUALS
IN ACR, COLON CANCER PRONE
AND CONTROL POPULATION GROUPS

| Subject | Age | Sex | Family | Number | % cells with actin cables |
|--------------------------------------|-----|-----|--------|--------|---------------------------|
| ACR (familial polyposis) symptomatic | | | | | |
| 1 | 43 | f | D | IV 1 | 73 |
| 2 | 28 | m | A | II 3 | 55 |
| 3 | 21 | m | B | IV 1 | 41 |
| 4 | 31 | f | E | III 7 | 34 |
| 5 | 19 | f | B | IV 2 | 25 |
| 6 | 17 | m | C | II 3 | 19 |
| 7 | 31 | f | A | II 1 | 11 |
| ACR (familial polyposis) potential | | | | | |
| 8 | 25 | f | A | II 2 | 78 |
| 9 | 47 | m | H | II 1 | 73 |
| 10 | 17 | m | B | IV 4 | 69 |
| 11 | 20 | f | B | IV 5 | 58 |
| 12 | 14 | m | C | II 4 | 48 |
| 13 | 10 | m | A | III 2 | 35 |
| 14 | 19 | m | E | IV 2 | 31 |
| 15 | 17 | m | B | IV 3 | 21 |
| 16 | 8 | m | A | III 1 | 17 |
| 17 | 9 | m | F | IV 2 | 15 |
| 18 | 17 | f | G | III 4 | 7 |
| ACR (familial polyposis) not at risk | | | | | |
| 19 | 35 | m | B | III 16 | 93 |
| 20 | 24 | m | B | IV 7 | 83 |
| 21 | 37 | f | B | III 18 | 82 |
| 22 | 36 | f | B | III 12 | 82 |
| 23 | 20 | m | B | IV 8 | 82 |
| 24 | 79 | f | B | II 8 | 79 |
| 25 | 34 | m | B | III 11 | 72 |
| 26 | 44 | f | B | III 17 | 48 |
| Colon cancer prone (affected) | | | | | |
| 27 | 46 | m | J | III 4 | 86 |
| 28 | 61 | f | J | III 9 | 83 |
| 29 | 50 | m | I | III 2 | 73 |
| 30 | 51 | m | J | III 1 | 56 |
| 31 | 27 | f | K | III 1 | 56 |
| 32 | 73 | m | J | II 7 | 48 |
| Colon cancer prone (50% risk) | | | | | |
| 33 | 22 | m | I | IV 3 | 89 |
| 34 | 57 | f | J | III 10 | 88 |
| 35 | 20 | m | K | III 3 | 87 |
| 36 | 17 | m | I | IV 4 | 86,12 |
| 37 | 24 | f | K | III 2 | 82 |
| 38 | 64 | m | J | III 7 | 68 |
| 39 | 38 | m | J | III 5 | 65 |
| Spouse controls | | | | | |
| 40 | 49 | f | I | III 3 | 90 |
| 41 | 62 | f | J | III 8 | 88 |
| 42 | 34 | m | B | III 5 | 83 |
| 43 | 39 | f | J | III 6 | 83 |
| 44 | 65 | f | J | II 13 | 68 |
| 45 | 48 | f | J | III 2 | 64 |
| 46 | 39 | m | C | I 1 | 61 |
| 47 | 51 | f | A | I 2 | 61 |
| 48 | 56 | f | B | III 8 | 58 |
| 49 | 54 | m | G | II 1 | 53 |
| Population controls | | | | | |
| 50 | 25 | m | | | 97 |
| 51 | 42 | m | | | 93 |
| 52 | 21 | m | | | 92 |
| 53 | 25 | f | | | 76 |
| 54 | 28 | m | | | 68 |
| 55 | 22 | m | | | 66 |

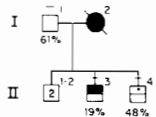
FAMILY A



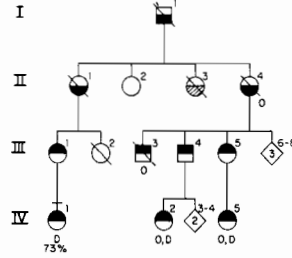
FAMILY B



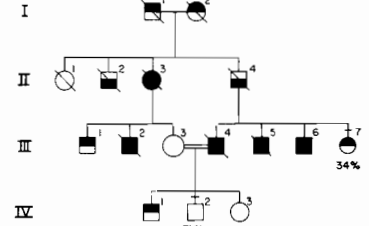
FAMILY C



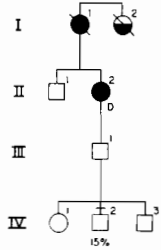
FAMILY D



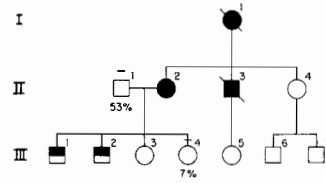
FAMILY E



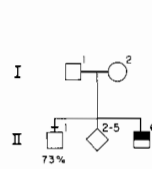
FAMILY F



FAMILY G

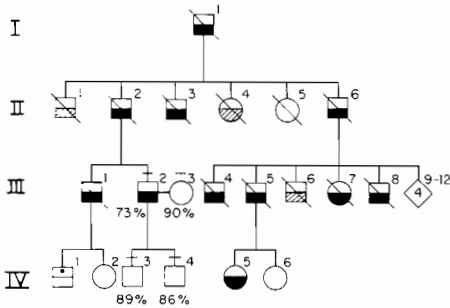


FAMILY H

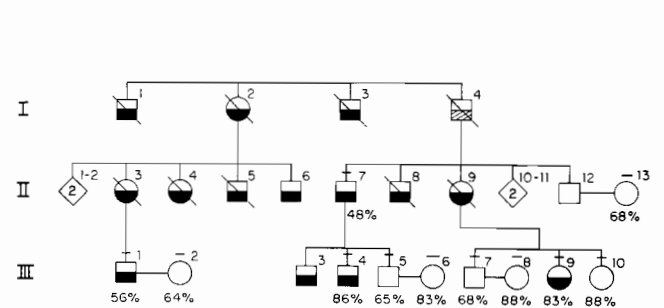


- Male with familial polyposis
- Female with colorectal cancer
- ◻ Discrete colon polyps
- ☒ Deceased Family member with other malignancy
- ⊕ Actin level measured with % of cells with actin cables
- 56%
- 73%
- Consanguineous mating
- D: Desmoid
- O: Osteoma

FAMILY I



FAMILY J



FAMILY K

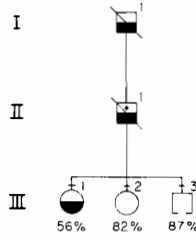


FIGURE 1 — ACR pedigrees encompass families A through H. CCP pedigrees encompass families I through K. Dashed line in family B separates ACR at risk branch from ACR not at risk branch (depicted to right of line).

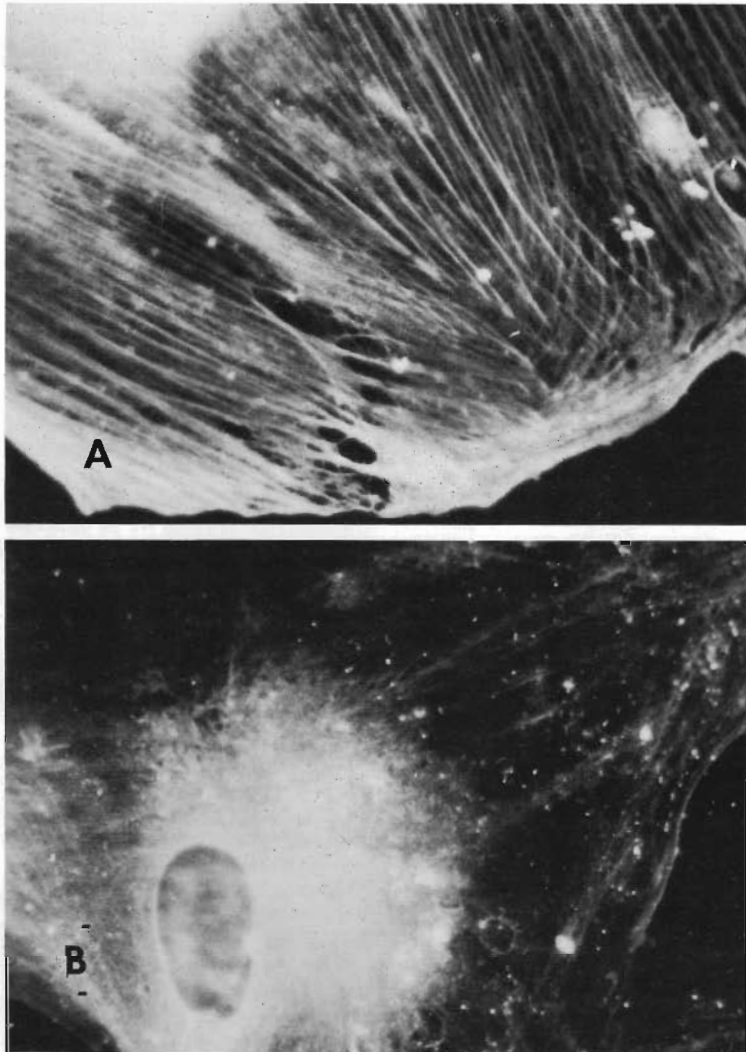


FIGURE 2 — Cytoskeletal actin organization at plane of adhesion in cultured skin Fibroblasts. (a) From a normal individual and (b) from an ACR patient.

RESULTS

The extent to which ACC were present in SF derived from individuals representing ACR and non-ACR phenotypes was examined (Fig. 2). Immunofluorescent visualization of individual cells showed that SF from normal subjects contained a multitude of long and well-organized ACC. These frequently ran for many micrometers in length and were up to 1 μ m in width (Fig. 2a). In contrast, SF from most ACR patients were deficient in ACC (Fig. 2b). As in a previous study (Kopelovich *et al.*, 1977), we found that, compared to cells from normal persons, less than one-half as many cells from ACR patients show sufficient ACC at the adherent plane in this assay.

Among seven affected ACR family members were two cases in which the percentage of actin cables was relatively high (Table I). Subject 1 is a 43-year-old female with's Gardner's syndrome from Family D (IV-1), whose mother is also affected. Subject 2 is a 28-year-old male from Family A (II-3) with classical ACR, whose affected older sister had a very low

percentage of actin-positive cells. These two patients had no clinical or pathologic features to distinguish them from other ACR cases in the series.

Among the 11 ACR family members at risk of being affected, four had values indistinguishable from those of the control groups, while seven had values falling in the range of the ACR patients. All at-risk persons with low actin cable values were under 20 years of age, compared to only 1 of the at-risk individuals with a normal pattern.

In one extended family (Family B), it was possible to compare actin cable values between an ACR-positive and an ACR-negative branch, consisting of a 70-year-old woman (II-8) without polyps plus her unaffected children and grand-children. The values for the ACR-negative branch resembled those for control individuals and were significantly higher ($p < 0.01$) than in the affected or at risk ACR groups (Table II).

The three CCP families (I, J, K) had normal actin cable values in all affected and at-risk individuals

TABLE II
ANALYSIS OF DISTRIBUTION OF ACTIN CABLES IN SF FROM ACR,
FAMILIAL COLON CANCER AND CONTROL POPULATION GROUPS

| Group | Number of individuals | Number of experiments | % of cells with actin cables (Mean \pm SEM) |
|------------------------|-----------------------|-----------------------|---|
| (1) ACR, symptomatic | 7 | 8 | 36.86 \pm 8.15 ^{1,2} |
| (2) ACR, potential | 11 | 11 | 41.09 \pm 7.66 ^{1,3} |
| (3) ACR, not at Risk | 8 | 8 | 77.63 \pm 4.70 |
| (4) Colon cancer prone | | | |
| Affected | 6 | 10 | 67.00 \pm 6.47 ⁴ |
| 50% Risk | 7 | 10 | 80.71 \pm 3.78 ⁴ |
| (5) Spouse control | 10 | 15 | 70.90 \pm 4.33 |
| (6) Population control | 6 | 8 | 82.00 \pm 5.58 |

¹ACR group cited differs significantly from the spouse control group ($p < 0.01$), and also from the population control group ($p < 0.01$), by the Student t-test. - ²ACR group differs significantly from the ACR not-at-risk group ($p < 0.001$). - ³Significant difference between the ACR potential group and the ACR not-at-risk group ($p < 0.01$). - ⁴CCP-affected and CCP 50% risk groups were not significantly different from controls or from ACR not-at-risk group.

(Table I). The distribution resembled that seen in the spouse and control populations (Table II). The age and sex of subjects did not appear to affect the results. A possible exception is individual IV-4 in family I (Table I) who, in repeated experiments, had very low actin levels (12%). Interestingly, this individual, who is clinically asymptomatic, has been shown to have the ACR-risk profile by other tests as well (Kopelovich, 1979, 1980).

Figure 3 plots the distribution of ACC values in all study and control groups. The affected and at-risk members of ACR families had lower mean values than did other groups, and some overlap was apparent in a few cases.

DISCUSSION

Fibroblastic cell strains from ACR individuals demonstrate phenotypic expressions often associated with transformed cells. These include growth disorders (Kopelovich, 1977a; Kopelovich *et al.*, 1977b; Pfeffer *et al.*, 1976), increased proteolytic activity (Kopelovich, 1977), altered cytoproteins (Kopelovich *et al.*, 1977), susceptibility to transformation by the Kirsten murine sarcoma virus (Pfeffer and Kopelovich, 1977) and simian virus 40

(Kopelovich and Sirlin, 1980). These cells probably represent an initiated state and in the presence of a tumor promoter show a partial loss of anchorage sensitivity (Kopelovich and Bias, 1980) and an ability to grow *in vivo* in athymic mice (Kopelovich *et al.*, 1979a).

We have previously suggested that a link might exist between the defective growth control of ACR fibroblasts and the occurrence of colonic adenocarcinoma (Kopelovich, 1980). In this regard, it was recently demonstrated (Delpech *et al.*, 1979) that the appearance of mesenchyme-associated antigen was topologically tightly coupled to the development of colonic adenocarcinoma. The significance of this observation remains to be established.

The present study of ACR patients and at-risk offspring provides further evidence that ACR is also associated with a systemic defect in the organization of cytoplasmic ACC. This defect was not found in the unaffected branch of an ACR family, in CCP families who lack the ACR gene, or in spouse and normal controls. Although the actin cable phenotype appears genetically linked to ACR, high values were found in two of seven patients. At this point, the test cannot be used with certainty as a marker of ACR risk, though low values may be suggestive in

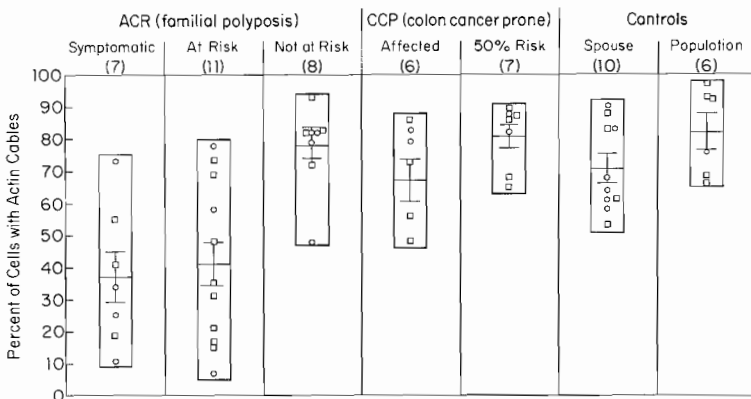


FIGURE 3 - Distribution of actin cable values in the various experimental groups. Figures in parenthesis indicate number of individuals examined in each group. Means \pm 1 SEM noted by lines within bars; squares are males, circles are females.

an individual case. It is of interest that all young family members at risk of ACR had low actin cable values. Follow up of the asymptomatic progeny with abnormal cytoskeletons will help clarify the potential utility of this assay as a marker of ACR risk. Follow up assays will no longer be totally dependent upon supplies of antibody to actin. Recently the fluorescent chemical actin probe FL-phalloidin has been shown to generate actin cytoskeletal patterns identical to those seen with anti-actin (Wulf *et al.*, 1979; Verderame *et al.*, 1980).

Although actin-containing elements were largely normal in CCP individuals, other transformation-related parameters associated with ACR (Kopelovich, 1979; Kopelovich *et al.*, 1979a) are being investigated for wider applicability in CCP families. For example, recent studies suggest that a tumor promoter may be used to distinguish skin fibroblasts of both ACR and CCP patients from normal subjects *in vitro* (Kopelovich and Gardner, 1980 submitted Int. J. Cancer). Carcinoma of the large bowel may arise from one of several different mutations, each inher-

ited in a dominant manner (Anderson and Romsdahl, 1977; Fraumeni, 1977; Knudson, 1977; Lynch, 1976). A possible exception is Turcot's syndrome (polyposis with brain tumors), which may be recessively inherited (Lynch, 1976). Whether these mutations are allelic or non-allelic is not known, but susceptibility mechanisms involving ACR and CCP would seem heterogeneous if the behavior of fibroblast cell strains is any indicator. The variability of expression in autosomal dominant forms of colon cancer may also result, in part, from different epigenetic control mechanisms (Riccardi, 1977).

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