

Reprint from

**International Cell Biology 1980—1981**

Edited by H. G. Schweiger

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Printed in Germany. Not for Sale.



Springer-Verlag  
Berlin Heidelberg New York

# F-Actin Patterns Quantitated with F1-Phalloidin in Skin Fibroblasts of Individuals Genetically Predisposed to Colon Cancer

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## Abstract

F1-phalloidin is a chemical probe for F-actin distribution in fixed cultured cells. Skin fibroblast cells from an individual can be distributed into four classes according to number and size of structures containing F-actin visualized with fluorescence microscopy after F1-phalloidin staining. The distribution shifts significantly when skin fibroblasts of persons with the inherited colonic neoplasm ACR are compared with cells from unaffected individuals. Skin fibroblasts from cancer patients of non-ACR colon cancer-prone families, and from cancer patients in families with multiple primary tumors, show normal F-actin distribution. These data confirm earlier studies which used antibody to actin (Kopelovich et al. 1980). Skin fibroblasts from one child of an ACR patient have an abnormal F-actin distribution, even though this child is currently free of symptoms of the disease. F1-phalloidin permits a prospective study of such individuals to determine whether F-actin distribution detected by a chemical probe can be used prognostically.

## Introduction

Most human cancers are of unknown etiology. The relative weight of environmental factors and host genetic susceptibility is thought usually to be tipped toward the environment. In rare cases however, the disease occurs as the result of a host mutation. In such cases it is reasonable to hope that all cells of an affected individual might reveal an altered phenotype when appropriately examined. Detection of such an abnormality in easily cultured cells might permit prognosis of asymptomatic children of individuals affected by an inherited cancer.

Patients with the autosomal dominant mutation ACR develop colonic polyps and adenocarcinomas of the lower intestine by middle age. One half of their children carry the mutation as well. In 1977 we reported that cytoskeletal actin patterns in forearm skin biopsy fibroblasts from ACR patients and in some of their children were abnormal. Bundles of actin were replaced in many cells by a diffuse actin distribution (Kopelovich et al. 1977). Recently, we reported that this change in actin pattern does not occur in skin fibroblasts from patients with other non-ACR, familial colonic neoplasms (Kopelovich et al. 1980).

Both these earlier studies depended upon antibody to actin as a probe of cytoskeletal organization. In 1979 Wulf and his co-workers (Wulf et al. 1979) described F1-phalloidin, a fluorescent derivative of a phallo-toxin which binds to F-actin with a dissociation constant of  $2.7 \times 10^{-7}$  M. F1-phalloidin generates quantifiable F-actin distribution patterns in fixed cultured cells. The quantitative distribution of cells by number and size of F-actin containing structures is altered by oncogenic transformation (Verderame et al. 1980). We use this defined chemical probe to reveal the quantitative distribution of F-actin patterns in skin cultures from patients with ACR and other inherited colonic neoplasms.

## Methods

### Cell and Culture Conditions

Biopsies were obtained from patients and their relatives seen at Memorial Sloan-Kettering Cancer Center and were processed as previously described (Kopelovich et al.

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1977). Skin fibroblasts were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS, Gibco) and 100 units per ml of penicillin and streptomycin, and were kept at 37 °C in an atmosphere of 10% CO<sub>2</sub>, 90% air, and 100% humidity. All cultures were routinely checked for mycoplasma contamination (Chen 1977) and were found to be negative.

### Fixation and Staining

Cells were plated on coverslips at a density of  $2-4 \times 10^3$  cells/cm<sup>2</sup> in DME 10% FCS. One day later the medium was changed to 1% FCS. The following day the cells were fixed in 10% formalin in phosphate-buffered saline pH 6.8 (PBS) for 20 min at room temperature, rinsed with PBS and extracted with 1% NP40 in PBS for 20 min. After three rinses in PBS, 5 min each, the cells were stained with 10 µl of F1-phalloidin (1 µg/ml in PBS) at 37° for 20 min. The coverslips were rinsed three times in PBS and mounted on microscope slides with Aquamount (Verderame et al. 1980).

### Fluorescence Microscopy

Stained coverslips were examined with a Leitz Orthoplan microscope using a Zeiss Planapo 63× oil immersion objective coupled to a Leitz I2 wide band exciter-barrier filter cube.

## Results

### Method of Scoring

Well-spread flat cells were scored for their actin cable content (Verderame et al. 1980). Each cell was placed in one of four distinct categories. Class I cells had heavy distinct cables crossing more than 90% of the central area of the cell, 50% of which span the breadth of the cell. Class II cells had fine cables and at least 2 heavy cables within the central half of the cell, extending more than half of its breadth. Class III cells had only fine cables and class IV cells had no detect-

able cables in the central area, but only a diffuse fluorescence; some class IV had fine cables solely at the periphery. 200 cells were scored for each culture by at least two individuals, using blind-coded slides.

### Comparison of Actin Pattern in Different Groups

Six groups of subjects were examined for their actin cable patterns: (1) normal subjects, either from the general population or spouses from the cancer families; (2) patients diagnosed with adenomatosis of colon and rectum (ACR); (3) asymptomatic children of ACR patients who have a 50% probability of developing the disease; (4) persons from colon cancer-prone families who have colon cancer (CCP+), (5) persons from colon cancer-prone families who have no symptoms but a 50% risk of developing colon cancer (CCP-), and (6) one patient with multiple primary colonic tumors.

Table 1 shows the percent of skin fibroblast cells at three different thresholds of cytoskeletal organization. The percent of cells with the largest cables are in category I. The percent of cells with fine cables but also at least some large cables are in categories I+II. The percent with any detectable cables are in categories I+II+III. Published data with anti-actin are based on a single threshold (+/-) score for the presence of cables (for example, Pollack et al. 1975). The percent (+) cells reported earlier in studies with anti-actin (Kopelovich et al. 1977; Kopelovich et al. 1980) is also given in Table 1. The general correlation of F1-phalloidin distribution with α-actin data is good in that population with more cells in categories I and II also have higher (+) scores.

By Student's t-test normal and ACR individuals have significantly different percentages of cells when scored by I+II or I+II+III thresholds (Table 2). In contrast, when cells are scored for the presence of only large cables (Category I) there is no statistical difference between normal and ACR individuals. The threshold of any detectable cables (I+II+III) depends upon the capacity of

**Table 1.** Percent of cells with cables: comparison of F1-phalloidin thresholds to anti-actin scores

Phenotype	Percent of cells in categories <sup>a</sup>				Percent of positive cells anti-actin <sup>b</sup>	
	N <sup>c</sup>	I	I+II	I+II+III	1977	1980
Normal	5	25 (28) <sup>d</sup>	48 (26)	89 (10)	77 (3)	76 (5)
ACR	7	4 (4)	15 (9)	68 (19)	32 (3)	37 (8)
CCP(-)	2	54 (14)	66 (11)	88 (6)	ND <sup>e</sup>	81 (4)
CCP(+)	2	26 (32)	53 (15)	88 (7)	ND	67 (6)
MPT	1	1 (2)	19 (21)	73 (9)	ND	ND

<sup>a</sup> Categories of F-actin distribution in fixed cells are shown in Verderame et al. 1980. Briefly, category I cells have large F-actin cables, category II cells have a mixture of large and fine cables, category III cells have only fine cables and category IV cells lack detectable cables with F1-phalloidin

<sup>b</sup> Positive cells contain at least two cables running the length of the cell (Kopelovitch 77), detectable with anti-actin

<sup>c</sup> Number of individuals

<sup>d</sup> Mean percent of cells (standard deviation)

<sup>e</sup> Not Done

F1-phalloidin to detect fine cables. The percentages using this threshold are clearly higher for each line than for the threshold I+II (Table 1). In both cases, the correlation with earlier, antibody-based data is good. From Table 1 it would appear that in general we were not visualizing as many fine cables with anti-actin as we do with F1-phalloidin.

The F1-phalloidin pattern change seems to be specific for ACR. Cells from the symptomatic and symptom-free CCP family members were not statistically different from normal cells even though these individuals were also at high risk for colon carcinoma (Tables 1, 2). Cells from one individual with multiple primary colonic tumors also showed a normal distribution (Tables 1, 2).

### Asymptomatic Children of ACR Patients

We examined cells from four children of ACR patients. While these children were all free of symptoms of ACR at the time of their skin biopsies, we found that cells from one child (subject 4) had an F1-phalloidin actin distribution much like that of cells from ACR patients (Table 3). That is, cells at the category I threshold were not different, but subject 4 had fewer cells with sufficient cables for the I+II threshold or the I+II+III threshold than did the other three subjects (Table 4). This distribution is significantly different from normal and also different from that of other non-ACR individuals or the other three asymptomatic children (Table 4).

**Table 2.** Significant differences among human fibroblast F-actin distributions, Student's t-test

Comparison	Categories (F1-phalloidin)		
	I	I+II	I+II+III
Normal vs ACR	0.2 > P > 0.1	0.01 > P > 0.001	0.01 > P > 0.001
Normal vs CCP(-)	P > 0.9	P > 0.9	0.8 > P > 0.7
Normal vs CCP(+)	P > 0.9	P > 0.9	P > 0.9
Normal vs MPT	0.6 > P > 0.5	0.1 > P > 0.05	0.2 > P > 0.1
CCP(-) vs CCP(+)	0.2 > P > 0.1	0.9 > P > 0.8	P > 0.9

**Table 3.** Percent of cells in F1-phalloidin categories, asymptomatic children of ACR patients

Subject	Age	Sex	Percent of cell in categories		
			I	I + II	I + II + III
1	20	F	13 (5)	48 (8)	91 (3)
2	10	F	7 (7)	51 (17)	92 (8)
3	19	M	33 (27)	64 (12)	97 (1)
4	8	M	10 (11)	20 (12)	40 (11)

**Table 4.** Significant differences among F-actin distributions in asymptomatic children of ACR patients

Comparison	Categories (F1-phalloidin)		
	I	I + II	I + II + III
Subj 1 - 3 Avg vs Normal Avg	0.7 > P > 0.6	0.9 > P > 0.7	P > 0.9
Subj 1 - 3 Avg vs ACR Avg	0.2 > P > 0.1	0.3 > P > 0.2	0.01 > P > 0.001
Subj 4 vs Normal Avg	0.7 > P > 0.6	0.4 > P > 0.3	0.01 > P > 0.001
Subj 4 vs ACR Avg	0.5 > P > 0.4	0.6 > P > 0.5	0.05 > P > 0.02
Subj 1 - 3 Avg vs Subj 4	0.9 > P > 0.8	0.1 > P > 0.05	0.01 > P > 0.001

All four children are under clinical observation, so it will be known in time whether any of them develop symptoms of ACR. Insofar as the F1-phalloidin pattern distribution of patient 4 is not significantly different from the average distribution found in ACR patients' cells (Table 4), it is reasonable to predict that subject 4 carries the ACR mutation and will in time develop symptoms of the disease.

## Discussion

Since ACR is inherited as an autosomal dominant trait, the progeny of a family in which one parent manifests ACR have a 50% probability of receiving the ACR gene. The F1-phalloidin cytoskeletal patterns of the children of ACR patients fell into two significantly different categories: individuals whose cells were statistically indistinguishable from normal ( $p > 0.90$ ) and those whose fibroblasts were clearly different from normal ( $p < 0.01$ ) and indistinguishable from those with ACR phenotype ( $p > 0.20$ ).

The similarity in pattern between cells from the ACR patients and those from subject 4 suggests that it would be useful to engage in a prospective study of actin patterns in cells of children of patients with ACR. This will require periodic examination of asymptomatic children of ACR patients for signs of the disease, as well as periodic biopsies to determine the actin organization of their skin fibroblasts. Future scans of actin cytoskeletons can be done reproducibly with the chemical reagent F1-phalloidin (Verderame et al. 1980).

The altered cytoskeletal patterns seen here are qualitatively similar to those seen in rodent fibroblasts transformed by the oncogenic virus SV40 (Pollack et al. 1975; Verderame et al. 1980). Cells from ACR individuals and asymptomatic progeny with an altered cytoskeleton have been reported to resemble transformed cells by the criteria of growth in low serum, loss of contact inhibition of division (Pfeffer et al. 1976) and secretion of large amounts of plasminogen activator (Kopelovich 1977).

The parallelism of the in vitro phenotypes of these two different genotypes suggests the possibility that the ACR gene product in humans and the early gene products of SV40 may in some yet unknown way intersect. To test this we are extending characterization of ACR cells to include their response to growth hormones, and their susceptibility to transformation by SV40 early gene sequences.

Given the small sample size of this scan the effect, if any, of the age of the individual on Fl-phalloidin visualized cytoskeletal distribution cannot be determined. In particular, there are no young (<20 years old) normal individuals in this study. We are in the process of obtaining such biopsies in order to confirm with Fl-phalloidin earlier reports (Kopelovich et al. 1977) that age is not a significant variable.

We plan to couple Fl-phalloidin fluorescence images to a vidicon based digitalized image analyzer (Sobel et al. 1980) resulting in signals which can be analyzed and compared by computer. This will make possible detailed prospective studies of cultured skin biopsy cells from persons at risk for cancer.

*Acknowledgements.* We would like to thank Drs. Th. Wieland and A. Deboben for their gift of Fl-phalloidin, Peggy Monaghan for the initial cell cultures, and Linda Sproviero and Marisa Bolognese for their excellent help with the manuscript. This work was supported by NIH grant CA-25066 and NRS Training Grant GM-07216.

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