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Flow cytometric analysis of DNA synthetic phase fraction of the normal appearing colonic mucosa in patients with colorectal neoplasms

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Abstract

DNA synthetic (S) phase fractions of normal appearing colonic mucosa in Japanese and British patients with colorectal neoplasms were compared with those in patients without colonic neoplasms. Normal crypts were isolated from fresh surgical specimens of the large intestine by the use of EDTA. After fixation with 70% ethanol, isolated crypts were digested with pepsin into single nuclei suspensions. These were stained with propidium iodide and examined by flow cytometry. S phase fraction was calculated from the flow cytometry DNA histogram using Baisch's method. S phase fractions of normal appearing crypts in Japanese and British patients with colorectal tumours were not significantly different and analysed together. S phase fraction of normal appearing colonic crypts in 14 patients with familial adenomatous polyposis (FAP) was 10·23 (2·59)% (mean (SD)) ranging from 5·8 to 18·8. S phase fraction of background normal mucosa in patients with large adenomas (over 2 cm) and adenocarcinomas were 9·74 (3·76)% (range, 2·7–16·1) and 8·93 (3·54)% (range, 2·9–18·9) respectively. In normal mucosa of patients without any colorectal neoplasms, S phase fraction was 8·99 (3·94)% (range, 3·9–17·7). There was no statistically significant difference in S phase fractions of normal mucosa in the four groups. Our results show that an increase in proliferative activity of background colonic crypts is not necessary for tumour development.

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Keywords: adenoma, carcinoma, DNA ploidy, familial adenomatous polyposis, flow cytometry, S phase fraction.

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Lipkin proposed that, in the morphogenesis of colorectal neoplasia, an expansion of the crypt proliferative zone with increased cellular proliferation preceded tumour development.[1] Many investigators have supported his proposal by examining the labelled cell position in colorectal mucosa using in vitro autoradiography[2 3] or in vitro bromodeoxyuridine labelling.[4] In familial adenomatous polyposis, sporadic cancer, and even adenoma patients, workers have shown an expanding proliferative zone with increasing proliferative activity in normal appearing colorectal mucosa.[5–7]

Using a complete serial sectioning technique, we found that adenomas in familial adenomatous polyposis patients begin to develop in the proliferative zone of the lower crypts in the form of atypical epithelial clusters – that is 'bud of adenoma'.[8] We also examined whether upward expansion of proliferative zone actually occurred in the normal appearing colonic mucosa using ex vivo autoradiography.[9] Our results conflicted with Lipkin's theory, and showed that in familial adenomatous polyposis patients the proliferative compartment was identical to sporadic colorectal cancer patients: the proliferative compartment was located in the lower two thirds of the crypts with a similar labelling index in the two groups.[10] Using cytophotometry, we compared the nuclear DNA content of epithelial cells in the lower two thirds and the upper one third of isolated colonic crypts in familial adenomatous polyposis and sporadic colorectal cancer groups. This showed that the upper one third of crypts had only a diploid DNA content and the lower two thirds also had a diploid population as well as a proliferative compartment in both groups.[11]

Both autoradiographic and cytophotometric techniques are tedious and score only a small number of cells. DNA flow cytometry, however, can analyse a large number of cells rapidly. It has been used to study DNA ploidy and cellular proliferative activities in various neoplasms.[12 13] Conventional flow cytometry techniques have inevitably analysed both epithelial and normal stromal cells and pure epithelial DNA ploidy and proliferative activities have rarely been ascertainable.[14 15] To improve this inadequacy we have applied the crypt isolation technique to flow cytometry. Crypt isolation has allowed us to analyse the accurate DNA ploidy of various colorectal neoplasms.[16]

In this paper, we report the S phase fraction of background non-neoplastic mucosa in groups of various colorectal neoplastic conditions in comparison with a control group without large bowel neoplasia using flow cytometry following crypt isolation.

Methods

Clinical material

Between 1989 and 1992, tissue for the study was taken from 93 patients with colorectal neoplasms undergoing resection of the large intestine in Hamamatsu University Hospital (Hamamatsu, Japan) and in related city hospitals. A further 29 patients undergoing

TABLE I *Clinical data of patients in two hospitals*

*Colorectal tumour**	*Patients (n)*	*Males/ females*	*Mean age (range)*	*Samples (n)*
Hamamatu University Hospital, Hamamatsu, Japan				
FAP	7	4/3	41·6 (22–56)	10
Adenoma	15	6/9	66·5 (41–81)	15
SCRC	71	36/35	63·4 (34–88)	71
St Mark's Hospital, London, United Kingdom				
FAP	7	5/2	22·9 (17–31)	19
SCRC	15	7/8	64·1 (43–83)	15
Control	7	3/4	38·4 (25–61)	13

*FAP, familial adenomatous polyposis; SCRC, sporadic colorectal cancer.

large bowel resection at St Mark's Hospital (London, UK) between July 1992 and March 1993, were also analysed.

Fourteen patients had colectomies for familial adenomatous polyposis, 15 patients had tubular or villous adenomas with mild to moderate atypia and more than 2 cm in size, 86 patients had adenocarcinomas, and seven patients had colectomies for dysfunctional colorectal diseases such as constipation, intussusception, megacolon and perineal pain (Table I).

Fresh specimens were obtained immediately after surgical excision. Normal mucosa at least 5 cm from the neoplasms was separated from the submucosa with scissors. Flat mucosa between multiple adenomas in familial adenomatous polyposis cases was also sampled. In the cases with non-neoplastic disease, normal mucosa without any inflammation was selected. In total colectomy cases, samples were routinely obtained from the ascending, transverse, and descending colons to be analysed separately. Rectal mucosal samples were not used in this study.

Crypt isolation

The method of Arai and Kino was used for crypt isolation.[17] Briefly, fresh mucosa was cut to a 5 mm square with a razor. This was then incubated at 37°C for 30 minutes in calcium and magnesium free Hanks's balanced salt solution containing 30 mM EDTA. After this the tissue was stirred in calcium and magnesium free Hanks's balanced salt solution. Normal crypts were separated from the lamina propria mucosa with 30 to 40 minutes. The isolated crypts were promptly fixed in 70% ethanol and stored at 4°C.

Identification of crypts

Fixed crypts were observed under a dissecting microscope (SZ60, Olympus, Tokyo) with normal and neoplastic crypts showing characteristic three dimensional features. Haematoxylin and eosin stained sections of paraffin wax embedded crypts confirmed their histological origins. Adenomatous crypts were usually intermingled with normal crypts in familial adenomatous polyposis patients and required careful separation.[11]

Flow cytometry

Normal crypts of each patient were incubated with 0·0125% pepsin (pH 2·0, Sigma, St Louis, MO) for five minutes at 37°C, washed twice with 0·2 M TRIS chloride buffered saline, and syringed through a 27 gauge needle. Normal nuclei was stained with propidium iodide (50 μg/ml, Sigma) containing ribonuclease (0·25 mg/ml, Sigma) in 0·2 M TRIS chloride buffered saline for 30 minutes in darkness at room temperature. After filtering through 37 μm nylon mesh (Tokyo Screen, Tokyo), samples were analysed on an EPICS PROFILE (Coulter, Hialeah, FL). An argon ion laser was used at 15 mW at a wavelength of 488 nm for excitation. Emission was measured using a 610 nm long pass filter. Ten thousand nuclei were counted in each sample. No extrinsic external control was used in this study.

Histogram interpretation

DNA histograms were analysed using the software package Cytologic (Coulter Corporation). All samples of normal mucosa have only one G0/G1 peak, which was regarded as diploid stemline. Overlapped nuclei of G0 or G1 phase – that is, doublet – have a lower peak pulse than that of single nuclei of G2 phase on measurement of DNA content by flow cytometer. These doublets can be excluded from single nucleus population on the scattergram. S phase fraction was calculated by using a rectangular computer software model of Baisch *et al.*[18] The coefficient of variation (CV) of each group was calculated as full width CV. All samples in this study were measured more than twice and the samples with a lower CV were used for S phase fraction analysis.

Histopathology

All resected surgical materials were routinely examined on paraffin wax embedded, haematoxylin and eosin stained sections. Diagnoses of epithelial tumours including familial adenomatous polyposis, adenoma, and carcinoma and of cases without colorectal neoplasms were made. The tissue fragments remaining after crypt isolation were fixed with 70% ethanol and examined histologically. These fragments show some remaining crypts that enabled estimation of the character of isolated crypts.

Statistical analysis

A non-parametric Mann-Whitney U test was used for statistical analysis using StatView-II software (Abacus Concepts, Berkeley, CA). Values of mean (SD) of CV and S phase fraction of each group were calculated. A value of significance of $p<0·05$ was assumed.

Results

Crypts of normal mucosa were easily separated from lamina propria mucosa by agitation of fragments obtained from incubation with EDTA. Under the dissecting microscope normal crypts had regular test tube like appearance and were up to 1 mm in length. This was confirmed histologically. Small or large crypts

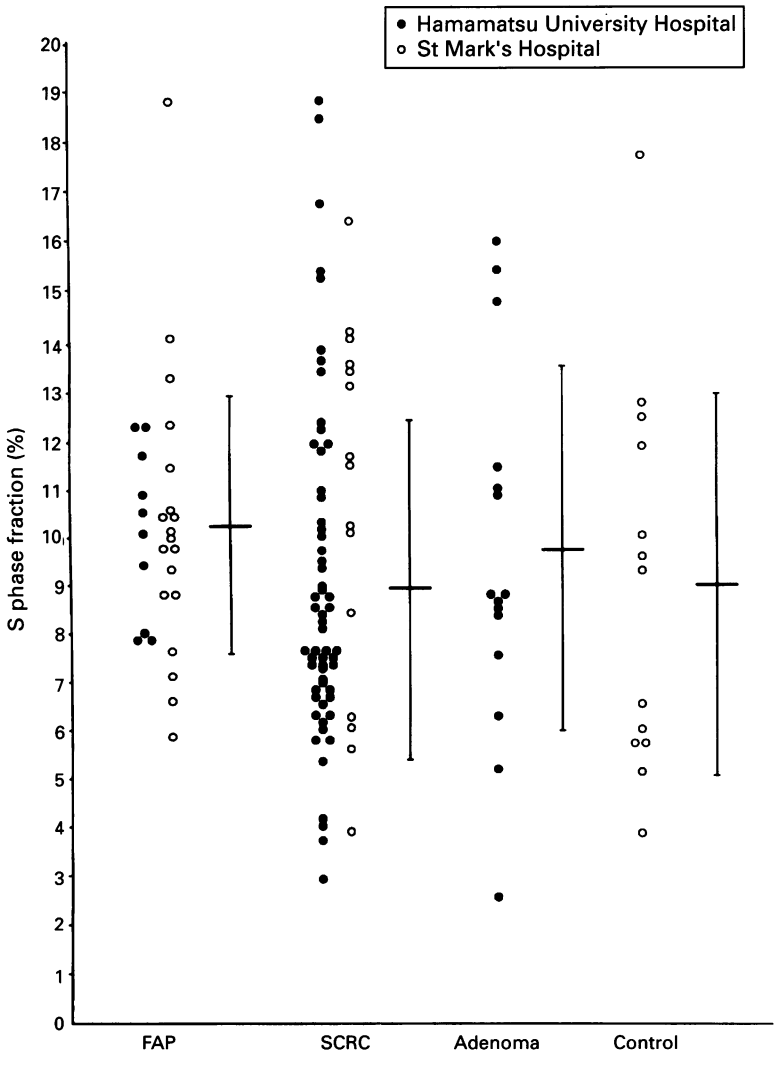
TABLE II *Values of S phase fraction and coefficient of variation (CV) of background colonic mucosa in 122 patients*

Tumour	Samples (n)	S phase fraction (mean (SD)) (range)	CV (mean (SD)) (range)
FAP	29	10·23 (2·59) (5·8–18·8)	2·65 (0·65) (1·40–3·86)
SCRC	86	8·93 (3·54) (2·9–18·9)	2·82 (0·54) (1·59–4·57)
Adenoma	15	9·74 (3·76) (2·7–16·1)	2·99 (0·52) (2·27–3·72)
Control	13	8·99 (3·94) (3·9–17·7)	2·38 (0·63) (1·60–3·40)

Abbreviations as in Table I.

and those having branches were avoided. Crypts of microscopic and single gland adenomas[8] found in familial adenomatous polyposis cases were usually irregular and larger than normal crypts. Their nature was confirmed on paraffin wax embedded, haematoxylin and eosin stained histological sections.[11]

A G0/G1 peak on DNA histogram of normal crypts measured by flow cytometry was defined as diploid and no multiple peaks were seen throughout the study. In the familial adenomatous polyposis group, values of S phase fraction of Japanese and British cases were as follows: 10·11 (1·77)% (range, 7·9–12·4) and 10·30 (2·97)% (range, 5·8–18·8). There was no statistically significant difference in the values of S phase fraction between the two countries.



All values of S phase fraction are plotted on a chart. Closed circles show the cases of Hamamatu University Hospital and open circles, St Mark's Hospital. Four vertical lines with median short lines show the means and standard deviations of each four groups. FAP=familial adenomatous polyposis; SCRC=sporadic colorectal cancer.

In the sporadic cancer groups, values of S phase fraction were also compared between Japanese and British cases as follows: 8·59 (3·42)% (range, 2·9–18·9) and 10·58 (3·74)% (range, 3·9–16·4). There was no significant statistical difference in the values of S phase fraction between the two countries. Consequently, Japanese and British cases were analysed together in the familial adenomatous polyposis and cancer groups. There were no British cases of large adenoma and Japanese cases were used for the comparison. There were no Japanese control cases and British cases were used as control.

The mean values of S phase fraction of the four groups were calculated as follows: familial adenomatous polyposis, 10·23 (2·59); sporadic colorectal cancer, 9·83 (3·54); adenoma, 9·74 (3·76); and control, 8·99 (3·94) (Table II). The Figure shows the values of S phase fraction of all cases in the four groups. No significant difference of S phase fraction was found statistically between all four groups.

The mean CV of each of the four groups was as follows: familial adenomatous polyposis, 2·65 (0·65); cancer, 2·82 (0·54); adenoma, 2·99 (0·52); and control, 2·38 (0·63) (Table II).

Discussion

Most investigators supporting Lipkin's proposal, have used an in vitro autoradiography or bromodeoxyuridine labelling method to examine the distribution of proliferative zones of colorectal mucosa obtained by biopsy.[2–7] We have pointed out several disadvantages in the application of in vitro autoradiography to biopsy material, and consequently developed ex vivo autoradiography, which has enabled us to use surgically resected colon for autoradiography.[9] Well oriented crypts cut longitudinally were analysed using this method and no expansion was found in either familial adenomatous polyposis or sporadic colorectal cancer. The labelling index was also the same in both groups.[10] Distribution of the DNA content of epithelial cells in isolated normal crypts was also analysed using cytophotometry and no expansion of proliferative zone was found in either familial adenomatous polyposis or the sporadic colorectal cancer groups.[11]

In both of our previous experiments we had no 'control' colons without neoplasms. Total or partial colectomies are performed in patients who suffer from constipation, volvulus of the sigmoid colon, megacolon and some other dysfunctional disorders of colorectum at St Mark's Hospital. These resected colons, which had no incidental epithelial neoplasia, were used as a control group in this experiment.

Nsien *et al* reported the flow cytometric analysis of normal appearing colorectal mucosa in normal, adenoma, and carcinoma patients. They failed to show abnormalities of DNA content in groups with adenoma or carcinoma.[15] They mentioned some possibilities why they failed to disclose abnormal proliferative activity in tumour groups; one being that

abundant normal stromal cells would mask abnormal epithelial populations on the flow cytometry DNA histogram.

We have pointed out several problems of conventional flow cytometry and to improve the method we applied the crypt isolation technique to flow cytometry.[16] Using this method we can calculate S phase fraction of epithelial cells accurately without contamination by interstitial cells. The values (8·9–10·2%) of S phase fraction of our study were comparable with the labelling indices (9·9–11·6%) by in vitro autoradiography[5] and the overall mean labelling index (10·3%) by in vivo bromodeoxyuridine labelling.[19]

The death rates of colorectal malignant neoplasms per 100 000 population in Japan versus England and Wales were: men, 22·1 *v* 34·5 and women, 18·3 *v* 33·4.[20] Regional differences of the incidence of colorectal malignancy between the two countries might be caused by dietary factors because high protein and fat, and low fibre in the diet may be regarded as possible risk factors of colorectal carcinomas.[21]

Cell kinetic studies of colorectal mucosa exposed to chemical carcinogens has been conducted in experimental animals with both expansion and hyperproliferation of the proliferative compartment demonstrated in normal appearing crypts. This has been considered as a preceding event in carcinogenesis and therefore as a risk factor for cancer.[22 23] According to these results, proliferative activity of the crypts in British cases might be higher than in Japanese cases. Our results, however, showed no significant differences in familial adenomatous polyposis and sporadic colorectal cancer groups from both countries.

We examined the proliferative compartment and activities of colonic crypts of mice treated with dimethylhydrazine and found that the proliferative compartment was slightly expanded in the experimental group compared with the control group but proliferative activity was the same in both groups. Direct toxic effect of chemical carcinogen might cause temporary damage of crypt epithelia and following the regenerative process result in the slight expansion of the proliferative compartment.[24]

We have previously pointed out the limitation of cell kinetic studies using biopsy material by in vitro autoradiography.[10] We also stress that 'normal' crypts have to be used for interpretation of cell kinetic analysis. We avoided rectal mucosa in this study because rectal crypts are often hyperplastic, branching, and tortuous even in healthy patients. Minor mucosal injury or focal mucosal prolapse may occur at defecation and the subsequent reparative process may cause 'abnormal' crypts. It is essential to evaluate the 'normal' crypts for the analysis of cell kinetics of the crypt epithelium.

The conclusion of this study confirmed our previous results and showed no hyperproliferation of histologically normal crypts in the colorectum in patients with neoplasia.[8 10 11 24]

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