DNA content and the adenoma-carcinoma sequence in the colorectum

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SUMMARY DNA content was measured in 269 benign adenomas and 203 adenocarcinomas of the large bowel by flow cytometry. Nuclear suspensions were prepared by pepsin digestion of paraffin sections, and an adjacent section was graded histologically by one observer. Aneuploid adenomas included five of 134 (4%) showing mild dysplasia, 19 of 107 (18%) showing moderate dysplasia, and 10 of 28 (36%) showing severe dysplasia. The association between aneuploidy and grade of epithelial dysplasia was highly significant. This gives support to the adenoma (dysplasia)-carcinoma hypothesis.

Carcinomas showing aneuploidy included 18 of 29 (62%) well differentiated, 92 of 144 (64%) moderately differentiated, and 19 of 30 (63%) poorly differentiated cases. There was no relation between aneuploidy and grade of carcinoma. These findings indicate an important biological difference between adenoma and carcinoma.

There are two contrasting views of the adenoma-carcinoma sequence in the colorectum. The traditional view contends that adenoma and carcinoma are fundamentally different lesions. The adenoma is interpreted as a circumscribed focus of dysplastic epithelium. Dysplasia may be graded low or mild (when it deviates minimally from normal), through to high grade or severe (approximating carcinoma in situ).¹ It is assumed that the progression from adenoma to carcinoma is accompanied by increasing epithelial dysplasia; this would represent a morphological counterpart of the multistep theory of carcinogenesis. According to this theory, malignant transformation becomes more likely as the severity of dysplasia increases.

The opposing faction emphasises the biological similarity of adenoma and carcinoma, regardless of the grade of epithelial dysplasia of adenoma. Riddell states that, "adenomas do not need to go through the full sequence of changes from mild to moderate to severe dysplasia to in situ and finally invasive carcinoma; the fact that they are adenomas provides all the required potential to invade."² Similarly, Isaacson writes, "severe dysplasia is not a necessary precursor of invasion," and, "in terms of their morphology there is no difference between cells comprising colorectal adenomas and carcinomas."³ Iden-

tical concepts have been proposed for the cervix⁴ and other sites. Fig. 1 summarises the essential differences between the two theories.

Recent studies have shown the existence of abnormal nuclear DNA content both in adenomas⁵⁻⁸ and carcinomas⁹⁻¹² of the colorectum. If the second hypothesis were true then one might expect to find similar DNA ploidy patterns in appropriately graded series of adenoma and carcinoma.

Fig. 1 Contrasting hypotheses governing adenoma (dysplasia)-carcinoma sequence shown schematically. Traditional view emphasises qualitative differences between dysplasia and carcinoma whereas second hypothesis emphasises similarity of a malignant neoplasm and its pre-invasive counterpart. Intensity of stippling signifies increasing dysplasia or loss of differentiation.

Fig. 2 Mild (a), moderate (b), and severe (c) dysplasia. (Haematoxylin and eosin.) Original magnification $\times 150$.

Material and methods

The material included 269 adenomas (from 213 patients) and 203 adenocarcinomas of the large intestine. There were 184 tubular, 73 tubulovillous, and 12 villous adenomas.

HISTOPATHOLOGICAL GRADING OF ADENOMA AND CARCINOMA

This was performed by a single observer using the criteria of Konishi and Morson¹³ and Jass *et al.*,¹⁴ respectively. Mild dysplasia deviated minimally from normal. Nuclei were slightly enlarged, elongated, and

Fig. 3 Well (a), moderately (b), and poorly (c) differentiated adenocarcinoma. (Haematoxylin and eosin.) Original magnification $\times 150$.

crowded, but uniform in size and shape. Nuclear polarity and mucus secretion were retained. In severe dysplasia nuclei were enlarged, ovoid, and stratified with loss of polarity. Mitoses were numerous. Nucleoli were prominent and mucus was reduced or absent. Moderate dysplasia showed an intermediate pattern (Fig. 2). Well differentiated adenocarcinoma resembled adenoma. Tubule configuration was regular and nuclear polarity retained. In moderately differentiated cases tubule configuration was regular or slightly irregular and nuclear polarity was just discerned or lost. In poorly differentiated carcinoma tubules were highly irregular or absent (Fig 3).

SAMPLING

Three 50 μ m sections were cut from the paraffin embedded tissue blocks. These thick sections were adjacent to the 4 μ m sections used for histopathological grading. This method of sampling was an important feature of this study; thick sections from different parts of the same adenoma sometimes showed clones with differing ploidy.

NUCLEAR SUSPENSION

These were prepared using a modification of the method described by Hedley *et al.*¹⁵ The sections were dewaxed with two treatments of 4 ml xylene incubated at 37°C for 10 minutes, followed by graded rehydration with 3 ml of 100%, 95%, 75%, 50% ethanol and distilled water (10 minutes each). Regular vortex mixing was carried out. The tubes were then centrifuged (500 g, five minutes) and the supernatant aspirated. The pellets were resuspended in 2 ml of pepsin (1% in 0.9% sodium chloride, pH 1.5) (Sigma) and incubated at 37°C for 90 minutes. Single nuclear suspensions were obtained by vortex agitation and pipetting. The suspensions were assessed and counted in a Neubauer chamber before filtering through a

20 μ m nylon mesh. The filtrates were immediately put on ice and centrifuged (500 g, 10 minutes).

STAINING

The pellets were resuspended in 1 ml propidium iodide (0.25 mg/ml in phosphate buffered saline) (Sigma) with 1 ml ribonuclease-A, type 1-AS (0.5 mg/ml in phosphate buffered saline (Sigma) to remove double stranded RNA stained by the propidium iodide and incubated for 15 minutes at 37°C. The tubes were again centrifuged (500 g, 10 minutes) and the resultant pellets resuspended in 1 ml phosphate buffered saline. Selected samples were checked by phase contrast and fluorescence microscopy. A permanent record was made by putting a drop from each sample on a slide, drying it in air, and staining it with haematoxylin.

MEASUREMENT OF FLUORESCENCE

Each sample was filtered through a 75 μ m nylon mesh immediately before flow analysis. Flow cytometry was carried out on FACS I cell sorter (Becton Dickinson, FACS System, Sunnyvale, California). Argon-ion laser excitation, 200 mW at 488 nm was used (Spectral-Physics, Mountain View, California) and fluorescence measured using a 610 nm long pass filter. The instrument was aligned by using chicken red cells fixed in glutaraldehyde. Ten thousand nuclei were analysed at a rate of 500 per second.

CONTROLS

Each set of samples to be analysed was started on a sample of normal mucosa or diploid adenoma to fix the diploid G1/0 peak at a standard channel (channel 60). (G2M peak would be at channel 120 and the intervening area would represent S phase). In addition, each sample possessed an internal control such as lymphocytes, or stromal cells, which enabled fine

Fig. 4 DNA histograms of (a) normal rectal mucosa (b) normal lymph node.

Fig. 5 DNA histograms of (a) diploid (above) and aneuploid (below) adenoma (b) diploid (above) and aneuploid (below) carcinoma.

Fig. 6 Aneuploid G1/0 peak coinciding with diploid G2M peak.

adjustments in the instrument to be made. The mean lymphocyte content of an adenoma sample was 16% ($n = 13$, range 3%–47%) and of a carcinoma sample was 20% ($n = 10$, range 7%–27%). Carcinoma sections also contained a proportion of normal mucosa, which, when dissected free, gave a diploid histogram, as did lymphocytes from lymph nodes that contained no metastasis (Fig. 4).

CRITERIA FOR ANEUPLOIDY

Tumour cells sharing a common G1/0 peak with that of lymphocytes and stromal cells were defined as diploid, while those possessing one or more population(s) distinct from diploid were defined as aneuploid (Fig. 5). Aneuploid cells with their G1/0 peaks coinciding with diploid G2M peaks were easily discernible because they were taller with distinctive tetraploid S and G2M phases (Fig. 6). A few equivocal cases (three of 203 cases of cancer) were resolved by repeating the analysis with more nuclei (70000) to detect any separate S and G2M phases. The Pearson χ^2 test and the test for trend in proportions were used.

Table Incidence of aneuploidy in graded series of adenoma and carcinoma (figures in parentheses are numbers (%))

Adenomas (n = 269)			Carcinomas (n = 203)		
Mild dysplasia	Moderate dysplasia	Severe dysplasia	Well differentiated	Moderately differentiated	Poorly differentiated
5/134 (4)	19/107 (18)	10/28 (36)	18/29 (62)	92/144 (64)	19/30 (63)

Results

Thirty four of the 269 adenomas (13%) were aneuploid. The Table shows the incidence of aneuploidy in adenomas (stratified according to grade of epithelial dysplasia). The association between grade of dysplasia and aneuploidy was highly significant (χ^2 for heterogeneity = 22.42, $p < 0.001$; trend $Z = 5.1$, $p < 0.001$). This trend exceeded that for size and aneuploidy ($Z = 4.2$, $p < 0.001$). No significant trend was observed for architectural type and aneuploidy. One hundred and twenty nine of the 203 adenocarcinomas (64%) were aneuploid. The incidence of aneuploidy in adenocarcinomas (stratified according to grade of differentiation) is collated with the adenoma data in the Table. There was no association between aneuploidy and grade of differentiation in adenocarcinoma (χ^2 for heterogeneity, $p = \text{NS}$; trend Z , $p = \text{NS}$).

Discussion

It has been suggested that the only difference between adenoma (or dysplasia) and carcinoma is that adenoma does not show malignant infiltration, in spite of having acquired the potential to invade.² Such a view would seem to deny the existence of any fundamental qualitative difference between adenoma and carcinoma. Isaacson extends this hypothesis in his assertion that mild, moderate, and severe dysplasia represent the intraepithelial counterparts of well, moderately, and poorly differentiated adenocarcinoma.³

In this study a large series of adenomas and carcinomas were graded according to the criteria described in detail by Konishi and Morson¹³ and Jass *et al*¹⁴ respectively. For adenomas the association between grade of dysplasia and aneuploidy was highly significant, whereas no such association was seen for grade of differentiation in rectal carcinoma. This finding suggests that the grading of adenoma and carcinoma are fundamentally different exercises. This also contrasts with Isaacson's statement that the terms differentiation and dysplasia (in context of the grading of neoplasia) are interchangeable.³ The results of the present study are not altogether surprising, as the histopathological criteria for grading

dysplasia and carcinoma are not, in fact, identical. In dysplasia equal emphasis must be placed on cytology, differentiation, and architecture,¹³ whereas architecture (tubule configuration) is of paramount importance in the grading of carcinoma.¹⁴ Isaacson could not distinguish (either morphologically or immunocytochemically) between adenoma and carcinoma stratified into their three respective grades.³ The present study, however, highlights a striking difference in ploidy patterns when the same exercise is undertaken. The main disparity is seen with mild dysplasia in adenoma (4% aneuploid) and well differentiated adenocarcinoma (62% aneuploid). It must be emphasised that flow cytometry provides a rapid but fairly crude measurement of DNA content and is likely to underestimate the chromosomal differences of adenoma and carcinoma. Direct chromosome analysis is time consuming but provides a far more sensitive measure of chromosomal aberration. It has been shown, for example, that diploid adenomas are associated with an entirely normal karyotype.^{16 17} On the other hand, the concept of a diploid carcinoma is more apparent than real; studies of karyotype show a constellation of trisomies and rearrangements.¹⁸

The concept of adenoma and carcinoma as a neoplastic continuum seems attractive and is certainly supported by recent advances in the field of molecular biology. Of particular interest have been the presence of an increased expression of the *ras* oncogene in the course of neoplastic transformation¹⁹ and similar patterns of gene hypomethylation in adenoma and carcinoma.²⁰ Such studies need to be confirmed and expanded; they will ultimately answer the fundamental question posed in this paper. Most mucin and enzyme histochemical studies support the stepwise concept, with mild dysplasia deviating minimally from the normal, and only severe dysplasia showing cancer associated phenotypes.²¹ Studies on cell culture and animal transplantation studies also highlight important biological differences between adenoma and carcinoma cells.^{17 22}

The prevalence and distribution of adenomas within necropsy specimens of colorectum have been studied in geographical areas with differing risk of colorectal cancer. Such studies provide powerful evidence that adenoma and carcinoma are funda-

mentally different lesions. Firstly, the prevalence of adenoma may be the same in areas of high and low risk of colorectal cancer.²³ Secondly, adenomas (unlike carcinomas) are distributed evenly within the colon with a predilection, if any, for the caecum.²⁴ It has been argued that environmental factors responsible for the conversion of adenoma to carcinoma are concentrated in the left colon; this would account for the sigmoid colon and rectum being the preferred sites for carcinoma, as well as large or severely dysplastic adenomas.¹³

It could be argued that the differences in the two hypotheses examined in this paper are more apparent than real. Both accept that the adenoma is neoplastic. One emphasises the differences between adenoma and carcinoma and the other their similarities. We believe that the second hypothesis falters at the clinical, biological, and epidemiological level. The experimental and epidemiological evidence strongly supports the traditional view that places the adenoma firmly in an intermediate position between normal tissue and carcinoma.

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