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The relationship between aneuploidy and *p53* overexpression during genesis of colorectal adenocarcinoma

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Abstract This paper describes the investigation of nuclear DNA content and *p53* immunoreactivity in normal mucosa (n=25), mildly (n=15), moderately (n=28) and severely atypical (n=22) colorectal adenomas and in colorectal adenocarcinomas (n=116). Twenty-seven per cent of the mildly atypical, 43% of the moderately, 77% of the severely atypical adenomas and 91% of the colorectal carcinomas were distinctly aneuploid. In the aneuploid lesions *p53* immunoreactivity was not observed in mildly atypical adenomas, whereas 17% of the moderately atypical, 24% of the severely atypical adenomas and 66% of the adenocarcinomas were *p53* positive. None of the diploid lesions were *p53* immunoreactive. These data are interpreted to indicate that genomic instability as reflected by crude aneuploidy occurs early during genesis of colorectal carcinoma and represents a high risk factor for *p53*-gene mutation.

Key words Ploidy · *p53* · Carcinogenesis · Colorectal neoplasia

Introduction

Carcinogenesis in the colorectal mucosa is suggested to be a multistep process including a number of specific genetic alterations ultimately resulting in a malignant transformed epithelium. According to Fearon and Vogelstein (1990) colorectal tumours appear to develop as a consequence of the mutational activation of oncogenes coupled with the inactivation of tumour-suppressor genes. Essential mutations in at least four or five genes seem to be required to produce malignant neoplasm. The total accumulation of genetic alterations rather than their chronologic order is suggested to determine the biological properties of tumour.

Lawrence Loeb (1991) hypothesized that "normal" spontaneous mutation rates (about 10^{-10} mutations/nucleotide/cell generation) cannot account for the four or more independent and specific mutations required for malignant transformation. He proposed the necessity of a hypermutation phenotype in order to achieve the critical frequency of genetic alterations. In line with this hypothesis in a number of chemically induced experimental malignancies (Konaka et al. 1982; Näslund et al. 1987) gross aneuploidy, indicating increased genetic instability, was observed to regularly precede invasive growth. Thus, for example, in beagle dogs (Konaka et al. 1982) 20-methylcholanthrene-induced bronchogenic squamous cell carcinoma only occurred in mucosal areas exhibiting longlasting aneuploidy (one-tenth–one-sixth of the expected beagle dog life time).

Aneuploidy has also been observed in the vast majority of colorectal carcinomas (Böhm and Sandritter 1975). Interestingly, aneuploidy was found to be already present in more than one-third of the small colorectal adenomas with mild histopathological atypia and in as many as about 90% of the adenomas with severe atypia. These data suggest that increased genetic instability as indicated by gross aneuploidy, generally precedes final malignant transformation of the colorectal mucosa.

Alteration of the *p53* tumour suppressor gene is the most frequently observed genetic lesion in human malignancies (Levine 1992). *p53* is a nuclear phosphoprotein which is thought to control cell growth at the G_1/S checkpoint (Levine et al. 1991). Numerous studies have demonstrated a high incidence of *p53* alteration in colorectal adenocarcinoma (Vogelstein et al. 1988; Baker et al. 1990; Bartek et al. 1991; Hollstein et al. 1991; Pignatelli et al. 1992). Since allelic loss as well as point mutations of the *p53* gene were found in colorectal adenomas, especially in large and/or severely atypical adenomas – frequent precursors of adenocarcinoma – *p53* alteration was thought to play a central role in the transition of

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premalignant to malignant colorectal lesions (Fearon and Vogelstein 1990).

The aim of the present work was to study the relationship between genetic instability as reflected by crude aneuploidy and *p53* mutation during pathogenesis of colorectal adenocarcinoma. Our results indicate that aneuploidy precedes *p53* mutation and that aneuploid clones might represent one category of the proposed hypermutatic phenotype, increasing the risk for multistage tumorigenesis.

Material and methods

The tumour material comprised 65 colorectal adenomas from 51 patients (27 females, 24 males, mean age 63.7 years, age range 20–90 years) and 116 colorectal adenocarcinomas from 116 patients (63 females, 53 males, mean age 68.8 years, age range 36–92 years) living in the state of Schleswig-Holstein (Germany). Surgical material from curative treatment was fixed in buffered neutral formalin and paraffin-embedded. The size of the adenomas was measured as the largest diameter (mm) in formalin-fixed tissue. The size ranged from 2 to 20 mm (mean: 5.5 mm).

From each tumour specimen four contiguous, 4 µm histological sections were prepared: one for haematoxylin and eosin staining for diagnostic histology; two contiguous sections for Feulgen staining and DNA analysis; and one for *p53* immunohistochemistry.

The adenomas were classified into tubular, villous and tubulovillous types according to the recommendation of the World Health Organization (1972). The degree of morphologic alterations of the epithelium and glands was recorded as mild, moderate and severe atypia. In mild atypia there was a mostly monolayered epithelium with mainly basally aligned small nuclei, exhibiting varying degrees of hyperchromasia; the glandular arrangement mostly regular. In moderate atypia minor loss of polarity with enlarged and irregularly shaped nuclei exhibiting varying degrees of hyperchromasia was seen. Increased nuclear-cytoplasmic ratio was evident with some folding of epithelial cells into the glandular lumen. Severe atypia showed loss of polarity, enlarged, hyperchromatic nuclei, enlarged nucleoli and abundant mitotic figures, a pronounced increase in nuclear-cytoplasmic ratio and distorted glandular structures with the so-called glandular "back-to-back" arrangement. There was frequent intraglandular bridging and budding. For DNA analysis 4 µm sections were cut from formalin-fixed, paraffin-embedded specimens, deparaffinized and refixed in formalin. The cell and tissue preparations were rehydrated in decreasing ethanol concentrations and exposed to acid hydrolysis in 5 N hydrochloric acid (HCl) at 22° C for 60 min. Then the specimens were rinsed in distilled water and stained with Schiff's reagent for 90 min at room temperature. After rinsing in distilled water again, the samples were washed three times in sulfide rinsing solution (10 ml sodium metabisulphite, 10 ml HCl, 180 ml distilled water). Finally, they were rinsed under running tap water, dehydrated in an increasing ethanol scale, transferred to xylene, and mounted in Eukitt (refractory index 1.494).

The tumour area for DNA measurement was selected based upon morphological criteria. Within the selected areas, DNA measurement in individual cell nuclei was performed at random. The specimens were evaluated on a television-based image analysis system, using a microscope (Nikon, plan objective 40/0.95) equipped with a video-CCD camera. In each case at least 100 neoplastic cell nuclei were measured, avoiding nuclei with indistinct nuclear membranes, and nuclei lying too close together, or overlapping. As an internal standard, at least 20 normal colorectal epithelial cells were assessed, and their median value was accepted as the "diploid" or "2c" DNA value.

DNA histograms obtained by image cytometry of 4 µm thick histological sections were interpreted according to a modified subjective method. The original method was used for the classification of DNA profiles obtained in fine-needle aspiration specimens from breast tumours (Auer et al. 1980). DNA histograms characterized by a single peak in the "diploid" or "near-diploid" region (1.5c–2.5c) of normal cells were classified as type I. The total amount of cells with DNA values exceeding the "diploid" region (>2.5c) is ≤10%. Type II shows a single peak in the "tetraploid" region (3.5c–4.5c) or a peak in both the "diploid" and "tetraploid" region (>20% of the total cell population). The number of cells with DNA values between the "diploid" and "tetraploid" region and those exceeding the "tetraploid" region (> 4.5c) is ≤10%. Type III histograms were characterized by DNA values ranging between the "diploid" and "tetraploid" region. Only a minor number of cells with DNA values exceeding 4.5c were present (<5%). Type IV shows increased and/or distinctly scattered DNA values exceeding the "tetraploid" region (>5%).

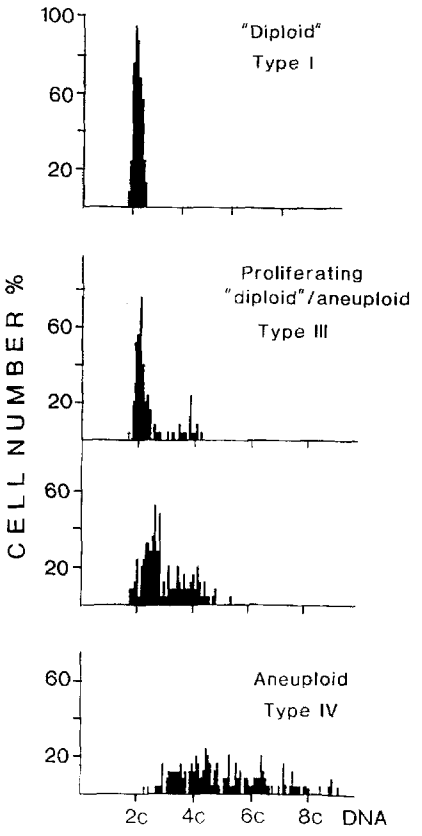

Fig. 1 Examples of "diploid" (type I), proliferating "diploid"/aneuploid (type III) and distinct aneuploid (type IV) DNA content histograms. The 2c-value denotes the DNA content in normal diploid G_0/G_1 colorectal mucosa cells

In the present investigation only type I, type III and type IV histograms were observed. Type I histograms were suggested to indicate the existence of "near-diploid" populations with relatively high genomic stability, whereas type IV histograms were suggested to reflect distinctly aneuploid populations with decreased genomic stability. Type III histograms are difficult to interprete and may comprise both highly proliferating "near-diploid" populations and aneuploid populations (Fig. 1).

Immunohistochemical staining for *p53* was performed using an avidin-biotin-peroxidase complex technique. Tissue sections, 4-µm-thick, were prepared from formalin-fixed, paraffin-embedded specimens, dewaxed and dehydrated. The slides were treated in 0.05% protease (Sigma P5417) at 22° C. Endogenous peroxidase activity was blocked by immersion of slides in 0.5% hydrogen peroxide in aqua dest. for 20 min. Non-specific staining was blocked by incubating sections in 1% bovine serum albumin (BSA) in TRIS-buffered saline for 45 min. The sections were in-

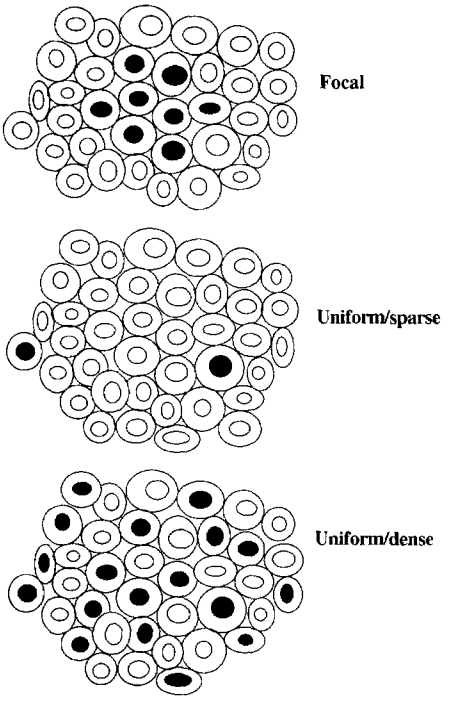

Fig. 2 Schematic illustration of the main distribution patterns of *p53* immunoreactive cells

cubated with *p53* CMI (Novocastra, 1: 700) in BSA overnight at 4° C. After that the sections were washed in TRIS-buffered saline. They were processed according to the routine avidin-biotin-peroxidase complex technique. Biotinylated anti-rabbit affinity-purified IgG (Vector BA-100, Vector Laboratories, Burlingame, Calif.: diluted 1: 200 in TRIS-buffered saline) was applied for 45 min, followed by a similar incubation with avidin-biotin-peroxidase complex (Vectastain Elite, Vector). Diaminobenzidine was used as chromogen. After the slides were counterstained with haematoxylin, they were dehydrated and mounted.

Only cells with a distinct brown staining confined to the nuclei were regarded as *p53* positive. MDA 231 human breast cancer cells were used as *p53* positive external staining control and morphologically normal cells in each specimen served as internal negative controls. *p53* immunoreactivity in histopathologically selected normal, dysplastic and neoplastic tissue was scored arbitrarily. The lowest numbers of scored cells were between 1,000 and 2,000. The following *p53* distribution types were recorded (see Fig. 2): uniform/dense (UD), uniform/sparse (US) and focal (F).

Results

Ploidy and *p53* overexpression was investigated in normal mucosa (n=25), mildly (n=15), moderately (n=28) and severely (n=22) atypical colorectal adenomas and in invasive adenocarcinomas (n=116). It can be seen from Fig. 3 that 100% of the normal mucosa specimens exhibited a "diploid" pattern. This number decreased to 53% in adenomas with mild atypia, 39% in adenomas with moderate atypia, 18% in adenomas with severe atypia and zero in colorectal adenocarcinomas. None of the "diploid" adenoma specimens showed immunohistochemical *p53* positivity.

A minority of the adenoma and adenocarcinoma cases exhibited a DNA distribution pattern comparable with

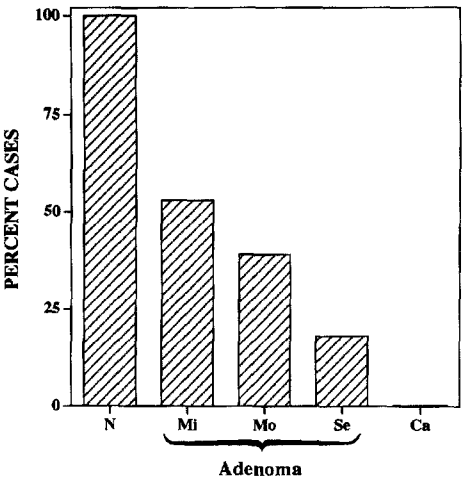

Fig. 3 Percent of cases with "diploid" (type I) DNA profiles in normal colorectal mucosa (*N*), in adenomas with mild (*Mi*), moderate (*Mo*), severe (*Se*) atypia and in colorectal adenocarcinomas (*Ca*). None of these "diploid" cases showed *p53* immunoreactivity

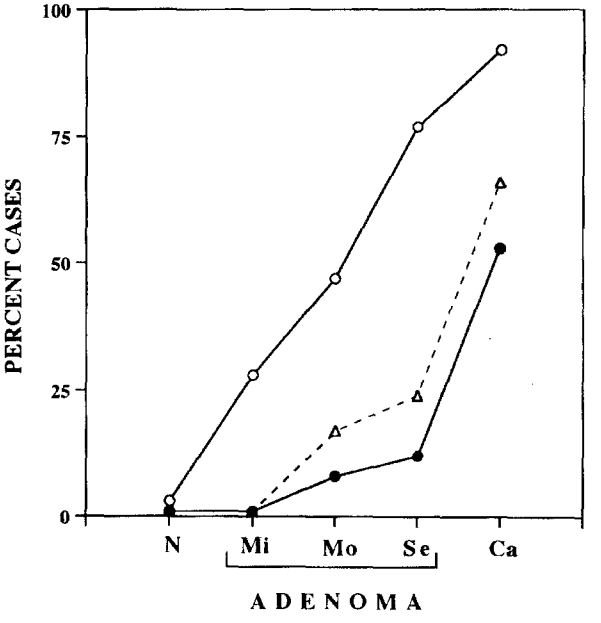

Fig. 4 Percent of cases with distinctly aneuploid (type IV) DNA profiles (○–○) and *p53* immunoreactivity [(△–△) all p53 positive cases, i.e. focal, uniform/sparse and uniform/dense distribution types; (●–●) only uniform/dense distribution type] in normal colorectal mucosa (*N*), in adenomas with mild (*Mi*), moderate (*Mo*), severe (*Se*) atypia and in colorectal adenocarcinomas (*Ca*)

that found in proliferating "diploid" populations. This type of DNA histogram may include cases with moderate aneuploidy which cannot be discriminated from proliferating "diploid" cases since they lack a detectable stemline in the region between 2c and 4c. In adenomas histopathologically graded as mildly, moderately or severely atypical 20%, 18% and 5% respectively showed a proliferating "diploid"/moderately aneuploid DNA profile. *p53* immunoreactivity was demonstrated in none of these cases. Nine percent of the colorectal adenocarcinomas exhibited proliferating "diploid"/moderately aneuploid

DNA profiles. Only 6% of these adenocarcinomas were *p53* positive (all of F- or US-type). It is also clear from Figure 4 that the percentage of neoplastic lesions with pronounced aneuploidy increased progressively from 27% in mildly atypical adenomas to 43% in moderately atypical adenomas, 77% in severely atypical adenomas and 91% in invasive adenocarcinomas. The percentages of *p53* positive cases were zero in mildly atypical adenomas, 17% (8% of UD-type, 9% of F- and US-type) in moderately atypical adenomas, 24% (12% of UD-type, 12% of F- and US-type) in severely atypical adenomas and 66% (53% of UD-type, 13% of F- and US-type) in invasive adenocarcinomas.

Discussion

Human malignancies can be subdivided by means of quantitative DNA cytochemistry into euploid and distinctly aneuploid types. Euploid tumours are characterized by minor quantitative or qualitative chromosomal aberrations or quantitative DNA alterations hardly detectable by means of karyotyping or DNA cytochemistry. Distinctly aneuploid tumours however are characterized by pronounced chromosomal aberrations and DNA distribution patterns clearly differing from those found in normal cells.

In invasive malignancies, aneuploidy has been demonstrated to correlate with aggressive tumour behaviour. Tumours generally associated with fatal outcome, such as bronchogenic squamous carcinoma (Nasiell et al. 1978), small cell lung cancer (Yoneyama et al. 1987), squamous carcinoma of the uterine cervix (Böhm and Sandritter 1975) and oesophageal carcinoma (Munck-Wikland et al. 1989), are highly aneuploid, whereas tumours associated with long survival, such as papillary thyroid carcinomas (Cohn et al. 1984) or carcinoids (Nobin et al. 1987) show diploid DNA profiles. Data from experimental animal model systems (bronchial and uterine cervical mucosa in dogs and mice) indicate that aneuploidy occurs early during carcinogenesis and probably reflects disturbance of DNA replication and/or mitosis in mortal cells (Ono et al. 1984; Näslund et al. 1987). Removal of the carcinogen (20-methylcholanthrene, 3,4-benzopyrene) at an early stage of carcinogenesis always resulted in the replacement of the aneuploid populations by normal cells (Ono et al. 1984). In contrast, a minor number of chemically induced more advanced premalignant lesions, histologically generally classified as severe atypia/carcinoma in situ and exclusively all invasive lesions exhibited autonomous expansion of the aneuploid clone independent of ongoing exposure to the carcinogen (Ono et al. 1984). This type of experiment also showed that in the organs studied (lung and cervix uteri) preexisting aneuploidy was a prerequisite for final malignant transformation.

Although the mechanism of *p53* tumour-suppressor gene involvement in the regulation of the normal cell cycle remains uncertain, increasing evidence indicates that functional inactivation of this gene by mutation or allelic loss is one of the most frequently observed gene alterations in human malignancies (Bartek et al. 1991). In colorectal adenocarcinomas, mutated *p53* has been demonstrated in 80%–90% of all tumours. In addition, studies in premalignant colorectal lesions suggest that an alteration of *p53*-gene function usually occurs late during carcinogenesis, generally close to the stage of final malignant transformation (Fearon and Vogelstein 1990). This observation is in agreement with the results reported herein. With the exception of two cases (adenomas with moderate atypia), all *p53* immunoreactive lesions belonged to lesions histopathologically defined as adenomas with severe atypia and invasive adenocarcinomas. The most important observation of the present study is that *p53* immunoreactivity was only observed in aneuploid lesions. Taken together the data suggest that *p53* overexpression is associated with pronounced genomic disturbance and instability as reflected by crude aneuploidy and that this alteration of the genome precedes *p53* immunoreactivity.

In this study we have not performed *p53*-gene sequence analysis. We are therefore not able to claim that *p53* overexpression examined by an immunohistochemical technique in formalin-fixed paraffin-embedded specimens as done herein in fact reflects mutated *p53*. However, the level of cellular wild-type *p53* in normal tissue is quite low probably because of the short half-life of the protein in most tissues. This is suggested to be the reason why wild-type *p53* is not usually detectable by means of immunocytochemistry. In contrast, all of the mutant *p53* proteins examined to date have strikingly prolonged half-life times resulting in increased protein amounts detectable by immunohistochemical methods. It is, therefore, reasonable to suggest that the immunohistochemical assay used herein detects mutated *p53* preferentially. If this is the case, our data support the hypothesis that cells exhibiting genomic instability as indicated by aneuploidy represent "hypermutation phenotype cells" with increased mutation rates, thereby increasing the risk of *p53*-gene mutations. Nevertheless, it has been suggested that cells in which the *p53* pathway is inactivated by mutation of *p53* or by host (MDM2) or viral oncoprotein, replicate damaged DNA, resulting in mutation, aneuploidy, mitotic failure and cell death Lane 1992; Carder et al. 1993). Failure of normal *p53* function may thus be one of the mechanisms through which aneuploid cells originate. Furthermore, it has been reported that treatment of normal cells with ultraviolet light induced the accumulation of wild-type *p53* through a posttranslational protein stabilization mechanism (Maltzman and Czyzyk 1984). Damage of the genome in colorectal cells by carcinogens could therefore result in increased amounts of normal *p53*. The biological reason for this is to stop cells from passing through the cell cycle and to keep altered cells growth-arrested until the damaged DNA is repaired so that cell division can result in healthy daughter cells.

It is unlikely that posttranslational stabilization and increase of wild type *p53* to immunohistochemically detectable levels is a reasonable explanation for the results reported herein. Ultraviolet light induced elevated wild-type *p53* occurs early after cell exposure, whereas the results of the present investigation indicate *p53* alterations as reflected by *p53* overexpression after longlasting genetic instability. Thus, distinct genomic disturbance, most probably induced by carcinogens, was observed in as many as 27% of the mildly atypical adenomas without detectable *p53* immunoreactivity. Furthermore, the marked correlation of increased *p53* positive lesions with the transition of severely atypical adenomas to invasive adenocarcinomas indicate that *p53* alterations as reflected by *p53* overexpression described in this work occurs late during malignant transformation.

Thus the addition of *p53* and ploidy studies of colorectal tumours to routine histological stains may provide additional information. Aneuploidy is suggested to characterize premalignant lesions while high risk for final malignant transformation is indicated by *p53* gene alteration.

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