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Numerical chromosome alterations in colorectal carcinomas detected by fluorescence in situ hybridization

Relationship to 17p and 18q allelic losses

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Abstract This study concerns DNA ploidy, numerical changes of chromosomes 7, 8, 10, 17 and 18, and allelic losses at chromosomes 17p13.3 (flanking the *p53* gene) and 18q21 (location of the *DCC* gene) in 31 freshly resected colorectal tumours. Cytological smears were used to determine DNA ploidy by image analysis, and chromosome numbers by fluorescence in situ hybridization (FISH) using chromosome-specific pericentromeric α-satellite DNA probes. Allelic losses were assessed by Southern blotting and by the polymerase chain reaction loss of heterozygosity method. Approximately 50% of the tumours were aneuploid. There was heterogeneity with respect to chromosome numbers, but gains and losses of chromosomes, or both, were detected in all carcinomas examined, including 10 that were nonaneuploid by image analysis. Trisomy 7 was found in 74% of the tumours, and monosomy of chromosome 18 in 32%. Allelic loss at chromosome 17p13.3 was evident in 13 of 26 informative cases, and only one case exhibited monosomy 17. In comparison monosomy 18 was found in 10 cases; 7 of them corresponded to approximately half of the cases with allelic loss within the *DCC* gene, and the other three were noninformative. These findings indicate that the loss of one chromosome 18 is an important mechanism producing allelic deletion of the *DCC* gene in colorectal carcinomas. Our data also suggest that monosomy 18 is a useful indicator for studying colorectal cancer progression on a cell by cell basis.

Key words Colorectal cancer · Fluorescence in situ hybridization · Polymerase chain reaction · *p53* · *DCC*

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Introduction

Nuclear abnormalities involving size, shape, and chromatin patterns are common in cancer and usually provide a means of identifying tumour cells. In addition, flow-cytometric [1, 10, 18, 19] and image analysis [2, 5–7, 42] studies have shown that alterations in the DNA content of cancer cells reflect the aggressiveness of a given tumour. Recent advances in molecular biology have established that a series of molecular events is involved in carcinogenesis, and these studies have led to the discovery of numerous oncogenes and tumour suppressor genes. They have confirmed that single nucleotide changes in these genes can be detected in tumour cells.

Colorectal carcinoma is one of the potentially most informative systems in this field, since the multistep involvement of molecular aberrations of the *APC*, *RAS*, *DCC* and *p53* genes in the genesis and progression of the tumour can be clearly defined [43]. Of these, *p53*, located on chromosome 17p13.1, is a tumour suppressor gene, and its mutation in conjunction with allelic loss plays a cardinal role in the development of many common human malignancies [43]. The *DCC* gene, located on 18q21, a chromosome in which allelic deletions are frequently detected in invasive colon cancer, encodes a product that shares high homology with neural cell adhesion molecules. Moreover, it is highly likely that *DCC* acts as a metastatic suppressor [14].

The cytogenetic approach, by which chromosomal defects or changes are documented, represents a resolution level that falls between the estimation of gross nuclear DNA content and molecular abnormalities. Thus, by using banding techniques it has been shown that chromosomes 17 and 18 are nonrandomly involved in colorectal cancers [4, 13, 26, 27] and chromosomes 7 and 8, in colon adenomas and cancers [26, 35]. However, the application of conventional karyotyping procedures to solid tumours has been limited by the inherent technical difficulties [15, 41, 44, 45]. Since solid tumours have low mitotic indices and contain large amounts of necrotic tissue and connective tissue, the rate of successful initiation

of primary cultures of cancer cells to be used in cytogenetic studies is usually low [41]. These considerations are particularly pertinent to cancers of the gastrointestinal tract, in which the usual presence of bacteria and other microorganisms often leads to contaminated cultures. Moreover, the cell population that does grow may not necessarily be an in vitro reflection of the true representation of the tumour [9, 15].

To circumvent these technical problems we used fluorescence in situ hybridization (FISH) with specific pericentromeric DNA probes for the detection of chromosomal aberrations on cytological smears obtained from patients with colorectal carcinomas. The probes used recognize α-satellite repetitive DNA sequences that span the centromeric region and are unique for each individual human chromosome. Hence, their use permits chromosome enumeration, even though a positive signal may not necessarily represent an intact chromosome [9, 40, 45]. In the present study we compare DNA ploidy, numerical changes of chromosomes 7, 8, 10, 17, and 18, and allelic deletions at chromosome 17p13.3 and within the *DCC* locus of colorectal carcinoma cells.

Materials and methods

Tissue samples

This study was carried out on specimens from 31 consecutive patients with colorectal adenocarcinoma, who were operated at the Department of Surgery of the Cancer Research Institute Hospital of Kanazawa University. In 4 cases Dukes' stage A was recorded; in 9, B; and in 18, C (Table 1). Tumour tissues and noncancerous mucosa distant from the cancer (each specimen about 0.8 cm^3) were dissected immediately after the operation. One portion of the tissue was used for DNA extraction, and the remainder, minced with a scalpel and incubated for 1 h at 37° C in Eagle's minimum essential medium (ICN Biochemicals, Tokyo, Japan) containing 5% calf serum and 0.1% collagenase (Wako Chemicals, Tokyo, Japan), and vortexed intermittently. Following filtration through 50-μm nylon mesh (Swiss Silk Bolting Cloth, Thal, Switzerland), the cell suspension was washed with phosphate-buffered saline, fixed with Carnoy's solution (methanol:acetic acid, 3:1) and stored at −20° C until use.

Image analysis

Smears of the nuclear suspensions were prepared on silanized glass slides (Dako, Glostrup, Denmark) and stained by the Feulgen reaction. Identically prepared material from a nontumorous part of the colon was used as a control. The nuclear DNA content was measured with the MIAMED DNA Image Analyzer (Leitz, Wetzlar, Germany) [2]. To establish the diploid value, the nuclei of 100 normal colon epithelium cells were analysed on the same slide. The tumours were classified according to Böcking et al. [5] as (a) nonaneuploid, when the DNA value of the stemline was within 2c±cv of the reference cells; (b) suggestive of aneuploidy, when the DNA value of the stemline was within i±cv of the reference cells (where i=4c, or 16c), and (c) aneuploid, when the DNA value of the stemline was outside of i±cv of the reference cells (where i=2c, 4c, 8c or 16c).

Fluorescence in situ hybridization

Numerical changes affecting chromosomes 7, 8, 10, 17 and 18 were determined by using chromosome-specific pericentromeric α-satellite repeat DNA probes purchased from Oncor (Gaithersburg, Md.). Nuclear suspensions from cancer tissue and normal colon mucosa were smeared separately on the same silanized slide. Denaturation, hybridization and posthybridization washings were done according to the manufacturer's protocols. Subsequently, following blocking with 1% bovine serum albumin (BSA)/4×SSC at room temperature for 5 min, the slides were incubated with fluorescein isothiocyanate (FITC)-labelled streptavidin (1:100, Vector Laboratories, Burlingame, Calif.) for 20 min at 37° C. The slides were then washed three times with 4×SSC/0.1% Triton X-100 at room temperature for 10 min, stained with propidium iodide/antifade (Oncor) and examined with an epifluorescence microscope (Olympus, Tokyo, Japan). Parallel slides were routinely stained with Giemsa stain and used for cell identification. The number of hybridization signals with each of the five probes was determined by examining more than 200 nuclei per case. Hybridization of a given DNA probe to nuclei of normal colonic mucosa cells served as control.

Numerical chromosome aberrations were considered as a chromosome loss (monosomy) when the percentage of nuclei with one signal exceeded 25%, and as a chromosome gain (trisomy, tetrasomy etc.) when the percentage of nuclei with three, four, or more signals exceeded 15%. These cut-off levels were based on the upper limit (mean+2SD) of the decreased fraction of normal colon mucosa in 24.9% and increased fraction in 14.2% (Table 2). When the percentage of nuclei with two signals exceeded 25%, it was considered that a disomic clone was present. Although it may detect smaller subpopulations, the cut-off level chosen does not differ significantly from that used by Waldman et al. [44].

Because of possible poor probe penetration, loss of target DNA, and incomplete denaturation of the target DNA [15, 37], it was reasonable to consider that some loss of chromosomal signals could occur. Hence, to avoid misinterpretation that might result from such possible artifacts, two-colour FISH was performed in the cases in which a subpopulation of nuclei with only one signal for chromosome 18 was identified by the one-colour procedure. For this purpose, a chromosome 10-specific probe was used as the internal hybridization efficiency control. For two-colour FISH a slight modification of the protocol described by Sauter et al. [36] was used. Briefly, the denatured hybridization mixture of biotin-labelled chromosome 18-specific probe and digoxigenin-labelled chromosome 10-specific probe was applied to denatured cells on slides. The conditions of denaturation, hybridization and post-hybridization washing were the same as in one-colour FISH. After blocking with 1% BSA/4×SSC at room temperature for 5 min, the slides were incubated for 15 min at room temperature with rhodamine-labelled sheep anti-digoxigenin Fab fragment (13 μg/ml; Boehringer Mannheim, Mannheim, Germany), followed by three 10-min washings at room temperature with 4×SSC/0.1% Triton X-100. The slides were then incubated with FITC-labelled streptavidin (1:100; Vector) at room temperature for 60 min, washed three times at room temperature with 4×SSC/0.1% Triton X-100, stained with 4′,6-diamino-2′-phenylindole dihydrochloride (DAPI)/antifade (Oncor) and examined with an epifluorescence microscope equipped with a triple band filter (Chroma Technology, Brattleboro, Vt.).

Southern blot hybridization

Allelic loss on chromosome 17p was assessed by using a probe for the highly polymorphic variable number of tandem repeat (VNTR) locus YNZ22, which is located at 17p13.3 [28]. DNA was extracted from tumour tissue and normal colon mucosa using the DNA extraction kit purchased from Stratagene (La Jolla, Calif.) and following the manufacturer's protocol. Digestion with *BamH1*, agarose gel electrophoresis, Southern transfer to nylon membranes and hybridization were performed by using standard procedures [31]. The DNA probe pYNZ22 was obtained through the Japanese

Cancer Research Resources Bank (Tokyo, Japan). The hybridization signals were quantitated with a densitometer (Vilber Lourmat, Marne la Vallée, France). A reduction of more than 30% of the normal signal, obtained with DNA from the respective normal tissue, was recorded as an allelic loss in accordance with Matsumura et al. [22].

Polymerase chain reaction based loss of heterozygosity assay

Allelic loss in the *DCC* gene was examined by polymerase chain reaction-based loss of heterozygosity (PCR-LOH) analysis [23] of the VNTR region [12, 16] and the *MspI* restriction site (M2) [12, 16, 34] within the gene. The respective PCR primers pairs were:

5′-GATGACATTTTCCCTCTAG-3′ (VNTR sense)
5′-GTGGTTATTGCCTTGAAAAG-3′ (VNTR antisense) and
5′-TGCACCATGCTGAAGATTGT-3′ (M2 sense)
5′-AGTACAACACAAGGTATGTG-3′ (M2 antisense)

Genomic DNA (500 ng) was added to 50 µl buffer [containing 50 µM of each deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 µg/µl BSA and 1 µM each of the sense and antisense primers] and denatured at 94° C for 5 min. Then, 2.5 U of recombinant *Taq* polymerase (Takara, Otsu, Japan) was added, and PCR was run for 35 cycles at 94° C for 1 min, 57° C for 30 s and 72° C for 30 s. Negative reaction controls from which template genomic DNA was omitted were included in each PCR run. VNTR polymorphism was determined by separating the respective PCR products on 2.5% agarose gels (Nippon Gene, Toyama, Japan). To determine M2 polymorphism, the PCR products were electrophoresed on 2.5% agarose gels, and the approximate 396-bp bands excised under ultraviolet light and their DNA extracted with SUPREC-01 (Takara, Othu, Japan). The DNA thus obtained was digested with the restriction endonuclease *Msp-1* (Toyobo, Osaka, Japan) in accordance with the manufacturer's instructions, and the resultant fragments, separated on 2.5% agarose gels. The intensity of individual bands was measured by densitomeric scanning, and a reduction of more than 30% was judged as representing allelic loss.

Results

The smears usually had variable numbers of inflammatory cells and connective tissue cells. However, it was not difficult to identify cancer cells by referring to Giemsa-stained preparations and to the configuration of propidium-iodine-stained nuclei.

FISH with each of the five pericentromeric probes used revealed that approximately 90% of the nuclei of normal colonic epithelial cells and connective tissue cells had two signals (Table 2). Even though it is not clear why 2.2±6.0% of normal cells had more than two signals with the chromosome 7 probe [3], these observations indicated the high probability that each probe reacts with its true target, and that the counting of the resulting fluorescent signals provides valid information with respect to the copy number of a given chromosome. Example of FISH on colon cancer cells are shown in Fig. 1. Examination of >200 nuclei of each tumour sample per probe disclosed numerical abnormalities (in the form of gains

Table 1 Chromosome copy numbers[a] determined by FISH analysis and ploidy patterns of colorectal carcinomas (*A* aneuploid, *SA* suspicion of aneuploidy, *N* nonaneuploid)

Case no.	Dukes' stage	Ploidy	Chromosome				
			7	8	10	17	18[b]
1	A	N	2	2/1	2	2	1 (74%)
2	A	N	3/2	2	2	2	2
3	A	SA	2/3	2/3	2	2	1/2 (58%)
4	A	A	3/2	2	2	2	2
5	B	N	2	2/1	2	2	2
6	B	N	2/3	2	2	2	1/2 (58%)
7	B	N	2	3/2	2	2	2
8	B	N	3/4/2	3/2	2	3/2	3/2
9	B	SA	5/4	2	2/3	4/2/3	2
10	B	A	2	2	1/2	2/1/3	2
11	B	A	4/2/3	4/2/3	2/3	4/2/3	1/2 (70%)
12	B	A	3/4	5/7/6/3	3/2	4	2
13	B	A	4/3/5/2	4/5/3	5/4/3/2	5/4	2
14	C	N	2	3/2	2	2	2/1 (39%)
15	C	N	2/3	2	2	2	2/1 (34%)
16	C	N	2/3	2	2	2	1/2 (72%)
17	C	N	4/2/3	3/4	2	2	2
18	C	SA	2/3	2	2	3/2	2
19	C	SA	3/4/2	3/2/4	2	2	2/3
20	C	SA	5/4/3/2	5/4/3/2	3/2	4/3/2	2
21	C	SA	4/3	4/3	3/2	4/3	3/2
22	C	A	3	2	2	2	2
23	C	A	3/4/2	2	2	2/3	2
24	C	A	3/2	3/2	2	2	2
25	C	A	3/2	1	2	2	1 (89%)
26	C	A	3/2	2/3	2	2/3	2
27	C	A	5/3/2	3/2	2	2/3	1/2 (52%)
28	C	A	5/4	3/2	3/4	4/2	2
29	C	A	2/3	2/3	2/3	4/3/2	2
30	C	A	2	4/2/3	2/3	4/2/3	2/1 (27%)
31	C	A	5/4/3	5/4/3/2	3/2/4	2/4/3	2

[a] Values indicating chromosomal copy numbers are arranged in order of predominance
[b] Percentages of tumour cells with monosomy 18 are in parentheses

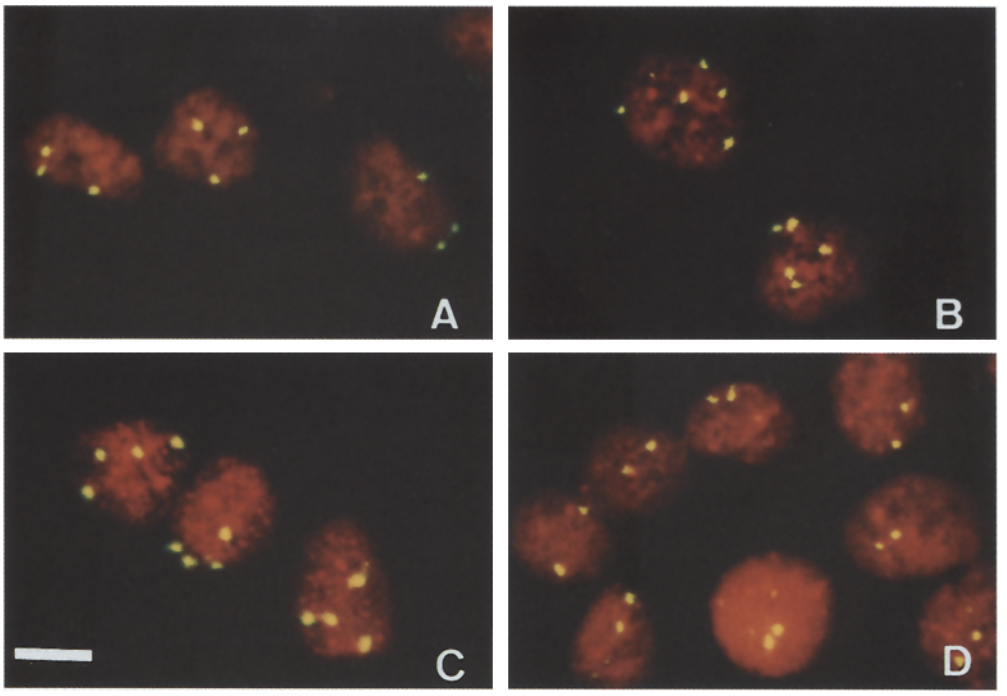

Fig. 1A–D Examples of fluorescence in situ hybridization (FISH) on nuclear smears of colon cancer using chromosome-specific DNA probes (case 12). This case shows **A** three signals for chromosome 7, **B** five for chromosome 8, **C** four for chromosome 17, and **D** two for chromosome 18. *Bar* 10 μm

or losses, or both) of chromosome 7, 8, 10, 17 and 18 in all 31 tumours, irrespective of their DNA ploidy (Table 1). However, the aneuploid tumours and those suggestive of aneuploidy had significantly more nondisomic chromosomes (average: 3.3) than the nonaneuploid tumours (average: 1.9); (Mann-Whitney test, $P<0.05$). Furthermore, numerical changes greater than tetrasomy were not found in the nonaneuploid cases. Intratumour heterogeneity with respect to the copy number of a given chromosome was evident in some cases (Table 1). In certain instances, abnormal copy numbers were seen concurrently in different chromosomes within individual tumours (e.g., chromosomes 7, 8 and 17 in case 11), although all tumours had some imbalance with respect to chromosome numbers (Table 1). Corroborative evidence that the results obtained with tumour cells were not due to a different affinity of a given probe for its target chromosome was provided by the absence of significant differences in the chromosome copy numbers of the normal cells (Table 2). Our results indicate that a single tumour cell may gain or lose different chromosomes.

Monosomy 18 was found in 10 of the 31 patients; the respective percentage are indicated in Table 1. Subjecting these 10 cases to two-colour-FISH verified that more than 25% of the tumour cells of each had a single signal for chromosome 18, and two or three signals for chromosome 10 (Fig. 2). From the results of two-colour FISH it can be concluded that the finding of a single copy of chromosome 18 in 32% of the tumours was not due to low hybridization efficiency, and that the 10 cases are indeed monosomic with respect to this chromosome. Monosomy of chromosomes 8, 10 and 17 was infrequent, and there was no case with monosomy 7 (Table 1).

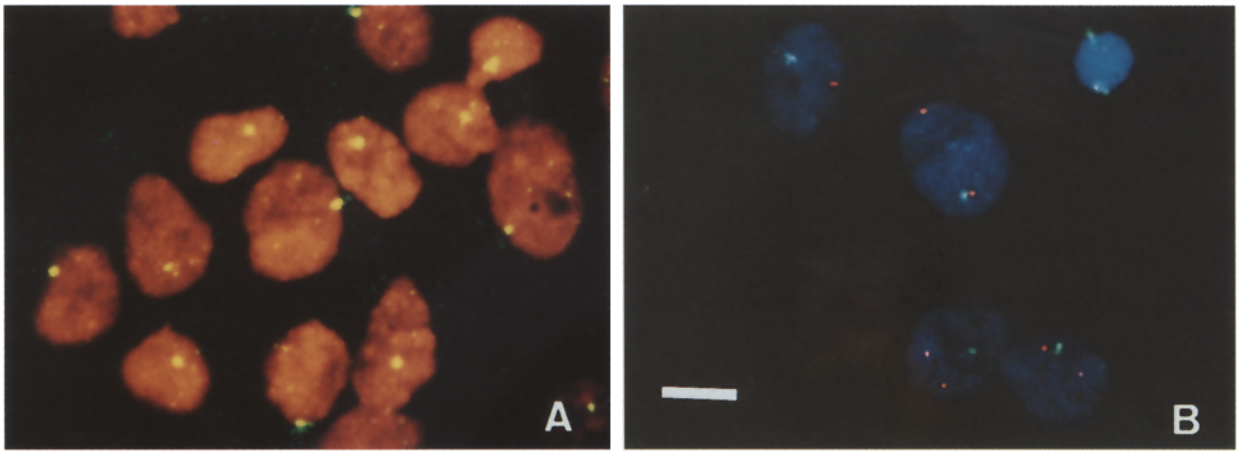

Fig. 2 **A** FISH for chromosome 18 and **B** two-colour FISH for chromosomes 18 (FITC detection, green signals) and 10 (rhodamine detection, red signals). Monosomy 18 and disomy 10 are readily evident (case 6). *Bar* 10 μm

Table 2 FISH analysis of normal colorectal mucosa cells

Copy no.	Chromosome[a]				
	7	8	10	17	18
0	0.5±0.8	0.5±0.9	1.2±2.1	0.7±0.5	1.3±1.9
1	5.8±5.8	6.4±6.4	6.9±6.3	8.9±6.3	5.1±6.0
2[b]	92.0±8.0	92.3±8.2	89.9±8.8	89.4±6.8	92.9±6.6
3	2.2±6.0	0.5±1.1	0.8±1.6	1.3±1.5	0.4±1.4
>4	0	0.1±0.2	0.3±0.4	0.1±0.2	0.5±0.5

[a] Percentage of nuclei with a given copy number of chromosomes (mean±SD)
[b] No significant difference between any two of the chromosomes (Mann-Whitney U test)

Table 3 Loss of heterozygosity (*He* heterozygous, *LOH* loss of heterozygosity, *Ho* homozygous)

Case no.	17 p 13.3 polymorphic site	*DCC* polymorphic sites	
	YNZ 22	VNTR	M2
1	He	LOH[a]	Ho
3	He	Ho[a]	Ho
4	Ho	Ho	Ho
5	He	Ho	He
6	He	LOH[a]	He
7	He	He	Ho
8	He	He	Ho
9	He	Ho	Ho
10	He[a]	Ho	Ho
11	LOH	Ho[a]	Ho
12	LOH	Ho	Ho
13	He	LOH	LOH
14	He	Ho[a]	Ho
15	LOH	LOH[a]	Ho
16	LOH	LOH[a]	Ho
17	Ho	He	He
18	He	Ho	Ho
19	He	Ho	Ho
20	LOH	LOH	LOH
21	LOH	He	He
22	LOH	Ho	Ho
23	LOH	LOH	Ho
25	LOH	LOH[a]	He
26	He	Ho	He
27	LOH	LOH[a]	Ho
28	LOH	Ho	LOH
29	Ho	LOH	Ho
30	LOH	LOH[a]	He
31	LOH	LOH	Ho

[a] Cases with monosomy according to fluorescence in situ hybridization

Trisomy of chromosome 7 was found in 23 cases and in three of them it was the sole chromosome numerical change. Although it was the most frequent single chromosomal alteration, the overall frequency of chromosome 7 trisomy did not differ significantly from that of chromosome 8 trisomy.

Allele analysis was performed on samples of the 29 patients from which adequate amounts of DNA could be extracted. LOH at 17p13.3 was found in 13 of 26 informative cases (Table 3). Figure 3 shows the results of Southern blot analysis of representative cases. Of the 13 tumours with the 17p deletion, there was only one (case 10) with monosomy 17 as determined by FISH analysis.

Allelic loss at the *DCC* gene was assessed by LOH at the VNTR and M2 sites, or both (Fig. 4). Of the 29 tumours analysed, 19 were informative and 13 of them (68%) had allelic loss. Of 10 cases with a monosomic population of chromosome 18, 7 (cases 1, 6, 15, 16, 25, 27 and 30) had LOH at the *DCC* gene; the other 3 were not informative.

Discussion

Colorectal carcinoma is one of the cancers in which the molecular events occurring in genesis and progression have been clarified [43], and numerous molecular genetic studies have focused on the biological behaviour of this cancer [20, 30, 39]. Several investigations have shown that DNA aneuploidy is associated with aggressive behaviour of colorectal carcinomas, and it has been proposed that this abnormality is a good parameter for indicating a poor prognosis [1, 10, 18]. Both DNA aneuploidy and molecular genetic alterations represent genetic changes of cancer cells, albeit at different levels, and

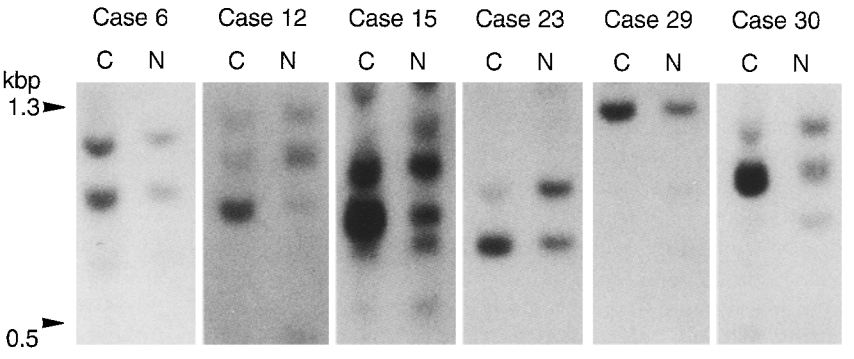

Fig. 3 Southern blot hybridization with pYNZ22. A spectrum of polymorphic *Msp*1 restriction fragments, ranging from 0.5 to 1.3 kbp, are shown in the autoradiograph. There is loss of heterozygosity (LOH) in cases 12, 15, 23 and 30. No LOH is seen in case 6, and case 29 is uniformative. *C* Carcinoma, *N* normal tissue

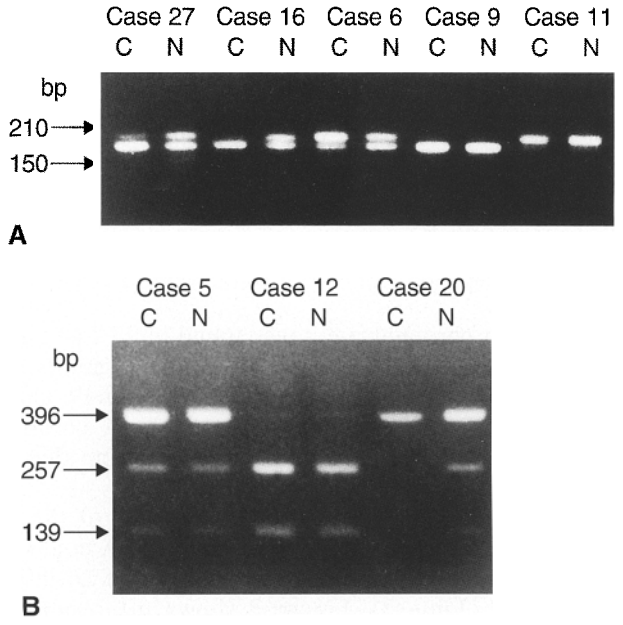

Fig. 4A, B LOH of *DCC* gene in colorectal carcinomas, revealed by polymerase chain reaction (PCR). Representative cases of LOH at the VNTR (**A**) and M2 (**B**) sites are shown. **A** A spectrum of fragments ranging from 150 to 210 bp is generated by PCR. Allelic imbalance is seen in the carcinomas, but not in the normal tissues of cases 27, 16 and 6. Cases 9 and 11 are uninformative. **B** PCR amplification of the M2 site within the *DCC* gene produces a 396 bp fragment, which *Msp*1 cleaves into a 257 and a 139 bp fragment. There is no LOH in the cancer tissue from case 5. In case 20, the 257- and 139-bp fragments, detected in normal tissue, are lost in the cancer tissue, indicating LOH. Case 12 is uninformative because the amplified 369 DNA fragments are homogeneously cut by *Msp*1

they are tightly intertwined. For example, genetic alterations appear to be linked to chromosomal instability and to the rather frequent occurrence of chromosomal aberrations [10]. Conversely, the various numerical chromosome abnormalities induced by nondisjunction or mitotic recombination are thought to cause amplification and deletion of a variety of oncogenes and tumour suppressor genes.

However, few studies have addressed the relationship of DNA aneuploidy and molecular genetic alterations in colorectal carcinomas [11, 32, 35]. This is probably due to the extreme complexity of the karyotypic alterations in fully developed cancer, which in turn has thus far prevented the application of conventional methods for such investigations. However, as shown here, this problem can be overcome by choosing the appropriate techniques. In the present study we first evaluated the relationship between DNA aneuploidy and chromosome number abnormalities, determined by image analysis and FISH, respectively. As our data indicate, all 31 cases, including the 10 that were nonaneuploid, had some numerical chromosome abnormality. These observations demonstrated that FISH was a more sensitive method for detecting nuclear DNA changes than image analysis of Feulgen-stained smears. This is not surprising, since the ploidy patterns determined by flow cytometry or by image analysis reflect total nuclear DNA content. However, as shown here, a loss or gain of a variety of chromosomes can occur in a single nucleus. Moreover, the DNA content of a single chromosome is equivalent to only a very small portion of the total genomic DNA that is detectable by image analysis. It is relevant to indicate in this context that in an earlier study it was calculated that the DNA content of chromosome 18 represents only 1.4% of the total DNA concentration of a normal diploid cell [24].

Although our results showed that the numerical chromosome changes were rather complex and associated with inter- and intratumour heterogeneities, two relatively common abnormalities were evident. The first, trisomy of chromosome 7, found in 23 of the 31 cases, was the most frequent numerical chromosome alteration, even though it was not significant compared with trisomy of chromosomes 8. Other studies have shown that trisomy 7 is one of the most frequent changes seen in several types of solid tumours [9, 26], and it has been speculated that when present in an extra copy number, genes on chromosome 7 (epidermal growth factor receptor, for example) confer a growth advantage [44]. Trisomy 7 is especially frequent in adenomas of the large intestine. According to Mitelman's tabulation of conventional karyotype analysis of 29 colon adenomas, 28 had trisomy 7 and in 13 of these it was the sole chromosome change [20]. Longy et al. [21] reported that trisomy 7 was one of the three chromosomal aberrations seen in carcinoma in situ of the colon. Trisomy 7 was found in about one-half of 20 colorectal carcinomas analysed in direct preparation or after short-term culture, but it was not the only change [26]. It is thus possible that trisomy 7 is the first nonrandom numerical chromosome change that occurs in the adenoma→carcinoma sequence and that this alteration is kept in some fully developed cancers.

The second frequent change observed by us was monosomy of chromosome 18. It is of significance in this context that Muleris et al. [27], upon reviewing the results of chromosome analyses of 100 cases of colorectal cancer, found that the most frequent alterations were a rearrangement of chromosome 17 and the loss of one chromosome 18. They grouped the cases with a chromosome 18 loss into two categories, and indicated that 28 cases had a single chromosome 18 (hemizygous cases) and 42, a relative deficiency of the chromosome [27]. By comparison, we found monosomy 18 in 10 patients, and monosomy 17 in 1. Monosomies of chromosome 17 and 18 are particularly important in cancer, since these chromosomes carry the tumour suppressor genes *p53* and *DCC*, respectively. Moreover, the loss of function of these genes is associated with allelic loss, even though the allelic loss is not essential for a *p53* gene with a negative dominant type mutation [11].

Allelic loss cannot be detected on a cell-by-cell basis at the present time without using such an elaborate method as microdissection, but it is safe to assume that a tumour cell with a monosomal chromosome will have lost

genes located on the chromosome in question. Since it is very unlikely that a clonal tumour is composed of a mosaic of cells having a particular chromosome of paternal and maternal origin, allelic losses should be detectable by PCR-LOH if the tumour is composed predominantly of monosomic cells. When our FISH and PCR-LOH results are compared, we see that 7 (cases 1, 6, 15, 16, 25, 27 and 30) of the 10 monosomy 18 cases corresponded to 54% of the PCR-LOH-positive cases, with the other 3 being noninformative. We adopted a reduction of 30% as the cut-off level for PCR-LOH, because this reportedly parallels Southern blot analysis [22, 23]. If the allelic loss found in these 7 cases is explained only by monosomy 18 cells, theoretically more than 30% of the monosomic population is necessary for such a result, and certainly more if we consider that the extracted DNA is diluted by nontumour DNA. In 5 of the 7 cases (cases 1, 6, 16, 25 and 27) in whom more than 50% of cells were affected by monosomy 18, the FISH and PCR-LOH results were in agreement. However, the positive PCR-LOH results in cases 15 and 30 cannot be explained by monosomy 18 alone. It is probably that the disomic tumour cells in these 2 cases also had a deleted *DCC* gene. Partial deletion is known to be a major mechanism in the allelic loss of *p53*. Two-colour FISH studies using a 17-α-centromeric probe and a *p53*-specific cosmid probe have shown that allelic loss of p53 in breast cancer [22] and in bladder cancer [36] takes place in most instances by way of the deletion of the short arm or a small portion of chromosome 17. We used the same experimental strategy and found that gastric cancers with *p53* allelic loss have a diminished number of *p53* signals compared with the 17 centromeric number, and also absence of monosomy 17 (in preparation). Therefore, we conclude that monosomy 18 is one of the major mechanisms of allelic loss of the *DCC* gene in colorectal cancers, but not the only one.

In the tumour progression model of Nowell [29], a successful mutation is followed by local clonal expansion, and the continued expansion, associated with successive generations of the mutation expansion cycle, is thought to account for the formation of fully developed cancers. Hence, the analysis of the spatial relationships of the clonal populations permits the documentation of the manner in which the evolution of related and unrelated clones produces regional karyotype differences [8]. It is of interest in this context that case 10 showed no LOH of 17p despite the presence of a monosomy 17 clone as the second major subpopulation. There are several possible explanations for the discordance between Southern blotting and FISH data. First, the major population of heterozygous disomic cells may have masked the allelic loss because the monosomic population was small. The second possibility is that the monosomic clone and the third clone of trisomy 17 were derived from a heterozygous tetrasomic cell through nondisjunction. In this case, Southern blot hybridization would not detect LOH. As FISH is applicable to histological sections, this technique can provide information with respect to chromosomal changes on a cell by cell basis. Thus, Micale et al. [25] have recently used FISH to detect losses of chromosomes 16, 17 and 18 in both breast cancer tissues and the neighbouring hyperplastic epithelia and proposed that precancerous lesions can be identified by this technique. Whether this also applies to colorectal tumours remains to be investigated. Nevertheless, as our data indicate, monosomy 18 represents a clonal change of advanced colorectal cancer and an indirect marker for allelic loss at the *DCC* gene locus.

Recent cytogenetical and molecular genetics studies have clearly shown that there are relationships among the type of chromosomal alterations, the pathological data, and the clinical behaviour of colorectal cancers [4, 13, 17, 30, 33, 38]. Thus, Takahashi et al. [38] found a significant correlation between LOH on chromosome 17p and the presence of lymphatic and/or vascular microinvasion. Moreover, Gerdes et al. [13] indicated that cytogenetic and/or molecular alterations of chromosomes 17 and 1 were predictive of poor survival, but that an alteration of chromosome 18 or 5 was not an important prognostic indicator. Ookawa et al. [33] suggested that concordant *p53* and *DCC* alterations play important roles in the acquisition of metastatic potential by colorectal carcinomas, since LOH and/or rearrangement at the *p53* and *DCC* loci were detected in all patients from whom metastatic tumours were examined. In another study [17] it was shown that patients with Dukes' stage A cancer usually have a normal life span, whereas those with disseminated disease (stage D) have a very poor survival rate. The prognosis of patients with Dukes' stages B and C cancer is somewhere in between, and they are potential candidates for adjuvant chemotherapy and radiation therapy [17]. Furthermore, O'Connell et al. [30], on the basis of an analysis of disease-free survival after surgical resection in 60 patients, concluded that allelic loss on chromosome 18q is indicative of poor prognosis for individuals with Dukes' B or C tumours, suggesting the possibility of adjuvant chemotherapy for such patients. Moreover, Jin et al. [17] also found that the patients whose Dukes' stage B cancer exhibited 18q allelic loss had a less favourable prognosis than those with tumours without the allelic loss, and that the chromosome 18q allelic loss had no significant prognostic value in patients with the more advanced stage C disease, even though the frequency of the allelic loss was higher in Dukes' C than in Dukes' B tumours. This set of observations suggests that early detection of clones with 18q allelic loss, documented by FISH as monosomy 18, may benefit patients with potentially poor prognosis by identifying their need for adjuvant therapy. Consequently, the finding of monosomy 18 by FISH would indicate that the application of this technique can not only provide information on the clonal progression of colorectal cancers, but also help the clinician in making appropriate therapeutic choices.

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