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Tamotsu Sugai · Shin-ichi Nakamura
Wataru Habano · Noriyuki Uesugi · Hajime Sato
Osamu Funato · Shunichi Sasou · Seishi Orii
Chuichi Itoh

Analysis of subclonal expansion of colorectal carcinomas by flow cytometry

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Abstract DNA heterogeneity of colorectal carcinomas has been investigated by flow cytometry; most studies have focused on the clinical usefulness of DNA ploidy analysis. Since cancers consist of predominant subclones with proliferative advantage due to clonal expansion, we attempted to analyse the clonal expansion of colorectal carcinomas within a tumour by measuring DNA ploidy. The DNA ploidy and heterogeneity of multiple fresh samples obtained from 164 colorectal adenocarcinomas were analysed by flow cytometry. Each tumour was divided into an average of six specimens, which were analysed separately. For 146 of the tumours (89%) at least one DNA aneuploid population was found within the cancer tissue examined. DNA multiploidy was detected in 26 cases (17.8%) among the cancers with aneuploidy. Based on the DNA index (DI), hypertriploid aneuploidy (1.7<DI<1.8) was found most frequently in the aneuploid colorectal cancers examined. DNA ploidy heterogeneity was seen in 75 (51.4%) of the DNA aneuploid tumours. There were only 3 cases with more than three subclones including a diploid line. The present results indicate that colorectal carcinomas consist of a few dominant subclones and have a DNA content (hypertriploid aneuploid) that confers a proliferative advantage.

Key words Subclonal expansion · DNA ploidy · Colorectal carcinoma

T. Sugai · S.-i. Nakamura (✉) · W. Habano · N. Uesugi
H. Sato · O. Funato · S. Sasou
Division of Pathology, Central Clinical Laboratory,
School of Medicine, Iwate Medical University,
Uchimaru 19-1, Morioka, 020-8505 Japan
e-mail: nakamurashin@jsn.justnet.or.jp
Tel.: +81-19-651-5111, Fax: +81-19-629-1437

S. Orii
First Department of Internal Medicine, School of Medicine,
Iwate Medical University, 19-1, Morioka, 020-8505 Japan

C. Itoh
Department of Clinical Pathology, School of Medicine,
Iwate Medical University, 19-1, Morioka, 020-8505 Japan

Introduction

Intratumour heterogeneity in DNA content has been demonstrated in colorectal carcinoma [12, 26]. Carcinoma of the colorectum is characterized by homogeneous histological appearances in different areas of the tumour, but primary malignant tumours are not always uniform, being composed of different subpopulations with different properties [21].

Recent molecular genetic data indicate that most neoplasms arise from a single altered cell, which expands as a neoplastic "clone" within a tumour mass [3, 16, 21, 22]. The clone evolves into variant subclones with additional genetic alteration, and these subclones possess new and different characteristics that enable local invasion and metastasis. The presence of multiple subclones within the neoplasm results in intratumour heterogeneity [21, 22], since a tumour mass consists of multiple expanded subclones derived from the original clone. The subclones may have different DNA content and may acquire a growth advantage. Consequently, these dominant subclones occupy the whole tumour. Intratumour DNA heterogeneity may result from the selection of predominant subclones, and the phenomenon may represent the expansion of multiple subclones in individual tumours.

Intratumour DNA heterogeneity has had a substantial impact on the study of DNA ploidy, as information obtained from a single part of the tumours may not be representative of the entire specimen [24]. DNA heterogeneity may provide valuable information regarding not only the clinical behaviour, but also the biological features of the tumour, such as clonal expansion. Because recent studies have focused on the clinical usefulness of intratumour DNA heterogeneity [12, 26] in prognosis, the biological aspects of this phenomenon have not been fully delineated. Therefore, using DNA ploidy as an indicator of tumour clonal expansion, we studied DNA heterogeneity in multiple fresh tissue samples from colorectal carcinomas.

Materials and methods

A total of 164 patients with colonic cancer who had undergone colectomy at the Iwate Medical University Hospital, Japan, between 1994 and 1997 were examined. They included 107 men and 57 women, with an age range of 35–92 years (mean age, 61.6 years).

Of the 164 tumours evaluated, 68 were located in the rectum and the remainder (96 cases) in the colon. Because no differences in the clinicopathological variables examined in this study were observed between the rectal and colonic carcinomas, both groups were analysed together.

Tumor location was classified as right-sided (proximal to splenic flexture; n=130) and left-sided (n=34) colon. Each histopathological diagnosis was made according to the criteria proposed by the Japanese Research Society for Cancer of the Colon and Rectum [13], and tumour stage was determined according to the modified Dukes' classification system [30].

Histological analysis revealed that all tumour samples were from adenocarcinomas. Histological grading of the differentiation revealed that 64 cases were well differentiated, 92 moderately differentiated and 5 poorly differentiated, and that the remaining 3 were mucinous carcinomas. Application of the Dukes' classification system for the evaluation of tumour stage revealed that 31 carcinomas were in stage A, 56 in stage B, 55 in stage C and 22 in stage D.

Three to nine tumour samples were taken from the central and peripheral parts of a freshly resected tumour specimen for DNA flow cytometry (mean: 6 samples). Normal colonic mucosa taken from the oral surgical margin of the resected colon was used for the control samples. Every tumour sample analysed was confirmed as cancerous by histology. To correlate flow cytometric results with histological assessment, the paraffin-embedded tissue used for histology was obtained from regions adjacent to the fresh samples.

The samples were minced with a scalpel into minute pieces, and then suspended in ice-cold phosphate-buffered saline (PBS) containing 0.1% Triton X-100 to isolate nuclei from the cytoplasm. The samples were filtered through a nylon mesh (37 µm, Tokyo screen, Tokyo). After treatment with RNAase (0.1 µg/ml, Sigma, St. Louis, Mo.), isolated nuclei were stained with propidium iodide (PI; Calbiochem, La Jolla, Calif.) at a final concentration of 50 µg/ml. The samples were analysed using a flow cytometer (EPICS XL, Coulter, Fla).

For each sample, 10000 cells were analysed. Normal stromal cells such as lymphocytes and fibroblasts present in each sample served as the internal standard. The DNA index (DI) was calculated as the ratio of the mode of the aneuploid peak to that of the normal peak in the DNA histogram. DNA diploid/near-diploid tumours were characterized by the presence of one G0/G1 peak, and their DIs were equal to 1.00. If there was a distinct shoulder on the slope of the diploid peak but no split extra peak, the case was considered to be near-diploid. Tumours that had at least one G0/G1 peak separate from a diploid peak were defined as DNA aneuploid. DNA multiploidy was regarded as the presence of two or more distinct aneuploid G0/G1 peaks. To confirm the presence of the near-diploid aneuploid peak, a mixture of tumour and normal isolated nuclei was measured. An appropriate mixture ratio sometimes identified two distinct peaks in near-diploid aneuploid cases. For confirmation of the accuracy of DNA measurement, the coefficient of variation (CV) was calculated for the tumour G0/G1 peak of each DNA histogram. Tumours with a CV greater than 5.0% were excluded from subsequent analysis.

When DNA indices of different samples in the same tumour differed by more than 10%, it was considered to be composed of heterogenous clones [31].

The data were analysed using the Chi-square test, with the aid of StatView-II software (Abacus Concepts, Berkeley, Calif.). Statistical significance was accepted at $P<0.05$.

Results

Flow cytometric analysis of DNA content in the 164 carcinomas showed that 146 were aneuploid (89%) and 18 were diploid/near-diploid (11%). Among the aneuploid tumours, 26 (17.8%) were classified as multiploid: 24 multiploid tumours each contained two aneuploid stemlines, and 2 each had three different aneuploid stemlines in the one specimen. The DI of the co-existing second or third DNA aneuploid stemlines (higher DI) were less than twice the DI of the first tumour aneuploid stemlines (lower DI) on the same histogram in 24 of the 26 cases (Fig. 1). The co-existing second or third DNA aneuploid stemlines showed random DIs on the same histogram.

Clinicopathological differences between diploid and aneuploid tumours are shown in Table 1. The two groups did not differ with respect to age, tumour location, histological grade or tumour stage. The clinicopathological features did not differ between diploid and aneuploid cancers.

The distribution of the DIs of all samples is shown in Fig. 2. Two peaks of DI values were found between 1.0 and 1.70 to 1.80. Aneuploid DIs ranged from 1.05 to 3.35 (mean 1.72±0.25). There were only 4 tumours in which the DIs exceeded 2.3.

A total of 75 carcinomas (51.4%) consisted of intratumour heterogeneous clones of DNA ploidy, and 56 of these each contained two different subclones: 29 cases contained aneuploid and diploid subclones and 27 contained aneuploid subclones showing different DIs within a tumour. Sixteen tumours each contained three different

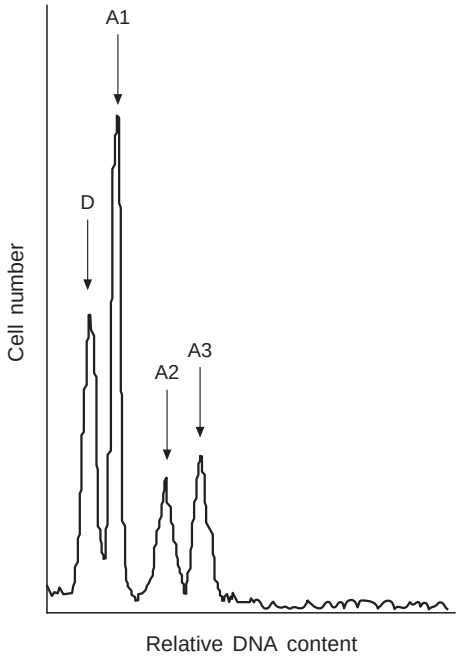

Fig. 1 Multiple DNA aneuploid (*A*) subclones in a specimen (aneuploid G1 peaks indicated by *arrows*, A1, DNA index 1.32; A2, 1.78; A3, 2.25). Neither the A2 nor the A3 subclone has a DNA index twice that of the A1 subclone (*D* diploid)

Table 1 Clinicopathological features in diploid and aneuploid colorectal cancers (*WDA* well-differentiated adenocarcinoma, *MDA* moderately differentiated adenocarcinoma, *PDA* poorly differentiated adenocarcinoma, *MC* mucinous carcinoma)

	Diploid (%)	Aneuploid (%)
Age		
50>	2 (11.1)	14 (9.6)
≥50	16 (88.9)	132 (90.4)
Location		
Left-sided colon	15 (83.3)	115 (78.8)
Right-sided colon	3 (16.7)	31 (21.2)
Histological grade		
WDA	7 (38.9)	57 (39.0)
MDA	11 (61.1)	81 (55.5)
PDA	0	5 (3.4)
MC	0	3 (2.1)
Dukes' stage		
A	6 (33.3)	25 (17.1)
B	5 (27.8)	51 (34.9)
C	5 (27.8)	50 (34.3)
D	2 (11.1)	20 (13.7)
Total cases	18 (100)	146 (100)

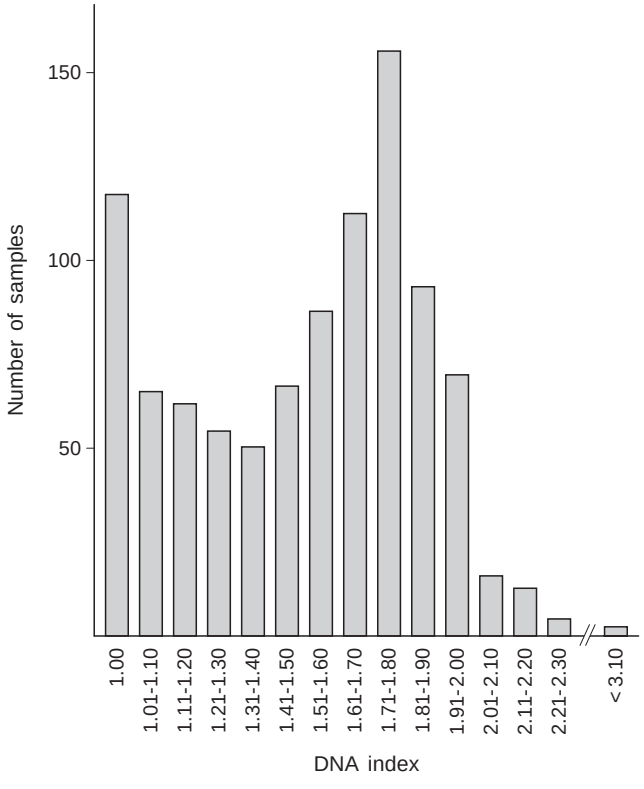

Fig. 2 Distribution of DNA indices in the 972 samples. The histogram indicates all detectable DNA stemlines

subclones: 6 tumours contained two different aneuploid and one diploid subclones and 10 tumours contained three different aneuploid subclones. Three tumours had more than three different subclones (including a diploid subclone). No more than five different subclones were observed in any one tumor (Table 2).

Table 2 Number of subclones within individual tumours (*A* aneuploid stem line, *D* diploid stem line, *A*, *A1*, *A2*, *A3*, *A4* DNA aneuploidies with different DNA indices)

Number of subclones		Number of cases	Total cases
One	D	18	89
	A	71	
Two	A, D	29	56
	A1, A2	27	
Three	A1, A2, D	6	16
	A1, A2, A3	10	
Four	A1, A2, A3, D	1	1
	A1, A2, A3, A4	0	
Five	A1, A2, A3, A4, D	1	2
	A1, A2, A3, A4, A5	1	

No correlation existed between histological differentiation and DNA content for heterogeneous and homogeneous tumours. With random selection of one sample from each tumour the probability of finding an aneuploid clone was 82%; to achieve a probability of 100% at least 5 samples were required.

Discussion

The DNA ploidy heterogeneity of colorectal carcinomas was 51.4% in the present study, a higher frequency than has been found in any previous study of colorectal carcinomas [12, 14, 25, 26, 32, 33]. Presumably this is the result of the large number of samples examined and also of the use of fresh surgical samples for the detection of near-diploid aneuploidy. Intratumour DNA heterogeneity has been demonstrated by flow cytometry in other solid tumours, including lung carcinomas [2], breast cacinomas [7], gastric carcinomas [8], renal cell carcinomas [18], and ovarian carcinomas [6]. If only a single sample is investigated, the presence of aneuploid clones of the tumour can easily be overlooked with consequent false assignment of aneuploid tumours to the diploid tumour group. Thus, multiple samples should be taken for accurate ascertainment of a tumour's ploidy status. Our study has demonstrated that at least five samples are required for accurate determinations of tumour DNA ploidy status.

DNA aneuploidy is frequently observed and may be a characteristic finding in colorectal carcinoma [4, 11, 17]. This phenomenon is thought to favour tumour growth and invasion. A previous study has indicated that aneuploid tumours may have higher proliferative activity than diploid tumours [1]. Moreover, mutation of the *p53* gene is associated with loss of wild-type *p53* function, including growth suppression [19]. Thus, cells that have acquired a *p53* mutation may have a selective growth advantage. Inactivation of p53 has been reported to be associated with the development of genomic instability and DNA aneuploidy [19, 23].

Recently, hereditary nonpolyposis colonic cancer (HNPCC), which frequently demonstrates microsatellite instability (MSI), has been the focus of attention because of its clinicopathological features. These include a young age at onset, right-sided predominance, and a tendency to a mucinous or poorly differentiated histological pattern [27]. Furthermore, this syndrome has generally been associated with a diploid pattern [27, 29]. In the present study, the incidence of these features in sporadic diploid colorectal carcinomas was similar to that in aneuploid colorectal cancers. MSI was found in 9–17% of seemingly sporadic colorectal carcinomas, and thus constitute only a small fraction of sporadic colorectal cancers. However, DNA ploidy data may explain the observation that colonic MSI-positive carcinomas are associated with a better prognosis than tumours without MSI.

We have shown that most colorectal carcinomas consist of a few neoplastic subclones. However, more than three different heterogeneous aneuploid subclones were found in only three cases, indicating that many tumours do not contain multiple dominant subclones. Interestingly, we found that an advanced colorectal carcinoma has five subclones at most. Consequently, these results are consistent with the histology of colorectal carcinomas, which show a homologous appearance.

On the basis of the distribution of DIs in human cancer, which usually shows a peak in the hypertriploid region, doubling of the nuclear DNA content is thought to be the first event in the development of DNA aneuploidy [10, 15]. Tumours with a high DI (more than 2.2) were rare in our study, suggesting that high-DI subclones may not acquire a proliferative advantage and therefore cannot be selected as dominant subclones. Our findings support the hypothesis that a decrease in genetic material in tumour cells with a tetraploid DNA content appears to be related to more pronounced anaplasia and enhanced malignant potential [9, 10, 11]. We believe that hypertriploid subclones acquire a selective proliferation advantage, resulting in predominance as subclones in a tumour mass. These subclones comprise different regions and expand within a single tumour. Flow-cytometric analysis using multiple sampling is a useful method to study such subclonal expansion in tumours.

We used the contaminating nonneoplastic cells as the internal diploid standard. If the sample examined contains a tumour diploid stemline, discrimination between the tumour diploid stemline and the nonneoplastic one is difficult on a DNA histogram. Nakamura et al. resolved this problem by using the crypt isolation technique [20]. The crypt isolation technique allows easy separation of the cancerous crypts from stroma in colorectal cancer tissue [20], thus enabling easy recognition of a tumour diploid stemline. Most colorectal cancers contain diploid and aneuploid stemlines [20]. However, the diploid tumour stemline cannot be analysed with our previous methods, since the tumour and nontumour diploid stemlines are mixed. The present study emphasizes intratumour expansion of aneuploid subclones within an individual tumour, and we did not use crypt isolation in this DNA analysis. Compared with crypt isolation, our method is an easy and simple technique for analysis of DNA aneuploid status.

Twenty-six of our the 164 tumours (17.8%) had at least one DNA multiploid sublcone (more than one different aneuploid subclone). Hiddemann et al. [12] found that the DI of the second DNA aneuploid stemline was twice that of the first in one-third of the multiploid cases examined on the same histogram. However, in the present study, in most multiploid cases the DI of the second aneuploid lines was not twice that of the first aneuploid line, and the DIs were variable. This indicates that selection of DNA aneuploid clones occurs randomly. Furthermore, we also detected three cell lines with different DNA aneuploidies in each of 2 carcinomas. Thus, it is not possible for many aneuploid subclones to coexist in the small area of the tumour.

The present study demonstrates a high incidence of aneuploidy and a high degree of intratumour ploidy heterogeneity in colorectal cancers. We suggest that DNA aneuploidy represents a secondary chromosome aberration and that DNA ploidy heterogeneity reflects increasing DNA instability [10, 11, 28]. In addition to its clinical significance, flow-cytometric DNA analysis may identify the basic aspects of tumour clonal development [5]. Our results suggest that the biological characteristics of malignant cell populations are variable.

References

1. Askensten UG, von Rosen, Nilsson RS, Auer GU (1989) Intratumoral variations in DNA distribution patterns in mammary adenocarcinomas. Cytometry 10:326–333
2. Carey FA, Lamb D, Bird CC (1990) Intratumoral heterogeneity of DNA content in lung cancer. Cancer 65:2266–2269
3. Dexter DL, Spremulli EN, Fligiel Z, Barbosa JA, Vogel R, Van Voorhees A, Calabresi P (1981) Heterogeneity of cancer cells from a single human colon carcinoma. Am J Med 71:949–956
4. Emdin SO, Stenling R, Roos G (1987) Prognostic value of DNA content in colorectal carcinoma. Cancer 60:1282–1287
5. Fischbach W, Zidianakis Z, Lüke G, Kirchner T, Mössner J (1993) DNA mapping of colorectal neoplasms: a flow cytometric study of DNA abnormalities and proliferation. Gastroenterology 105:1126–1133
6. Friedlander ML, Taylor IW, Russel P, Tattersall MHN (1984) Cellular DNA contents a stable feature in epithelial ovarian cancer. Br J Cancer 49:173–179
7. Fuhr JE, Ashley FBS, Kattine AA, Meter SV (1991) Flow cytometric determination of breast tumor heterogeneity. Cancer 67:1401–1405
8. Fujimaki E, Sasaki K, Nakano O, Chiba S, Tazawa H, Yamashiki H, Orii S, Sugai T (1996) DNA ploidy heterogeneity in early and advanced gastric cancers. Cytometry 26:131–136
9. Giaretti W (1994) A model of DNA aneuploidization and evolution in colorectal cancer. Lab Invest 71:904–910
10. Giaretti W, Santi L (1990) Tumor progression by DNA flow cytometry in human colorectal cancer. Int J Cancer 45:597–603
11. Giaretti W, Danova M, Geido E, Mazzini G, Sciallero S, Aste H, Scivetti P, Riccardi A, Marsano B, Merlo F, d'Amore ESG (1991) Flow cytometric DNA index in the prognosis of colorectal cancer. Cancer 67:1921–1927

12. Hiddemann W, Von Bassewitz DB, Kleinemeier HJ, Schulte-Brochterbeck E, Hauss J, Lingemann B, Büchner T, Grundmann E (1985) DNA stemline heterogeneity in colorectal cancer. Cancer 58:258–263
13. Japanese Society for Cancer of the Colon and Rectum (1997) Japanese Classification of Colorectal Carcinoma, 1st end. Kanehara, Tokyo, pp 30–63
14. Kallioniemi OP (1988) Comparison of fresh and paraffin embedded tissue as starting material for DNA flow cytometry and evaluation of intratumoral heterogeneity. Cytometry 9:164–169
15. Kallioniemi OP, Punnonen R, Mattila J, Lehtinen M, Koivula T (1987) Prognostic significance of DNA index, multiploidy, and S-phase fraction in ovarian cancer. Cancer 61:334–339
16. Kimball PM, Brattain MG (1980) Isolation of a cellular subpopulation from a human colonic carcinoma cell lines. Cancer Res 40:1574–1579
17. Kokal W, Sheibani K, Terz J, Harada R (1986) Tumor DNA content in the prognosis of colorectal carcinoma. JAMA 255:3123–3127
18. Ljungberg B, Stenling R, Roos G (1985) DNA content in renal cell carcinoma with reference to tumor heterogeneity. Cancer 56:503–508
19. Meling GI, Lothe RA, Borresen AL, Graue C, Hauge S, Clausen OPF, Rognum TO (1993) The TP 53 tumor suppressor gene in colorectal carcinomas. II. Relation to DNA ploidy pattern and clinicopathological variables. Br J Cancer 67:93–98
20. Nakamura S, Goto J, Kitayama M, Kino I (1994) Application of the crytp-isolation technique to flow-cytometric analysis of DNA content in colorectal neoplasms. Gastroenterology 106: 100–107
21. Nowel PC (1976) The clonal evolution of tumor cell populations. Science 194:23–28
22. Nowell PC (1986) Mechanism of tumor progression. Cancer Res 46:2203–2207
23. Offerhous GJA, De Feyter EP, Cornelisse CJ, Tersmette KWF, Floyd J, Kern SE, Vogelstein B, Hamilton SR (1992) The relationship of DNA aneuploidy to molecular genetic alteration in colorectal carcinoma. Gastroenterology 102:1612–1619
24. Okada S, Ishii H, Nose H, Okusaka T, Kyogoku A, Yoshimori M, Sakamoto M, Hiroshashi S (1994) Intratumoral DNA heterogeneity of small hepatocellular carcinoma. Cancer 75: 444–450
25. Petersen SE, Bichel P, Lorentzen M (1978) Flow cytometric demonstration of tumor-cell subpopulations with different DNA contents in human colorectal carcinomas. Eur J Cancer 15:383–386
26. Quirke P, Dyson JED, Bird CC, Joslin CAF (1985) Heterogeneity of colorectal adenocarcinomas evaluated by flow cytometer and histopathology. Br J Cancer 51:99–106
27. Rüschoff J, Bocker T, Schlegel J, Stumm G, Hofstaedter F (1995) Microsatellite instability: new aspects in the carcinogenesis of colorectal carcinoma. Virchows Arch 426:215–222
28. Sasaki K, Hashimoto T, Kawachino K, Takahashi M (1988) Intratumoral regional differences in DNA ploidy of gastrointestinal carcinomas. Cancer 62:2569–2575
29. Thibodeau SN, Bren G, Schaid D (1993) Microsatellite instability in cancer of the proximal colon. Science 260:816–819
30. Turnbull RB, Kyle K, Watson FR, Spratt J (1967) Cancer of the colon; the influence of the no-touch isolation technique on survival rates. Ann Surg 166:420–427
31. Tytor M, Wingren S, Olofsson J (1987) Heterogeneity of squamous cell carcinomas of the oral cavity studied by flow cytometry. Pathol Res Pract 187:30–35
32. Wersto RP, Liblit RL, Deitch D, Koss LG (1990) Variability in DNA measurements in multiple tumor samples of human colonic carcinoma. Cancer 67:106–115
33. Wolley RC, Schreiber K, Koss LG, Karas M, Sherman A (1982) DNA distribution in human colon carcinomas and its relationship to clinical behavior. JNCI 69:15–22