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Colorectal Cancer in Colonic Crohn's Disease – High Frequency of DNA-Aneuploidy

U. SJÖQVIST[1], R. BEFRITS[2], S. SÖDERLUND[1], A. ÖST[3], P. KARLÉN[1], B. TRIBUKAIT[4], C. RUBIO[4], P. RUTGEERTS[5], K. GEBOES[6] and R. LÖFBERG[7]

[1]*Department of Medicine, Karolinska Institutet, Stockholm Söder Hospital,;*
[2]*Department of Gastroenterology, Karolinska Institutet, Karolinska University Hospital;*
[3]*Department of Pathology, Medilab AB, Karolinska Institutet;*
[4]*Department of Pathology, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden;*
[5]*Department of Gastroenterology and* [6]*Department of Pathology, Leuven University Hospital, Belgium;*
[7]*IBD-Unit, HMQ Sophia Hospital, Department of Medicine, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden*

Abstract. *Background: The risk of colorectal cancer (CRC) in colonic Crohn's disease (CCD) seems to be of the same magnitude as in extensive, longstanding ulcerative colitis (UC) and colonoscopic surveillance has been advocated. Mucosal dysplasia and DNA-aneuploidy are early warning markers of malignant transformation in UC. Data concerning the occurrence of such premalignant lesions in CCD are scarce. Aims: The objective of this study was to investigate the DNA ploidy pattern in CCD-patients with manifest CRC, both in the tumour, as well as in the adjacent and distant colorectal mucosa. The results from DNA-flow cytometry analyses (FCM) prior to the development of a CRC in CCD were also investigated. Materials and Methods: Biopsies obtained at colonoscopy and surgical specimens from 43 patients with colonic or ileocolonic CD developing CRC between 1988 and 1998 were reviewed. The CRC histological phenotype, and the occurrence of dysplasia were registered. CRC-tissue and tissue from areas with dysplasia adjacent to and/or distant from the tumour were obtained from paraffin-embedded blocks and were analysed by FCM after preparation. Results: Twenty-four CRCs in 21 patients (14 men) were suitable for FCM-analyses. The median age at CRC-diagnosis was 53 years (21-73) and the median CCD-duration was 14.5 years (1-50). A predominance of CRC was found either in the cecum (9/24) or in the rectum (7/24). DNA-aneuploidy was found in 62.5% (15/24) of the tumours, in 25% (2/8) in adjacent and/or distant mucosa, and in 50% (2/4) of the patients that had been subjected to colonoscopic surveillance prior to the CRC-diagnosis. In 7 patients (29%), definite dysplasia was detected adjacent to and/or distant from the tumour. Of the 6 patients undergoing colonoscopic surveillance, 3 (50%) displayed definite dysplasia prior to the colectomy. Conclusion: Since DNA- aneuploidy is a common feature in CRCs in CCD and precede the development of invasive carcinoma, inclusion of FCM-analyses of colorectal biopsies may enhance the sensitivity of identifying high-risk CCD-patients prone to develop CRC within the frame of colonoscopic surveillance programs.*

The first case of cancer of the large intestine occurring in Crohn's disease (CD) was reported by Warren and Sommers in 1948 (1). Weedon *et al.* (2) reported, 30 years ago, that the cancer risk in the small and large bowel in patients with CD was nearly 20-fold increased when compared to the general population. Since then, several population-based reports in the literature have suggested that patients with Crohn's disease affecting the colon and/or rectum are at an increased risk of developing colorectal cancer (CRC) (3-5). However, some epidemiological-based studies have failed to demonstrate an increased risk of CRC in CD (6).

In 1984, Hammarberg and co-workers (7), using DNA-flow cytometry (DNA-FCM), found a good correlation between abnormal cellular DNA-content (aneuploidy) and the presence of cancer in colonic biopsies in patients with extensive ulcerative colitis (UC). A subsequent 10-year

Abbreviations: CD, Crohn's disease; CRC, colorectal cancer; DNA-FCM; DNA-flow cytometry analyses; IBD, inflammatory bowel disease; UC, ulcerative colitis.

Correspondence to: Dr Urban Sjöqvist, Department of Medicine, Karolinska Institutet at, Stockholm Söder Hospital, SE-118 83 Stockholm, Sweden. Tel: 46-8-616 35 57, Fax: 46-8-616 31 46, e-mail: u.sjqvist@telia.com

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follow-up study (8) indicated that DNA-aneuploidy could be detected many years before histological dysplasia, and it was suggested that DNA-aneuploidy could be an adjunct marker to histology to predict neoplastic transformation in longstanding UC (8-10). Only limited experience exists from prospective colonoscopic surveillance of patients with colonic Crohn's disease (CCD) using both DNA-FCM and histopathology (11) or just dysplasia (12).

In later years, an increased number of CRCs in patients with CD was noticed in the Stockholm County (13). Because of that increase, a colonoscopic surveillance program for patients with CCD was suggested.

In this light, a retrospective collection was initiated in Stockholm, Sweden and Leuven, Belgium to analyse the DNA-ploidy pattern in CD-patients with a colonic or rectal carcinoma. This study included the DNA of the tumour, as well as the DNA of the non-neoplastic mucosa near to and distant from the tumour. The DNA studies prior to the development of the CRC (*i.e.* during the surveillance period) were also available in some patients. The results of this investigation are reported below.

Materials and Methods

Patients. Biopsies and surgical specimens from 43 patients having colonic or ileocolonic CD, who developed a CRC between 1988 and 1998, were investigated. Patients were assembled either from Stockholm (Sweden) (n=34), or Leuven (Belgium) (n=9). All histological specimens were reviewed and material from the paraffin blocks analysed by FCM for assessment of DNA-ploidy pattern. The material from 21 patients (13 males and 8 females) was suitable for FCM-analyses. The median age at CRC-diagnosis was 53 years (range 21-73, mean 53.7±14 years) and the median duration of CD was 14.5 years (range 1-50, mean 16.5±12.8 years), with no differences between males and females.

Histopathology. The haematoxylin-eosin sections were reviewed in a blinded manner with regard to IBD-type, dysplasia and cancer by three of the authors (AÖ, CR and KG). Internationally accepted histological criteria (14) were strictly applied to differentiate CD from UC and indeterminate colitis. Dysplasia was classified using the criteria established by Riddell *et al.* (15) and was recorded only when the mucosa showed no active inflammation (defined as no more than infiltration of lymphocytes and plasma cells in the lamina propria) to avoid misinterpretation of reactive, inflammatory changes (16). Epithelial dysplasia was classified as either not present (NP), indefinite; probably negative (probably reactive), indefinite; unknown, indefinite; probably positive (probably dysplastic), low-grade dysplasia, high-grade dysplasia or dysplasia with an associated lesion or mass (DALM) or cancer.

DNA-flow cytometry. The paraffin-embedded biopsies were analysed as previously described (17). After deparaffination with xylene and graded alcohols, the biopsies were put in 3 ml of 95% ethanol. After 1 h at room temperature, the ethanol was removed and the biopsies were rehydrated in 2 ml water for about 1 h at room temperature. The water was replaced by 0.2 ml subtilisin Carlsberg solution (0.1% (w/v) Sigma protease XXIV (Sigma P8038, St. Louis, MO, USA), 0.1 M Tris, 0.07 M NaCl (Merck), pH 7.2) and the samples were incubated for 2 h at 45˚C in order to digest the cytoplasm of the cells. The residual pieces of connective tissue were removed, and a droplet of the resulting cell suspensions was stained with DAPI-Sulforhodamine and examined microscopically for assessment of representativeness (*e.g.* epithelial cells and absence of cytoplasm). In the vast majority of the suspensions, no or less than 5% of lymphatic cell elements were found. Biopsies with ≥10% lymphatic cell elements were excluded from cell cycle composition analysis. The incubation was continued if cytoplasm linked to cell nuclei was still seen. If bare nuclei without any residual cytoplasm were present, then the whole suspension of nuclei was stained by directly adding 0.2 ml DAPI-SR101 solution (8 mM DAPI (Sigma D9542), 50 mM Sulforhodamine 101 (SR101) (Sigma S7635), 0.1 M Tris, 0.07M NaCl, pH 7.5). After a staining time of at least 30 min, the samples were analysed using a PAS II flow cytometer (Partec, Munster, Germany) equipped with a mercury arc lamp HBO100. The suspended cell nuclei showed minimal damage and, by avoiding any centrifugation steps, showed extremely low frequencies of aggregates and background level. For cell cycle analysis, the sliced nuclei option for background subtraction of the Multicycle program (Version 3.0, Phoenix Flow System, San Diego, CA, USA) was used. The histograms were classified as diploid or non-diploid. Non-diploid cell populations were further classified by their DNA-index (position of the aneuploid G1 peak in relation to that of the diploid G1 peak (DNA-index 1.0). Tetraploidy (DNA-index 2.0) was only assumed to be present if the corresponding octaploid G2 peak was found.

Mucosal dysplasia (n=7) (histology) and DNA-aneuploidy (n=8) were also studied in biopsies near to or distant from the tumour, respectively. All available biopsies prior to surgery were also investigated (n=6).

Statistical analysis. Mean values ± SD or median values with range were used in the text for the descriptive tables.

Ethical considerations. The study was performed in accordance with the principles stated in the Declaration of Helsinki and the study was approved by the local ethics committee in Stockholm and in Leuven.

Results

The paraffin blocks in 21 of the 43 patients initially planned for this investigation could be retrieved and altogether comprised 24 CRCs suitable for DNA-FCM. Two patients were excluded due to findings of carcinoids. Six of the CRCs had been fixed in Bouin's preparation making the FCM uncertain. In 14 of the tumours, deficiency of remaining tissue from the CRCs made it impossible to perform DNA-FCM.

Dukes' classification. There were 24 adenocarcinomas of which 3 were Dukes' A, 12 Dukes' B, 7 Dukes' C and 2 Dukes' D (Table I). There was a good correlation between the location of the tumours and the extent of CD (Table II) and a cecal-rectal predominance of the cancers was found (13/24).

Table I. *Dukes' classification of the CRCs.*

Dukes' classification	No.
Dukes' A	3
Dukes' B	12
Dukes' C	7
Dukes' D	2

Table II. *A cecal-rectal predominance of CRCs in longstanding CD was found as well as good correlation between extent of CD and localisation of CRC.*

CD-extent	CRC-site
Ileocaecal (5)	Caecum (3), right colon (1), not specified (1)
Ileocolonic (3)	Caecum (1), splenic flexure (1), descending colon (1), rectum (4)
Colonic (11)	Caecum (5), right colon (2), transverse colon (1), splenic flexure (1), rectum (2)
Rectal (1)	Rectum (1)

DNA-content. Fifteen (62.5%) of the tumours were aneuploid and 9 (37.5%) were diploid. There was no correlation between ploidy status and Dukes' stage or localisation of the tumour. The median ploidy level of the aneuploid CRCs was 3.2c (range 1.6-3.8, mean 3.07±0.7c) and the median S-phase was 15.2% (range 5.9-25.8, mean 15.2±5.6%). For diploid tumours, the corresponding values for cells in S-phase were 2.3% (range 1.2-19.8, mean 6.2±7.8%) and differed significantly from aneuploid tumours (p=0.0269). A DNA-FCM-histogram of one of the patients, having an aneuploid cell-population at 2.8c with a very high proliferation (S-phase 25.8%), is provided in Figure 1.

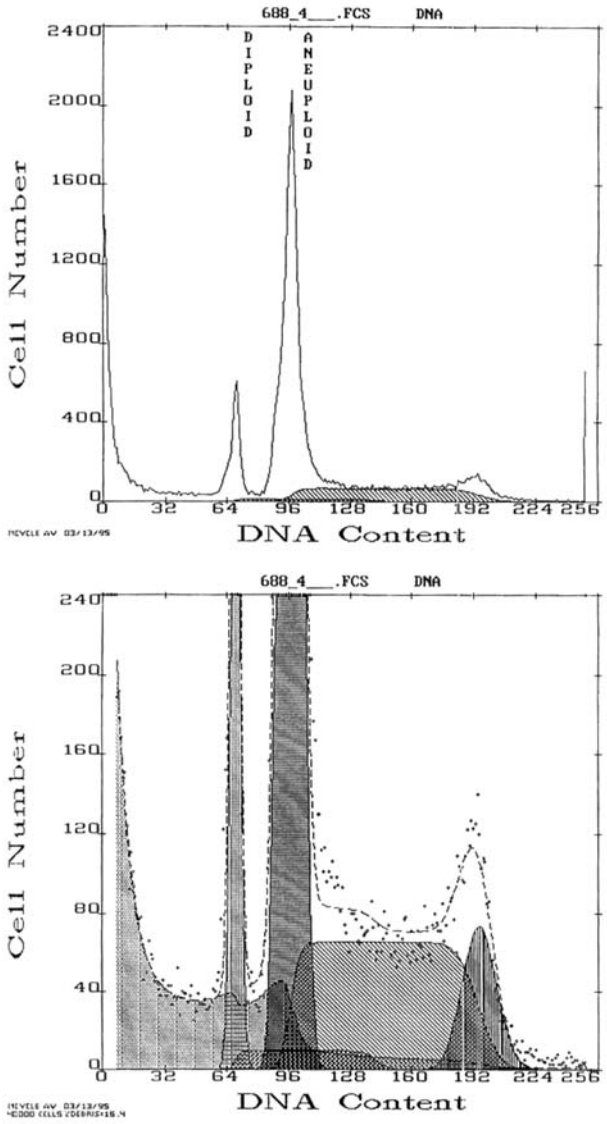

Figure 1. *A DNA-histogram in a patient with colonic CD and CRC in the transverse colon showing the aneuploid cell-population at 2.8c (88% of the cells), 25.8% of the cells were in the S-phase and 6.5% in G2-phase, indicating a high proliferation of the tumour. The lower part of the figure shows the histogram at higher magnification.*

Concomitant findings at surgery. In 5 (24%) patients, neoplasia or definite dysplasia was detected in the mucosa adjacent and distant to the tumour. One patient harboured 4 CRCs, one in the cecum, one in the splenic flexure, one in the descending colon and one in the rectum. Of these 4 tumours, all well- to moderately-differentiated (Dukes' B (n=2) and C (n=2)), the one in the caecum was aneuploid. Three patients had high-grade dysplasia (HGD) adjacent to (n=2) or distant from the index tumour (n=1). One patient had low-grade dysplasia (LGD) in the vicinity of the CRC.

Three colectomy specimens were available for a full DNA-FCM analyses work-up (meaning that biopsies of the entire colon and rectum could be obtained). One patient with an aneuploid CRC in the transverse colon was also found to have widespread aneuploidy throughout the colon and rectum (7/8 aneuploid biopsies). The remaining 2 patients (one with a diploid CRC and one with a aneuploid CRC) had diploid samples elsewhere in the colorectal mucosa.

Findings in patients subjected to prior colonoscopic surveillance. Four of the patients had been under colonoscopic surveillance under the diagnosis of UC and, thus, reclassified to CD. In these patients, 2-4 biopsies at 10-cm intervals along the entire colon and rectum were obtained for histopathology and DNA-FCM. Dysplasia was found in 3 patients of which one had LGD 7 years before the colectomy. Three of the patients had been subjected for surveillance with biopsies for DNA-FCM, of whom 2 had displayed aneuploidy 3 and 4 years before surgery, respectively.

Discussion

In this retrospective study, we studied the DNA-ploidy pattern in CD-patients with manifest CRC. We found that aneuploidy was a common feature in the tumours and that patients had concomitant dysplasia and aneuploidy in both near and distant mucosa. Furthermore, and perhaps more importantly, patients undergoing surveillance had both dysplasia and aneuploidy several years before the development of a CRC. Altogether 62.5% of the tumours displayed aneuploidy. This is in line with previous reports from studies of sporadic CRC (18-21). Similar cancer risk factors as in UC seem to be involved in CCD. Firstly, the duration of CCD was substantialy long with a median of 14.5 years. Secondly, the median age at CRC was relatively low (53 years). Thirdly, most of the patients had an extensive disease (20 of the 21 patients had ileocaecal, ileocolonic or colonic CD) and there was a good correlation between the localisation of the CRC and the extent of the disease. Caecal and rectal CRCs accounted for two-thirds of all the tumours in CCD (16/24).

Previous studies addressing DNA-FCM in CD are scarce. In one study of 24 patients with longstanding CD undergoing colonoscopic surveillance, 3 patients displayed DNA-aneuploidy; one of these subsequently developed a carcinoma (11). In another study in adolescents and young adults, 4 out of 17 patients with CD had aneuploidy during colonoscopic surveillance (22).

Only limited experience exists from prospective ongoing colonoscopic surveillance of patients with longstanding, extensive colonic CD. In fact, the only study published so far (12) reported 259 patients with chronic Crohn's colitis of a duration of 8 years or more who were surveyed for 18 years (mean 2.6 examinations per patient). This screening and surveillance program detected dysplasia or CRC in 16% of the patients (10 with indefinite, 23 with LGD and 4 with HGD and 5 CRCs).

The number of patients having CD involving the large bowel, with no major resection or colectomy, are likely to increase in the future as an effect of improved and more aggressive medical therapy (azathioprine, 6-mercaptopurine and infliximab). Furthermore, the incidence of the colonic form is steadily increasing (23) and it may thus be appropriate to consider surveillance in this subgroup of patients with CD.

Conclusion

In summary, CCD-patients with long duration, extensive colonic disease and young age at onset should be subjected, after 8-10 years of duration, to colonoscopic surveillance with biennial examinations with multiple biopsies for both histopathology and DNA-FCM. Both those procedures may improve the selection of CCD-patients with high cancer risk. Prospective studies in a larger material are needed to confirm these results.

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