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ORIGINAL ARTICLE

Chromosomal instability analysis and regional tumor heterogeneity in colon cancer

Vincenza Barresi [a,b], Sergio Castorina [c,d], Nicolò Musso [b], Carmela Capizzi [b], Tonia Luca [d], Giovanna Privitera [d], Daniele Filippo Condorelli [a,b,*]

[a] *Department of Biomedical and Biotechnological Sciences, Section of Medical Biochemistry, University of Catania, Italy;* [b] *Laboratory of Complex Systems, Scuola Superiore di Catania, University of Catania, Italy;* [c] *Department of Biomedical and Biotechnological Sciences, Section of Anatomy, University of Catania, Italy;* [d] *Fondazione Mediterranea G.B. Morgagni, Catania, Italy*

Chromosomal instability (CIN) is classically defined as an increase in the rate at which numerical or structural chromosomal aberrations are acquired in a cancer cell. The number of somatic copy number abnormalities (CNAs) revealed by high resolution genomic array can be considered as a surrogate marker for CIN, but several points, related to sample processing and data analysis, need to be standardized. In this work we analyzed 51 CRC samples and matched normal mucosae by whole genome SNP arrays and compared different bioinformatics tools in order to identify broad (>25% of a chromosomal arm) and focal somatic copy number abnormalities (BCNAs and FCNAs respectively). In 15 tumors, two samples, separated by at least 1 cm, were taken from the same tumor mass (double-sampling pairs) in order to evaluate differences in detection of chromosomal abnormalities between distant regions of the same tumor and their influence on CIN quantitative and qualitative analysis. Our data show a high degree of correlation of the quantitative CIN index (somatic BCNA number) between distant tumor regions. On the contrary, a lower correlation is observed in terms of chromosomal distribution of BCNAs, as summarized by a simplified cytogenetic table. Quantitative or qualitative analysis of FCNAs, including homozygous deletions and high level amplifications, did not add further information on the CIN status. The use of the index "somatic BCNA number" can be proposed for a robust classification of tumors as CIN positive or negative even in the presence of a significant tumor regional heterogeneity.

Keywords Colon cancer, copy number abnormalities, SNP array, chromosomal instability (CIN), microsatellite instability (MSI)
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Introduction

At least two forms of genomic instability have been described in colorectal cancer (CRC): *chromosomal instability* (CIN) and *microsatellite instability* (MSI) (1). CIN is classically defined as an increase in the rate at which numerical or structural chromosomal aberrations are acquired in a cancer cell and is present in the large majority of CRC (about 85%) (2–6). MSI is identified by somatic changes in the number of repeating units of microsatellite repeats due to defects in the DNA mismatch repair (MMR) genes, such as *MLH1, MSH2, MSH6*, and *PMS2*. MSI is detected in about 15% of all colorectal cancers; 3% of these are associated with Lynch syndrome (hereditary nonpolyposis colorectal cancer due to germline mutations in MMR proteins) and the other 12% are caused by acquired hypermethylation of the promoter of the *MLH1* gene (7,8). Although MSI and CIN have been considered mutually exclusive, it is now clear that MSI tumors also show varying degrees of CIN and that a percentage of the tumors can be considered positive for both form of instability (5,9).

The search for the presence of MSI in CRC is widely entered in the routine clinical practice due to the availability of convenient molecular test based on comparison between tumor and normal DNA from the same patient. Although the main clinical use of MSI testing is to provide a first step toward the identification of patients with Lynch syndrome, several

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* Corresponding author.
E-mail address: daniele.condorelli@unict.it

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studies have shown that MSI status can also be considered a prognostic marker, associated with a better prognosis, and a predictor of response to chemotherapy (no benefit of traditional 5-fluorouracil-based adjuvant chemotherapy in patients with MSI-associated CRC) (1,8,10,11). However, the simple distinction between MSI and microsatellite stable (MSS) tumors does not take into account the existence of MSI/CIN+ tumors, whose prognosis and response to therapy is suggested to be different from MSI/CIN− tumors (1,12).

On the other hand, the evaluation of CIN status has received less attention in routine clinical practice (1,13). One of the reasons is represented by the high rate of failure of standard metaphase cytogenetics in solid tumors such as CRC. Moreover, targeted molecular cytogenetics techniques, such as the search for some common loss of heterozygosity (LOH) by microsatellite analysis in specific chromosomal regions, provide only partial information and leads to an underestimate of CIN.

The sequential development of the technologies called “comparative genomic hybridization (CGH)”, “array-CGH” and “single nucleotide polymorphism (SNP)-arrays”, yielded a progressive improvement of the level of reproducibility, sensitivity and resolution in the field of genome-wide molecular cytogenetics. One of the major applications of these methodologies has been the characterization of microscopic and submicroscopic chromosomal abnormalities in tumoral tissues. In particular, last generation genomic arrays are able to detect chromosomal deletions or amplifications (copy number abnormalities, CNAs), at a lower limit of tens of kb, representing one of the best available option for a complete description of the chromosomal abnormality profile of CRC tumors (14–17). Although a rigorous evaluation of CIN requires single-cell technologies (2,3), the number of somatic CNAs, i.e. copy number gains and losses revealed by high resolution genomic array, can be considered as a surrogate marker for CIN in routine diagnostics. However, several points, related to sample processing and data analysis, need to be examined and standardized in order to improve feasibility and clinical usefulness of SNP-array analysis. One of the main limiting factors of CNA analysis in tumoral samples is tissue heterogeneity (18–22). Two types of tissue heterogeneity are particularly relevant to the routine clinical application of genomic arrays for cancer cytogenetics: *normal/cancer cell admixture* and *tumor clonal heterogeneity*. The first one is due to the fact that, in a tumor, cancer cells are admixed with normal cells, thus diluting the somatic cancer cell information. Indeed, normal/cancer cell admixture not only decreases the sensitivity for the detection of CNAs, but also seriously affects the proper identification of boundaries and size of CNAs. Although laser microdissection can improve this issue, this procedure is technically challenging in a routine context and does not ensure a reproducible and complete separation of cancer cells from normal ones. The second type of tissue heterogeneity in solid tumors is represented by the co-existence of different clonal subpopulations inside the same tumoral mass (23,24). These different clones could be homogeneously admixed or regionally segregated. In the latter case, the results of the molecular characterization might be deeply dependent on the topographical localization of the analyzed sample in the tumoral mass (25,26).

CNAs can be grossly distinguished in “broad copy number abnormalities” (BCNAs), defined in the present report as structural aberrations that involve more than 25% of a chromosomal arm, or numerical aberrations involving whole chromosomes, and “focal copy number abnormalities” (FCNAs), i.e. small size losses or gains of chromosomal DNA. BCNAs (involving a larger number of consecutive probe signals) could be identified more robustly than FCNAs in routine analysis aimed to detect the CIN status, even in the presence of tissue heterogeneity. In the present work we compared different methods for their ability to detect BCNAs and evaluated the number of BCNAs and isolated FCNAs in a collection of MSI and MSS CRC samples. Moreover, we compared the results obtained in two samples (double-sampling pairs) derived from the same CRC tumor at a distance of at least 1 cm, in order to evaluate differences in detection of chromosomal abnormalities between distant regions of the same tumor and their influence on quantitative and qualitative CIN analysis.

Finally, the analysis of somatic tumoral BCNAs allowed us to distinguish three types of FCNA: “*isolated FCNAs*” (somatic tumoral FCNAs outside BCNA regions), “*Homozygous Deletions*” (HoDs, somatic tumoral deletions with a calibrated log2ratio value less than 0.81), “*High Level Amplifications*” (HLAs, focal regions with a calibrated log2ratio higher than 5.2). Although such identifications are less robust to tissue heterogeneity their annotation in a concise clinical report can be useful for interpretation of mutation status of cancer genes. In this work we report an analysis of the recurrence of such chromosomal abnormalities in our series of colorectal cancer.

Materials and methods

Patients

50 patients underwent surgical resection for primary invasive colorectal cancer at the “*Centro Clinico Diagnostico S.r.l. G.B. Morgagni*” in Catania. Tumors were staged according to the tumor-node-metastasis (TNM) staging system of American Joint Committee on Cancer (AJCC). The sex distribution, the number of patients, average age, and the staging grade of tumor are reported in Table 1.

51 tumoral biopsies were collected directly after surgical resection (in one patient a sample from a synchronous colorectal adenocarcinoma was collected, tumoral sample 3). In 29 cases biopsies of adjacent normal mucosa (at distance of 3–6 cm from the tumor) were also collected (matched tumor/mucosa pairs). In 15 tumors, two samples were taken at arbitrary positions in the tumor area, separated by at least 1 cm (double-sampling pairs). All CRC specimens were frozen and stored at −80 °C until DNA extraction. Informed consent was obtained from all patients involved in this study. This project was approved by the Ethics Committee of ASL3 of Catania (Italy).

Table 1 Clinic-pathological characteristics of the cohort of 50 colorectal patients

			Stage		
Sex	N. patients	Age (mean)	II	III	IV
F	21 (42%)	70.81 (±12.8)	9	11	1
M	29 (58%)	66.86 (±14.29)	12	13	4

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