

Recent advances in molecular diagnostics of colorectal cancer by genomic arrays: proposal for a procedural shift in biological sampling and pathological report

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Summary

Two forms of genetic instability have been described in colorectal cancer: *chromosomal instability*, characterized by structural and numerical chromosomal abnormalities and associated to aneuploidy; and *microsatellite instability*, characterized by a deficiency in the mismatch repair system that leads to slippage in microsatellites and is associated to euploidy. Thirteen colorectal cancer sample DNAs were analyzed after colectomy. High-resolution genome-wide DNA copy number and Single Nucleotide Polymorphism genotyping analysis was performed by Affymetrix SNP 6.0 arrays that interrogates 906,600 single nucleotide polymorphisms and 945,826 copy number probes. We implemented this analysis as part of a routine procedure that includes the sampling of fresh tissue from the tumor mass without affecting the subsequent standard histopathological procedure. The novel molecular technology allows the determination of a genome-wide molecular karyotype using only 500 ng of high-quality tumor DNA; it distinguishes the two main types of genomic instability, discriminating between chromosomal instability positive and negative tumors. It also detects loss of heterozygosity (LOH) regions, called copy neutral-LOH. Tumor-associated copy neutral-LOH regions may play a pivotal role in oncogenesis when they determine duplications of either activating or loss of function gene mutation. We observed recurrent gains of chromosomes 2, 7, 8q, 9, 12, 13, 20 and losses of chromosomes 4, 5, 8p, 15, 17p, 18, 22, and Y, in agreement with previous cytogenetic studies. The use of such sampling procedure could stimulate the routine detection of point mutations in specific genes, thus avoiding subsequent sectioning of formalin-fixed and paraffin-embedded samples.

Key words

Colorectal cancer; genetic instability; genomic array

Introduction

Colorectal cancer (CRC) affects over 1 million people each year, accounting for 9% of all new cancer cases worldwide. It is the fourth most common cancer in men and the third most common cancer in women (Power et al., 2009). CRC was a disease primarily observed in longstanding developed nations, whose populations typically exhibit risk factors for colorectal cancer, as obesity, a diet low in fruits and vegetables, physical inactivity, and smoking. However, in recent years, high CRC rates have been reported

also in newly developed countries. Screening can reduce the mortality associated with the disease, but the participation rates are still suboptimal (Center et al., 2009).

It is well-known that at least two forms of genetic instability have been observed in CRC: *chromosomal instability* (CIN), that is present in 85% cases and is characterized by structural and numerical chromosomal abnormalities (aneuploidy); and *microsatellite instability* (MSI), characterized by a deficiency of the mismatch repair system that leads to slippage in microsatellites, and associated to a normal or quasi-normal karyotype (euploidy). The latter instability has been found to be associated with hereditary non-polyposis colorectal cancer (HNPCC) that involves mutation of the human mismatch repair genes hMSH2 and hMLH1 as well as many sporadic colon cancers. It is also clear that such molecular classification has a clinical impact both in prognostic and therapeutic terms. The prognostic value of CIN and MSI has been subject of large meta-analyses which established that patients with CIN+ disease have a poorer prognosis (hazard ratio for death = 1.45) and patients with MSI+ CRC have a better prognosis (hazard ratio = 0.65) (Walther et al., 2008; Popat et al., 2005). Moreover, several reports have suggested that the efficacy of adjuvant chemotherapy with 5-fluorouracil in colorectal cancer depends on the mismatch repair status and it has also been described a possible detrimental effect of adjuvant 5-FU therapy in loco-regional MSI+ colorectal cancer (Ribic et al. 2003; Watanabe et al. 2006; Jover et al. 2009). Finally, several of the chromosomal abnormalities described in CIN+ colorectal cancer may have an important prognostic and clinical impact. Nowadays there is still a substantial delay between novel molecular insights and established clinical practice. However, recent advances in molecular cytogenetics techniques are likely to introduce a revolution in the field (Sheffer et al. 2009). In particular, high resolution genomic array allows the determination of an accurate genome-wide molecular karyotype using only 500 ng of DNA extracted from the tumor mass after colectomy. However, the need of high-quality DNA does not allow the use of formalin-fixed and paraffin-embedded tissue for this type of analysis. Therefore, in order to exploit the advantages provided by novel molecular technologies, it is necessary to adopt some minor changes in the routine procedures for tumor sampling and sample storage.

In the present paper we report representative examples of results obtained from CIN+ and MSI+ colorectal cancer using last generation photolithographic DNA arrays and suggest a protocol for tumor sampling and pathological report.

Materials and Methods

Colorectal cancer tissue sampling for molecular biology after colectomy

The resected colon (Fig. 1) was washed with physiological solution, put it in a sterile kidney dish and cut in the longitudinal direction of the intestine to expose the lumen. The lumen was washed with physiological solution and two samples of tumor tissue (about 100 mg each) were taken in two different points of the tumor mass. Samples should fit the following requirements: a) they must derive from the principal tumor mass; b) they must be far from the area of transition between normal mucosa and tumor; c) they must be taken on the luminal surface of the tumor (<0.3 cm in depth); d) they must be far away from each other; e) they must not be part of

necrotic or suppurative inflamed areas. A sample of normal mucosa was also taken at least 3 cm far from the border of tumor mass. Samples were transferred to microtubes and immediately carried to the laboratory in a pre-refrigerated LabTop Cooler and stored in a -80°C freezer.

After tissue sampling, the excised colon was fixed in 40% formalin; the tissue was then embedded in paraffin for histopathological examination. Microscopic examination allowed a rough estimation of the fraction of tumor cells in the areas close to the tissue sampled for molecular biology.

Tumor samples and genomic DNA extraction

Thirteen colorectal cancer samples were analyzed after colectomy. Six cases were assigned at the pathological stage T3N0, six at T3N1, and one at T4N0.

Genomic DNA (gDNA) was extracted using the QIAamp DNA tissue Mini Kit. The concentration and the quality of the DNA was determined using a ND-1000 spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA).

High-resolution genome-wide DNA copy number and SNP genotyping analysis

High-resolution genome-wide DNA copy number and Single Nucleotide Polymorphism (SNP) genotyping analysis was performed according to the protocol supplied by the manufacturer (Affymetrix, Santa Clara, CA) for Affymetrix SNP 6.0 arrays that interrogates 906,600 SNPs and 945,826 copy number probes (SNP/CNV array). Briefly, 500 ng of gDNA (50 ng/ul) was digested with NspI and StyI restriction enzymes, ligated to respectively NspI and StyI adaptors, amplified by polymerase chain reaction (PCR) using a single primer with the TITANIUM DNA Amplification Kit (Clontech Laboratories, Mountain View, CA). PCR products were purified with Agencourt AMPure® Magnetic Beads (Agencourt Bioscience Corporation, Beverly, MA) and purified amplicons were fragmented, end-labeled and hybridized to a GeneChip Affymetrix SNP 6.0 arrays at 50°C for 16-18 hours in a GeneChip® Hybridization Oven 640 (Affymetrix). After washing and staining in a GeneChip® Fluidics Station 450 (Affymetrix), the arrays were scanned with a GeneChip® Scanner 3000 7G (Affymetrix). Array scanning and data analysis were performed using Affymetrix® "GeneChip Operating Software" (GCOS) and "Genotyping Console™" (GTC) version 3.0.1.

Results

The Single Nucleotide Polymorphism (SNP) array technology allowed for a clear discrimination of CIN+ and CIN- tumors (compare Figs. 2A and B). 85% of the analyzed tumors were CIN+. The average age of patients affected by aneuploid tumors was 65.64 ± 10.33 , while the age of patients affected by euploid tumors was 46.5 ± 3.53 .

As expected, the euploid tumor in Fig. 2B showed a high degree of microsatellite instability (MSI+), as detected by a specific PCR test according to NHI guidelines (Dietmaier et al. 1997).

The technology used in the present study performs also the genotyping analysis of almost 900,000 SNPs and allowed us the detection of loss of heterozygosity

ity (LOH) regions that occurs without concurrent changes in the gene copy number. Such defects, called copy neutral (CN) -LOH, are attributed to mitotic recombination occurring in somatic cells or to abnormalities following repair of DNA double-strand breaks. Tumor-associated CN-LOH regions may play a pivotal role in oncogenesis when they determine either duplications of activating genes or loss of function mutations.

Indeed, several chromosomal abnormalities were detected in CIN+ tumors in agreement with a previous report (Sheffer *et al.* 2009). An average (\pm SD) of 13 (\pm 6) chromosomal abnormalities (gain, loss or CN-LOH) were detected in tumors at stage T3N0, 18 (\pm 2) at stage T3N1 or T4.

An example of a report of all chromosomal abnormalities detected by SNP array in the same case of Fig. 2A is reported in Table I.

Discussion

In the present work we report the ability of recent molecular cytogenetic techniques, based on SNP DNA arrays, to distinguish the two main types of genomic instability in CRC (CIN and MSI) and describe a routine procedure that allows the sampling of fresh tissue from the tumor mass without affecting the following standard histopathological procedure. While efficient molecular tests for detection of MSI are already available (Dietmaier *et al.*, 1997), in routine analysis CIN has been mainly supposed indirectly by exclusion of MSI. The availability of such techniques and their wide application should clarify the existence of CIN- and MSI- tumors and provide a large amount of information on their molecular abnormalities. Among the more frequent chromosomal abnormalities in CIN+ tumors, we observed gain of chromosomes 2, 7, 8q, 9, 12, 13, 20 and loss of chromosomes 4, 5, 8p, 15, 17p, 18, 22, and Y.

Moreover, recent advances in analysis of SNP-array data allow for a quantification of tumor and normal cell fraction in the case of CIN+ tumors (Assie *et al.*, 2008; Goransson *et al.*, 2009). Indeed, even in the presence of a contaminant fraction of normal cells up to 50%, SNP arrays were sensitive enough to detect the main chromosomal abnormalities.

Another advantages of such standard procedure for tissue sampling is the possibility to extract undegraded RNA from the same frozen samples used for DNA extraction, thus allowing the preparation of a DNA and RNA Bank available for genomic and transcriptomic studies.

Finally, the use of such a sampling procedure dedicated to molecular biology analysis could stimulate the routine detection of point mutations in specific genes, thus avoiding subsequent sectioning of formalin-fixed and paraffin-embedded samples and allowing integration of information on point mutations with that derived by genome-wide analysis of chromosomal abnormalities and gene expression changes.

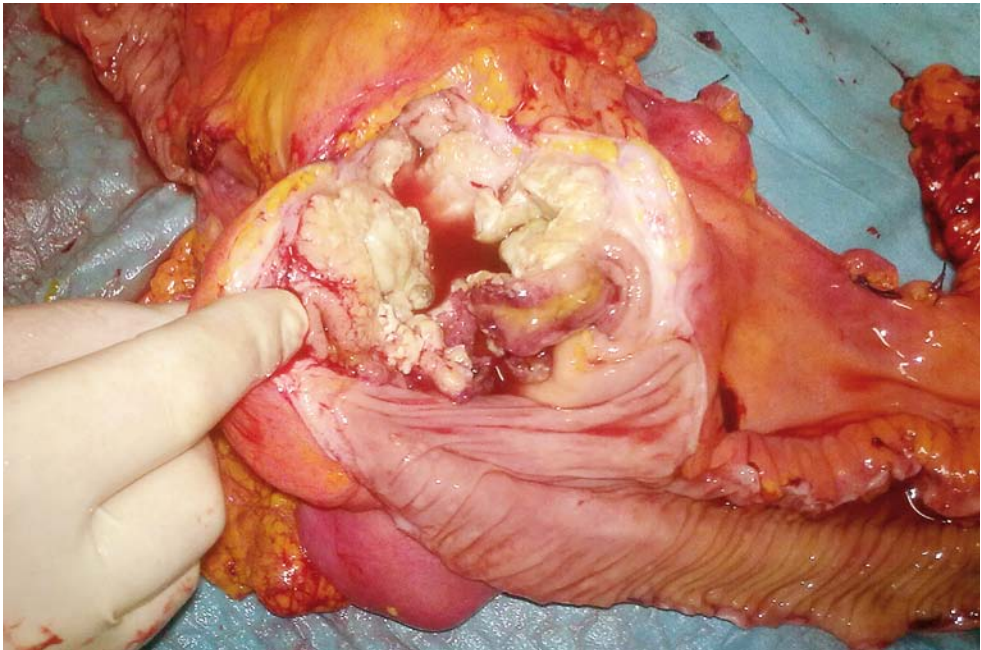
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Figures and tables

Chromosome	Gain (copy number in brackets)	Loss (copy number in brackets)	CN-LOH (copy neutral loss of heterozygosity)
3	q21.1-qtel (3)		
4		q31.3-qtel (1)	
5			CN-LOH whole chr.
6		whole chr. (1)	
7	whole (3)		
8	q (3)	p (1)	
9	whole (3)		
10			CN-LOH q
11			CN-LOH q
14	cen-q11.2 (3-5)		CN-LOH q11.2-qtel
17		p(1)	CN-LOH q
18		whole chr (1)	
20	q (3-4)	p(1)	

Table I – Report of chromosomal abnormalities detected by SNP array analysis in CIN+ colorectal cancer**Fig. 1** – Surgical specimen of colectomy (removal of the colon) showing a cancer.

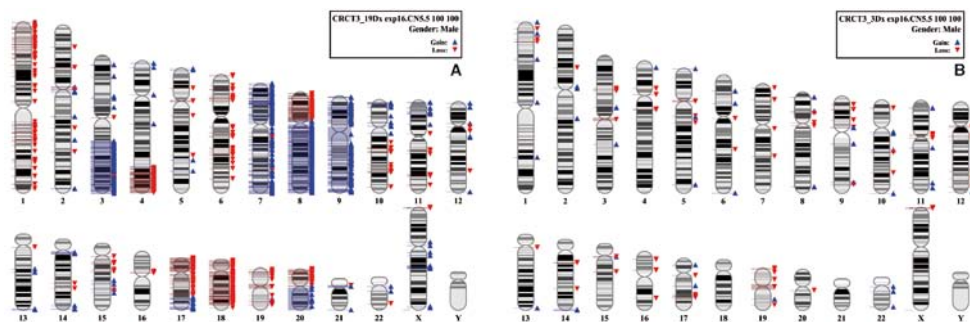


Fig. 2 – Overview of copy number changes in CIN+ (A) and CIN- MSI+ (B) colorectal cancer. Gains and losses are represented on chromosome ideograms by blue and red triangles respectively (karyoview by Affymetrix® Genotyping Console 3.0.1).