Brazilian Journal of Medical and Biological Research (1997) 30: 915-921
ISSN 0100-879X

Utilization of microsatellites for the analysis of genomic alterations in colorectal cancers in Brazil

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Abstract

Two different pathogenetic mechanisms are proposed for colorectal cancers. One, the so-called "classic pathway", is the most common and depends on multiple additive mutational events (germline and/or somatic) in tumor suppressor genes and oncogenes, frequently involving chromosomal deletions in key genomic regions. Methodologically this pathway is recognizable by the phenomenon of loss of heterozygosity. On the other hand, the "mutator pathway" depends on early mutational loss of the mismatch repair system (germline and/or somatic) leading to accelerated accumulation of gene mutations in critical target genes and progression to malignancy. Methodologically this second pathway is recognizable by the phenomenon of microsatellite instability. The distinction between these pathways seems to be more than academic since there is evidence that the tumors emerging from the mutator pathway have a better prognosis. We report here a very simple methodology based on a set of tri-, tetra- and pentanucleotide repeat microsatellites allowing the simultaneous study of microsatellite instability and loss of heterozygosity which could allocate 70% of the colorectal tumors to the classic or the mutator pathway. The ease of execution of the methodology makes it suitable for routine clinical typing.

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Research supported by CNPq and FAPEMIG.

Received February 28, 1997
Accepted June 3, 1997

Key words
- Polymerase chain reaction
- Colorectal carcinoma
- Microsatellite
- *p53*
- *DCC*
- Microsatellite instability

Introduction

The characterization of chromosomal and molecular alterations in many types of neoplasias has led to the current paradigm that cancer is a genomic disease (1). In colorectal carcinomas, which are among the most prevalent human cancers worldwide (2), a series of studies have established that additive mutations in *c-Ki-ras*, an oncogene, together with the inactivation of the tumor suppressor genes *p53* (located on chromosome 17p), *DCC* (deleted in colon cancer, located on chromosome 18q) and *APC* (adenomatous polyposis coli, located on chromosome 5q), occur in most patients and are implicated in the stepwise transformation of the normal mucosa into a malignant tumor (3). Inherited germline mutations in *APC* (present on chromosome 5q21) preclude one

of the necessary mutational steps and thus are associated with a strong familial predisposition to colorectal cancer (4). It is important to note that, while mutations in oncogenes are generally single dominant events, the inactivation of tumor suppressors is dependent on the functional loss of both copies of the relevant genes. While the first of the two losses occurs most frequently by gene mutations, the second one is more often a chromosomal event, generally a deletion (5-7). Since the deletion generally involves simultaneous loss of genetic loci near the tumor suppressor – and occasionally loss of the whole chromosome or chromosome arm – these events are strongly associated with loss of heterozygosity (LOH) of hypervariable polymorphisms (minisatellites and microsatellites) located in the deleted region.

Recently, studies of an autosomal dominant form of colorectal cancer known as "hereditary non-polyposis colorectal cancer" (HNPCC) have shown that this tumor is caused by germline mutations in human DNA repair genes, principally *hMSH* and *hMLH1* but also *hPMS1* and *hPMSH2* (reviewed in Refs. 8-10). Mutations in these genes lead to a defect in the correction of mismatches occurring during DNA replication, with the subsequent accumulation of mutations throughout the genome, including tumor suppressor genes and oncogenes, which can cause malignant transformation (9,11). Mismatch repair defects occur also in a substantial proportion (15-25%) of sporadic colorectal cancers (9).

Microsatellites are genomic sequences consisting of 2-6-bp motifs repeated in multiple tandem copies (reviewed in Ref. 12). Because of their repetitive nature, these sequences are prone to frequent changes in the repeat number by replication slippage (13). Since these mutations are almost always corrected by the DNA repair system, microsatellites, although polymorphic in populations, are somatically stable. In the case of tumors with defects in mismatch repair, however, new mutant alleles are frequently observed in microsatellites, a phenomenon termed "microsatellite instability" or "replication error phenomenon (RER)" (8). It is questionable if microsatellite instability has any direct relationship with carcinogenesis, but at any rate it serves as a marker for the presence of mismatch repair deficiency in the tumors (8,9).

In summary, two different pathways of colorectal carcinogenesis can be recognized. One, called the "classic pathway", is the most common and depends on multiple additive mutational events (germline and/or somatic) in tumor suppressor genes and oncogenes, frequently involving chromosomal deletions in key genomic regions (3,7). Methodologically this pathway is recognizable by the LOH phenomenon. On the other hand, the "mutator pathway" depends on early mutational loss of the mismatch repair system (germline and/or somatic) leading to accelerated accumulation of gene mutations in critical target genes and progression to malignancy (8-10). Methodologically this second pathway is recognizable by the phenomenon of microsatellite instability. The distinction between these pathways seems to be more than academic. There is mounting and convincing evidence that the tumors emerging from the mutator pathway have lower proliferative activity and overall better prognosis than those emerging from the classical pathway (14-16). Thus, laboratory analysis of colorectal tumors aimed at establishing the pathogenetic pathway may soon be incorporated into medical practice. Therefore, it will be necessary to develop simple methods applicable to the routine clinical laboratory.

In practice, microsatellites are typed by the polymerase chain reaction (PCR) using primers designed from the DNA sequence flanking the tandem repeat arrays. Most studies of microsatellite instability have been based on the study of $(CA)_n$ repeats, which

are abundant and sensitive, but cumbersome to type because alleles can only be easily resolved on sequencing gels and generally require isotopic labeling for visualization of the PCR products. In contrast, as we have shown elsewhere, microsatellites with repeats of more than 3 bp can be much more simply typed in short non-denaturing gels with non-isotopic silver staining (17-19). We report here a very simple methodology based on a set of tri-, tetra- and pentanucleotide repeat microsatellites which allows the simultaneous study of microsatellite instability and loss of heterozygosity and thus helps to allocate a given colorectal tumor to the classic or mutator pathway. The ease of execution of the methodology makes it suitable for routine clinical typing. Moreover, the present study provides the first evaluation of the relative importance of the two pathways in the pathogenesis of colorectal carcinoma in Brazil.

Material and Methods

Patients

A sequential sample of 20 colorectal adenocarcinomas was obtained at the Hospital das Clínicas, Federal University of Minas Gerais, and the Colo-Proctology Service of the Santa Casa de Misericórdia, Belo Horizonte, Brazil. There was no selection on the basis of age, location of the tumor, histopathological classification or family history. However, all tumors were subsequently found to be isolated cases in the family. All 20 samples consisted of paired fragments of normal colonic mucosa and tumoral tissue from the same patient and were subjected to microscopic analysis prior to DNA extraction to ensure the absence of neoplastic infiltration in the mucosa and the predominance (>80%) of neoplastic cells in the tumor samples. DNA was extracted from the tumor samples by an alkaline extraction procedure (20). To establish a population baseline, all tri-, tetra- and pentanucleotide loci were studied in DNA extracted from the blood of at least 100 unrelated individuals randomly chosen among those presenting for paternity tests at the Núcleo de Genética Médica de Minas Gerais, Belo Horizonte.

Microsatellite analysis

We used microsatellites to detect loss of heterozygosity in the *p53*, *DCC* and *APC* loci. For *p53* we used the pentanucleotide repeat *p53ALU* (21,22) (AAAAT repeats) which is located in the first intron of the *p53* gene; for *DCC* we used the closely linked tetranucleotide repeat *D18S51* (AAAG repeats; 23) and for *APC* we used the linked CA-repeat microsatellite *D5S299* (24). For the detection of microsatellite instability we utilized the previous three microsatellites plus the trinucleotide repeats *D13S308E* (17) and *D2S196E* (19) (containing CAT and ACA repeats, respectively) and the tetranucleotide repeat *D12S67* (25) containing GATA repeats. PCR reactions were carried out in 20-µl volumes with 10-100 ng of genomic DNA as a template. The primers used are listed in Table 1. For resolution of the alleles of the *D5S299* CA-repeat locus, the products were denatured and resolved on a sequencing gel (50 cm x 30 cm x 0.1 cm); after electrophoresis, a strip of the area expected to contain the products was cut and stained with silver salts for visualization (18). For the tri-, tetra- and pentanucleotide microsatellites, non-denaturing electrophoresis was performed on short gels (17 cm x 10 cm x 0.15 cm) followed by silver staining (18).

Results

Loss of heterozygosity

LOH was considered to have occurred when the tumor sample exhibited complete or almost total absence of one of the alleles

Table 1 - Details of the microsatellites studied and some of their population parameters.

*Observations made in the present study based on a random sample of N Brazilians (N = 408 for *D12S67*; N = 311 for *D18S51*; N = 100 for *p53ALU*).

Locus	Size of repeat	Chromosomal mapping	Primer sequences	No. of alleles	Hetero-zygosity	Ref.
D5S299	2	5q15-5q22	5′- GTAAGCAGGACAAGATGACAG - 3′ 5′- GCTATTCTCTCAGGATCTTG - 3′	10	0.80	24
D2S196E	3	2q11.2	5′- GGTTGATTTGTCATTGCTGCTCA - 3′ 5′- CACTCTCAGGACCAACAGACGTTC - 3′	4	0.74	19
D13S308E	3	13q11	5′- AGCTTGAATAAAGTGCCCAGC - 3′ 5′- GCATGTTGTCCTTAAAGCCCC - 3′	8	0.71	17
D12S67	4	12	5′- GCAACAGTTTATGCTAAAGC - 3′ 5′- GCCTATGCAGTTCAAATCTA - 3′	11*	0.80*	25
D18S51	4	18q21.33	5′- GAGCCATGTTCATGCCACTG - 3′ 5′- CAAACCCGACTACCAGCAAC - 3′	16*	0.89*	23
p53ALU	5	17p13.1	5′- ACTCCAGCCTGGGCAATAAGAGCT - 3′ 5′- AACAGCTCCTTTAATGGCAG - 3′	7*	0.65*	21,22

present in the normal mucosa (Figure 1A). Obviously, homozygous patients could not be informative for LOH. For this reason, we established the level of heterozygosity of *p53ALU* and *D18S51* in the Brazilian population. The location of the pentanucleotide repeat polymorphism *p53ALU* in the first intron of the *p53* gene (21,22) is ideal for studies of loss of heterozygosity. We typed this polymorphism in 100 randomly chosen individuals (Table 1) and found that 65 of them were heterozygous. This means that in 35% of cases the polymorphism will unfortunately not be informative. However, it still appears to be the best microsatellite marker for this purpose. In contrast, *D18S51*, which is not located as ideally but still closely linked to the *DCC* gene (23), exhibited a much higher heterozygosity of 89% among 311 individuals. The *D5S299* polymorphism is closely linked to the *APC* gene and has an expected heterozygosity of 0.70 in Europeans (24). This polymorphism has the disadvantage of being a dinucleotide repeat and of needing more complex techniques for allele resolution.

Searching for LOH in our series of 20 tumors (Table 2) we found that six samples (30%) were non-informative (homozygous) for *p53ALU*; in the 14 heterozygous samples LOH was observed in eight cases (57%). Loss of heterozygosity in *DCC* was analyzed using microsatellite *D18S51*, which is located on 18q21.3, in the proximity of the *DCC* gene on 18q21.1. At this locus, four samples were non-informative (20%) and in the 16 heterozygous samples LOH was observed in six cases (37.5%). For *D5S299* 17 patients were informative, but LOH was not observed in any of them.

Microsatellite instability

All 20 adenocarcinomas were successfully amplified using the six microsatellites (Table 2). The presence of extra bands in the amplification of tumoral DNA that were not present in the amplification of DNA from the normal mucosa defined microsatellite instability (Figure 1B) and was observed in five tumors. This frequency of 25% agrees well with those reported in previous studies, which range from 15% to 28% for sporadic colorectal tumors (8,9). Two of these five samples showed instability in two microsatellites, one in three loci and the remaining

two samples in four distinct loci.

Pathogenetic classification of colorectal tumors

Of the 20 tumors studied, 14 (70%) could be assigned to the classic or mutator pathway of carcinogenesis (Table 2). Nine cases (45%) showed LOH at *p53ALU* and/or *D18S51* and absence of microsatellite instability and could thus be assigned to the classic pathway. Five cases (25%) showed microsatellite instability and absence of LOH and could thus be allocated to the mutator pathway (including one case that presented both replication errors in two microsatellites and LOH in *D18S51*; see Discussion below). Six tumors could not be classified because they showed neither LOH nor microsatellite instability.

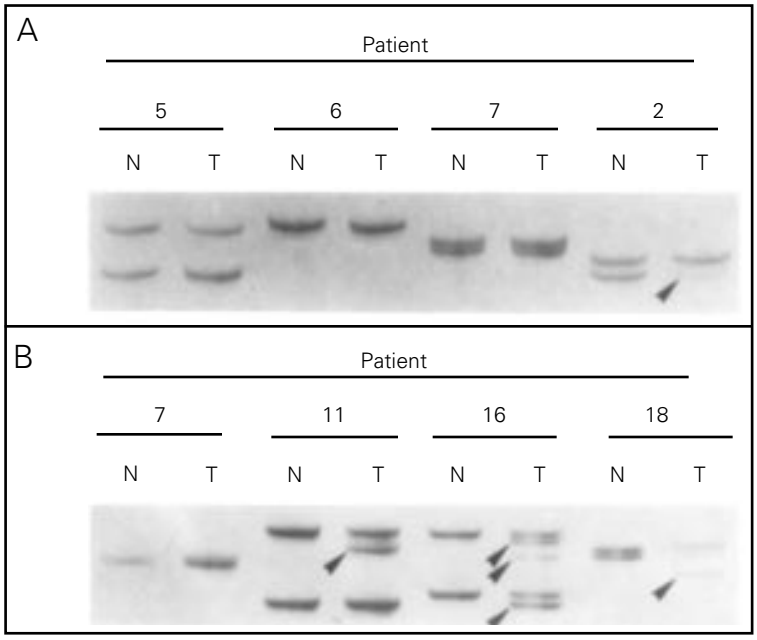

Figure 1 - Examples of the detection with microsatellites of loss of heterozygosity and microsatellite instability in colorectal carcinomas. *A*, Silver-stained polyacrylamide gel containing *D18S51* alleles amplified from the normal mucosa (N) and tumor (T) of four patients (identified by numbers) with colorectal adenocarcinoma. The tumor from patient 2 presents loss of heterozygosity (arrowhead). Patient 6 is homozygous and consequently is not informative. *B*, Silver-stained polyacrylamide gel containing *D12S67* alleles amplified from the normal mucosa (N) and tumor (T) of four patients (identified by numbers) with colorectal adenocarcinoma. The tumors from patients 11, 16 and 18 present microsatellite instability identified by the appearance of new mutant alleles (arrowheads).

Discussion

In the present study we were able to assign 70% of the colorectal tumors to the classic or the mutator pathway (9). There are both theoretical and methodological reasons why it was not possible to establish the pathogenetic pathway of all colorectal tumors. First, in addition to the established so-called classic and mutator pathways, there may exist (and probably do exist) as yet unknown other route(s). Another reason for uncertainty is that microsatellite instability may be present in the tumor but may not be detected (10).

The fact that LOH is thought to indicate the classic pathway of tumorigenesis depends on probabilistic considerations. In normal states the chromosomal mutation rate is higher than the genic one. Thus, we expect to see frequent chromosomal deletions in tumors with an intact repair system undergoing the classic pathway of tumorigenesis. In the mutator pathway, gene mutation rates are elevated by hundred- or thousand-fold and thus much more likely to occur than the chromosomal accidents that lead to LOH. These predictions were largely observed experimentally (11). However, there is no compelling reason why LOH may not occur in tumors with microsatellite instability (11), as shown by the one instance of a tumor with both microsatellite instability and LOH at *D18S51* in the present study.

It is significant that we could assign 14 of the 20 tumors with only six microsatellites, five of which are tri-, tetra- or pentanucleotide repeat microsatellites and thus were very easy to type using non-denaturing short gels and silver staining. For the dinucleotide repeat *D5S299* we adapted a silver staining procedure to the large sequencing gels, but the technique is still cumbersome. However, based on the results in Table 1, we can actually demonstrate that if *D5S299* had not been typed, our classification of the tumors

Table 2 - Microsatellite findings in each of the 20 colorectal tumors studied.

LOH = Loss of heterozygosity; NI = not informative (only indicated for the *p53ALU*, *D18S51* and *D5S299*); MSI+ = microsatellite instability present.

Tumor number	*p53ALU*	*D18S51*	*D5S299*	*D2S196E*	*D13S308E*	*D12S67*	Pathway classification
1	LOH	LOH	NI				Classic
2	LOH	LOH	NI				Classic
3	LOH						Classic
4			MSI+	MSI+	MSI+	MSI+	Mutator
5	LOH						Classic
6	NI	NI					?
7	NI		NI				?
8	LOH	LOH					Classic
9							?
10	NI		MSI+	MSI+			Mutator
11	MSI+	LOH				MSI+	Mutator
12	LOH	LOH					Classic
13	LOH	NI					Classic
14	NI						?
15	NI	LOH					Classic
16	NI		MSI+		MSI+	MSI+	Mutator
17	LOH						Classic
18	MSI+		MSI+		MSI+	MSI+	Mutator
19		NI					?
20		NI					?

would still remain the same. Hence, for clinical studies we can apparently limit ourselves to the five other microsatellites, considerably simplifying the problem.

The great majority of studies focusing on colorectal cancer have come from First World countries where this tumor is the second or third most common cancer and has shown a rising incidence (2). In Third World countries, colorectal cancer seems to be less common and it was important to ascertain whether the relative importance of the two recognized pathogenetic mechanisms was the same in both environments. The rates of DNA replication errors, as well as the rates of LOH linked to the tumor suppressor genes *p53* and *DCC* obtained in this study, were closely similar to the frequencies reported by others. We observed loss of heterozygosity at *p53* in 8 of 14 informative tumors (57%), a value similar to published data ranging from 40 to 76% (26-28). We observed loss of heterozygosity at *D18S51* in 6 of 16 informative tumors (38%), a value similar to published data ranging from 34 to 52% (27,28). On the other hand, we did not observe any LOH in 17 tumors informative for *D5S299*. However, when compared with the expected frequency (16-20%; Refs. 27-29) this is not significantly different (χ^2 = 2.5; 0.2>P>0.1), suggesting that, at least at this data level, the epidemiology of colorectal cancers in Brazil does not differ significantly from that of Europe and the United States.

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