Analysis of genomic instability in adult-onset celiac disease patients by microsatellite instability and loss of heterozygosis

Ariela F. Fundia[a], Alejandra S. Cottliar[a], Graciela La Motta[b], Adriana Crivelli[b], Juan Carlos Gómez[b], Irma R. Slavutsky[a] and Irene B. Larripa[a]

Background and Aims **Malignant complications of celiac disease (CD) include carcinomas and lymphomas. The genetic basis behind cancer development in CD is not known, but acquisition of genetic abnormalities and genomic instability has been involved. The aim of this study was to explore molecular characteristics of genomic instability in CD patients by analyzing microsatellite instability (MSI) and loss of heterozygosis (LOH) with carefully selected microsatellites.**

Methods **We genotyped small bowel biopsies and peripheral blood samples from 20 untreated CD patients using five microsatellites related to MMR genes (panel A), and five repeats associated with tumor suppressor genes, chromosome instability, inflammation, and cancer (panel B).**

Results **Genomic instability was found in seven out of 20 (35%) cases at: D5S107, D18S58, GSTP, TP53 or DCC, being TP53 the most frequently affected (five out of seven cases; 71%). Microsatellite alterations were significantly found using panel B markers ($P=0.04$). No cases with high frequency of MSI and replication error phenotype were detected. Only one case displayed MSI-L alone. Three patients exhibited LOH and three other cases showed LOH with low level of MSI, being classified as having chromosome instability phenotype.**

Conclusion **Two novel observations were found in this study: first, the finding that non-neoplastic cells from a group of untreated CD patients present genomic instability at nucleotide level; and second, the advantage to use carefully selected microsatellites to identify celiac patients with molecular instability. Our data support the existence of chromosome instability phenotype in CD, suggesting that stable and unstable patients are genomically distinct subtypes that may follow a different evolution.** ***Eur J Gastroenterol Hepatol*** **20:1159–1166 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]Genetics Department, Hematological Research Institute '*Mariano R Castex*', National Academy of Medicine, Buenos Aires and [b]Nutritional Support and Malabsorption Unit, San Martín Hospital, UNLP, La Plata, Argentina

Correspondence to Dr Ariela Freya Fundia, PhD, Genetics Department, National Academy of Medicine, Pacheco de Melo 3081, Capital Federal 1425, Buenos Aires, Argentina
Tel: +54 11 4805 5759 x241/291; fax: +54 11 4803 9475;
e-mail: affundia@hematologia.anm.edu.ar

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Introduction

Celiac disease (CD) is a common autoimmune disorder characterized by intestinal inflammation and mucosal atrophy triggered by dietary gluten in genetically predisposed individuals [1]. Although, most patients improve with a gluten-free diet (GFD) [2], a small percentage (2–5%) of adult-onset CD patients develop refractoriness or pre- and malignant complications [3–7]. The association between CD and malignancy, particularly for intestinal nonHodgkin lymphoma and different carcinomas of the gastrointestinal tract is well established [3,5,7–10]. The genetic basis behind cancer development in CD is not known [10], but chronic stimulation of intraepithelial lymphocytes by gluten, impropriate immune responses to gluten and acquisition of genetic abnormalities as well as genomic instability have been suggested [11–18].

Genomic instability reveals an increased rate of mutation in the genome and is considered to be involved in the origin of many human cancers. Two molecular phenotypes associated with distinct pathways of genome destabilization have been identified in human cancer: the mutator phenotype with high-level of microsatellite instability (MSI) and the chromosome instability (CIN) phenotype. The high-level MSI phenotype display intragenic mutations caused by an altered repair of DNA replication errors, because of germline mutations or methylation-induced silencing of key DNA mismatch repair genes (MMR) [19,20]. CIN phenotype arising from mutations in genes controlling DNA segregation is characterized by an imbalance in chromosome number (aneuploidy) and an enhanced rate of loss of heterozygosis (LOH), which is an important mechanism for inactivating tumor suppressor genes [19,21]. The analysis of MSI and LOH with

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microsatellites polymorphic markers, also named short tandem repeats (STR), is useful to test the mutational status of MMR and tumor suppressor genes. MSI and LOH had been shown in a wide variety of human malignancies as well as in non-neoplastic, chronically inflamed tissues [22]. Genomic instability in adult CD patients has, however, not been extensively studied. To explore molecular characteristics of genomic instability in CD patients, MSI, and LOH were analyzed at loci related to MMR genes as well as selected loci associated with tumor suppressor genes, CIN, and genes related to inflammation and cancer.

Materials and methods

Patients and DNA isolation

Small bowel biopsy (SBB) and peripheral blood lymphocyte (PBL) samples from 20 CD patients (14 females and six males, mean age 35.2 years; range 21–66 years) were analyzed (Table 1). None of the patients were complying with the GFD at the time of sample collection. Thirteen patients were studied at diagnosis. The remaining seven had been earlier diagnosed, having a mean evolution time of 145 months (range: 11–326 months). The long period of time without adherence to GFD in this group of patients was related to cultural and socio economical conditions. Five patients reported smoking and none alcohol consumption. CD diagnosis was based on clinical, laboratory, and histological parameters. All small bowel mucosal samples were obtained from the distal part of the second duodenum region by duodenoscopy and traditionally processed. Histological and quantitative morphometric evaluations (intraepithelial lymphocyte counts) were performed by an independent qualified observer. Histological characteristics of intestinal mucosa were assessed by conventional microscopy. Intestinal biopsies were categorized according to Marsh's criteria [1]. All newly diagnosed and the seven rebiopsed CD patients had a flat duodenal mucosa (III of Marsh's criteria). Clinical forms of CD presentation were classified as: asymptomatic, monosymptomatic, and polysymptomatic. All patients gave informed consent and the study was approved by the Ethics Committee of our Institution.

Table 1 Clinical characteristics from celiac patients

Patient	Sex/age (years)	Clinical form	Duration of disease (months)	Nutritional status	Malabsorption syndrome
1	F/26	A	0	NN	No
2	M/24	A	0	NN	No
3	M/30	A	0	NN	No
4	F/42	A	0	NN	No
5	F/31	A	23	NN	No
6	F/38	A	0	NN	No
7	F/21	M′	0	NN	No
8	M/24	M′	285	NN	No
9	M/30	M′	0	MoM	No
10	F/48	M′	0	NN	No
11	M/54	M′	11	NN	No
12	F/26	P	0	SM	Yes
13	F/27	P	0	MM	Yes
14	M/27	P	0	MoM	No
15	F/30	P	117	MM	Yes
16	F/26	P	230	MM	Yes
17	F/39	P	0	MoM	Yes
18	F/66	P	0	MoM	Yes
19	F/55	P	326	MoM	Yes
20	F/40	P	23	MM	Yes

A, asymptomatic; F, female; M, male; M′, monosymptomatic; MM, mild malnutrition; MoM, moderate malnutrition; NN, normal nutrition; P, polysymptomatic; SM, severe malnutrition.

For microsatellite studies, genomic DNA samples from biopsies of abnormal flat duodenal mucosa were compared to PBL from the same patient. DNA extraction was performed from SBB and PBL using a standard phenol/chloroform extraction method. DNA concentration and purity was measured by spectrophotometry and integrity of DNA was checked by agarose gel electrophoresis.

Short tandem repeats selection

Ten different STR, grouped into two panels (A and B), were chosen at loci related to MMR genes or loci with known involvement in inflammation, cancer or CD chromosome instability, considering a reported heterozygosity higher than 0.70 (Table 2). Panel A includes microsatellite markers on MMR associated loci chosen from the Bethesda reference panel recommended for MSI testing in the workshop on HNPCC at the National Cancer Institute [20]. Panel B contains five additional markers located at loci associated with *p53* and *DCC* tumor suppressor genes [23,24], fragile site FRA6F nonrandomly involved in CD CIN [15,16] and *PLA2* and *GSTP1* genes linked to inflammation and cancer [25,26]. The use of markers PLA2 and GSTP1 was preferred because they contain tri-nucleotide and penta-nucleotide repeats that would allow maximizing the sensitivity of the MSI testing. The quasimonomorphic mononucleotide BAT 25 and BAT 26 repeats were not scored for LOH. Primer sequences were obtained from the Human Genome Database (GDB, URL: *http://www.gdb.org*) and the specificity of amplified loci was confirmed by the Basic Local Alignment Search Tool from the National Center for Biotechnology Information (URL: *http://www.ncbi.nlm.nih.gov/*).

Short tandem repeat analysis

The procedure used for STR analysis was published earlier [27]. Briefly, amplification was performed by touchdown PCR in a 25 µl reaction mixture containing 1.5 mmol/l $MgCl_2$, 100 µmol/l dNTPs, and 0.4–0.8 µmol/l of each primer. Electrophoresis was performed on 15% nondenaturing polyacrylamide gels in a Biorad Protean II xi Cell (gel size 20 cm × 17 cm × 0.75 mm) at constant voltage (250–400 V) overnight for 15–20 h, depending on the average size of each marker. Gels stained with 0.1%

Table 2 Microsatellite loci and primers

STR	Map	Type	Het	Size	Gene[a]	Primer sequences	GDB[b]
Panel A							
D2S123	2p16	DI	0.77	197–227	*hMSH2, hMSH6*	5′ AAACAGGATGCCTGCCTTTA 3′ 5′ GGACTTTCCACCTATGGGAC 3′	187953
BAT 26	2p16.2	MONO	NR	120–125	*IVS 5, hMSH2*	5′ TGACTACTTTTGACTTCAGC 3′ 5′ AACCAATCAACATTTTTAACC 3′	9834505
BAT 25	4q12	MONO	NR	120–125	*IVS 16, cKIT*	5′ TCGCCTCCAAGAATGTAAGT 3′ 5′ TCTGCATTTTAACTATGGCTC 3′	9834508
D5S107	5q13.3	DI	0.805	133–155	–	5′ GATCCACTTTAACCCAAATAC 3′ 5′ GGCATCAACTTGAACAGCAT 3′	177304
D18S58	18q22.3–q23	DI	0.74	144–160	–	5′ GCTCCCGGCTGGTTTT 3′ 5′ GCAGGAAATCGCAGGAACTT 3′	188140
Panel B							
D6S267	6q21–q22.1	DI	0.76	235–245	*FRA6F*	5′ AGAGGTAGCTTAGAATGTGCTC 3′ 5′ TAGACAAAGAATGTCGCAAA 3′	187999
GSTP1	11q13	PENTA	0.82	190–235	*Promotor, GSTP1*	5′ AGCCTGGGCCACAGCGTGAGACTACGT 3′ 5′ TCCCGGAGCTTGCACACCCGCTTCACA 3′	270066
PLA2	12q23–qter	TRI	0.73	122–137	*PLA2G1B*	5′ TTGAGCACTTACTATGTGCC 3′ 5′ CTAGGTTGTAAGCTCCATGA 3′	177862[c]
TP53	17p13.1	DI	0.90	103–135	*TP53*	5′ AGGGATACTATTCAGCCCGAGGTG 3′ 5′ ACTGCCACTCCTTGCCCCATTC 3′	191095
DCC	18q21.3	DI	0.86	106–160	*IVS 7, DCC*	5′ TCCCTCTAGAAATTGTGTG 3′ 5′ TGACTTTATCTCATTGGAG 3′	196684

Type: MONO, DI, TRI, and PENTA -nucleotidic repeat sequences.
Het, heterozygosity; NR, nonreported heterozygosity.
[a]Gene sequences located at or near the microsatellite.
[b]GDB identification number (*www.gdb.org*).
[c]Primer sequences modified respect to GDB data.

silver nitrate were analyzed under white light by two independent authors and digitalized with HP Photosmart 735 camara Q2210A (Hewlett-Packard Company, Palo Alto, California, USA). The sensitivity to detect minor cell populations in mixed samples reported for this technique was 0.8–1.6% [27]. Any sample pair observed to have an STR alteration underwent a second test with a new PCR amplification and gel electrophoresis at that locus.

Scoring of MSI and LOH was undertaken according to standardized international criteria [20]. A change of any length because of either insertion or deletion of repeating units, at a given marker within abnormal cells (SBB) compared with normal cells (PBL) was classified as MSI. LOH was defined as loss of one of the preexisting alleles in abnormal DNA compared with normal tissue [20] or when it showed at least a 50% reduction in the relative intensity [28]. Cases with heterozygous allele pattern were considered informative for LOH study [20,28,29]. MSI was classified into three groups: high frequency of MSI (MSI-H) if more than 30–40% of markers exhibited instability, low frequency MSI (MSI-L) if less than 30% STR were altered, and microsatellite stable if no instability was observed on evaluated loci [20]. Patients presenting at least one MSI and/or at least one LOH were considered to have genetic instability. According to Kazama *et al.* [29], the phenotype of genomic instability was defined considering that patients with MSI-phenotype should exhibit MSI-H and patients with CIN-phenotype would present LOH at one or more loci but did not exhibit MSI-H [29].

Statistical analysis

The two proportion test was used to analyze statistical significance between STR groups, considering the *P* value of less than 0.05 as statistically significant. The Primer of Biostatistics Program, Version 3 (1992) was used.

Results

Microsatellite screening was systematically performed to detect band shifts and/or losses on matched samples of DNA isolated from SBB and the corresponding PBL from 20 patients with CD. STR markers without a successful PCR amplification of both samples from the same individual were not considered for the analysis. On average, at least 7–10 microsatellites could be amplified per patient. For each marker, most cases presented detectable heterozygous allele patterns being informative for the LOH study. The highest frequency of heterozygosity was observed at D5S107, TP53, and PLA2 loci (0.95), followed by GSTP1 (0.85), D6S267 (0.80), DCC (0.77), D18S58 (0.75), and D2S123 (0.73). These frequencies are similar to those reported (Table 2).

Overall genomic instability was found in seven out of 20 (35%) cases showing MSI or LOH in at least one microsatellite marker. Five unstable cases were studied at diagnosis and the other two were analyzed during the evolution of disease but they do not comply with the GFD. Stability of the microsatellite loci evaluated was found in 13 out of 20 patients (65%). In addition, no STR

changes were identified in three normal small bowel biopsies (data not shown).

A total of 12 microsatellite alterations were found, where nine (75% of all instabilities) were identified with markers from the panel B and three (25%) were revealed with panel A (Table 3). Results obtained with both groups of STR were analyzed by the two proportion test demonstrating a significant difference between the number of STR changes revealed by panel B (nine out of 12) in respect to those from panel A (three out of 12) ($P = 0.04$). Examples of microsatellite alterations of our CD study group are shown in Fig. 1. To test if these changes correlate with some clinicopathological variables of these patients, we compared age, sex, clinical form, malabsorption, and nutritional status between stable and unstable patients. No significant differences, however, were observed.

Six of the unstable cases (85%) exhibited LOH and four patients (57%) showed MSI. The phenotype of genetic instability identified is analyzed in Table 3. No patients with MSI-H and replication error phenotype were detected, as none of them presented alterations in more than 30% of the analyzed STR. All patients with band shifts at 1 or 2 STR loci per patient (10–25% of the informative STR) met the definition of MSI-L. One case displayed MSI-L phenotype alone at marker GSTP1 and did not show LOH on any other evaluated loci. Six cases were classified as having CIN phenotype: three patients with LOH alone and three cases presenting LOH associated with MSI-L. Table 4 describes the mutation frequency of each STR. The highest frequency of genomic instability was found in 71% of unstable samples at TP53, followed by GSTP1 (43%), D18S58 (29%), DCC (25%), and D5S107 (17%). LOH was mainly observed at TP53 locus, affecting four individuals, with scarce allelic losses in other loci. MSI was detected in two patients at marker GSTP1, whereas D5S107, D18S58, and TP53 were unstable only once.

Discussion

Malignancies are the most serious complications of CD especially in undiagnosed patients or when responses to GFD are not achieved or sustained. Malignant tumors include gastrointestinal carcinomas and non-Hodgkin lymphoma at any site, being enteropathy-type T-cell lymphoma (ETL), a rare high-grade T-cell non-Hodgkin lymphoma of the small intestine, almost exclusively observed in CD patients [3,5,7–10]. Besides cancer, refractory CD type II and ulcerative jejunitis are two recently described premalignant complications of CD that may progress to lymphoma [5,6]. To date, no known genetic mechanisms have been identified to explain cancer predisposition in CD, but the acquisition of genetic abnormalities as well as genomic instability have been implied [14–18]. Recently, Isaacson and Du proposed that the development of ETL is a multistage process spread out from a background of CD, based on the clonal expansion of monoclonal neoplastic T-cell

Table 3 **Microsatellite analysis in celiac patients with genetic instability**

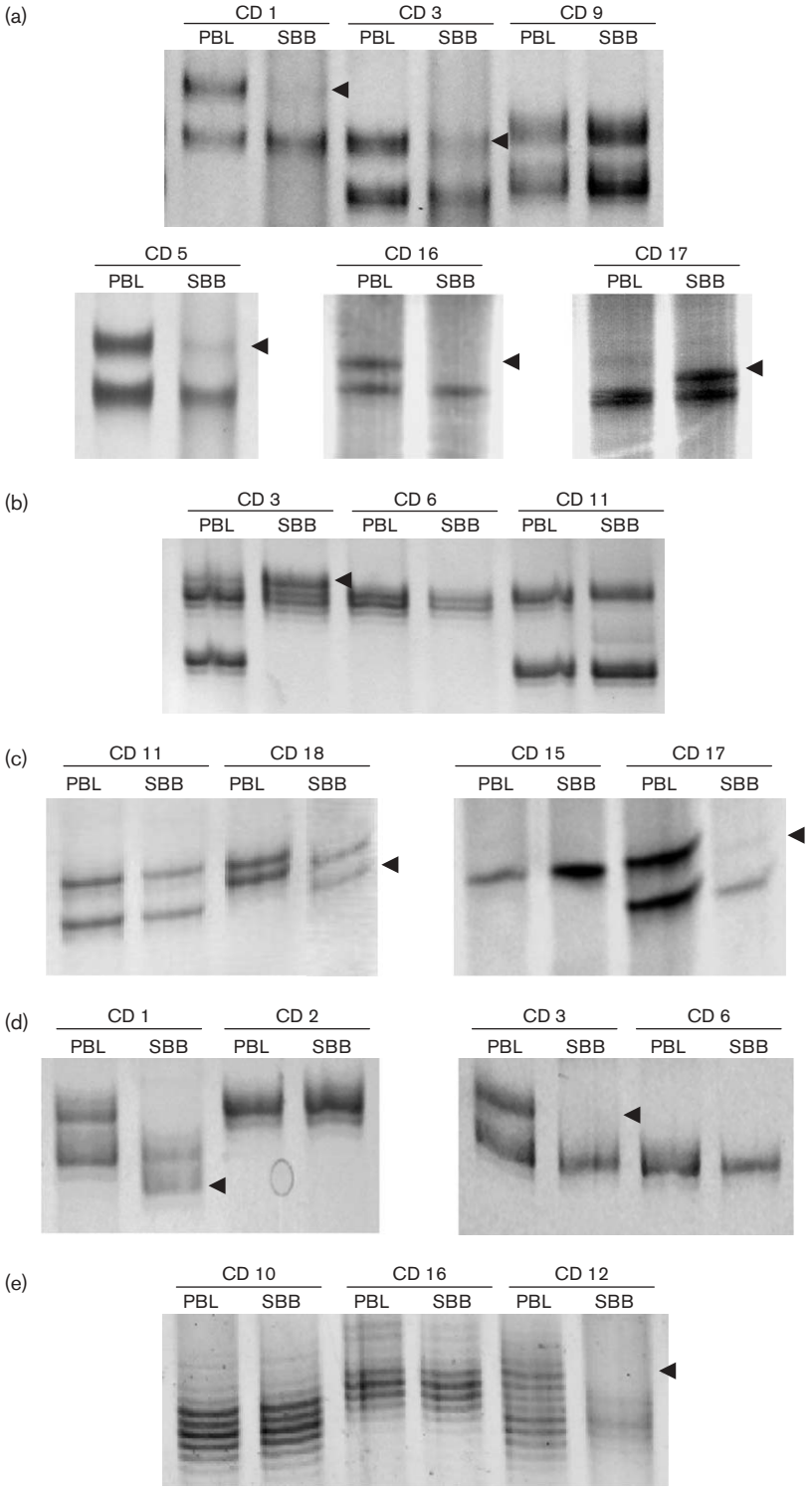
Cases with genetic instability	STR from panel A					STR from panel B					MSI[a]	LOH[b]	Genetic instability phenotype
	D2S123	BAT26	BAT25	D5S107	D18S58	D6S267	GSTP1	PLA	TP53	DCC			
1						–				–	2/8	1/6	CIN with MSI-L
3		–						–		–	1/7	2/6	CIN with MSI-L
5	–							–			0/8	1/6	CIN
12											0/10	1/8	
										/			
													CIN; with MSI-L
18	/									/	1/10	0/8	MSI-L

□, microsatellite stable; ▒, microsatellite instability; ■, loss of heterozygosis; ⊘, homozygous alleles; ⊟, sample pairs without successful PCR amplification;

[a] Number of unstable markers/Total STR analyzed in each patient.

[b] Number of unstable markers/Total informative STR in each patient excluding homozygosity.

Fig. 1



Microsatellite genotyping on celiac disease (CD) patients comparing allelic patterns from small bowel biopsies (SBB) respect to the corresponding normal peripheral blood cells (PBL) showing: (a) Amplification of TP53 locus displaying loss of heterozygosis (LOH) (cases 1, 3, 5, and 16); microsatellite instability (MSI) evidenced by short tandem repeats (STR) amplification (case 17), and normal genotype (patient 9); (b) MSI at D5S107 locus evidenced by STR amplification (case 3) and normal patterns (patients 6 and 11); (c) MSI at GSTP1 locus evidenced by STR deletion (patient 18), LOH (case 17), and normal genotypes (cases 11 and 15); (d) MSI at D18S58 locus evidenced by STR deletion (patient 1), LOH (case 3), and normal genotypes (cases 2 and 6); (e) LOH at DCC locus (case 12) and normal patterns (patients 10 and 16). Black arrows point the mutated allele band.

Table 4 Frequency of mutation at each STR locus in the unstable CD group

	Genetic instability[a]		
STR	LOH	MSI	Total (%)
TP53	4/7	1/7	5/7 (71)
GSTP1	1/7	2/7	3/7 (43)
D18S58	1/7	1/7	2/7 (29)
DCC	1/4	0/4	1/4 (25)
D5S107	0/6	1/6	1/6 (17)

[a]Number of cases showing MSI or LOH/total unstable CD patients.
CD, celiac disease; LOH, loss of heterozygosis; MSI, microsatellite instability; STR, short tandem repeats.

lymphocytes emerging from intraepithelial lymphocytes in refractory CD and leading to an overt ETL. These events include impropriate immune responses to gluten, such as the expression of IL 15 by epithelial cells, and the acquisition of genetic abnormalities [14]. To study the genetic events linked to the development of cancer in CD, in the first cytogenetic studies of celiac patients we have proposed that CIN affecting fragile sites and lymphoma breakpoints could be related to CD cancer proneness [15,16]. Using a molecular approach, Cottliar *et al.* [17] confirmed CIN phenotype in CD suggesting that telomere reduction may be also related to the malignant predisposition. In line with these findings and others, Verkarre *et al.* [18], indicated that a CIN pathway might operate during lymphoid transformation in CD.

This study is the first to clearly indicate that a group of untreated CD patients presents genomic instability at nucleotidic level, affecting specific STR loci. The minimal number of loci recommended for MSI studies is five [20] but we used another panel of five STR (panel B) chosen by virtue of their association to tumor suppressor genes, chromosome instability, inflammation, and cancer. The use of this precise panel allowed the identification of seven unstable cases instead of two cases found with Bethesda markers (panel A). Moreover, the proportion of STR changes identified with panel B was significantly higher than those revealed by panel A, suggesting that Bethesda repeats are not useful to study genomic instability in these patients. Earlier reports suggested that the ability to detect instability depends on the type of microsatellite examined [20,30–33]. Furthermore, we detected different frequencies of instability at each microsatellite loci studied. This variation was already demonstrated experimentally to depend on the genomic context revealing differences in the efficiency of DNA mismatch repair [34]. Interestingly, according to the 'Real Common Target Genes' model for MSI carcinogenesis, mutations in microsatellites inside genes involved in tumor cell growth were found more frequently than in microsatellites without involvement in malignant transformation [35,36]. Thus, our data demonstrate that a careful selection of markers near or within genomic loci containing genes important in CD should be performed to study genomic instability in this disease.

Another interesting finding of this study was the higher involvement of microsatellite alterations at TP53 loci in the unstable CD cases. *TP53* is the most commonly mutated tumor suppressor gene in human cancers [24]. The *TP53* tumor suppressor pathway is well known to be crucial for maintaining genomic integrity and preventing cells from undergoing oncogenic transformation. Lost of normal *TP53* function is commonly caused by loss of one allele and missense mutation in the other. Mutations of this gene can lead to overexpression of TP53 protein, which suppresses cell growth and transformation. Accumulation of TP53 protein was detected in the duodenal mucosa of children with CD [37], as well, in different non-Hodgkin lymphoma and carcinomas of the gastrointestinal tract [38–40]. It is unclear whether TP53 microsatellite mutations, among other factors, would provide the necessary mechanism to generate an unstable background in non-malignant mucosal cells from untreated patients.

Finally, we established that most of the CD unstable cases exhibit CIN phenotype and are not associated with MMR defects. Genetic abnormalities associated with CD or with the tumors related to the disease are not fully characterized. Originally, we observed increased frequencies of chromosome aberrations in PBL of adult patients with untreated CD [15,16]. CIN was also described by Kolaček *et al.* [41] in PBL from children with CD and other inflammatory enteropathies, and proposed that the abnormality is not specific for CD [41]. In the follow up of children with CD, these authors confirmed their hypothesis, showing that a strict GFD decreases the rate of chromosomal aberrations, suggesting that genomic instability is a secondary phenomenon, possibly caused by chronic intestinal inflammation [42]. By cytogenetic analysis of the immunophenotypically abnormal T-cell clones isolated from patients with refractory sprue, Verkarre *et al.* [43] showed partial trisomy of the 1q22–q44 region, suggesting that gain of chromosome 1q might be an early event during the development of ETL. As well, comparative genomic hybridization showed multiple chromosomal imbalances in ETL, including more frequently the gain of 9q33–q34 observed in 64% of the cases examined. Further recurrent chromosomal gains were observed at 1q32, 5q35, 7q22, 8q24, and recurrent losses were found at 8p22–p23, 9p21, 13q22, and 18q22 [44]. High frequency of genetic aberrations was demonstrated by microsatellite testing in ETL, including LOH at TP53 repeat, MSI-L phenotype and more frequently amplification of 9q34 region encompassing c-abl and Notch-1 gene loci [45]. Obermann *et al.* [40] demonstrated frequent deletions at 9p21 in ETL cases, which were accompanied by loss of p16 expression, suggesting

inactivation of the *p16* tumor suppressor gene. Potter *et al.* [46] found that CD cases with small bowel adenocarcinoma exhibit MSI-H and MMR defects, suggesting that these may be caused by the chronic inflammation of the intestine.

The link between inflammation and cancer is well known. Inflammation contributes to about 25% of all cancer cases worldwide. Several mechanisms might cooperate to facilitate the emergence of tumors in inflamed tissues [18]. Mediators of the inflammation response, for example, cytokines, free radicals, prostaglandins, and growth factors, can induce genetic alteration including point mutations in tumor suppressor genes, change gene-expression profile and induce posttranslational modifications leading to genetic and physiological instability and cancer [47]. Considering that the major carcinogenic pathways that lead to malignancy, namely CIN and MSI were also found in inflammatory conditions, chronic inflammation could be considered as a risk factor for neoplastic development through a genetic instability pathway. To our knowledge, genetic instability by revealing MSI and LOH status were not studied earlier in intestinal mucosa from CD patients. Evidence of authentic molecular alterations in CD cells was shown by Cottliar *et al.* [17] and the finding of specific STR alterations identified in this study. Altogether, most genetic studies, as was suggested earlier by Verkarre *et al.* [18], point to the role of chronic inflammation in the induction of genomic instability and malignant emergence, particularly in those patients prone to develop complications such as refractoriness or ulcerative jejunitis.

In conclusion, there are two novel observations in this study: firstly, the finding that a group of CD patients present genomic instability at nucleotide level previously to initiate the GFD; and secondly, the advantage to use carefully selected microsatellite markers to identify celiac patients with molecular instability. Probably, stable and unstable patients are genomically distinct subtypes that may follow a different evolution. These findings highlight the existence of molecular alterations in CD non-neoplasic cells that may possibly provide the necessary mechanisms required for malignant complications in at-risk individuals. Further research at basic science level will improve the understanding of CD and the identification of those individuals at higher risk of adverse events who might benefit from further intervention.

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'Primer Simposio Latinoamericano de Enfermedad Celíaca' performed in Buenos Aires, 28-30 June 2007, published in a CD form.

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