Introduction of Cytogenetic Tests in Colorectal Cancer Screening

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Abstract

Background and aim. The existing tests practiced on a large scale in colorectal cancer (CRC) screening do not fully accomplish the goal of best specificity and sensitivity or either an optimal cost/efficiency ratio. We aimed to analyze genetic mutations diagnosed in the DNA of exfoliated cells in the stool of the patients diagnosed with CRC through screening. We also aimed to demonstrate the similarity between the detected mutations in tumor samples and in exfoliated cells in the stool in order to prove the reliability of these cytogenetic tests, so that they could be introduced in CRC screening program. **Methods**. We studied 200 patients diagnosed with CRC from whom we prelevated biopsy and stool samples. Samples were submitted to genetic analysis through denaturing gradient polyacrylamide gel electrophoresis method, and through polyacrylamide gel electrophoresis method for the heteroduplex analysis. Analyzed genes were *APC, COL11A1, MLH1, MSH2* and *MSH6*. The chromosomal study was carried out using the PRINS technique. **Results.** We discovered mutations in the *APC* gene (exons 4, 9, 13, and 15c) and *COL11A1* gene (exon 54). Our chromosomal study detected instability of chromosomes 1, 7, 9, 20, and in 10 achrocentric chromosomes. **Conclusions**. Our results plead for the implementation of proposed cytogenetic tests in CRC screening programs.

Key words

Colorectal cancer – tumor biopsy DNA – stool DNA, noninvasive screening tests.

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Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed cancer in both men and women and the second leading cause of cancer death after lung cancer. CRC is curable if diagnosed at an early stage [1]. Screening for CRC lowers both the mortality and the incidence of the disease and is widely recommended for people over 50's.

Colorectal cancer screening still implies improvements in order to ensure reliable tests to be practiced on a large scale, with better specificity and sensitivity and with an optimal cost/efficiency ratio. The existing tests do not fully accomplish these goals. Each existing screening option (i.e., fecal occult-blood testing [FOBT], sigmoidoscopy, colonoscopy and barium enema) has advantages and limitations and no option is ideal. As a result, molecular genetic screening analysis of stool DNA has been proposed as an alternate, noninvasive screening tool for CRC [2, 3].

This study was designed to analyze the genetic mutations identified in the DNA of exfoliated cells in the stool of the patients diagnosed with CRC through screening and to demonstrate the similarity between the detected mutations in tumor samples and in exfoliated stool cells. Finally, we aimed to prove the reliability of these cytogenetic tests in order to implement them in CRC screening programs.

Material and method

We studied 200 patients diagnosed with CRC within the screening program. We obtained both biopsy and stool samples that have been submitted to genetic analysis in order to detect mutations through DNA electrophoresis technique in denaturing gradient polyacrylamid gel. Subjects were given detailed instructions for stool collection; no dietary or medication modifications were required and a minimal 30g sample was required. Fresh samples of colorectal biopsy and of feces were immediately frozen at -20^0C.

The analysed genes and their mutations were selected based on the ground of genetic studies of CRC patients with a positive family history [4-6]. The studied genes in both types of prelevated samples were: the *APC* gene, the *COL11A1*

gene, and the genes responsible for hereditary nonpolyposis colorectal cancer: *MLH1, MSH2* and *MSH6*.

A chromosomal study was carried out in 20 patients with tumor masses detected by screening. We analyzed all chromosomes and used the PRINS technique to diagnose chromosomal microsatellite changes in studied cases.

The genetic methods of processing samples were the denaturing gradient gel electrophoresis method and the polyacrylamid gel electrophoresis method for the heteroduplex analysis.

Denaturing gradient gel electrophoresis (DGGE) [7]

The method (Fig. 1) allows a rapid screening for single base changes in DNA fragments.

The technique is based on the migration of double stranded DNA molecules through polyacrylamide gels containing linearly increasing concentrations of denaturating agent, such as formamide and ureea, although increasing temperature gradients have also been successfully applied. The position in the gradient where a domain of a DNA fragment melts and thus nearly stops migrating is dependent on the nucleotide sequence in the melted region. Sequence differences in otherwise identical fragments often cause them to partially melt at different positions in the gradient and therefore "stop" at different positions in the gel.

By comparing the melting behavior of the polymorphic DNA fragments side-by side on denaturing gradient gels, it is possible to detect fragments that have mutations in the first melting domain.

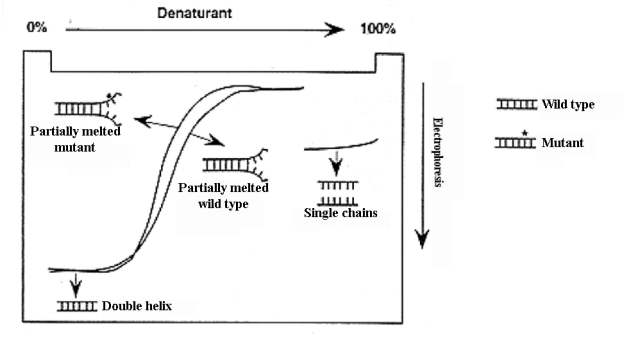


Fig 1. The DGGE technique.

Polyacrylamide gel electrophoresis method for the heteroduplex analysis (HA) [7]

Heteroduplex analysis (Fig. 2) has been used to enhance the sensitivity of DGGE in the detection of point mutation [12-14]. DNA fragments for HA can be visualized via a variety of methods including bromide staining, labelling with radioisotopes, and silver staining. The mutation detection rate of HA under ideal conditions is near 90%.

By using PCR amplification from individuals heterozygous for any sequence difference, the single strands do not necessarily rehybridize exactly with the complementary strand. They can alternatively form a DNA hybrid (heteroduplex) consisting of a sense strand with one sequence variant and an antisense strand with another variant.

As a consequence, the heteroduplex DNA has a region

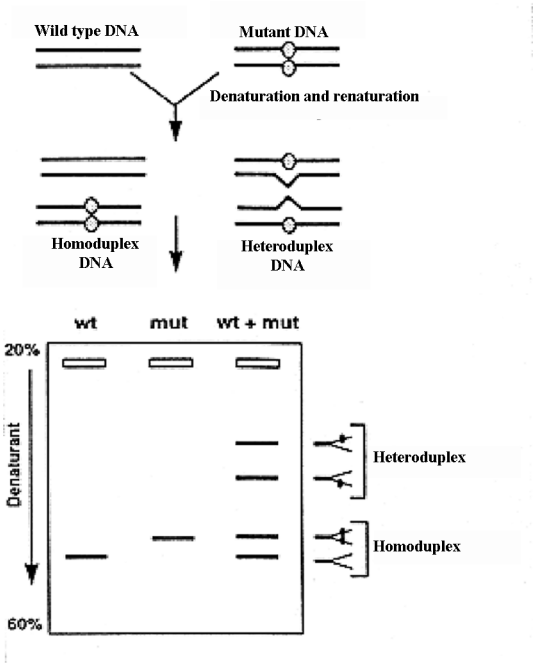


Fig 2. The polyacrylamide gel electrophoresis method for heteroduplex analysis.

of at least one base pair mismatch. The region of mismatch can elicit a mobility shift, which is usually small but can be visualized after prolonged electrophoresis on native polyacrylamide gels.

Technical principles

Detection of a certain gene mutation includes the following steps:

1. genomic DNA extraction from the analyzed samples;
2. amplification of the interested gene amplicons through PCR reaction;
3. amplification check up through electrophoresis in agarosis gel and bromide ethidium staining;
4. mutation identification through DGGE technique and silver or ethidium bromide staining.

DNA extraction from proposed samples

DNA extraction from the stool has been made by a specific kit for stool extraction (QIAGEN GmbH, Hilden, Germany) [8, 9]. DNA tissue extraction from the colorectal cancer has been performed using DNA IQ (TM) System kit (Promega, USA) [10].

Validation of human origin of DNA extracted from stool by STR loci typing

In the preliminary phase of our study, we comparatively analyzed the DNA extracted from the biopsy samples of the patients with the DNA extracted from the stool samples of the same patients. This comparative analysis was performed by investigating 9 human STR loci ("short tandem repeat" or "microsatellite repeats"), frequently used in the DNA typing techniques of forensic medicine. Determination of the 9 loci has been made by using kits of molecular biology, namely GenePrintSTR Systems of Multiplex type (Promega, USA).

The PRINS technique [7, 11]

The advent of molecular genetic techniques has brought forth new procedures for in situ chromosomal analysis. One of these techniques is the primed in situ labeling (PRINS) procedure (Fig. 3). The PRINS method combines the high sensitivity of the PCR reaction with the cytological localization of DNA sequences. The technique has thus proved to be a useful tool for in situ chromosomal screening, and has become a simple and efficient complement to conventional cytogenetic and FISH methods [11, 12].

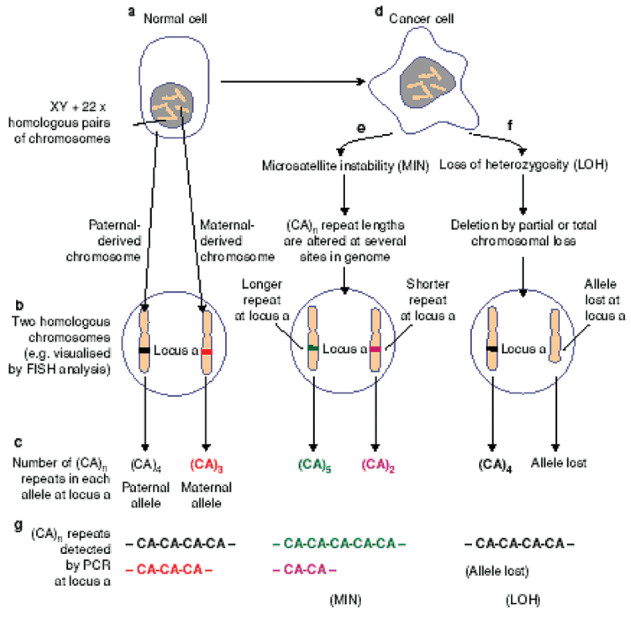
The PRINS method produces the same type of results as FISH. However, the main advantages of the PRINS design are faster (complete procedure, including fluorescence detection, is less than two hours) and cheaper detection of target sequences, probes are readily available from any oligonucleotide synthesis facility, and can be designed to suit this purpose.

Results

The aspect of the electrophoresis plaque obtained in order to point out the STR alleles for the studied cases is shown in Fig. 4. In all analyzed cases, the STR alleles from the "b" sample (biopsy), respectively the "f" sample (stool) are identical. This fact proves that the DNA samples have the same origin, belonging to the same patient, and the extracted DNA quantity is sufficient for PCR amplification that is required for further testing.

1. DGGE analysis of the APC gene mutations

The *APC* gene was studied by DGGE technique in 50 patients. As most of the identified *APC* gene mutations are localized around specific "hot points" of the gene, the DGGE study of the *APC* gene is usually performed on exons 1-15.



The effects of microsatellite instability (MIN) and loss of heterozygosity (LOH) on $(CA)_n$ dinucleotide tandem repeats

Fig 3. The PRINS technique.

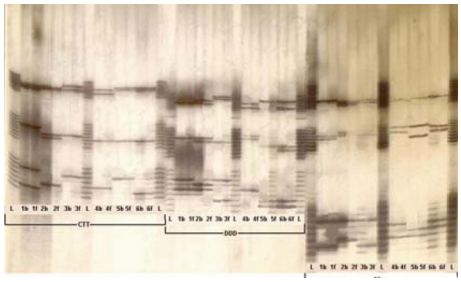


Fig 4. DGGE plaque of the PCR amplification products for the analyzed loci. "L" – allelic "ladders" for each locus. "1b, 2b,….6b" – amplified biopsy DNA . "1f, 2f,….6f" – amplified stool DNA. .

Among the 15 exons of the *APC* gene, we analyzed the mutations of 10 exons, which are amplified in 12 PCR reactions.

PCR reaction. The 12 amplicons of the *APC* gene were amplified through PCR reaction, in three groups, according to the common characteristics of the melting point and migration in the electrophoresis process. After amplification, samples were examined through electrophoresis in agarosis gel to confirm the successful outcome of the amplification. Two samples were applied for migration, one being considered as the "normal" type (the same in all the cases), and the other representing the analyzed case.

DGGE analysis of exons 4, 5 and 7. We detected mutations at the level of the exon 4 in 9 patients. The DGGE analysis of the exons 5 and 7 showed no electrophoretic pattern pointing out a mutation in all the analyzed cases.

The DGGE analysis of the exon 4 clearly proves the presence of a displaced bands pattern in three cases, when comparing with control (Fig. 5) which indicates the presence of mutations. The same gap in the sample, as compared to the control, can be seen in both DNA samples, from biopsy and stool.

DGGE analysis of exons 8, 9, 10 and 12. By respecting the "melting" properties of the PCR fragments obtained through exons 8, 9, 10 and 12 amplification, we discovered a mutation of exon 9, using the melting point at 69ºC. The mutation became obvious in both the DNA samples, from biopsy and stool, in 5 cases (Fig. 6).

The DGGE analysis of the exons 8, 10 and 12 showed no electrophoretic pattern evidencing a mutation in all the analyzed cases.

DGGE analysis of exons 13, 14 and 15. The DGGE analysis of the exon 13 detected different mutations in 6 patients. Two types of specific mutations were detected in two cases (Fig. 7). As we observed, the pattern of mutation differed in these two cases, but the mutation was identical in both samples, from stool and biopsy.

The DGGE analysis of the exon 14 showed no electrophoretic pattern pointing out for the presence of any mutation in all the analyzed cases.

The analysis of the exon 15, with the size of 6.5 bp, was made with three pairs of primers, for the amplicons c, d and

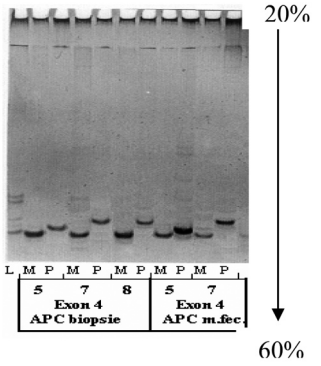


Fig 5. DGGE of the amplified DNA for exon 4 of the APC gene (M – control; P – sample), using silver staining. First 6 fronts – biopsy DNA (*APC* biopsy). Last 4 fronts – stool DNA (*APC* stool). L – molecular weight ladder. Arrow – direction of migration and increasing concentration of the denaturation gradient.

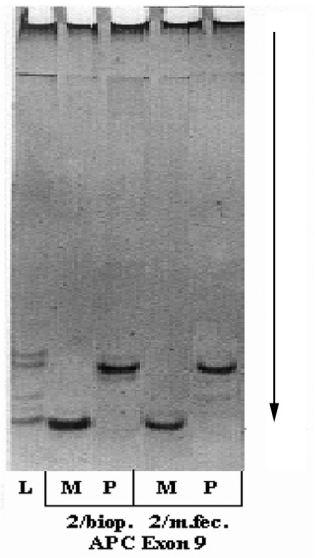


Fig 6. DGGE of the amplified DNA for exon 9 of the *APC* gene (M – control; P – sample).

e. Two mutations of the amplicon c were identified in five patients (Fig. 8). Even in these cases, the mutations were of a different type, but identical in biopsy and stool.

2. DGGE analysis of *MLH1, MSH2* and *MSH6* genes mutations

The *MLH1* gene consists of 3 exons and 22 amplicons and the *MSH2* gene, consists of 2 exons and 22 amplicons.

All three analyzed genes, on all amplicons groups, in all 90 studied cases (30 cases for each studied gene) showed no mutations. (The primers for the PCR amplification of these amplicons were provided by INGENY, Holland). We noticed no difference in the migration speed through DGGE of the control compared to the samples in any studied case.

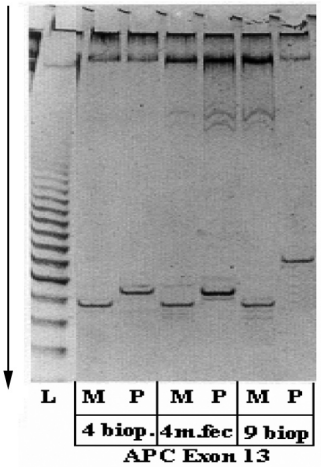


Fig 7. DGGE of the amplified DNA for exon 13 of the *APC* gene (M – control; P – sample).

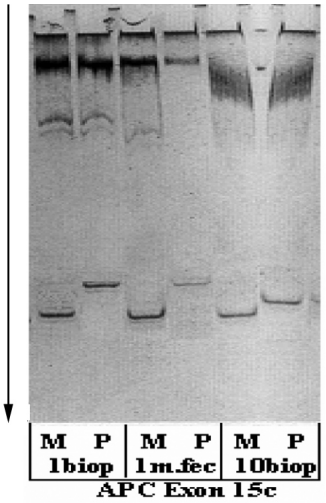


Fig 8. DGGE of the amplified DNA for exon 15c of the *APC* gene (M – control; P – sample).

3. DGGE analysis of *COL11A1* gene mutations through DNA heteroduplexes

COL11A1 contains 68 exons, yet not wholly sequenced, of which exons 38, 41, 16, 54, 55, 56 and 57 were studied.

Heteroduplex analysis for exons 38, 41 and 16. For all 3 analyzed amplicons in all 40 studied cases, the migration speed was identical for both control and samples.

Heteroduplex analysis for exons 54, 55, 56 and 57. It clearly demonstrated in 6 cases the presence of a displaced bands pattern in the biopsy samples compared with control. We noticed no mutations in the rest of the analyzed cases.

For case no. 6, the fronts marked with "M" and "P" present a single migrating band (Fig. 9). The "M+P" migrating front presents two bands, out of which the slowest part generates the heteroduplexes obtained through denaturation-renaturation, and the faster one, the homoduplexes. The samples differ from the wild type due to the fact that they contain amplified mutant type DNA, which through electrophoresis leads to speed migration modification. The mutation detected by us is

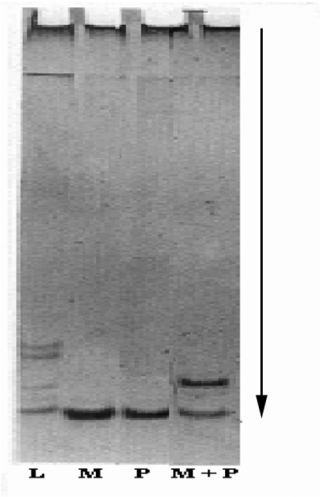


Fig 9. Electrophoresis in 6% polyacrylamid gel of the processed samples in order to point out heteroduplexes for exon 54, using silver staining. L – molecular weight ladder. M – healthy individual allele (control). P – unprocessed allele for exon 54. M+P – denatured-renatured sample.

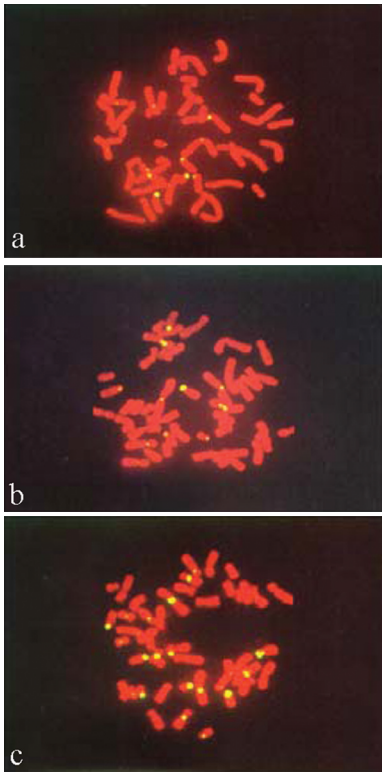


Fig 10. PRINS amplification with primers for the β-satellite region
a. Chromosome 1 and a pair of achrocentric chromosomes are marked after a single amplification cycle. b. A greater number of chromosomes are marked after 10 amplification cycles and the original signal is stronger. c. After 20 amplification cycles, 10 achrocentric chromosomes plus chromosomes 1, 7, 9 and 20 are marked.

a substitutive type one as a series of two bands was evident on the migration front.

4. Chromosomal aberrations

The chromosomal study was carried out in 20 patients. We analyzed all chromosomes and consequently determined by means of the PRINS technique and of the kits provided by Boehringer-Mannheim (Germany), the microsatellite changes of chromosomes 1, 7, 9, 20 and in 10 achrocentric chromosomes. We obtained the following results: the primer derived from β-satellite sequence (AGTGCAGAGATAGTCACAATG-CCCC) marked 6 situses with a strong signal in PRINS reaction – it was the chromosome 1 and another two pairs of achrocentric chromosomes (Fig. 10). Six other situses were well marked with the satellite III area primer –chromosome 9, 7, and the third pair of the achrocentric ones (Fig. 11).

After 20 amplification cycles, the number of marked situses doubled, and the signal became stronger. For example, a single repetitive sequence located on the chromosome 9 is easily detected through PRINS reaction (Fig. 11a). The amplification in several cycles amplifies the initial sequences of the chromosome 9 and also detects other situses that include fewer copies of the sequence, respectively chromosome 1, and the achrocentric chromosomes 13-15 and 21-22 (Fig. 11b).

Discussion

Stool DNA analysis is an emerging non-invasive diagnostic tool for CCR [13]. Although companies that produce kits for DNA extraction from such biologic samples warrant individual genomic DNA presence, there are no studies proving that this DNA effectively belongs to the host. It is obvious that the DNA extracted from stools may originate not only from the colorectal mucosa, but also from the colorectal microbial flora, from the potential eukaryotic parasites, and even from undigested food remains of animal origin. Direct analysis of DNA extracted from stools supposes that, through PCR amplification procedure, the DNA of the host is specifically amplified due to the use of specific primers. Nevertheless, a successful PCR reaction does not guarantee that the matrix DNA belongs to the individual from whom the stool probe provides.

We found no studies aiming to clarify this crucial aspect for a correct interpretation of the results. Therefore, in our study we comparatively analyzed the DNA extracted from the biopsy samples of the patients with the DNA extracted from the stool samples of the same patients, by investigating a number of 9 human STR loci. We consider this method as the most emphatic in proving that the DNA origins from the same person, both in biopsy tissue and stool. Our results plead for the validation of the stool samples (from the exfoliated tumor cell) as belonging to the subject from whom

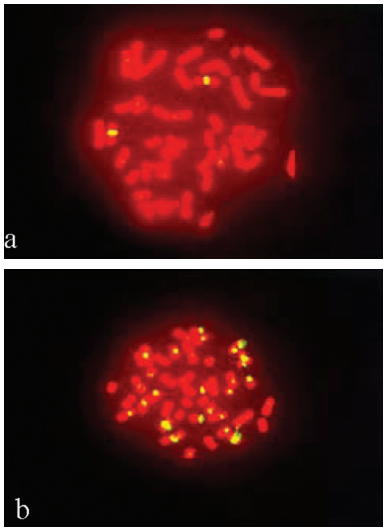


Fig 11. PRINS amplification with primers for the satellite III region. a. After a single amplification cycle, only chromosome 9 is marked. b. After 5 amplification cycles, chromosome 9 is intensely marked, but achrocentric chromosomes and even some metacentric chromosomes are also marked.

the probe was collected, because they expressed the same genetic profile as the ones obtained from tumor biopsy.

Diagnosing CRC by testing stool for DNA is based on identifying the oncogene mutations characteristic of colorectal neoplasia that are detectable in exfoliated epithelial cells in the stool, as a healthy adult excretes approximately 1,010 epithelial cells every day [14]. However, no single mutation has been identified as being expressed universally. For this reason, multiple target assay panels (MTAP) currently being studied have the potential to attain higher detection rates than current screening methods.

In our study, we found mutations involving the *APC* gene, and the *COL11A1* gene. Our chromosomal study detected instability in the cases of chromosomes 1, 7, 9, 20 and in 10 achrocentric chromosomes.

Stool-based DNA testing is a promising, single, noninvasive test that is intended to identify the presence of genetic mutations known to be associated with CRC. The accuracy of the test in asymptomatic persons (e.g., the target population for screening) is unclear.

There is a lack of consensus on how many or which markers are necessary for the test to achieve acceptable sensitivity and specificity level [15].

Conclusions

The genetic tests performed in our study argue for the validation of the stool samples as belonging to the subject from whom the probe was collected, due to the fact that they have the same genetic profile as the ones obtained from tumor biopsy.

By studying *MLH1, MSH2, MSH6, APC* and *COL11A1* gene mutations, we identified the presence of *APC* gene mutations, at the level of exons 4, 9, 13 and 15c, and the presence of *COL11A1* gene mutations at the level of exon 54, mutations that can be recommended as genetic tests with great specificity in CRC screening.

We detected instability in the cases of chromosomes 1, 7, 9, 20 and in 10 achrocentric chromosomes.

Conflicts of interest

None to declare.

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