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Genetic Biomarkers for Neoplastic Colorectal Cancer in Peripheral Lymphocytes

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ABSTRACT

Background: *Loss of genomic stability appears as a key step in colorectal carcinogenesis. Micronucleus (MN) designates a chromosome fragment or an entire chromosme which lags behind mitosis. MN may be noticed as an additional nucleus within the cytoplasm cell during the intermediate mitosis phases. We tested the hypothesis that MN and its related anomalies may be associated with the presence of neoplastic colorectal lesions.*

Method: *Peripheral blood lymphocytes were cultured and microscopically examined. The frequency of micronuclei (FMN) and the presence of nucleoplasmic bridges (NPB) in binucleated cells were compared in patients with of without colorectal neoplastic lesions.*

Results: *We included 45 patients undergoing colonoscopy, 23 males and 22 females, with a median age of 59. 17 patients had polyps, 11 colorectal cancer (CRC) and 17 had a normal colonoscopy. The FMN was significantly higher in women than in men (8.14 vs 4.17, p=0.008); NPB were significantly less frequent in patients with advanced adenomas (>10mm or vilous) or CRC (p=0.044) when compared with patients with normal colonoscopy, hiperplastic polyps or non-advanced adenomas.*

Conclusion: *Micronuclei are more frequent in women, but its frequency was not significantly different in patients with advanced adenomas or CRC. Null or low frequency values for nucleoplasmic bridges presence in peripheral lymphocyte may be predictive for advanced adenomas and colorectal cancer.*

Keywords: micronucleus, nucleoplasmic bridges, colon cancer

INTRODUCTION

In 1988 a model of colorectal carcinogenesis reflecting the molecular alterations appearing successively from normal mucosa to adenoma and carcinoma was proposed (1). The mechanisms involving genomic instability in colon cancer are only partially understood. Clarifying these steps may have important implications in screening approach, prognostic stratification and treatment strategies.

Colorectal cancer (CRC) is a result of progressive accumulation of genetic and epigene-

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tic alterations of colorectal epithelial cells towards adenocarcinoma. The loss of genomic cell stability seems to be the main pathogenic key, appearing early in the carcinogenesis process.

Genetic instability supposes in fact, the loss of DNA integrity. There were described 3 major pathways of genomic instability in CRC. The first one, described in 1988, named *Chromosomal Instability,* and is the most important and most frequent type, being in the same time, the earliest anomaly in colonic carcinogenesis (2, 3). It is also called *Suppressor type*. More than 85% of CCR have this somatic mutation (both sporadic forms and genetic syndromes, as Familial Adenomatous Polyposis). This mechanism involve the inactivation of some tumoral suppressor genes, as APC -5q, p53- 17p, DDC, SMAD2 or SMAD4-18q. Tumoral cells that results through this way expresses a lot of cytogenetic anomalies. In the last years many genes were identified as triggers of chromosomal instability (4-6).

The second pathway of genomic instability, named *Mutator* type was discovered in 1993, and it originates from the inactivation of a reparatory genes system which involved in fixing the errors that appear during DNA replication (7-9). It includes the accumulation of somatic mutations at the level of certain repetitive small nucleotidic base sequences, called microsatelites. The most frequent genes involved in CRC are MLH1 and MSH2. Tumoral cells that results from this mechanism do not express many cytogenetic anomalies. This type is mainly involved in hereditary non polypous cancers (HNPCC), but also in 10-12% of sporadic CRC.

In 1999 a third mechanism of colorectal carcinogenesis was discovered, the *Methylator* type (CpG island methylator phenotype), which presumes the hypermetilation of one promoter region sequence of a "reparatory" DNA gene (MLH1 in most cases). It is associated with serrated adenoma (with an accelerate adenoma – carcinoma sequence). The characteristics of this type are female predominance, proximal localization, mucinous and poorly differentiated forms in histology (2,4,6).

As the access to complex genetic tests is low, the researchers are looking for simple biomarkers to evaluate cancer risk in a general population or in patients with predisposing conditions or exposure to environmental risk factors.

Micronucleus (MN) is a fragment or a whole chromosome that is left behind during cellular mitotic division, appearing in the cytoplasm as a small additional nucleus (10). Its formation in dividing cells is the result of chromosomal breakage or loss, due to unrepaired or mis-repaired DNA lesions (11,12). Similarities between the level of chromosomal alterations in oral mucosa or peripheral lymphocytes and correspondent levels in tumoral tissues guided their utilization as biomarkers of cancer risk. So, the frequency of MN was proposed as a marker of chromosomal lesions, of genomic instability and cancer risk, integrating both, acquired mutations and genetic susceptibility (13-16).

Nucleoplasmic bridges (NPB) are thought to originate from rearranged chromosomes with more than one centromere, eg. dicentric chromosomes whose centromeres are pulled apart to opposite poles of the cell at anaphase. Binucleated cells with NPB often contain MN. Their importance is that provides direct evidence of genomic damage resulting from mis-repaired DNA breaks (17).

The aim of our study was to characterize a cohort of patients undergoing colonoscopy with either normal aspect, neoplastic or non neoplastic colonic polyps or colorectal cancer, searching associations with frequency and number of micronuclei and with the presence and frequency of nucleoplasmic bridges.

METHOD

A prospective cohort study was performed, including consecutive patients with colorectal cancer and/or colonic polyps and/or normal colonoscopy. Patients were signed the inform consent to participate in the study and we obtained the approval of local ethic committee.

We considered as excluding criteria the concomitance of neoplasm, other than colorectal, age under 18, genetic colorectal neoplasia and a recent exposure to radiations (therapeutically or professionally). Two pathologists confirmed the diagnosis. The cytogenetic analysis was performed by a single specialist who also prepared and scored the slides.

Heparinized blood samples collected by venipuncture were obtained from patients after informed consent. Blood was kept at room temperature for the shortest time possible, until

the samples were processed (within 4 hours). The total volume of each culture was 5 ml. A peripheral blood karyotyping medium with phytohemagglutinin (PHA) M was used (Biological Industries,Israel). Whole blood cultures were used, with a 10% ratio of whole blood to culture medium. For each sample duplicate cultures were set up. Cells were incubated at 37° C. Cytochalasin-B (Sigma; dissolved in dimethylsulphoxide) was added to cultures 44 hours after PHA stimulation, in a concentration of 6.0 μg Cyt-B/ml. Culture tubes were re-incubated.

Cells were harvested 70-72 hours after PHA stimulation. First, cells were treated with a hypotonic solution for 5 minutes, at room temperature. After that, cells were fixed with methanol and acetic acid at a 3:1 ratio. Cells were transferred to slides by dropping and they were stained with Giemsa. Two slides were prepared from each of the duplicate cultures in order to obtain a measure of experimental variation, i.e., coefficient of variation.

Each slide was scored for the following: number of micronuclei (MNi), the distribution of binucleate (BN) cells with zero, one or more MNi, the frequency of micronucleated BN cells, the frequency of nucleoplasmic bridges (NPB) found in 1000 BNCs, and also the proportion of mononucleated, binucleated, trinucleated and tetra-nucleated cells per 500 cells scored. Scoring criteria for selection of BNCs, MN and NPB (Table 1) were described previously in detail (17). In the case of NPB scoring, we were careful to include only those BNCs in which the main nuclei were clearly separated because it is difficult to determine the presence of a NPB when nuclei are touching. Criteria for selecting binucleated cells which can be scored for the presence of micronuclei and nucleoplasmic bridges were standardized and published in 2003 by a multicentric evaluation (17), as seen in the table below.

We used SPSS 11.0 package for statistical analysis, applying the non parametric U Mann-Whitney test for comparison of quantitative

Binucleated cells (BNc)	1. The two nuclei in a BNc should have intact nuclear membranes and be situated within the same cytoplasmic boundary. 2. The two nuclei in a BNc should be approximately equal in size, staining pattern and staining intensity. 3. The two nuclei within a BNc may be attached by a fine nucleoplasmic bridge which is no wider than one-forth of the largest nuclear diameter. 4. The two main nuclei in a BNc may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable. 5. The cytoplasmic boundary or membrane of a BNc should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.
Micronuclei (MN)	1. The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei. 2. MN are round or oval in shape. 3. MN are non-refractile and they can therefore be readily distinguished from artifact such as straining particles. 4. MN are nor linked or connected to the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary. 5. MN usually has the same staining intensity as the main nuclei but occasionally staining may be more intense.
Nucleoplasmic bridges (NPB)	1. NPB are continuous nucleoplasmic link between the nuclei in a binucleated cell. 2. The width of a nucleoplasmic bridge may vary considerably but usually does not exceed one-fourth of the diameter of the nuclei within the cell. 3. NPB should have the same staining characteristics of the main nuclei. 4. On rare occasions more than one nucleoplasmic bridge may be observed within one binucleated cell. 5. A binucleated cell with a nucleoplasmic bridge may or may not contain one or more micronuclei.

TABLE 1. Criteria for scoring BN cells, MN and MPB

variables between two groups. A p value of less than 0.05 was considered statistically significant.

RESULTS

50 patients undergoing colonoscopy were included in the study. 5 patients were excluded from the statistical analysis, 2 with history of neoplasia (haemopathy in remission), one suspected for attenuated familial adenomatous polyposis syndrome, one with previous professional exposure to radiations and one with a controversial histology.

An increased number of MN/BN cells and in MN total number was observed in this one patient with prior radiation exposure. While in other patients the maximum number of MN was 3 per cell, in this patient particularly we found even 11 MN in binucleated lymphocytes (Figure 1 and 2), and a significant higher number of MN (17, vs. an average of 7.11 in the rest of the patients).

Of the assessable 45 patients, 23 were men and 22 were women, with a median age of 59. We found 17 patients with polyps, 11 with colorectal cancer and 17 with normal colonoscopy.

The number of MN was significantly higher in women (8.14 vs 4.17, p = 0.008), and so was the number of binucleated cells with 1 MN (Figure 3). There were no differences by sex in patients with cancer or polyps in comparison with those with a normal colonoscopy, so that the difference is real an independent to diagnostic.

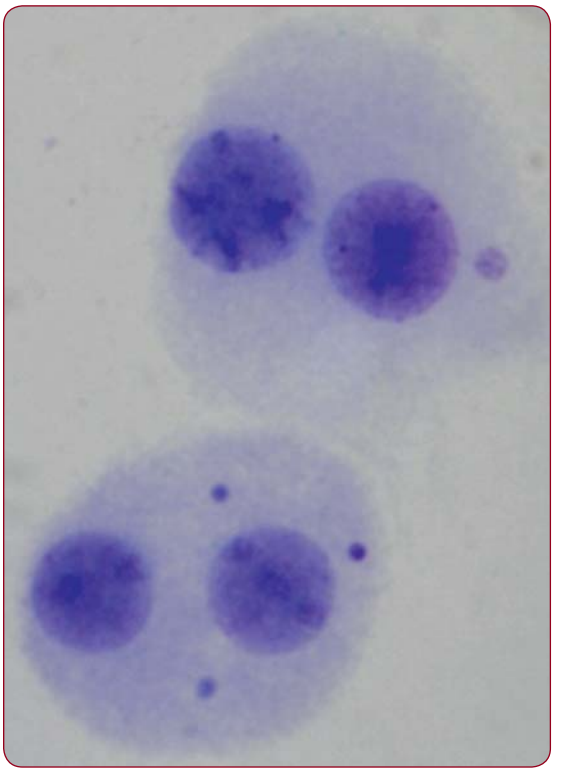

FIGURE 2. Photomicrograph of two binucleated cells (BN) one with a single micronucleus and one with three micronuclei (MN) in the same patient

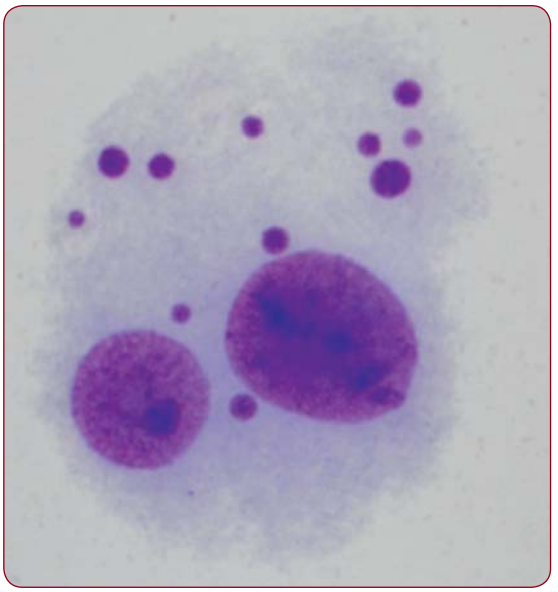

FIGURE 1. Photomicrograph of a binucleated cell (BN) with 11 micronuclei (MN) with various sizes in one patient with professional exposure to ionized radiations

In patients with advanced adenomas (size > 10mm or vilous) or colorectal cancer, nucleoplasmic bridges were significantly less frequent (p = 0.044) when compared with patients with normal colonoscopy or with hiperplastic polyps or non-advanced adenomas. Moreover, nucleoplasmic bridges were not at all found in any patient with colorectal cancer, as opposed with patients with normal colonoscopy or with hyperplastic or adenomatous polyps (0/11 vs 13/34, p = 0.018).

DISCUSSION

The facility and low cost of MN frequency analysis of in peripheral lymphocytes allowed the design and publication of many *in vivo* and *in vitro* studies on genomic lesions in different cancer sites.

The association between an increase of MN frequency and cancer is sustained by many ob-

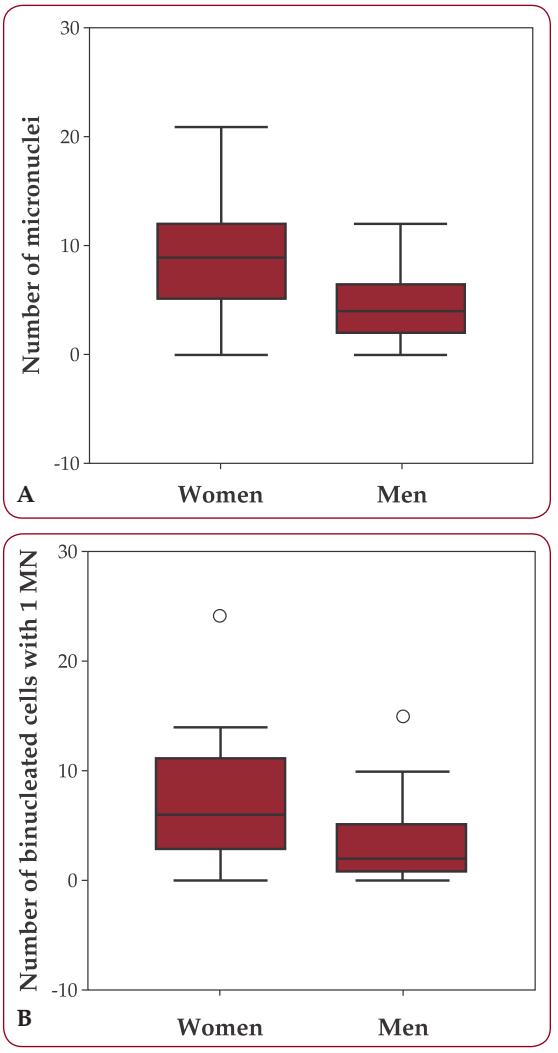

FIGURE 3. Comparison of MN number (a) and frequency of binuceated cells with 1 MN (b) by sex groups

servational studies: a high frequency of MN in untreated lung cancer patients, in multiple hereditary neoplasms, in patients exposed to ionized irradiations (18-20). An inverse correlation between MN frequency and folate acid levels was described (21,22).

The Cytokinesis-block micronucleus assay (CBMN) was already validated as a useful tool for *in vivo* or *in vitro* studies of nuclear and cellular dysfunctions caused by aging, excess or deficit of certain micronutrients as well as genotoxines exposure. More recent studies show that this method may be also used in nutrigenomics and toxicogenomics. But its ultimate use is disease prediction. The HUMN international project aims of finding pathological variables which modifies MN frequency in humans and their signification. A recent cohort study on 6718 patients form 10 countries has shown that there is positive correlation of MN frequency and cancer frequency in mean risk group (RR = 1.84, 95% CI =1.28-2.66) and high risk group (RR = 1.53, CI 1.04-2.66) as opposed to low risk group [23]. These observations are sustained by cohort studies demonstrating fair correlation between MN frequency and cancer risk, higher for urogenital and digestive tract sites [24-32]. A case control study on lung cancer risk in smokers has shown that both nitrosamine and nicotine derived from 4 - (methylnitrosamino) -1 - (3-pyridyl)-1-butanona (NNK) induce MN presence and are associated with lung cancer (OR 2.06 şi 2.32, respectiv); they also found a positive association between NPB and lung cancer (OR 29.05 and 45.52, respectiv) (28).

One previous paper assessed MN frequency in patients with colon cancer, neoplastic or non neoplastic polyps or with normal colonoscopy (10). Their results showed an increase of MN frequency in patients with cancer and adenomatous polyps when compared with patients with hiperplastic polyps or normal colonoscopy, in contradiction with our results. In this previous study nucleoplasmic bridges were not evaluated.

Studies have shown that NBP may be broken in more than one region in late anaphasis leading to acentric chromosome formation and eventually MN formation. Therefore, some MN may origin from broken NPB, although if this really happens in cytokinesis blocked cells it is actually unclear. NPB significance should not be underestimated as it gives direct proofs of genome alteration by DNA repairing defects or telomere fusion, processes which may not be assessed by exclusive MN evaluation originating form acentric or chromosome loss (33,34).

Thus, our result seem more concordant, as we did not find higher MN frequency, nor NPB frequency, and this may be used for colorectal cancer screening.

CONCLUSION

Micronuclei in peripheral lymphocytes are more frequent in women, but their frequency was not proved to be predictive for significant adenoma or colon cancer in this study. Conversely, the absence of nucleoplasmic bridges in peripheral lymphocytes may be predictive for the presence of advanced adenomas (vilous or larger than 10 mm) or colorectal cancer.

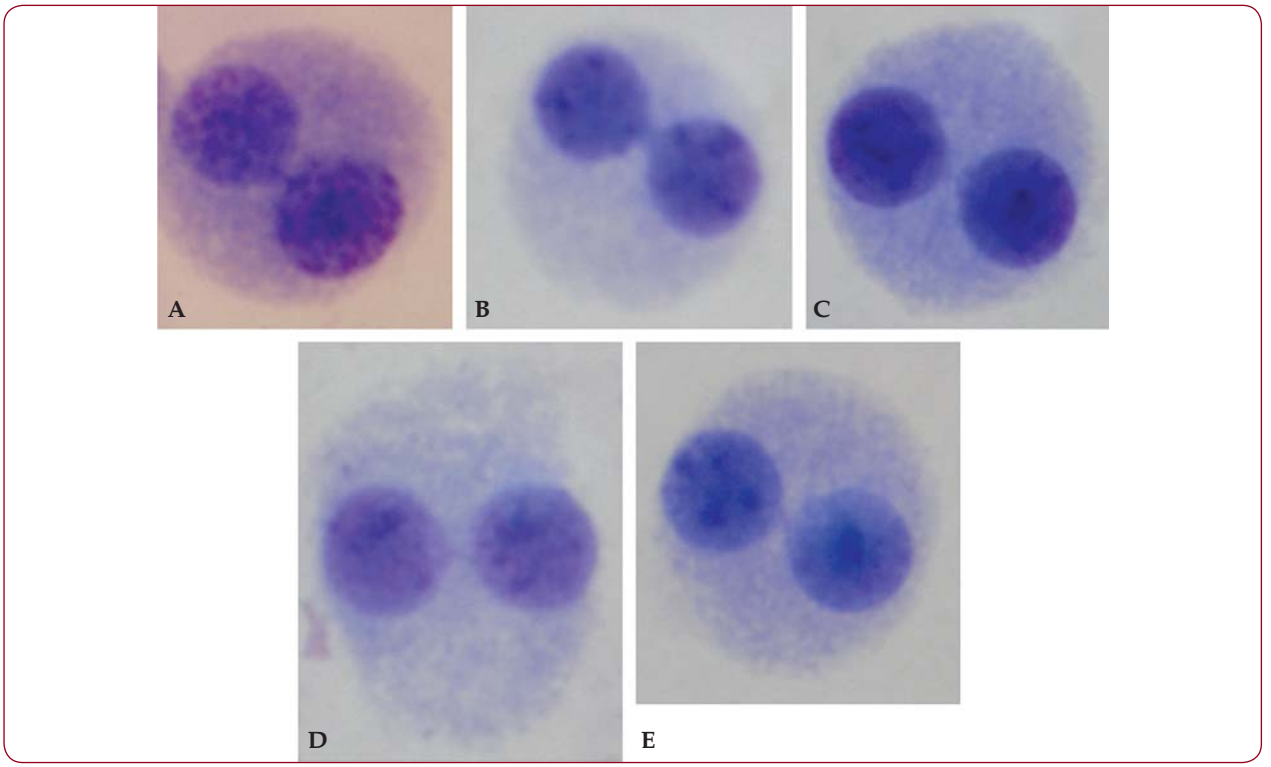

FIGURE 4. (A-E) Photomicrograpfs of typical binucleated cells (BN) with nucleoplasmic bridges (NPB). (B) Ilustrates a relatively wide nucleoplasmic bridge.

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