

## Micronucleus analysis in patients with colorectal adenocarcinoma and colorectal polyps

Ali Karaman, Doğan Nasır Binici, Mehmet Eşref Kabalar, Züleyha Çalığışu

Ali Karaman, Department of Medical Genetics, Erzurum Training and Research Hospital, Erzurum 25240, Turkey  
Doğan Nasır Binici, Department of Internal Medicine, Erzurum Training and Research Hospital, Erzurum 25240, Turkey  
Mehmet Eşref Kabalar, Department of Pathology, Erzurum Training and Research Hospital, Erzurum 25240, Turkey  
Züleyha Çalığışu, Department of Medical Oncology, Erzurum Training and Research Hospital, Erzurum 25240, Turkey  
Author contributions: Karaman A performed micronucleus analysis in the lymphocytes of all subjects, analyzed all of the data and wrote the article; Binici DN performed colonoscopic operations; Kabalar ME analyzed all biopsy materials; Çalığışu Z performed physical examinations.

Correspondence to: Ali Karaman, MD, Erzurum Training and Research Hospital, (Erzurum Numune Hastanesi) Department of Medical Genetics, Erzurum 25240, Turkey. [alikaramandr@hotmail.com](mailto:alikaramandr@hotmail.com)  
Telephone: +90-442-2321139 Fax: +90-442-2321390  
Received: September 23, 2008 Revised: November 11, 2008  
Accepted: November 18, 2008  
Published online: November 28, 2008

### Abstract

**AIM:** To determine, by counting micronucleus (MN) frequencies, whether chromosomal or DNA damage have an effect on the pathogenesis of early colorectal adenocarcinoma (CRC).

**METHODS:** We analyzed MN frequencies in 21 patients with CRC, 24 patients with colon polyps [10 neoplastic polyps (NP) and 14 non-neoplastic polyps (NNP)] and 20 normal controls.

**RESULTS:** MN frequency was significantly increased in CRC patients and in NP patients compared with controls ( $3.72 \pm 1.34$ ,  $3.58 \pm 1.21$  vs  $1.97 \pm 0.81$ ,  $P < 0.001$ ). However, there was no difference in the MN frequency between CRC patients and NP patients ( $P > 0.05$ ). Similarly, there was no difference in the MN frequency between NNP patients ( $2.06 \pm 0.85$ ) and controls ( $P > 0.05$ ).

**CONCLUSION:** Our results suggest increased chromosome/DNA instabilities may be associated with the pathogenesis of early CRC.

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**Key words:** Colorectal adenocarcinoma; Colon polyp; Micronucleus; Genetic instability

**Peer reviewer:** Dr. Bernd Sido, Department of General and Abdominal Surgery, Teaching Hospital of the University of Re-

gensburg, Regensburg D-93049, Germany

Karaman A, Binici DN, Kabalar ME, Çalığışu Z. Micronucleus analysis in patients with colorectal adenocarcinoma and colorectal polyps. *World J Gastroenterol* 2008; 14(44): 6835-6839 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6835.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6835>

### INTRODUCTION

Genomic instability plays an essential role in the development and progression of human colorectal cancer (CRC)<sup>[1]</sup>. Two major types of genetic instability have been described in CRC: chromosomal instability and microsatellite instability<sup>[1]</sup>. About 60% of CRCs develop through the chromosomal instability pathway, which is characterized by losses and gains of chromosomes (aneuploidy), as well as losses of heterozygosity<sup>[1]</sup>.

CRC progresses through four distinct clinical stages that are described as dysplastic crypts, small benign tumors, malignant tumors invading surrounding tissues, and finally metastatic cancer. This progression involves several genetic changes such as inactivation of tumor suppressor genes and activation of oncogenes<sup>[2]</sup>. Mutations of the adenomatous polyposis coli (*APC*) gene are considered the earliest<sup>[3]</sup> and most prevalent genetic changes in colorectal tumorigenesis. More than 85% of colon cancers are estimated to have a somatic mutation of *APC*<sup>[4]</sup>. Furthermore, a large number of genes that trigger chromosomal instability have been identified in yeast in the past<sup>[5]</sup>. The underlying mechanisms leading to chromosomal instability in colorectal cancer remain to be characterized. The DNA double-strand break (DSB) is regarded as the most critical of all DNA lesions<sup>[6,7]</sup>, and it has been shown that defects in the cellular response to DSBs can lead to genetic alteration, chromosomal instability, and ultimately malignant transformation<sup>[8]</sup>.

The genome damage to the lymphocytes of peripheral blood has been widely used as a biomarker of genotoxic environmental factors, and long-term studies have demonstrated its validity and high clinical productivity<sup>[9]</sup>. Micronucleus (MN) is an acentric chromosome fragment or whole chromosome that is left behind during mitotic cellular division and appears in the cytoplasm of interphasic cells as a

small additional nucleus<sup>[10]</sup>. The formation of MN in dividing cells is the result of chromosome breakage due to unrepaired or mis-repaired DNA lesions, or chromosome malsegregation due to mitotic malfunction. These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell cycle checkpoint and/or DNA repair genes, as well as deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation machinery<sup>[11-14]</sup>. All these events can cause the formation of MN through chromosomal rearrangements, altered gene expression or aneuploidy, effects associated with the chromosome instability phenotype often seen in cancer<sup>[15,16]</sup>.

The MN frequency test, widely accepted for *in vitro* and *in vivo* genotoxicity investigations, is a sensitive marker of genomic damage<sup>[17,18]</sup>. The presence of an association between MN induction and cancer development is supported by a number of observations. The most substantiated include: the high frequency of MN in untreated cancer patients and in subjects affected by cancer-prone congenital diseases, e.g. Bloom syndrome or ataxia telangiectasia<sup>[15,19]</sup>, the presence of elevated MN frequencies in oral mucosa, used as a surrogate biomarker of cancer in clinical chemoprevention trials<sup>[20]</sup>, the correlation existing between genotoxic MN-inducing agents and carcinogenicity, e.g. ionizing and ultraviolet radiation<sup>[21,22]</sup>.

A major question in cancer genetics is to what extent chromosome or genetic instability is an early event and thus a driving force of tumorigenesis<sup>[23,24]</sup>. The aim of this study was to determine, by counting MN frequencies, whether chromosomal or DNA damage has an effect on the pathogenesis of early CRC.

## MATERIALS AND METHODS

### Patients

This study was conducted between May 2008 and September 2008 in the Erzurum Training and Research Hospital. Twenty-one patients with colorectal adenocarcinoma and 24 patients with colorectal polyps were studied. The study was conducted using colonoscopic specimens from subjects with the established diagnosis of colorectal polyps or colorectal adenocarcinoma in histologic analysis. Specimens were separated for each level and placed in 10% formalin solution. The pathologic specimens were reviewed independently by two pathologists.

Pathologists were blinded to the subject's clinical history, the colonoscopic findings, and the results of the Hematoxylin-Eosin staining assay. Pathologic reading was determined for each biopsy slide with an overall pathologic diagnosis determined for each subject.

We performed MN analysis in 21 (12 females and 9 males; mean age:  $57.62 \pm 10.84$  years) patients with CRC, in 10 (4 females and 6 males; mean age:  $52.44 \pm 8.36$  years) patients with NP, in 14 (6 females, 8 males; mean age:  $52.92 \pm 9.14$  years) patients with NNP and in 20 (8 females and 12 males; mean age:  $50.25 \pm 9.38$  years) healthy controls. The patients were selected

from non-smoking and nonalcoholic subjects. None of the subjects had a history of viral infection, bacterial infection or any metabolic diseases. The patients had not been treated with chemotherapy or radiotherapy during the last 4 mo. The patient and control groups were chosen for their similar habits. The hospital Ethical Committee approved the human study. All patients were analyzed prior to treatment.

### Micronucleus analysis

For MN analysis, 2 mL of heparinized blood was drawn from each individual. Lymphocyte cultures were established by adding 0.5 mL of whole blood to 5 mL karyotyping medium (Biological Industries, Beit Haemek, Israel) with 2% phytohemagglutinin M (PHA; Biological Industries) according to standard techniques. The culture was kept at 37°C for 72 h. Cytochalasin B (6 µg/mL, Sigma, USA) was added after 44 h of culture to block cytokinesis, allowing the identification of lymphocytes dividing in culture. Cells that had undergone the first mitosis were thus recognized as binucleated cells and were selectively screened for the presence of MN. The cells were then treated hypotonically with 0.075 mol/L KCl for 5 min at room temperature, and fixed in methanol/acetic acid (3:1). Cells were dropped onto slides and stained with 5% Giemsa in phosphate buffer (pH 6.8) for 5 min. A thousand binucleated cells from each case were examined for MN by an experienced observer<sup>[25]</sup>.

### Statistical analysis

The MN rates were analyzed statistically by student's *t*-test. To evaluate the correlations between the age, sex, and MN rates, the coefficients of Spearman  $\rho$  correlation were calculated. A *P* value less than 0.05 was considered to be significant.

## RESULTS

MN frequencies and clinical data obtained from the patient and control groups are shown in Table 1. According to these results, the mean MN frequency was significantly increased in CRC patients compared with controls ( $3.72 \pm 1.34$  vs  $1.97 \pm 0.81$ ,  $P < 0.001$ ). Similarly, the mean MN frequency was significantly increased in NP patients compared with controls ( $3.58 \pm 1.21$  vs  $1.97 \pm 0.81$ ,  $P < 0.001$ ). However, there was no difference in the mean MN frequency between CRC patients, and NP patients ( $P > 0.05$ ). Similarly, there was no difference in mean MN frequency between NNP patients and controls ( $2.06 \pm 0.85$  vs  $1.97 \pm 0.81$ ,  $P > 0.05$ ). On the other hand, the mean MN frequencies did not correlate with patients' age or sex in the CRC patients (for each,  $P > 0.05$ ). Similarly, the mean MN frequencies did not correlate with patients' age or sex in the colon polyp patients (for each,  $P > 0.05$ ).

## DISCUSSION

CRCs progress through a series of clinical and his-

**Table 1** Micronucleus (MN) results of the patients with colorectal cancer and colon polyps and healthy controls (mean  $\pm$  SE)

	Sex (F/M)	Age (yr)	Age at diagnosis (yr)	MN/1000 BN
CRC patients (n = 21)	12/9	57.62 $\pm$ 10.84	56.98 $\pm$ 9.45	3.72 $\pm$ 1.34
NP patients (n = 10)	4/6	52.44 $\pm$ 8.36	51.68 $\pm$ 8.54	3.58 $\pm$ 1.21
NNP patients (n = 14)	6/8	52.92 $\pm$ 9.14	50.48 $\pm$ 8.29	2.06 $\pm$ 0.85
Controls (n = 20)	8/12	50.25 $\pm$ 9.38		1.97 $\pm$ 0.81

CRC: Colorectal adenocarcinoma; NP: Neoplastic polyp; NNP: Non-neoplastic polyp.

topathological stages ranging from dysplastic crypts through small benign tumors to malignant cancers. This progression is the result of a series of genetic changes that involve activation of oncogenes and inactivations of tumor suppressor genes<sup>[26]</sup>. In colorectal cancer, chromosomal instability is the major form of genetic instability<sup>[27]</sup>. It is generally agreed that colorectal cancers develop as a consequence of accumulation of mutations in key genes such as *K-Ras*, *Apc*, and *p53* that are critical for regulating cell proliferation or cell cycle checkpoint control. In humans, the development from early adenomas to metastatic carcinomas takes somewhere from 20 to 40 years; it is believed that genetic instability plays a key role in accelerating the rate of mutation in cancerous cells<sup>[28]</sup>.

CRCs exhibit a defect in chromosome segregation, leading to frequent gains or losses of chromosomes ( $> 10^2$  per chromosome per generation)<sup>[28]</sup>. Chromosome instability has been detected in the smallest adenoma, suggesting that chromosome instability may occur at very early stages of colorectal carcinogenesis<sup>[29]</sup>. Extensive research during the past has led to the identification of genes that play a major role in the development of colorectal cancer. For example, mutations or deletions of the adenomatous polyposis coli (*APC*) gene, encoding a 310-kDa cytoplasmic protein<sup>[30,31]</sup>, are commonly found in inherited familial adenomatous polyposis patients and in sporadic colorectal cancers<sup>[32,33]</sup>. Such mutations appear to be an early event during colorectal tumorigenesis<sup>[4]</sup>.

The most commonly affected gene in sporadic colon cancer with defective DNA mismatch repair (MMR) is *hMLH1*, with the primary mechanism of gene inactivation being hypermethylation of the promoter<sup>[34]</sup>. These tumors account for approximately 15% of sporadic colon cancers. The majority of sporadic colon cancers (85%), however, are proficient in DNA MMR but show another form of genomic instability at the gross chromosomal level, which has been called chromosomal instability. Such chromosomal instability represents the end result of a number of processes, including mutations in mitotic checkpoint genes, microtubule spindle defects, and telomere dysfunction<sup>[35]</sup>.

Two types of genetic instability have been identified, with chromosomal instability predominating<sup>[1,36]</sup>. The molecular basis for chromosomal instability is just beginning to be explored<sup>[37]</sup>. A large number of gene alterations can give rise to chromosomal instability in *Saccharomyces cerevisiae*<sup>[5,38]</sup>. These genes include

those involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function, and microtubule formation and dynamics as well as checkpoints that monitor the progress of the cell cycle. To date, the only genes implicated in aneuploidy in human tumor cells are those of the latter class. Heterozygous mutations in the mitotic spindle checkpoint gene *hBUB1* were detected in a small portion of colorectal tumors with the chromosomal instability<sup>[39]</sup>.

The identification of aneuploidy at early stages of tumor formation in *MYH*- and *APC*-mutant polyps is interesting also in view of previous reports showing that loss of *APC* function in primary mouse cell lines results in chromosomal instability due to a kinetochore attachment defect at mitosis<sup>[36]</sup>. It is generally accepted that *APC*'s main tumor suppressing activity resides in its capacity to bind and regulate Wnt/ $\beta$ -catenin signal transduction<sup>[40]</sup>. However, additional *APC* functions in cytoskeletal organization, mitotic spindle assembly, cell migration, and apoptosis may play important roles in tumor progression and malignant transformation<sup>[40,41]</sup>.

It has been demonstrated that chromosomes display nonrandom changes in cancer cells. These include structural rearrangements, e.g. deletions, amplifications or translocations that arise from breaks in DNA, as well as alterations in the number of intact chromosomes, known as whole-chromosome missegregations, originating from errors in cell division (mitosis). As a result of the accumulation of such processes, chromosomal instability is thought to play a key role in tumor development<sup>[40]</sup>.

In the present study, we investigated whether cytogenetic abnormalities participate in the pathogenesis of early CRC. Cytogenetic endpoints are sensitive biomarkers that are widely accepted to evaluate chromosome damage<sup>[42,43]</sup>. MN assay provides a measure of both chromosome breakage and chromosome loss or nondisjunction in clastogenic and aneugenic events, respectively<sup>[11,13]</sup>.

MN assay is a sensitive indicator of exogenously or endogenously caused genetic damage and MN frequency has become an important end point in genotoxicity testing both *in vivo* and *in vitro*<sup>[17,18]</sup>. Elevated levels of MN are indicative of defects in DNA repair and chromosome segregation which could result in generation of daughter cells with altered gene dosage, or deregulation of gene expression that could lead to the evolution of the chromosome instability phenotype often seen in cancer<sup>[10,11,15,21]</sup>. These considerations give

mechanistic support to a possible causal association between MN frequency and the risk of cancer. A recently published cohort study linking the frequency of micronuclei in lymphocytes of healthy subjects to the risk of cancer reported stomach cancer among the sites most specifically associated with micronuclei frequency<sup>[44]</sup>. Similar findings have also been reported for preneoplastic lesions of colon<sup>[45]</sup>, esophagus<sup>[46]</sup> and cervix<sup>[47]</sup>. In particular, the higher risks noted for stomach and intestinal cancers, are in agreement with the literature, which emphasizes the role of chromosome rearrangements in the early stages of these tumours<sup>[47,48]</sup>.

Our study, which showed increased MN frequencies in the lymphocytes of CRC and colon polyp patients, could support these observations, as the induction of changes in DNA that lead to mutations plays a role in carcinogenicity. Establishment of inherited susceptibility factors is important in recognizing individuals at a higher risk of developing CRC, so that they may benefit from early detection and prevention programs. Many investigators have demonstrated genomic instability and abnormalities in patients with CRC<sup>[49-51]</sup>. Further, experimental evidence shows that early colorectal adenomas have allelic imbalance<sup>[52]</sup>. *bCDC4* mutations have been shown to occur early in colorectal tumorigenesis<sup>[53]</sup>.

An association between MN and cancer has been reported<sup>[19]</sup>. The causes of this association may be structural chromosomal aberrations and aneuploidy<sup>[19]</sup>. The presence of an association between the frequency of micronuclei in lymphocytes and cancer risk has been suggested<sup>[13,44]</sup>. Our findings of a high level of MN frequency in patients with CRC or NP seem to support this association. Thus, MN assay may be performed in lymphocytes as an indicator of genomic instability relevant to colorectal tumorigenesis.

In conclusion, our results indicate that the increased MN frequency in lymphocytes of patients with CRC and NP may reflect genomic instability or deficiency of DNA repair capacity. Further, these results suggest increased chromosome/DNA instabilities may be associated with the pathogenesis of early CRC.

## COMMENTS

### Background

It is known there is an increased micronucleus (MN) frequency rate in neoplastic disease. Colorectal adenocarcinoma (CRC) is a common cause of cancer-related deaths worldwide, despite improved diagnostic and therapeutic implications. Hence, early diagnosis has critical importance. The aim of this study was to determine, by counting MN frequencies, whether chromosomal or DNA damage has an effect on the pathogenesis of early CRC.

### Research frontiers

The MN frequency test, widely accepted for in vitro and in vivo genotoxicity investigations, is a sensitive marker of genomic damage. Therefore, in this study, we aimed to determine, by assessing MN rates, whether genetic impairment and DNA damage have an effect on the pathogenesis of CRC.

### Innovations and breakthroughs

Our results suggest increased genomic instability may be associated with the pathogenesis of early CRC. The identification of increased MN frequency rate in patients with colorectal lesions may be helpful in the early diagnosis of CRC.

### Applications

MN analysis has come into use as a sensitive means of monitoring DNA damage. MN analysis may be used as a marker to estimate the risk of CRC.

### Terminology

Micronucleus (MN): MN is an acentric chromosome fragment or whole chromosome that is left behind during mitotic cellular division and appears in the cytoplasm of interphasic cells as a small additional nucleus.

### Peer review

This study indicated genetic impairment and genetic instability may play an important role in CRC. Further, MN frequency is a promising biomarker for assessing the risk of neoplastic progression in colorectal adenocarcinoma.

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