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Genomic instability in patients with Barrett's esophagus

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Abstract Our study aimed to determine, by counting sister chromatid exchange (SCE) and micronucleus (MN) frequencies, whether genetic impairment and DNA damage have an effect on the pathogenesis of Barrett's esophagus (BE). This study was conducted between June 2007 and November 2008 in the Erzurum Training and Research Hospital. We analyzed SCE and MN frequencies in 30 patients with BE, and in 30 control cases. SCE was significantly increased in BE patients compared with controls (6.89 ± 1.04 vs. 5.01± 0.88, $P < 0.001$). Similarly, MN was significantly increased in BE patients compared with controls (3.48 ± 1.08 vs. 1.83 ± 0.64, $P < 0.001$). Our data indicate that the increased SCE and MN rates in lymphocytes of patients with BE may reflect genomic instability or deficiency of DNA repair capacity. © 2010 Elsevier Inc. All rights reserved.

1. Introduction

The major risk factor for esophageal adenocarcinoma (EAC) is the presence of Barrett's esophagus (BE), a premalignant neoplastic lesion that is characterized by intestinal metaplasia replacing the normal squamous esophageal epithelia [1]. The presence of BE increases the overall risk of adenocarcinoma by 40-fold [2]. In addition, similarities in the gene expression profile of Barrett's esophageal adenocarcinoma further support a close relationship between the two tissues [3]. Clinical studies have identified esophageal acid reflux as a risk factor for BE and EAC [2]. In vitro experiments using adenocarcinoma and non-neoplastic Barrett's esophageal cell lines established that short-term exposure to an acidic environment results in increased cell proliferation [4].

A variety of environmental and host factors are thought to play a role in the etiology of esophageal adenocarcinoma, including acid reflux [2], gender, race, obesity [5], *Helicobacter pylori* colonization, and cigarette smoking [5]. The mechanisms of action of these factors are likely to directly or indirectly involve DNA damage. Increased levels of DNA damage have been detected in Barrett's mucosa [6] and may be associated with progression [7]. Impaired ability to repair such damage may therefore play a role in progression to esophageal adenocarcinoma, as has been suggested in a study of polymorphisms in DNA repair genes in persons with Barrett's esophagus [8]. On the other hand, the involvement of reactive oxygen specimens (ROS) would also fit well with observations that tissues from patients with esophagitis and Barrett's esophagus show elevated levels of ROS [9], and that Barrett's tissues bear p53 mutation types indicative of ROS exposure [10].

A comprehensive understanding of the basic mechanisms in the progression of esophagitis, BE, and EAC is urgently needed to develop a management strategy for BE. It is generally accepted that BE progresses in a stepwise fashion: esophagitis to metaplasia to dysplasia to EAC, with the histologic changes leading to EAC being alterations at the cellular and molecular levels. Abnormalities in apoptosis, proliferation, and differentiation have proven to be common cellular events in BE and EAC [11]. Acquired genetic abnormalities, such as gene mutation, gene deletion, loss of heterozygosity, aberrant methylation, aberrant gene expression, and chromosomal aberrations, have also been reported [12].

The SCE phenomenon is widely used as a reliable and sensitive indicator of chromosome (DNA) instability because the sister chromatid exchange (SCE) patterns can reveal a general genome instability [13]. SCE in peripheral lymphocytes has been widely used to assess exposure to mutagens and carcinogens [14]. The frequency of SCE is

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increased in patients with nasopharyngeal carcinoma and gastric cancer [15–17].

Micronuclei (MN) are acentric chromosome fragments or whole chromosomes that are left behind during mitotic cellular division and appear in the cytoplasm of interphasic cells as small additional nuclei [18]. 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 [19–21]. All these events can cause the formation of MN through chromosomal rearrangements and altered gene expression or aneuploidy, effects associated with the chromosome instability phenotype often seen in cancer [22,23]. The frequency of MN is increased in untreated cancer patients and in subjects affected by cancer-prone congenital diseases, e.g., Bloom syndrome or ataxia telengiectasia [22,24].

The aim of this study was to determine, by counting SCE and MN frequencies, whether genetic impairment and DNA damage have an effect on the pathogenesis of Barrett's esophagus.

2. Materials and methods

2.1. Patients

This study was conducted between June 2007 and November 2008 in the Erzurum Training and Research Hospital. Thirty patients with BE of the distal esophagus diagnosed were studied. The study was conducted using endoscopic specimens from subjects with the established diagnosis of BE, the presence of specialized intestinal metaplasia (SIM) in histologic analysis. The length of columnar lined epithelial was defined as the distance from the proximal margin of the gastric fold to the squamous columnar junction. Short-segment BE was defined as a columnar lined epithelium length less than or equal to 2 cm, and long-segment BE was defined as a columnar lined epithelium length greater than 2 cm. The columnar lined epithelium was biopsied using jumbo biopsy forceps in a four-quadrant fashion at 2-cm intervals starting at the distal margin and proceeding approximately to the squamous columnar junction. Specimens were separated for each level and placed in 10% formalin solution.

2.2. Pathologic analysis

The pathologic specimens were reviewed independently by two pathologists. Pathologists were blinded to the subject's clinical history, the endoscopic findings, and the results of the immunohistochemistry staining assay. Alcian blue staining was used to identify any presence of SIM. Hematoxylin-eosining was used to identify any presence of dysplasia. The specimens had not any dysplasia. Both pathologists had to concur on all specimens with SIM. Pathologic reading was determined for each biopsy slide, with an overall pathologic diagnosis determined for each subject.

We performed SCE and MN analysis in 30 patients with BE (8 females, 22 males; mean age: 42.66 ± 15.33 years; age range: 28–67 years), and in 30 healthy controls (10 females and 20 males; mean age: 42.86 ± 13.38 years; age range: 26–65 years). The mean age was not statistically different between patient and control groups ($Z = 0.112$, $P > 0.05$). We selected from nonsmoking 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 months. The patient and control groups were chosen for their similar habits. The hospital Ethical Committee approved the human study. All patients were analyzed before treatment.

2.3. Cell culture

For SCE and MN analysis, 3 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 [25]. Duplicate cultures were incubated at 37°C for 72 hours. All slides were coded and read blind.

2.4. Sister chromatid exchange analysis

A total of 5 μg/mL of 5-bromodeoxy-uridine (BrdU; Sigma Chemical Co., St. Louis, MO) were added after 24 hours of culture. Cells were cultured in the dark for 48 hours and metaphases were blocked during the last 2 hours with Colcemid (0.1 μg/mL; Biological Industries). Further processing included hypotonic (0.075 molar KCl) treatment, fixation (methanol/acetic acid: 3/1), slide preparation, and fluorescein plus Giemsa (FPG) staining for detection of SCE [25]. Fifty second division metaphases were scored on coded slides by a single observer as the number of SCE/cell per individual.

2.5. Micronucleus analysis

Cytochalasin B (6 μg/mL; Sigma) was added after 44 hours of culture to block cytokinesis, allowing identifying lymphocytes dividing in culture. The culture was kept at 37°C for 72 hours. Cells that have undergone the first mitosis are thus recognized as binucleated cells and are selectively screened for the presence of MN. The cells were then treated hypotonically with 0.075 molar KCl for 5 minutes 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 minutes. About 1,000 binucleated cells from each case were examined for MN by an experienced observer [26].

2.6. Statistical analysis

The SCE and MN rates were analyzed statistically by the Mann–Whitney *U*-test. To evaluate the correlations between the age, sex, SCE, and MN rates, the coefficients of Spearman ρ correlation were calculated. $P < 0.05$ was considered to be significant.

3. Results

The mean SCE/cell value was significantly higher in the patients with BE (6.89 ± 1.04; range 5.14–9.75) compared with the controls (5.01 ± 0.88; range: 3.6–6.74; $Z = 5.300$; $P < 0.001$). Similarly, there was significant difference in mean MN/1,000 BN frequency between patients with BE (3.48 ± 1.08; range: 1.52–5.74) and controls (1.83 ± 0.64; range: 0.85–3.20; $Z = 5.530$; $P < 0.001$; Table 1). On the other hand, the SCE frequency did not correlate with patients' age or sex in the BE patients ($r = 0.115$, $P > 0.05$ and $r = 0.057$, $P > 0.05$, respectively). Similarly, the MN frequency did not correlate with patients' age or sex in the BE patients ($r = 0.093$, $P > 0.05$ and $r = 0.120$, $P > 0.05$, respectively). The prevalence of SCE in the metaphase chromosomes of one peripheral blood lymphocyte of a patient with Barrett's esophagus is shown in Fig. 1. Similarly, the micronuclei in the peripheral blood lymphocytes of a patient with Barrett's esophagus are shown in Fig. 2.

4. Discussion

Barrett's esophagus is characterized by chronic inflammation, cellular damage/repair, and increased proliferation [27]. Chronic inflammation is associated with oxidative damage and increased levels of double-strand DNA breaks. Thus, diminished DNA repair in Barrett's esophagus could lead to accelerated progression to esophageal adenocarcinoma [28]. Progression in persons with Barrett's esophagus is associated with increasing chromosomal instability [29]. Multiple genetic abnormalities have been reported in Barrett's esophagus-associated adenocarcinoma and its precursors [30].

Many investigators have demonstrated karyotypic imbalances and abnormalities in adenocarcinomas and adjacent metaplastic epithelium from esophagectomy specimens [12]. A widespread gain of DNA chromosomal material has been implicated in the progression of Barrett's esophagus–associated dysplasia, and the presence of aneuploidy, as assessed by flow cytometry, has been shown to predict the subsequent development of adenocarcinoma [28]. A variety of gains and losses of specific chromosomes and chromosome regions have also been detected in esophageal adenocarcinoma specimens and adjacent mucosa by both traditional cytogenetics and by comparative genomic hybridization [31–33]. The studies consistently show an accumulation of chromosomal abnormalities as the histology changes progressively from intestinal metaplasia to dysplasia and carcinoma [34,35].

Some authors have examined the frequencies of chromosomal aberration (CA), SCE, and MN frequencies in cancer patients to understand the genetic mechanism. They found that frequency of SCE was not high, but that CA and MN rates were observed in patients with cancer [36]. Furthermore, Norppa et al. [14] reported that increased CA rates, but no high SCE frequencies, were observed in gastrointestinal cancers. However, Paulson [37] reported that increased chromosomal instability and copy number alterations were rare in the early stages, but that high-level amplifications were observed frequently in the later stages (high-grade dysplasia and EAC). The results suggest increased chromosomal instability may be associated with the pathogenesis of EAC.

In this study, we investigated whether cytogenetic abnormalities participate in the pathogenesis of BE. Sister chromatid exchange is known to be increased by exposure to various genotoxic carcinogens [38], and seem to reflect the repair of DNA lesions by homologous recombination [39]. Important sources of exposure include diet, general environment, medical exposure to ionizing radiation, and internal generation of genotoxic species. Internal phenomena such as metabolism, errors of DNA replication, inflammation, and oxidative stress may be of importance. Inflammatory diseases, oxidative stress, and radiation exposure have been associated with the generations of clastogenic factors, which may be quite persistent [40,41]. The frequency of SCE is increased in patients with chronic atrophic gastritis, chronic pancreatitis, and Crohn's disease [17,42,43]. These disesases are premalignant conditions.

The micronuleus assay is a sensitive indicator of exogenously or endogenously caused genetic damage, and has become an important endpoint in genotoxicity testing both in vivo and in vitro [24]. Elevated levels of MN are indicative of defects in DNA repair and chromosome segregation, which could result in generation of daughter cells

Table 1
SCE and MN results of patients with BE and controls

	Sex (Female/ male)	Age (years) Mean ± SD	SCE Mean ± SD	MN/1,000 BN Mean ± SD
BE patients (n = 30)	8/22	42.66 ± 15.33	6.89 ± 1.04[a]	3.48 ± 1.08[b]
Controls (n = 30)	10/20	42.86 ± 13.28	5.01 ± 0.88	1.83 ± 0.64

[a] Significant at $P < 0.001$.
[b] Significant at $P < 0.001$.

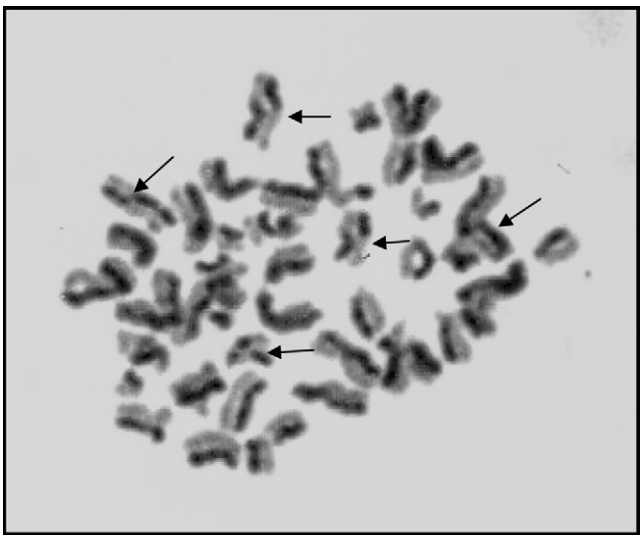


Fig. 1. A metaphase plaque of peripheral blood lymphocyte stained by the FPG technique showing differential staining of chromatids. The arrows show exchange points.

with altered gene dosage, or deregulation of gene expression that could lead to the evolution of the chromosome instability phenotype often seen cancer [18,19,22,24]. 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 more specifically associated to micronuclei frequency [44]. Similar findings have also been reported for preneoplastic lesions of the colon [45], esophagus [46], and cervix [47]. In particular, the higher risks for stomach and intestinal cancers are in agreement with the literature,

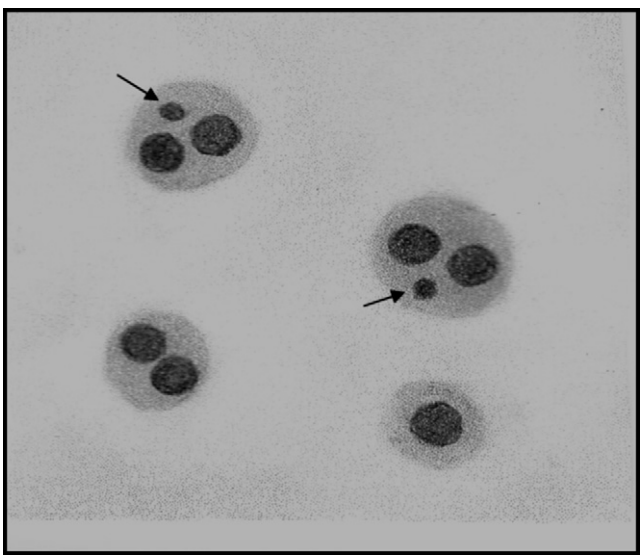


Fig. 2. The peripheral blood lymphocytes stained by Giemsa staining of nuclei. The arrows show micronuclei.

which emphasizes the role of chromosome rearrangements in the early stages of these tumors [48,49].

In our study, no significant relationship was found between SCE and MN frequencies and age or sex in patients with BE. Age and sex, however, have been reported to influence SCE and MN frequencies in the cultured peripheral blood lymphocytes of humans [50].

Our study, which showed increased SCE and MN frequencies in the lymphocytes of BE patients, could support these observations, as the induction of changes in DNA that lead to mutations play a role in carcinogenicity. Establishment of inherited susceptibility factors is important to recognize individuals at a higher risk for developing esophageal adenocarcinoma, so that they may be benefit from early detection and prevention programs. Many investigators have demonstrated genomic instability and abnormalities in patients with BE [12,19,30]. Patients with BE have a markedly increased risk of EAC, but the mechanism underlying this increased risk is not well understood. Chronic inflammation has been associated with the development of chromosomal aberrations in both disorders that progress to neoplasia, such as ulcerative colitis [51] and Barrett's esophagus [52]. Our results confirm and extend this conception to Barrett's esophagus.

There is increasing evidence that oxidative stress plays a key role in the progression of BE to dysplasia to EAC, with normal aerobic cellular metabolism producing several ROS, including superoxide anion, hydroxyl radical, and nonradicals such as hydrogen peroxide [53]. Oxidative damage to DNA over time can cause changes to both the structure and function of chromosomes. These changes in the genetic code may lead to cancer and other chronic diseases [54]. When normal cells are inflamed through chronic reflux, the production of ROS can be overwhelming, resulting in injury to the cells, which can induce gene mutations and lead to the progression from normal cells to cellular dysplasia and cancer [55].

In conclusion, our results suggests that genomic instability is present at very early stages of neoplastic progression in BE patients.

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