Effect of systemic treatment on the micronuclei frequency in the peripheral blood of patients with metastatic colorectal cancer

TAXIARCHIS KONSTANTINOS NIKOLOUZAKIS[1], POLYCHRONIS D. STIVAKTAKIS[2], PARASKEVI APALAKI[3], KATERINA KALLIANTASI[3], THEODOROS MARIOLIS SAPSAKOS[4], DEMETRIOS A. SPANDIDOS[5], ARISTIDIS TSATSAKIS[3], JOHN SOUGLAKOS[6] and JOHN TSIAOUSSIS[1]

[1]Laboratory of Anatomy-Histology-Embryology, Medical School, University of Crete, 71110 Heraklion;
[2]Laboratory of Toxicology, Medical School, University of Crete, 71409 Heraklion;
[3]Department of Forensic Sciences and Toxicology, Medical School, University of Crete, 71003 Heraklion;
[4]Laboratory of Anatomy and Histology, Nursing School, National and Kapodistrian University of Athens, 11527 Athens;
[5]Laboratory of Clinical Virology, Medical School, University of Crete, 71003 Heraklion;
[6]Department of Medical Oncology, University General Hospital of Heraklion, 71110 Heraklion, Greece

Received November 2, 2018; Accepted December 12, 2018

DOI: 10.3892/ol.2019.9895

Abstract. Colorectal cancer (CRC) is the third most diagnosed type of cancer affecting males, and the second most diagnosed type of cancer affecting females, and one of the leading causes of cancer-related mortality globally. The estimation of the micronuclei (MN) frequency in peripheral blood lymphocytes (PBLs) from patients with CRC is proposed as a prognostic/predictive easy-to-use biomarker. In this study, we aimed to investigate the effects of systemic treatment on the MN frequency in PBLs from patients with CRC in order to determine the effectiveness of the MN frequency as a biomarker. For this purpose, from 2016 to 2018, we quantified the MN frequency as a prognostic/predictive biomarker in serial samples from 25 patients with metastatic CRC (mCRC) using cytokinesis block micronucleus assay (CBMN assay). The MN frequency in the PBLs of the patients was evaluated before, during the middle and at the end of the therapy (approximately 0, 3 and 6 months). The results revealed a common pattern regarding the fluctuation in the MN frequency. Statistical analysis confirmed that when the disease response was estimated with radiological criteria, a good response was depicted at the MN frequency and vice versa. Consequently, the findings of this study suggest that the MN frequency may serve as a promising prognostic/predictive biomarker for the monitoring of the treatment response of patients with CRC.

Correspondence to: Dr John Tsiaoussis, Laboratory of Anatomy-Histology-Embryology, Medical School, University of Crete, Voutes, 71110 Heraklion, Greece
E-mail: tsiaoussis@uoc.gr

Professor Aristidis Tsatsakis, Department of Forensic Sciences and Toxicology, Medical School, University of Crete, 71003 Heraklion, Greece
E-mail: tsatsaka@uoc.gr

Abbreviations: CRC, colorectal cancer; MN, micronuclei; MNf, micronuclei frequency; CBMN assay, cytokinesis block micronucleus assay; CIN, chromosomal instability; MSI, microsatellite instability; CIMP, CpG island methylation phenotype; LCC, left-sided colon cancer; RCC, right-sided colon cancer; mCRC, metastatic colorectal cancer; BN cells, binucleated cells; CBPI, cytokinesis block proliferation index; BNMN, binucleated cells with micronuclei; FOLFIRI, folinic acid with 5-fluorouracil and irinotecan; FOLFOX, folinic acid with 5-fluorouracil and oxaliplatin; PBLs, peripheral blood lymphocytes

Key words: metastatic colorectal cancer, micronucleus, prognostic biomarker, predictive biomarker, micronuclei frequency, cytokinesis block micronucleus assay

Introduction

Colorectal cancer (CRC) is third most commonly diagnosed type of cancer affecting males, following lung and prostate cancer, and the second one affecting females, following breast cancer (1). In fact, CRC is a polygenic disease, which arises both from epigenetic, as well as genetic alterations in a variety of oncogenes, tumor suppressor genes, mismatch repair genes and cell cycle regulating genes in colon mucosal cells (2). Due to late diagnosis, approximately a quarter (20-25%) of CRC cases at the time of diagnosis present distant metastases, and another quarter of patients with early resectable CRC will eventually develop metastatic disease, most often in the liver.

It has been described that different pathways lead to carcinogenesis in the colonic epithelium; however, the majors ones are the following: Chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylation phenotype (CIMP) (3). All these pathways attribute to the transformation of an adenoma to carcinoma, a multistep carcinogenic process known as the adenoma-carcinoma sequence (4), which is considered to be a common process in all CRCs (5). As CRC is an heterogeneous disease, it exhibits various clinical manifestations, biological behavior and an in-tumor variety of mutations (6), making it a true

challenge for the clinician. Despite the fact that left-sided colon cancer (LCC) accounts for the majority of CRC cases, the number of cases with right-sided colon cancer (RCCs) is constantly rising (7). The female sex, age, a previous history of cancer and insulin resistance are some of the risk factors that have been associated with RCC, while a low-fiber diet, smoking and alcoholism have been associated with LCC (8,9). Moreover, LCC is commonly associated with metastasis to the liver and lungs, while RCC tends to be more differentiated and is associated with metastasis to the regional lymph nodes and the peritoneal cavity (10). In fact, it is estimated that approximately 22% of patients with CRC present with stage IV metastatic CRC (mCRC) at the time of diagnosis, indicating that, if treated, the expected 5-year-survival rate is only 13% (11). In this setting, chemotherapy is mainly used as a palliative measure in order to improve the quality of life and achieve the optimum survival. However, not all patients with stage IV disease exhibit the same response to treatment, even if the underlying genetic status is the same (12). This is the cornerstone of the research for prognostic and predictive biomarkers; the in-group difference.

Micronuclei (MN), or Howell-Jolly bodies, are small intracellular particles enwrapped in a nuclear envelope. They are formed as a result of acentric chromatid/chromosome fragments (mainly due to extensive DNA damage) or whole chromatids/chromosomes (mainly due to mitotic spindle failure, kinetochore damage, centromeric DNA hypomethylation and defects in the cell cycle control system) that during the anaphase of dividing cells do not follow the rest of the chromosomes and are not included in the nucleus during telophase. Instead, enwrapped by the nuclear membrane, they form daughter nuclei-like structures that are just a fraction of the size of the mother nucleus (13,14). Numerous studies have evaluated the use of MN frequency (MNf) in different cell types and lines in order to determine whether it can be used as an effective biomarker for various types of cancer (including lung, bladder and colorectal cancer) (15-17). Almost all of these studies agree that MNf is a sensitive indicator of cancer since, compared to healthy controls, there is a significant increase in MN formation regardless of the type of cancer. However, for patient's convenience, peripheral blood lymphocytes (PBLs) are preferably used. What is more, those who evaluated MNf in CRC did prove an increased MNf (thus indicating its possible use as a diagnostic biomarker), but did not evaluate their patients in the long-term and did not include cases with metastatic disease. Hence, the importance of MNf as a prognostic and/or predictive biomarker in mCRC has not yet been investigated in detail, at least to the best of our knowledge. The only published attempt to illuminate the prognostic properties of MNf, to the best of our knowledge, comes from a team which evaluated MNf in urothelial cells of patients with bladder cancer (18,19).

Thus, under this scope, the present study aimed to assess the efficiency of MNf as a biomarker for the prognosis and disease/treatment prediction of patients with mCRC.

Patients and methods

Patients and study protocol. The protocol of this study was approved by the Ethics Committee for Patients and Biological Material of the University Hospital of Heraklion (Heraklion, Greece). During the period between December, 2016 and February, 2018, 27 patients referred to the Department of Medical Oncology of the University Hospital of Heraklion were enrolled in this study. All patients signed a written consent. The inclusion criteria were as follows: i) Patients with mCRC treated with 1st line systemic treatment according to the Hellenic Society of Medical Oncologists (HeSMO) guidelines (20); and ii) an age between 50-75 years. The exclusion criteria were as follows: i) Failure to complete the therapeutic regimen for any reason (toxicity, refusal of the patient, or death); and ii) the refuse of the patient to attend the study. Based on the chemotherapeutic protocol that was selected [folinic acid with5-fluorouracil and oxaliplatin (FOLFOX) or folinic acid with5-fluorouracil and irinotecan (FOLFIRI) with or without a biological factor], patients were further divided into subgroups. Another division of the patients was made based on their body mass index (BMI) before treatment (BMI <25, BMI ≥25 but ≤30, and BMI >30). The RECIST criteria version 1.1 were used for the evaluation of the treatment response (21). According to these criteria, patients were evaluated at the end of the therapy and were divided into 3 subgroups as follows: Good response, stable disease and no response. Peripheral blood samples were collected at fixed time-points, namely before the beginning of the therapy, 3 months after the initiation of treatment and at the end of treatment (at 0, 3 and 6 months of treatment, respectively) for the evaluation for MNf using the cytokinesis block micronucleus assay (CBMN assay). By June, 2018, 25 out of the 27 patients had completed the study. One patient presented with increased toxicity and terminated the therapy and the other one died due to a heart failure as a result of a lower respiratory tract infection. Finally, 10 healthy individuals (5 male and 5 female) were recruited from the Health Center of Agia Varvara, Heraklion, Crete, after receiving a thorough explanation about the study, how their samples would be handled and signing a written consent. The inclusion criteria were an age between 55 and 70 years and a personal history free of cancer, autoimmune diseases and COPD. Exclusion criteria were the presence of the above-mentioned diseases, direct exposure at any time to pesticides and/or herbicides and the lack of will of the participant.

MN test. The MN test is an official regulatory 'tool' in the European Legislation (B.12, Regulation 440/2008/EC) validated by OECD (22). Whole blood (0.5 ml) was added to 6.5 ml Ham's F-10 medium (Gibco/Thermo Fisher Scientific, Waltham, MA USA), 1.5 ml fetal bovine serum (Standard Fetal Bovine Serum, certified, US origin, Gibco/Thermo Fisher Scientific), and 0.3 ml phytohemagglutinin M (PHA-M; 10 ml, Thermo Fisher Scientific). Cultures were incubated at 37˚C for a period of 72 h. Six micrograms per milliliter of cytochalasin-B (white to off-white powder, ≥98% 5 mg; Acros Organics, Inc./Thermo Fisher Scientific) was added 44 h following culture initiation. Peripheral blood lymphocytes (PBLs) were collected by centrifugation at 400 x g (1,500 rpm) at 20˚C for 25 min at 72 h post-incubation. A mild hypotonic solution of Ham's F-10 medium and milli-Q water (1:1, v/v) was added to the cell solution and left for 3 min at room temperature. The cells were fixed with a methanol:acetic acid solution (5:1, v/v) placed on microscope slides and stained with Giemsa (Gibco/Thermo

Table I. Patient characteristics (age, sex, ECOG performance status, location of the primary tumor, number of metastatic sites, BRAFV600E status, KRAS exon 2 status, NRAS status, MMR status).

Characteristic	N=25	%
Median/mean age (range), years	67/66.04 (50-75)	
Sex		
Male	12	48
Female	13	52
Performance status (ECOG)		
0	22	88
1	3	12
Location		
Right-sided	6	24
Left-sided	19	76
Median/mean number of metastatic sites (range)		
Liver	3.5/4.8 (0-20)	
Lung	3/3.4 (0-10)	
Lymph nodes	0/2.33 (0-13)	
Peritoneum	0/1.33 (0-7)	
BRAS^{V600E} status		
WT	13	52
Mutant	2	10
Unknown	10	40
KRAS exon 2 mutation		
WT	13	52
Mutant	10	40
Unknown	2	8
NRAS mutation		
WT	11	44
Mutant	2	8
Unknown	12	48
MMR status		
Proficient	9	36
Deficient	2	8
Unknown	14	56

Fisher Scientific) 15% at 25˚C for 30 min, as previously described (23,24). The slides were then placed under a Nikon Eclipse E200 microscope (Nikon Holdings Europe B.V., Amsterdam, The Netherlands) where the binucleated cells (BN cells) and MN were viewed. One thousand BN cells with an intact cytoplasm were scored per slide for each sample, in order to calculate the MNf. Standard criteria were used for scoring the MN (25). The cytokinesis block proliferation index (CBPI) is given by the following equation:

$$CBPI = \frac{M_1 + 2M_2 + 3(M_3 + M_4)}{N}$$

where M1, M2, M3 and M4 correspond to the number of cells with 1, 2, 3, and 4 nuclei and 'N' is the total number of cells.

Table II. Patient data regarding the therapeutic protocol, biological agent and BMI.

Patient no.	Chemotherapy	Biologic factor	BMI
1	FOLFIRI	No	34.7
2	FOLFOX	Cetuximab	25.76
3	FOLFIRI	Bevacizumab	20.68
4	FOLFIRI	Bevacizumab	29.17
5	FOLFIRI	No	32.46
6	FOLFOX	Cetuximab	32.46
7	FOLFIRI	No	20.44
8	FOLFIRI	Aflibercept	25.24
9	FOLFIRI	Aflibercept	36.48
10	FOLFIRI	Aflibercept	37.63
11	FOLFOX	Aflibercept	41.59
12	FOLFOX	Cetuximab	25.71
13	FOLFIRI	Cetuximab	31.16
14	FOLFOX	Bevacizumab	26.21
15	FOLFOX	No	32.0
16	FOLFIRI	Aflibercept	30.77
17	FOLFIRI	Cetuximab	24.14
18	FOLFIRI	Bevacizumab	21.87
19	FOLFOX	No	24.03
20	FOLFOX	Bevacizumab	25.83
21	FOLFOX	Bevacizumab	32.71
22	FOLFOX	No	26.44
23	FOLFOX	No	25.53
24	FOLFOX	Panitumumab	18.36
25	FOLFOX	Panitumumab	18.75

BMI, body mass index; FOLFIRI, folinic acid with 5-fluorouracil and irinotecan; FOLFOX, folinic acid with 5-fluorouracil and oxaliplatin.

These parameters were calculated by counting 2,000 cells, in order to determine the possible cytotoxic effects, as previously described (26).

Statistical analysis. Statistical analysis of the MN data was performed with the G-test for independence on 2x2 tables. The Chi-squared test was used for the analysis of the CBPI data. The level of significance was set at 0.05. One-way ANOVA was applied to estimate differences between 3 groups. Mean plots with error bars and bar charts were used for the graphical presentation of the data. The IBM SPSS Statistics Package 21.0 was used for data analysis and for the graphic representation of the data. The level of acceptance of null hypotheses was set at the 0.05 level.

Results

The patient characteristics are shown in Table I, while the type of chemotherapy, the biological factor and the BMI of each patient are presented in Table II. Out of the 25 patients, 12 were treated with FOLFIRI and 13 with FOLFOX, while 18 of them were additionally treated with a biological agent

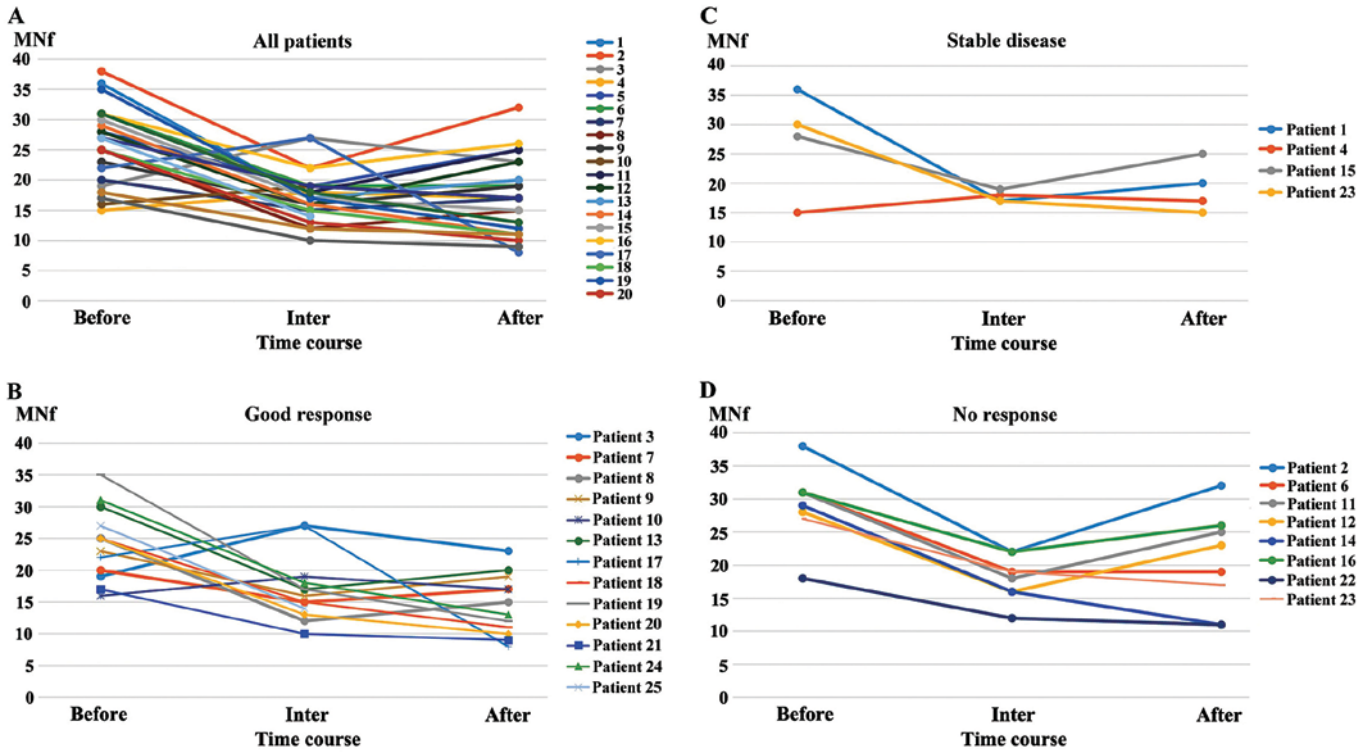

Figure 1. (A) MNf of all patients for the three time-points exhibiting a mixed 'v' and 'Λ' trend. (B) MNf of the good response group presenting a shallow 'v' trend. (C) MNf of the stable disease group presenting a very shallow 'v' trend. (D) MNf of the no response group presenting a deep 'v' trend. MNf, micronuclei frequency.

(cetuximab, aflibercept, bevacizumab or panitumumab). The mean BMI was 28.07 (ranging from 18.36 to 41.59).

In the control group (10 individuals), the mean values of binucleated cells with micronuclei (BNMN), and MN and CBPI values were 6.91±1.14, 7.91±1.14 and 1.34±0.04 respectively. Fig. 1 illustrates the MNf trends of each patient when interpreted as one group (Fig. 1A), as a good response group (Fig. 1B), as a stable disease group (Fig. 1C) and as a no response group (Fig. 1D) across their treatment (at the beginning, middle and end). Fig. 1A exhibits a mixed 'v' and 'Λ' trend, Fig. 1Ba shallow 'v' trend, Fig. 1C a very shallow 'v' trend, and Fig. 1Da deep 'v' trend.

Data regarding the mean values of BNMN, MN and CBPI and the related P-values when patients were treated as a solid group are presented in Table III. Table IIIA shows the data from the comparison of all the patient mean MNf, BNMN and CBPI values to those of the controls. For all time-points (before, middle and after treatment) the patient mean BNMN and MNf values [BNMN: Before, 23.84±5.58 (P<0.001); middle, 15.56±3.54 (P=0.004); and after, 15.21±5.53 (P=0.006); MNf: Before, 26.28±6.30 (P<0.001); middle, 17.40±4.08 (P=0.003); and after, 17.29±6.19 (P=0.004)] were significantly higher compared to those of the controls. However, no significant differences were observed for CBPI (before, 1.30±0.05; middle, 1.32±0.06; and after, 1.31±0.02).

Table IIIB shows the results from the comparison between patients with BMI <25 (7 patients) and BMI ≥25 but ≤30 (8 patients) before therapy. The mean BNMN and MNf values were as follows: (BNMN: BMI <25, 24.00±5.69; BMI ≥25 but ≤30, 23.88±6.79; MNf: BMI <25, 25.57±5.88; BMI ≥25 but ≤30, 26.13±7.26) and did not exhibit any significant differences (BMI 25-30 vs. BMI <25; P=0.98 and P=0.91, respectively). Furthermore, the results from the comparison between patients with BMI <25 and BMI >30 (10 patients) before therapy (BNMN: BMI <25, 24.00±5.69; and BMI >30, 23.70±5.06; MNf: BMI <25, 25.57±5.88; and BMI >30, 26.50±7.97) also did not exhibit any significant difference (P=0.95 and P=0.85, respectively). The mean CBPI was almost the same for all the BMI groups (BMI <25, 1.29±0.05; BMI ≤25 but ≤30, 1.31±0.05; and BMI >30, 1.32±0.06).

Table IIIC shows the results when all the patient mean BNMN, MNf and CBPI values at the middle (BNMN, 15.56±3.54; MNf, 17.40±4.08; CBPI, 1.32±0.06) and at the end (after) (BNMN, 15.21±5.53; MNf, 17.29±6.19; CBPI, 1.31±0.02) were compared to those before treatment (BNMN, 23.84±5.58; MNf, 26.28±6.30; CBPI, 1.30±0.05). The comparison of the mean BNMN, MNf and CBPI values at the middle against those at the beginning of treatment revealed that the mean BNMN values were not significantly lower (P=0.05), while the mean MNf values were (P=0.04). The comparison of the mean BNMN, MNf and CBPI values at the end against those at the beginning of treatment revealed that both the mean BNMN and MNf values were significantly lower (P=0.04 and P=0.04, respectively). The CBPI values were again almost the same for both time-points.

Data regarding the mean values of BNMN and MN and the related P-values when patients were divided into subgroups are presented in Table IV. Table IVA shows the results from the comparison of the samples before treatment from the patients with a good response (13 patients) against those who were stable (4 patients) and those with no response (8 patients). The mean BNMN values before treatment for the good, stable and no response groups were 22.31±5.28, 23.00±6.38 and 26.75±5.20, respectively. The comparison between the groups did not reveal any

Table III. Statistical analysis of MN assay in cultures of peripheral blood lymphocytes showing BN scored, mean frequency of BNMN, mean frequency of MN and CBPI, for the mean BNMN, MNf and CBPI values.

A, all vs. controls

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value	CBPI (means ± SE)
Control	10,000	6.91±1.14			7.91±1.14			1.34±0.04
Before	25,000	23.84±5.58	25.47	**<0.001**	26.28±6.30	26.71	**<0.001**	1.30±0.05
Middle	25,000	15.56±3.54	8.03	**0.004**	17.40±4.08	8.54	**0.003**	1.32±0.06
After	25,000	15.21±5.53	7.47	**0.006**	17.29±6.19	8.37	**0.004**	1.31±0.02

B, BMI 25-30 vs. <25 and >30 vs. <25

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value	CBPI (means ± SE)
BMI <25	7,000	24.00±5.69			25.57±5.88			1.29±0.05
BMI 25-30	8,000	23.88±6.79	0.006	0.98	26.13±7.26	0.012	0.91	1.31±0.05
BMI >30	10,000	23.70±5.06	0.004	0.95	26.50±7.97	0.03	0.85	1.32±0.06

C, middle vs. before and after vs. before therapy

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value	CBPI (means ± SE)
Before	25,000	23.84±5.58			26.28±6.30			1.30±0.05
Middle	25,000	15.56±3.54	3.85	**0.05**	17.40±4.08	3.99	**0.04**	1.32±0.06
After	25,000	15.21±5.53	4.24	**0.04**	17.29±6.19	4.11	**0.04**	1.31±0.02

Values in bold font indicate statistically significant differences (P<0.05) compared with the controls or as indicated. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored; 25,000 cells in total for each endpoint); BNMN, binucleated cells with micronuclei; CBPI, cytokinesis block proliferation index; BMI, body mass index.

significant differences (good vs. stable, P=0.88; and good vs. no response, P=0.36). The mean MNf values before treatment for the good, stable and no response group were 24.23±5.60, 27.25±8.85 and 29.13±5.59, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.54; and good vs. no response, P=0.33).

Table IVB shows the results from the comparison of the samples at the middle of treatment from the patients with a good response against those which were stable and those with no response. The mean BNMN values for the good, stable and no response groups at the middle of therapy were 15.23±4.53, 15.75±0.96 and 16.00±2.62, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.89; and good vs. no response, P=0.84). The mean MNf before treatment for the good, stable and no response group were 16.92±5.11, 17.75±0.96 and 18.00±3.34, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.84; and good vs. no response, P=0.79).

Table IVC shows the results from the comparison of the samples at the end of the treatment from the patients with a good response against those with a stable response and those with no response. The mean end BNMN values for the good, stable and no response group were 12.67±4.21, 17.00±3.74 and 18.57±7.04, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.24; and good vs. no response, P=0.12). The mean MNf values before treatment for the good, stable and no response group were 14.50±4.76, 19.25±4.34 and 21.00±7.85, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.70; and good vs. no response, P=0.11).

Table IVD shows the results from the comparison of the mean BNMN and MNf values from the good response group before therapy against those at the middle and after therapy. The mean BNMN values before, middle and after therapy were 22.31±5.28, 15.23±4.53 and 12.67±4.21, respectively. The mean MNf values for the same time-points were 24.23±5.60, 16.92±5.11 and 14.50±4.76, respectively. The comparison between time-points revealed a significant decrease only when after treatment was compared with before treatment, with an insignificant decrease at the middle (BNMN: Before vs. middle, P=0.09; and before vs. after, P=0.01; MNf: Before vs. middle, P=0.09; and before vs. after, P=0.02).

Table IV. Statistical analysis of the mean BNMN and MNf at different time-points.

A, before therapy: Stable vs. good and no response (No res vs. good).

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Good	13,000	22.31±5.28			24.23±5.60		
Stable	4,000	23.00±6.38	0.02	0.88	27.25±8.85	0.37	0.54
No res	8,000	26.75±5.20	0.85	0.36	29.13±5.59	0.95	0.33

B, at the middle of treatment: Stable vs. good and No res vs. good.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Good	13,000	15.23±4.53			16.92±5.11		
Stable	4,000	15.75±0.96	0.02	0.89	17.75±0.96	0.04	0.84
No res	8,000	16.00±2.62	0.04	0.84	18.00±3.34	0.07	0.79

C, after therapy: Stable vs. good and No res vs. good.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Good	13,000	12.67±4.21			14.50±4.76		
Stable	4,000	17.00±3.74	1.35	0.24	19.25±4.34	0.15	0.70
No res	8,000	18.57±7.04	2.43	0.12	21.00±7.85	2.60	0.11

D, good response: Middle vs. before and after vs. before.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Before	13,000	22.31±5.28			24.23±5.60		
Middle	13,000	15.23±4.53	2.93	0.09	16.92±5.11	2.84	0.09
After	13,000	12.67±4.21	6.06	**0.01**	14.50±4.76	5.52	**0.02**

E, stable response: middle vs. before and after vs. before.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Before	4,000	23.00±6.38			27.25±8.85		
Middle	4,000	15.75±0.96	2.97	0.08	17.75±0.96	4.45	**0.03**
After	4,000	17.00±3.74	1.94	0.16	19.25±4.34	3.01	0.08

F, no response: middle vs. before and after vs. before.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Before	8,000	26.75±5.20			29.13±5.59		
Middle	8,000	16.00±2.62	6.11	**0.01**	18.00±3.34	5.91	**0.02**
After	8,000	18.57±7.04	3.24	0.07	21.00±7.85	2.87	0.09

Values in bold font indicate statistically significant differences (P<0.05) compared with the controls or as indicated. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored; 25,000 cells in total for each endpoint); BNMN, binucleated cells with micronuclei.

Table IVE shows the results from the comparison of the mean BNMN and MNf values from the stable group before therapy against those at the middle and after therapy. The mean BNMN values before, middle and after therapy were

23.00±6.38, 15.75±0.96 and 17.00±3.74, respectively. The mean MNf values for the same time-points were 27.25±8.85, 17.75±0.96 and 19.25±4.34, respectively. The comparison between time-points revealed a significant decrease only for MNf when middle was compared with before treatment, while BNMN for the same time-point exhibited an insignificant decrease. At the end of the therapy, both the BNMN and MNf values increased so that there were no significant difference between before and after therapy (BNMN: Before vs. middle, P=0.08; and before vs. after, P=0.16; MNf: Before vs. middle, P=0.03; and before vs. after, P=0.08).

Table IVF shows the results from the comparison of mean BNMN and MNf values from the no response group before therapy against those at the middle and after therapy. The mean BNMN values before, middle and after therapy were 26.75±5.20, 16.00±2.62 and 18.57±7.04, respectively. The mean MNf values for the same time-points were 29.13±5.59, 18.00±3.34 and 21.00±7.85, respectively. The comparison between time-points revealed a significant decrease only when middle was compared with before therapy both for BNMN and MNf. On the contrary, at the end of the therapy, both BNMN and MNf increased so that there was no significant difference between before and after treatment (BNMN: before vs. middle, P=0.01; and before vs. after, 0.07; MNf: before vs. middle, P=0.02; and before vs. after, P=0.09).

Discussion

The results of the current study indicated that patients diagnosed with metastatic CRC, regardless of sex and BMI, had high rates of BNMN and MNf. This was found both before and throughout the systemic therapy, even though they tended to decrease after therapy, but never to the degree of the individuals without cancer. In parallel, they had the same CBPI with healthy individuals that remained stable throughout treatment, while no change in the CBPI was evidenced at any point time or for any group.

It is well established that MN assay is a sensitive indicator of genomic damages of exogenous and endogenous origin (23,27). MNf in PBLs represents an indirect, intracellular indicator of chromosomal and genomic instability (high levels of MN are indicative of extended damages of the DNA repair system and in chromosomal division) (17,28-30). It has been proven that, even though MNf does not differ between the two sexes (31), it does between young and older and between normal-weight and obese individuals as a result of the accumulation of genetic damage (31,32). These facts support the hypothesis that the CBMN assay can be used as an indicator of the genotoxic and cytotoxic state (33). Indeed, it has previously been concluded that high levels of MN are linked to cancer (16). Moreover, a number of theories support the hypothesis that MNf can be used as a tool for cancer prognosis (18,19,34); however, they all agree that further investigations are required to verify this claim.

This study focused on the evaluation of MNf as a potential prognostic/predictive biomarker for CRC monitoring in a rather common group of patients with CRC, those with distant metastases (stage IV disease). For this purpose, 25 patients with stage IV CRC from a single oncologic center were included. Based on the current therapeutic guidelines for stage IV CRC, these patients underwent treatment with either FOLFOX or FOLFIRI with an addition of a biological factor based on their underlying genetic status (RAS and BRAF mutations). Folinic acid and 5-fluorouracil are the common compounds of the FOLFOX and FOLFIRI regimens, while oxaliplatin and irinotecan are the compounds that differentiate them, respectively. Bibliographic data have indicated that FA is an anti-clastogenic agent which significantly reduces the percentage of BNMN (35). It has been found that oxaliplatin induces cytogenetic damage (BNMN) through its clastogenic action, possibly through interfering with topoisomerase II (36). As regards irinotecan, Kopjar *et al*, using the CBMN assay, observed a dose-dependent increase in MNf in an *in vitro* study with human lymphocytes (37). Another study on irinotecan also found a significant increase of BNMN, but in a non-dose-dependent manner (38). However, to the best of our knowledge, there is no study available to date estimating the MNf and BNMN using the actual combination of FOLFOX or FOLFIRI with or without biological agents. Moreover, to the best of our knowledge, this study is the first one conducted with such a patient group and, thus, any interpretation of the data presented will be based mainly on data coming from different patient groups and thus should be treated accordingly.

CBPI is a tool widely used not only to better understand the BNMN results, but also to estimate any cytotoxic effect from chemical agents on cell cultures that use cytochalasin B expressed by an altered proliferation cells (38). As regards the best understanding of BNMN results, when CBPI is indifferent between time-points, then MNf results are comparable and any fluctuation of MNf can be attributed solely to the disease and/or the systemic treatment. As for the cytotoxicity, when the CBPI value is close to one, there is no cytotoxic event. However, in order to extract safer conclusions regarding cytotoxicity, patient CBPIs are compared to those of the control and not to the unit. In this study, if we address all patients as one solid group, before the beginning of the therapy, we can see that there was no difference in their CBPI values compared with the healthy individuals, suggesting no cytotoxicity from the disease. Moreover, we can see that CBPI remained almost the same throughout the duration of therapy. Thus, the combination of the disease and chemotherapy again did not lead to cytotoxicity. Therefore, it is safe to say that the MNf results are indicative of the patients' condition. Since sex does not affect MNf and the age group of our patients was the same (between 50 and 75 years old), the main parameters that had to be examined as to whether they affect MNf were BMI and malignancy per se. For the former case, patients were divided based on their BMI into 3 groups (BMI <25, BMI ≤25 but ≤30 and BMI >30). Statistical analysis of the MNf before the beginning of the treatment revealed no statistical differences (Table IIIB). For this reason, BMI was excluded from the final interpretation. The comparison of the MNf and BNMN scores between the patients before the systemic treatment and the healthy individuals (matched for sex, age and BMI) revealed significantly higher rates for both indexes (P<0.001). In fact, this significantly higher rate of MNf and BNMN was maintained throughout treatment (Table IIIA). Thus, it is reasonable to assume that the increased rates of MNf and BNMN are due to cancer. A following comparison of the mean BNMN and MNf of all patients revealed that BNMN decreased insignificantly at the middle and significantly at

the end (P<0.05), while MNf was significantly lower for both time-points (P=0.04 and P=0.04 respectively) (Table IIIC).

Based on the RECIST 1.1 criteria, we further divided the patients into the 'good response', 'stable disease' and 'no response' groups. The subsequent analysis revealed some very interesting data. First of all, when each subgroup was compared to the other for the same time-point, no significant differences were revealed both for the mean BNMN and MNf values. However, the subsequent comparison between time-points of the same group revealed that the 'good response' group had a declining trend for BNMN and MNf with an insignificant decrease at the middle (P=0.09 for both), and a significant one at the end of the therapy (P=0.01 and P=0.02, respectively) exhibiting a 'shallow v trend' (Fig. 1A). The same analysis was performed for the 'stable disease' group revealing a significant) decrease followed by an increase, making the MNf difference between before and after treatment insignificant (Fig. 1C). The 'no response' group exhibited a significant decrease at the middle both for BNMN and MNf (P=0.01 and P=0.02, respectively). Interestingly though, the trend was reversed at the end of the therapy, where both the BNMN and MNf values increased to such an extent, that no significant difference was evident anymore, exhibiting a 'deep v trend' (Fig. 1D).

Overall, there is a clear genotoxic state in the PBLs of patients represented by the very high mean MNf before therapy. This genotoxic state depicts the great cancer load at that time. After the first trimester of the therapy, the decrease in the mean MNf reflects the response of the organism to the treatment accomplished by the depletion of the sensitive cancer clone. However, the following increase of mean MNf (but never to the degree before treatment) raises a challenge for its clinical interpretation. The first scenario is that of a 'gradual emergence of a resistant clone'. First, sensitive cancer cells are depleted and so MNf and BNMN decrease. Subsequently, resistant ones emerge as they do not have to compete for energy or oxygen supply. In fact, this scenario could explain the fluctuations in MNf observed in the 3 response groups during the therapy. At the middle of therapy, both the good and no response groups began killing sensitive cells and decreased their MNf numbers, while the stable group did not. While the good responders then continued to deplete sensitive cancer cells, the non-responders began to increase cancer cells and their MNf increases accordingly, while the stable group maintained almost the same cell number and MNf. The second scenario is the 'long-term chemo-effect'. As mentioned before, both oxaliplatin and irinotecan increase MNf. Thus, while the cancer load decreases and the drug accumulation is not yet at its peak, the MNf also decreases. However, as the rate of cancer cells decrease diminishes and the accumulation of the drug reaches its zenith, the MN-increasing properties of oxaliplatin and irinotecan become evident. This scenario can also explain why at the end of the treatment the response group did not differ significantly, in terms of mean BNMN and MNf, than the other 2 groups, even though their cancer burden was reduced by >30%. In other words, systemic treatment increased MNf and prevented a cell number difference to be seen. Interestingly though, even if the majority of the patients exhibited the 'v'-shaped trend of MNf, there were 4 patients who exhibited a reverse 'v'-shaped trend, with an increase of mean MNf at 3 months, and a subsequent decrease at 6 months, as shown in Fig. 1. It is noteworthy that these 4 patients who did not follow the 'v'-shaped trend as the rest of the participants, but rather an inverted 'v', were proven to share the same therapy with a combination therapy of FOLFIRI and some type of biological agent. Whichever the case may be, as exhibited by the results from the good and the poor response groups, MNf was not associated with tumor response.

The key is to identify the exact time when the relapse or stability of the disease occurs and is first depicted in MNf by a certain increase of it. In doing so, we would be able to achieve a better tailoring of the therapy and at the same time we will be a step closer towards personalized treatment with a possible shortening of the chemotherapy duration. This in turn would positively affect not only patients, in terms of less side-effects as a consequence of tailored systemic therapy, but also the health care system due to the decreased financial burden of shortened systemic therapy. However, more patients and even more sampling points would be required in order to successfully identify the true nadir of MNf.

The findings of this study reveal an association, firstly between MNf and CRC per se, with significantly elevated MN rates at all time-points and, secondly between MNf and response to treatment, where a good response was evidenced by the significantly low rates at the end of treatment and a bad response by the maintenance of high rates at the end. Despite the fact that the results of the current study are in the same line of evidence with previously published data (15-19), they should be interpreted with caution and would be used as hypothesis-generated. We aim to continue this research in a prospective larger group of patients with metastatic CRC in order to validate the findings of the current study and establish the prognostic and predictive significance of MNf in this setting.

Acknowledgements

The authors would like to thank the Toxicology Laboratory of the Medical School, University of Crete for providing the necessary infrastructure, and the Department of Medical Oncology, University Hospital of Heraklion and the Health Center of Agia Barbara, Heraklion for providing blood samples from oncologic patients and controls, respectively

Funding

This study was supported by the Special Research Account of University of Crete (ELKE nos. 4658, 3392, 3963) and ToxPlus S.A.

Availability of data and materials

All data generated or analyzed during this study is included in this published article or are available from the corresponding author upon reasonable request.

Authors' contributions

TKN conducted the experiments, interpreted the data and wrote the manuscript. PDS performed the analysis and interpreted the data. PA conducted the experiments and wrote the manuscript. KK conducted the experiments and wrote the manuscript.

TMS drafted, interpreted the data and critically revised the article. DAS conceived and designed, critically reviewed and supervised the article. AT conceived and designed, critically revised, provided laboratory infrastructure and was responsible for the critical revision of the article for important intellectual content. JS provided blood samples, edited the manuscript and was responsible for the critical revision of the article for important intellectual content. JT conceived and designed the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Blood and information of patients were obtained with written informed consent. Procedures involving patients in this study were approved by the Human Ethics Committee at the University Hospital of Heraklion on December 2016.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Tsiaoussis J, Vassilopoulou L, Nikolouzakis T, Rakitskii VN, Vakonaki E, Fragkiadaki P, Stivaktakis P and Tsatsakis AM: Biomolecular profile of colorectal cancer - The role of telomerase as a potent biomarker. Farmacia 65: 643-659, 2017.
3. Nikolouzakis TK, Vassilopoulou L, Fragkiadaki P, Mariolis Sapsakos T, Papadakis GZ, Spandidos DA, Tsatsakis AM and Tsiaoussis J: Improving diagnosis, prognosis and prediction by using biomarkers in CRC patients (Review). Oncol Rep 39: 2455-2472, 2018.
4. Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B and Starling N: Colorectal cancer. Lancet 375: 1030-1047, 2010.
5. Engstrand J, Nilsson H, Strömberg C, Jonas E and Freedman J: Colorectal cancer liver metastases - a population-based study on incidence, management and survival. BMC Cancer 18: 78, 2018.
6. Souglakos J, Philips J, Wang R, Marwah S, Silver M, Tzardi M, Silver J, Ogino S, Hooshmand S, Kwak E, *et al*: Prognostic and predictive value of common mutations for treatment response and survival in patients with metastatic colorectal cancer. Br J Cancer 101: 465-472, 2009.
7. Richman S and Adlard J: Left and right sided large bowel cancer. BMJ 324: 931-932, 2002.
8. Haggar FA and Boushey RP: Colorectal cancer epidemiology: Incidence, mortality, survival, and risk factors. Clin Colon Rectal Surg 22: 191-197, 2009.
9. Koliarakis I, Psaroulaki A, Nikolouzakis TK, Sgantzos M, Kokkinakis M, Goulielmos G, Androutsopoulos V, Tsatsakis A and Tsiaoussis J: Intestinal microbiota and colorectal cancer: A new aspect of research. J BUON 23: 1216-1234, 2018.
10. Adam R, de Gramont A, Figueras J, Kokudo N, Kunstlinger F, Loyer E, Poston G, Rougier P, Rubbia-Brandt L, Sobrero A, *et al*; of the EGOSLIM (Expert Group on OncoSurgery management of LIver Metastases) group: Managing synchronous liver metastases from colorectal cancer: A multidisciplinary international consensus. Cancer Treat Rev 41: 729-741, 2015.
11. Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RG, Barzi A and Jemal A: Colorectal cancer statistics, 2017. CA Cancer J Clin 67: 177-193, 2017.
12. Charlton ME, Kahl AR, Greenbaum AA, Karlitz JJ, Lin C, Lynch CF and Chen VW: KRAS testing, tumor location, and survival in patients with stage IV colorectal cancer: SEER 2010-2013. J Natl Compr Canc Netw 15: 1484-1493, 2017.
13. Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD and Thomas P: Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis 26: 125-132, 2011.
14. Mateuca R, Lombaert N, Aka PV, Decordier I and Kirsch-Volders M: Chromosomal changes: Induction, detection methods and applicability in human biomonitoring. Biochimie 88: 1515-1531, 2006.
15. Pardini B, Viberti C, Naccarati A, Allione A, Oderda M, Critelli R, Preto M, Zijno A, Cucchiarale G, Gontero P, *et al*: Increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of bladder cancer. Br J Cancer 116: 202-210, 2017.
16. Ravegnini G, Zolezzi Moraga JM, Maffei F, Musti M, Zenesini C, Simeon V, Sammarini G, Festi D, Hrelia P and Angelini S: Simultaneous analysis of SEPT9 promoter methylation status, micronuclei frequency, and folate-related gene polymorphisms: The potential for a novel blood-based colorectal cancer biomarker. Int J Mol Sci 16: 28486-28497, 2015.
17. Maffei F, Zolezzi Moraga JM, Angelini S, Zenesini C, Musti M, Festi D, Cantelli-Forti G and Hrelia P: Micronucleus frequency in human peripheral blood lymphocytes as a biomarker for the early detection of colorectal cancer risk. Mutagenesis 29: 221-225, 2014.
18. Espinoza F, Cecchini L, Morote J, Marcos R and Pastor S: Micronuclei frequency in urothelial cells of bladder cancer patients, as a biomarker of prognosis. Environ Mol Mutagen: Oct 4, 2018 (Epub ahead of print). doi: 10.1002/em.22252.
19. Wang RC, Yang L, Tang Y and Bai O: Micronucleus expression and acute leukemia prognosis. Asian Pac J Cancer Prev 14: 5257-5261, 2013.
20. Dervenis C, Xynos E, Sotiropoulos G, Gouvas N, Boukovinas I, Agalianos C, Androulakis N, Athanasiadis A, Christodoulou C, Chrysou E, *et al*: Clinical practice guidelines for the management of metastatic colorectal cancer: A consensus statement of the Hellenic Society of Medical Oncologists (HeSMO). Ann Gastroenterol 29: 390-416, 2016.
21. Schwartz LH, Litière S, de Vries E, Ford R, Gwyther S, Mandrekar S, Shankar L, Bogaerts J, Chen A, Dancey J, *et al*: RECIST 1.1-Update and clarification: From the RECIST committee. Eur J Cancer 62: 132-137, 2016.
22. Hayashi M: The micronucleus test-most widely used in vivo genotoxicity test. Genes Environ 38: 18, 2016.
23. Stivaktakis P, Vlastos D, Giannakopoulos E and Matthopoulos DP: Differential micronuclei induction in human lymphocyte cultures by imidacloprid in the presence of potassium nitrate. ScientificWorldJournal 10: 80-89, 2010.
24. Fenech M: The cytokinesis-block micronucleus technique: A detailed description of the method and its application to genotoxicity studies in human populations. Mutat Res 285: 35-44, 1993.
25. Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S and Zeiger E; HUman MicronNucleus project: HUMN project: Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat Res 534: 65-75, 2003.
26. Surrallés J, Xamena N, Creus A, Catalán J, Norppa H and Marcos R: Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures. Mutat Res 341: 169-184, 1995.
27. Stivaktakis PD, Giannakopoulos E, Vlastos D and Matthopoulos DP: Determination of genotoxic effects of methidathion alkaline hydrolysis in human lymphocytes using the micronucleus assay and square-wave voltammetry. Bioelectrochemistry 113: 9-14, 2017.
28. Zhang CZ, Spektor A, Cornils H, Francis JM, Jackson EK, Liu S, Meyerson M and Pellman D: Chromothripsis from DNA damage in micronuclei. Nature 522: 179-184, 2015.
29. Bonassi S, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, Ban S, Barale R, *et al*: An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis 28: 625-631, 2007.
30. Schmid W: The micronucleus test. Mutat Res 31: 9-15, 1975.
31. Fenech M and Bonassi S: The effect of age, gender, diet and lifestyle on DNA damage measured using micronucleus frequency in human peripheral blood lymphocytes. Mutagenesis 26: 43-49, 2011.

32. Andreassi MG, Barale R, Iozzo P and Picano E: The association of micronucleus frequency with obesity, diabetes and cardiovascular disease. Mutagenesis 26: 77-83, 2011.
33. Bonassi S, Coskun E, Ceppi M, Lando C, Bolognesi C, Burgaz S, Holland N, Kirsh-Volders M, Knasmueller S, Zeiger E, *et al*: The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN(XL)): The role of life-style, host factors, occupational exposures, health status, and assay protocol. Mutat Res 728: 88-97, 2011.
34. Ramesh G, Chaubey S, Raj A, Seth RK, Katiyar A and Kumar A: Micronuclei assay in exfoliated buccal cells of radiation treated oral cancer patients. J Exp Ther Oncol 12: 121-128, 2017.
35. Scaglione F and Panzavolta G: Folate, folic acid and 5-methyltetrahydrofolate are not the same thing. Xenobiotica 44: 480-488, 2014.
36. de Souza AP, Lehmann M and Dihl RR: Comparative study on the induction of complex genomic alterations after exposure of mammalian cells to carboplatin and oxaliplatin. Drug Chem Toxicol 40: 410-415, 2017.
37. Kopjar N, Zeljezić D, Vrdoljak AL, Radić B, Ramić S, Milić M, Gamulin M, Pavlica V and Fucić A: Irinotecan toxicity to human blood cells in vitro: Relationship between various biomarkers. Basic Clin Pharmacol Toxicol 100: 403-413, 2007.
38. Kasuba V, Rozgaj R, Gamulin M and Trosić I: Assessment of cyto/genotoxicity of irinotecan in v79 cells using the comet, micronucleus, and chromosome aberration assay. Arh Hig Rada Toksikol 61: 1-9, 2010.