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BRIEF ARTICLE

Investigation of genome instability in patients with non-alcoholic steatohepatitis

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Abstract

AIM: To evaluate the occurrence of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in the mitogen-stimulated lymphocytes of patients with non-alcoholic steatohepatitis (NASH).

METHODS: The study was performed in 25 (9 females, 16 males) patients newly diagnosed with NASH, and 25 healthy subjects of similar ages and genders were used as a control group. None of the controls was known to be receiving any drugs for medical or other reasons or using alcohol. Hepatosteatosis was further excluded by abdominal ultrasound imaging in the control group. The numbers of MN, NPBs and NBUDs scored in binucleated (BN) cells were obtained from the mitogen-stimulated lymphocytes of patients and control subjects. Statistical comparisons of the numbers of BN cells with MN, NPBs and NBUDs and ages between the patients with NASH and control subjects were performed.

RESULTS: The mean ages of the patients and the control group were 41.92 ± 13.33 and 41.80 ± 13.09 years ($P > 0.05$), respectively. The values of the mean body mass index (BMI), HOMA-IR, hemoglobin, creatinin, aspartate aminotransferase, alanine aminotransferase, triglyceride, high density lipoprotein, and low density lipoprotein were 31.19 ± 4.62 kg/m^2 *vs* 25.07 ± 4.14 kg/m^2, 6.71 ± 4.68 *vs* 1.40 ± 0.53, 14.73 ± 1.49 g/dL *vs* 14.64 ± 1.30 g/dL, 0.74 ± 0.15 mg/dL *vs* 0.80 ± 0.13 mg/dL, 56.08 ± 29.11 U/L *vs* 16.88 ± 3.33 U/L, 92.2 ± 41.43 U/L *vs* 15.88 ± 5.88 U/L, 219.21 ± 141.68 mg/dL *vs* 102.56 ± 57.98 mg/dL, 16.37 ± 9.65 mg/dL *vs* 48.72 ± 15.31 mg/dL, and 136.75 ± 30.14 mg/dL *vs* 114.63 ± 34.13 mg/dL in the patients and control groups, respectively. The total numbers and frequencies of BN cells with MN, NPBs and NBUDs, which were scored using the CBMN cytome assay on PHA-stimulated lymphocytes, were evaluated in the patients with NASH and control group. We found significantly higher numbers of MN, NPBs and NBUDs in the BN cells of patients with NASH than in those of the control subjects (21.60 ± 9.32 *vs* 6.88 ± 3.91; 29.28 ± 13.31 *vs* 7.84 ± 3.96; 15.60 ± 5.55 *vs* 4.20 ± 1.63, respectively, $P < 0.0001$).

CONCLUSION: The increased numbers of MN, NPBs and NBUDs observed in the lymphocytes obtained from patients with NASH may reflect genomic instability.

Key words: Non-alcoholic steatohepatitis; Micronucleus; Nucleoplasmic bridges; Nuclear buds

Core tip: We aimed to evaluate the micronucleus, nucleoplasmic bridges and nuclear buds in the mitogen-

stimulated lymphocytes of patients with non-alcoholic steatohepatitis (NASH). Genomic instability may be a stage in the development of hepatic carcinogenesis. NASH is a major cause of so-called cryptogenic liver cirrhosis and can result in hepatocellular carcinoma (HCC). Our results support this suggestion; although none of the patients had liver cirrhosis in our study, there is high genomic instability in their mitogen-stimulated lymphocytes. Further prospective studies are needed to further clarify this topic, especially among patients with HCC, cirrhosis and NASH.

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INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is an underdiagnosed liver disease characterized by steatosis and necroinflammation with hepatocyte injury (ballooning), with or without fibrosis. Non-alcoholic fatty liver (NAFL) is characterized by steatosis without inflammation and fibrosis[1]; its prevalence is 10%-30% in adults[2]. NASH is a major cause of so-called cryptogenic liver cirrhosis[3] and cause hepatocellular carcinoma[4,5].

The use of the cytokinesis-blocked micronucleus (CBMN) assay on peripheral blood lymphocytes is one of the most well-validated cytogenetic tests for measuring DNA damage, genome instability and cancer risk[6]. The CBMN assay allows once-divided cells to be recognized by their binucleated (BN) cell appearances after the inhibition of cytokinesis by cytochalasin B[7]. This method was initially proposed for the evaluation of the micronucleus (MN) in BN cells. However, the CBMN assay has more recently been considered a multipurpose test because it can analyze the proliferation index (a measure of cytostasis), cell death (a measure of cytotoxicity) and DNA damage[8-10]. It is often called a cytome assay[11,12]. The events of DNA damage are scored specifically in once-divided BN cells. The frequency of BN cells with MN, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) provides a measure of genome instability or DNA damage. MN is formed through different processes, such as chromosome breakage or complete chromosome loss that lags behind anaphase in cell division. NPBs originate from asymmetrical chromosome rearrangements and/or telomere end fusions. NBUDs are considered biomarkers of gene amplification[9,12].

In this study, our objective was to determine the spontaneous number of MN, NPBs and NBUDs in the phytohemagglutinin (PHA)-stimulated lymphocytes of patients with NASH.

MATERIALS AND METHODS

Patients and controls

This study was conducted between August 2012 and September 2012 in Kayseri Educational and Research Hospital Department of Gastroenterology. Written informed patient consent was obtained from each patient before the procedure, and the study was approved by the Ethics Committee of Kayseri Educational and Research Hospital. The study was performed on 25 (9 females, 16 males) patients newly diagnosed with NASH and on 25 healthy controls of similar ages and genders. None of the participants was known to be receiving any drugs for medical or other reasons or using alcohol. In addition, hepatosteatosis was excluded by abdominal ultrasound imaging in the control group.

Inclusion and exclusion criteria

Each subject in the NASH group had a history of chronic serum alanine aminotransferase (ALT) elevation, which was defined as an ALT > 40 U/L that occurred on two separate occasions separated by at least 3 mo (90 d). No patients or control subjects had an alcohol habit. The subjects also underwent a work-up for other causes of chronic hepatitis of unknown etiology, including serological evaluation for alpha-1-antitrypsin, hepatitis B surface antigen, hepatitis C antibody, copper, ceruloplasmin, antinuclear antibody, anti-smooth muscle antibody, anti-liver kidney microsomal antibody, and total immunoglobulin G. None of the patients, controls, or any of their first degree relatives had diabetes mellitus. Subsequently, the NASH subjects underwent a standard-of-care liver biopsy to identify the etiology and severity of NASH. To be included in the study, the biopsy had to show macrovesicular fat in a minimum of 5% of the hepatocytes, the absence of other etiologies identifying the presence of fat, and a pattern of injury consistent with NASH, as determined by a pathologist[13].

Our patients and control subjects were asked about and examined for conditions affecting MN frequency, including malnutrition, occupational or environmental exposure to known genotoxic agents, smoking, and tea or coffee drinking. None of the patients were receiving medication.

Body mass index (BMI), homeostasis model assessment insulin resistance (HOMA-IR), aspartate aminotransferase (AST), ALT, triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), hemoglobin (Hb) and creatinin were measured or calculated for both groups.

Lymphocyte cultures for CBMN assay

Three milliliter blood samples were collected in heparinized tubes from the antecubital vein after informed consent had been obtained from all patients and control subjects. Approximately 0.4 mL of heparinized whole blood samples was cultured for 72 h at 37 ℃ in 5 mL of

Table 1 Demographic and laboratory parameters of the patient and control groups

Parameter	*n*	mean	SD	*P*
Age (yr)				0.977
Patient	25	41.92	13.33	
Control	25	41.80	13.09	
BMI (kg/m^2)				0.001
Patient	25	31.19	4.62	
Control	25	25.07	4.14	
HOMA-IR				0.001
Patient	25	6.71	4.68	
Control	25	1.40	0.53	
Hb (g/dL)				0.80
Patient	25	14.73	1.49	
Control	25	14.64	1.30	
Creatinine (mg/dL)				0.12
Patient	25	0.74	0.15	
Control	25	0.80	0.13	
AST (U/L)				0.001
Patient	25	56.08	29.11	
Control	25	16.88	3.33	
ALT (U/L)				0.001
Patient	25	92.20	41.43	
Control	25	15.88	5.88	
TG (mg/dL)				0.001
Patient	25	219.21	141.68	
Control	25	102.56	57.98	
HDL (mg/dL)				0.52
Patient	25	46.37	9.65	
Control	25	48.72	15.31	
LDL (mg/dL)				0.02
Patient	25	136.75	30.14	
Control	25	114.63	34.13	

BMI: Body mass index; HOMA-IR: Homeostasis model assessment insulin resistance; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; Hb: Hemoglobin.

peripheral blood karyotyping medium that was supplemented with 1.5% phytohemagglutinin-M to stimulate T-lymphocytes (all from Biological Industries, Kibbutz Beit Haemek, Israel).

In our study, two parallel cultures were prepared simultaneously for each patient and control subject to determine their intra-individual differences. Different slides of two parallel cultures were prepared[14].

Forty-four hours after the initiation of the cultures, the cells were blocked from entering cytokinesis by the addition of cytochalasin-B to each culture tube at a final concentration of 3 μg/mL (Sigma-Aldrich)[14]. The cultures were stopped at 72 h after initiation, treated with hypotonic solution (0.1 mol/L KCl) for 4 min and fixed in two changes of methanol:acetic-acid (3:1)[15]. The fixed cells were spread onto glass slides and stained with 5% Giemsa (Merck) in Sorensen's buffer for 10 min.

CBMN cytome assay

Different slides of two parallel cultures from each patient and control subject were prepared and evaluated. All slides were evaluated blindly using a Nikon Alphaphot-2 light optical microscope. For each sample (patient and control subject), 1000 BN cells were scored for the numbers of micronucleus (MN), NPBs, and nuclear buds (BUDs) in the lymphocytes of the patients and control subjects. The published criteria for the determinations of BN cells, MN, NPBs and NBUDs were followed[12].

Statistical analysis

Statistical comparisons of the number of BN cells with MN, NPBs, NBUDs and the ages of the patients with NASH with those of the control subjects were performed using a non-parametric Mann-Whitney *U* test for two independent samples. Spearman's rho correlation analysis was used to determine the relationships among age and the numbers of MN, NPBs and NBUDs.

RESULTS

The mean ages of the patients and control group were 41.92 ± 13.33 and 41.80 ± 13.09 years, respectively ($P > 0.05$). The demographic and laboratory parameters of the patient and control groups are shown in Table 1. The total numbers and frequencies of BN cells with MN, NPBs and NBUDs scored using a CBMN cytome assay in PHA-stimulated lymphocytes from patients with NASH are shown in Table 2, and those of the control group are shown in Table 2. We found significantly higher numbers of MN, NPBs and NBUDs in the BN cells of patients with NASH than in those of the control subjects (21.60 ± 9.32 *vs* 6.88 ± 3.91; 29.28 ± 13.31 *vs* 7.84 ± 3.96; 15.60 ± 5.55 *vs* 4.20 ± 1.63, respectively, $P < 0.0001$) (Table 3).

DISCUSSION

In the present study, the numbers of MN, NPBs and NBUDs in the lymphocytes of patients with NASH showed a significant increase compared to the control group. Considering the lack of data in the literature related to our values obtained for MN, NPBs and NBUDs, it was not possible to make direct comparisons with other studies. However, the formation of nuclear anomalies, including MN, NPBs and NBUDs, have previously been reported as events commonly observed in the early stages of carcinogenesis[6]. Therefore, we believe that the increased presence of DNA damage biomarkers, including MN, NPBs and NBUDs, in the lymphocytes of patients with NASH may be associated with an increased risk of developing liver cancer.

Oxidative stress, genetic defects in cell cycle checkpoints, defects in DNA repair genes or environmental/dietary factors can each cause the formation of MN *via* chromosomal rearrangements, altered gene expressions or aneuploidy, all of which are associated with a chromosome instability phenotype that is observed primarily in cases of cancer[16,17].

Some previous studies have discussed the association between the induction of MN and the development of cancer. In untreated cancer patients and in subjects with cancer-prone congenital diseases, such as Bloom

Table 2 Total numbers and frequencies of binucleated cells with micronucleus, nucleoplasmic bridges and nuclear buds scored using the cytokinesis-blocked micronucleus cytome assay in phytohemagglutinin-stimulated lymphocytes from patients with non-alcoholic steatohepatitis and the control subjects

ID	Age (yr)	Sex	No. of MN in BN cells[1]	Distribution of BN cells with					No. of BN cells with NPBs	No. of BN cells with NBUDs
				1MN	2MN	3MN	4MN	5MN		
Patients with non-alcoholic steatohepatitis										
1	45	M	19	15	-	-	1	-	25	19
2	60	M	24	19	1	1	-	-	46	11
3	50	M	33	24	1	1	2	-	33	22
4	66	M	41	39	1	-	-	-	20	22
5	22	M	8	8	-	-	-	-	32	17
6	43	M	33	29	2	-	-	-	25	14
7	36	F	14	14	-	-	-	-	21	17
8	51	F	9	9	-	-	-	-	16	10
9	35	M	21	16	-	-	-	1	25	14
10	42	M	11	11	-	-	-	-	12	15
11	30	F	28	24	2	-	-	-	50	20
12	22	M	11	7	2	-	-	-	17	15
13	31	M	12	10	1	-	-	-	21	16
14	41	M	15	13	1	-	-	-	18	25
15	33	M	10	10	-	-	-	-	30	10
16	44	F	22	17	1	1	-	-	58	21
17	47	M	20	13	2	1	-	-	40	17
18	20	M	25	16	1	1	-	1	14	10
19	22	M	24	20	-	-	1	-	32	8
20	46	F	37	27	2	2	-	-	25	10
21	68	F	36	26	5	-	-	-	63	28
22	48	M	17	15	1	-	-	-	28	6
23	60	M	22	18	-	-	1	-	36	19
24	45	F	24	20	2	-	-	-	25	14
25	41	M	24	19	1	1	-	-	20	10
The control subjects										
1	45	M	5	5	-	-	-		11	3
2	60	M	8	6	1	-	-		10	5
3	50	M	7	7	-	-	-		6	5
4	66	M	11	9	1	-	-		12	6
5	22	M	2	2	-	-	-		2	4
6	43	M	6	4	1	-	-		1	4
7	36	F	3	3	-	-	-		9	4
8	51	F	9	5	2	-	-		5	3
9	35	M	4	2	1	-	-		4	1
10	42	M	6	6	-	-	-		7	5
11	30	F	8	8	-	-	-		1	4
12	22	M	2	2	-	-	-		8	4
13	31	M	2	2	-	-	-		10	5
14	41	M	7	5	1	-	-		16	8
15	33	M	7	7	-	-	-		12	6
16	44	F	11	8	-	1	-		5	6
17	47	M	9	7	1	-	-		4	2
18	20	M	1	1	-	-	-		8	4
19	22	M	4	4	-	-	-		9	1
20	46	F	11	7	2	-	-		8	3
21	65	F	17	15	1	-	-		9	5
22	48	M	12	10	1	-	-		14	6
23	60	M	6	6	-	-	-		13	3
24	45	F	11	9	1	-	-		5	3
25	41	M	3	3	-	-	-		7	5

The numbers of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored on 1000 binucleated (BN) cells per subject. [1]Total number of MN: (1MNX1) + (2MNX2) + (3MNX3) + (4MNX4) + (5MNX5). M: Male; F: Female.

Syndrome, an increased frequency of MN has been shown[16,18]. Clinical chemoprevention trials on oral premalignancies have used MN in the oral mucosa as a surrogate endpoint of cancer[19,20]. Another piece of corroborating evidence concerning the association between the MN frequency and the development of cancer is the correlation between genotoxic MN-inducing agents, such as ionizing and ultraviolet radiation, and carcinogenesis[21,22].

Bonassi *et al*[6] evaluated the MN frequency in a total of 6718 subjects selected from the database of Human

Table 3 The numbers of micronucleus, nucleoplasmic bridges and nuclear buds in phytohemagglutinin-stimulated lymphocytes from patients and controls (means ± SD)

Group	Age (yr)	No. of MN in BN cells	No. of BN cells with NPBs	No. of BN cells with NBUDs
Patients (n = 25)	41.92 ± 13.33	21.60 ± 9.32	29.28 ± 13.31	15.60 ± 5.55
Controls (n = 25)	41.80 ± 13.09	6.88 ± 3.91	7.84 ± 3.96	4.20 ± 1.63
P value	0.977	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
Z value	0.029	5.413	5.953	6.016

The numbers of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored on 1000 binucleated (BN) cells per subject. [1]Patients with non-alcoholic steatohepatitis exhibited statistically higher numbers of MN, NPBs and NBUDs in 1000 BN cells than controls, according to the two-tailed nonparametric Mann-Whitney *U*-test for the comparison of the means of independent variables.

Micronucleus Projects, and they followed the subjects for cancer incidence or mortality. After a median duration for follow-up of 8 years, 219 incident cancers and 56 cancer deaths were detected. The subjects in the medium/high MN frequency groups demonstrated a significant correlation between overall cancer incidence and MN frequency (the P values were 0.001 and 0.03, respectively). The risks associated with specific cancer sites were also tested. All cancer sites except for the hepatobiliary and pancreas primaries (RR = 0.163; 0.27-1.44) were shown to have higher relative risks in the medium/high MN groups. The most prominent risk increase was found for bladder and kidney cancers (RR = 8.23; 1.08-63.0). The group concluded that MN frequency in PBL is predictive of cancer risk[6].

The MN technique provides a convenient and reliable index of both chromosome breakage and chromosome loss[18,23]. No studies have been conducted regarding MN formation in the lymphocytes of patients with NASH. In our study, a significant increase in the number of MN was found in the stimulated lymphocytes of patients with NASH. These results strongly support the theory that genomic impairment is elevated in the lymphocytes of patients with NASH.

In addition, the number of MN may be related to other factors, such as micronutrients (folate and riboflavin concentration), occupational or environmental exposure, genetic polymorphisms, lifestyle, smoking and tea or coffee drinking[24-26]. Our patients and control subjects were free from any conditions affecting their MN frequency, such as malnutrition, occupational or environmental exposure. The smoking, tea and coffee habits of the patients and control subjects were similar.

It has been reported that patients who are affected by familiar cutaneous malignant melanoma[27] or cancer-prone congenital diseases, *e.g.*, Bloom syndrome or ataxia telangiectasia, have abnormally high MN frequencies[28]. Moreover, Karaman *et al*[29] observed a significant increase in the MN levels of the lymphocytes of patients with colorectal adenocarcinomas and neoplastic polyps. Hamurcu *et al*[15] showed a clear increase in the frequency of MN in the peripheral lymphocytes of untreated cancer patients. In our previous study, we reported high MN, NPB and NBUD ratios in patients with ulcerative colitis[30]. Additionally, increased MN frequency has been reported in patients with diseases with high cancer risks, such as acromegaly[31] and polycystic ovary syndrome[32].

There are some reports about NASH-related hepatocellular carcinoma (HCC)[4,33-35]. Takuma *et al*[36] reviewed the literature and reported 105 cases (11 of them were their patients) of NASH-associated HCC. They reported that patients with non-cirrhotic NASH may be a high-risk group for HCC. Our results support this suggestion; although none of the patients had liver cirrhosis in our study, there was high genomic instability in their mitogen-stimulated lymphocytes.

Further studies are required to understand the importance of MN, NPBs and NBUDs on NASH-related genomic damage and hepatocellular carcinoma.

COMMENTS

Background

Non-alcoholic steatohepatitis (NASH) is an underdiagnosed liver disease characterized by steatosis and necroinflammation with hepatocyte injury (ballooning), with or without fibrosis. NASH is a major cause of so-called cryptogenic liver cirrhosis and can result in hepatocellular carcinoma. The use of the cytokinesis-blocked micronucleus assay on peripheral blood lymphocytes is one of the most well-validated cytogenetic tests for measuring DNA damage, genome instability and cancer risk. Authors evaluated the risk of genomic instability in patients with NASH in this study.

Research frontiers

The micronucleus (MN) technique provides a convenient and reliable index of both chromosome breakage and chromosome loss. The technique is simple and inexpensive, but it provides important knowledge about genomic instability and DNA damage.

Innovations and breakthroughs

This is the first study that investigates DNA damage in patients with NASH using this method.

Applications

Patients with NASH show genomic instability, but further studies investigating genomic instability in patients with cirrhosis and hepatocellular carcinoma are necessary.

Terminology

MN is formed through several different processes, such as chromosome breakage or complete chromosome loss, that lag behind anaphase in cell division. Nucleoplasmic bridges originate from asymmetrical chromosome rearrangements and/or telomere end fusions; nuclear buds are considered biomarkers of gene amplification.

Peer review

This is a good study that investigates the micronucleus frequency in patients with NASH. A suggestion to the authors is that the patients with high micronucleus ratios should be followed to observe whether they develop hepatocellular carcinoma.

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