ORIGINAL ARTICLE

# Analysis of Xq27.3 Fragility Using the Micronucleus-Fluorescence *In situ* Hybridization Assay

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## ABSTRACT

Chromosome fragile sites tend to form gap or break in chromosomes when the cells are exposed to replication stress. Folic acid deprivation in the culture medium induces folate-sensitive rare fragile sites, such as FRAXA which is responsible for the fragile X mental retardation syndrome. Chromosome instability at fragile sites can be evaluated by biomarkers of genomic instability such as frequency of micronuclei (MN). It was aimed to analyse the chromosome content of MN in Fragile X cells during folate deprivation by the MN-fluorescence in situ hybridization (FISH) method. Samples from five Fragile X syndrome patients, diagnosed using cytogenetic and molecular methods, as well as from their parents and five controls were included in the study. Blood samples were cultured in two different culture media (folate-deficient and normal). Results of MN-FISH test were analysed in terms of MN frequency and chromosome content of MN. An accumulation of MN in Fragile X patients, mainly containing T (+) or C (+) MN or T (+) plus C (+) MN in binucleated cells was found. Finally, MN-FISH analysis allowed confirming that the increase in MN frequency is due to a higher sensitivity to chromosome breakage along the X chromosome.

**Key words:** Cytogenetics, DNA damage, fluorescence *in situ* hybridization, Fragile X syndrome, micronuclei

## Introduction

Chromosome fragile site is a chromosomal locus that tends to form a gap or break in condensed metaphase chromosome following exposure of cells to DNA replication stress. Based on their frequency, fragile sites are classified as rare fragile and common fragile sites.[1,2] Fragile sites can be observed as gaps, constrictions, or breaks on metaphase chromosomes that arise when cells are inhibited from undergoing DNA replication.[3,4] Folic acid plays a critical role in maintaining genomic stability; it is required for DNA repair, prevention of chromosome breakage and hypomethylation of DNA. In case of folate deficiency, the incorporation of uracil into DNA rather than thymine leads to the accumulation of dUMP excessive accumulation of uracil causes single-and double-stranded DNA breaks, chromosome breakage, and micronucleus formation.[5,6] Folate deficiency induces chromosome instability and DNA replication-associated DNA breakage as well as triggers off formation of micronuclei (MN). FRAXA fragile site is associated with Fragile X syndrome (FXS) and therefore, prone to form a break in folate deprivation. Although it is still unclear how the folate deficiency causes genomic instability, the trinucleotide repeat sequence might be considered the cause of genomic instability at rare fragile sites such as FRAXA on chromosome X (at Xq27.3). Expanded CGG repeats at fragile sites have the potential to form unusual secondary structures, including hairpins, triplex, and tetraplex structures, which can perturb the elongation of DNA replication.[3] CGG repeats influence nucleosome assembly and causes chromatin decondensation.[7,8] These studies suggest that DNA repair capacity, on the hairpin and tetra helical opening structure, may be defective in FXS. Triplet repeat instability causes DNA damage and DNA repair defects. Therefore, FXS cells become sensitive to DNA damage.

FXS is the most common type of inherited mental retardation (MR) disease, with a prevalence of 1/4000 in males and 1/7000 in females.[9] MR is less severe in female than male patients and only one in three female carriers exhibit signs of the disease. Most of the cases of FXS are caused by CGG trinucleotide

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Website: www.genome-integrity.org

DOI: 10.4103/genint.genint_4_20

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**How to cite this article:** Özer L, Ruhi HI, Bökesoy I. Analysis of Xq27.3 fragility using the micronucleus-fluorescence *in situ* hybridization assay. Genome Integr 2020;11:1.

repeats expansion at the untranslated region (5'UTR) of the fragile X mental retardation 1 (FMR1) gene on Xq27.3. Abnormal expansion of this triplet causes hypermethylation and the subsequent silencing of the FMR1 gene. The gene product, fragile X mental retardation protein (FMRP), is a widely expressed RNA-binding protein, found at particularly high levels in the brain and testes. FXS is caused by a deficiency or absence of FMRP.[10] Rare mutations (including deletions and point mutations) within FMR1 gene that disrupt RNA splicing, can lead to FXS, although it represents ≤1% of all cases.[11-14]

In 1969, a constriction in the long arm of the X chromosome in metaphase was observed in cultured cells obtained from patients with X-linked MR.[4] This constriction at Xq27.3 is one of the chromosomal fragile sites that can be induced when cells are cultured in the presence of folic acid or thymidine deficient medium,[13] and this cytogenetic finding was commonly used for diagnosis of FXS. However, nowadays, the use of molecular genetic methods for diagnosis of FXS is preferred.

The cytokinesis-block micronucleus (CBMN) assay has been used as a screening test to detect DNA damage. The frequency of MN in peripheral lymphocytes is a biomarker of genomic instability. MN's arise after mitosis by nuclear membrane formation around either a lagging chromosome (s) or chromosomal fragments. MN is formed as a consequence of whole chromosome/chromatid breaks or acentric chromosome/chromatid fragments left outside the main daughter nuclei. Chromosome fragments in MN could be the result of double-stranded DNA breakage, conversion of single-strand breaks into double-strand breaks, or inhibition of DNA synthesis.[15,16] The combination of the MN assay with the fluorescence *in situ* hybridization (FISH) technique, also named as MN-FISH, can be used to demonstrate the presence of damaged chromosome fragments using specific probes for them.[17] Besides, MN-FISH test allows the detection of chromosomal origin of spontaneous and induced MN.

In the present study, we have analyzed the frequency of MN occurring in human peripheral lymphocytes from FXS patients, as well as in their parents and a control group in normal and folate deprivation conditions. Besides, employing the MN-FISH technique with centromeric and telomeric probes from the X chromosome, it was analyzed the distribution of T (+) and C (+) MN in the patient group in comparison to their parents and the control group. In this respect, we have been found a higher frequency of T (+) fragments in the patient group respect to their parents and the control group, indicating that the X chromosome is prone to be involved in chromosome damage produced in FXS patients.

# Subjects and Methods

## Subjects

Five patients diagnosed using cytogenetic and/or molecular methods, as well as their parents were included in the current study. Five healthy male individuals were taken as control group. The patients' mothers were included in the study to detect whether premutant carriers have an increased DNA damage response to folate deprivation. The patient parents and controls were informed of the research purpose and accepted to sign an informed consent. The approval of the Ethics Committee (Ankara University Ethics Committee) was obtained. Blood samples were collected using venipuncture from all individuals into heparin-containing tubes for MN-FISH analysis.

## Cytokinesis-block micronucleus assay

The CBMN assay was performed according to Fenech[15] procedure. Whole blood cultures were established by mixing 0.4 ml whole blood with 5 ml RPMI medium (Biological Industries; Kibbutz Beit-Haemek, 25,115, Israel) (folate-deficient medium [without folic acid]; normal medium [with folic-acid]) supplemented with 10% fetal calf serum (Sigma-Aldrich, USA), 2 mM L-glutamine (Sigma-Aldrich, USA), antibiotics (100 IU penicillin and 100 µg/ml streptomycin), and 1% phytohemagglutinin (Sigma-Aldrich, USA). Cells were cultured at 37°C for 72 h and cytochalasin B (Sigma-Aldrich, USA) was added after 44 h (final concentration, 6 µg/ml).

Cells were centrifuged and treated with a cold hypotonic solution (0.075 M KCl) for 20 min. Cells were fixed in cold fixative (methanol:acetic acid [3:1]). The fixed cells were transferred to humidified slides using Pasteur pipettes, air-dried, and stored at −20°C for FISH analysis.

## Fluorescence *in situ* hybridization

To investigate the X chromosome content of MN we performed FISH analysis using X centromeric and X telomeric probes. A chromosome X centromeric probe (Vysis/DXZ1/CEPX: Spectrum Green/Xp11.1-q11.1) and Xq/Yqtelomeric probe (Vysis/TelVysion: Spectrum Orange/Xq/Yq) (Abbott Lab, Illinois, USA) were used. Chromosome spreads slides were treated with 10% pepsin diluted in 10 mM HCl for 10 min at 37°C. Briefly, the slides were washed in distilled water and phosphate-buffered saline (PBS) solution for 5 min and postfixed for 10 min at room temperature (RT) with 1% formaldehyde diluted in PBS. Slides were washed with PBS for 5 min at RT. DNA denaturation was performed in 70% formamide (Sigma-Aldrich, USA) ×2 saline–sodium citrate (SSC) buffer at 70°C for 2 min and slides were dehydrated in an ethanol serie (70%, 85%, 100%). After overnight hybridization at 37°C, posthybridization washes were performed using 50% formamide (Sigma-Aldrich, USA)/×2 SSC (10 min at 46°C), followed by one wash in × 2 SSC (10 min at 46°C) and one in 1% NP40 (Sigma-Aldrich, USA)/×2 SSC (5 min at 46°C). Then, slides were counterstained with DAPI (Sigma-Aldrich, USA) and examined under a fluorescence microscope equipped with the appropriate filter set (Applied Imaging Corp., San Jose, CA 95134-2302, USA). The micronucleus frequencies of binucleated (BN) cells were determined following scoring criteria from Fenech[15] in every sample. The number of telomere-positive (T+) and centromere-positive (C+) MN was determined in BN lymphocytes for every sample. Results were given as a total number of MN and number of C(-) MN, C(+) MN, T(+) MN, and T(-) MN in 2000 BN cells (from 2 analyzed

slides) for every sample. Pooled data from patients, as well as from mothers, fathers, and healthy donors was analyzed.

### Statistical analysis

Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS Inc., Chicago, IL, USA) version 11.5. The Shapiro–Wilk test was used to assess whether continuous variables were normally distributed or not. The frequency of BN, MN, and total number of MN was shown as descriptive statistics. The differences among groups were evaluated using the Kruskal–Wallis test. The Wilcoxon signed-rank test was performed to compare differences between folate-deficient and normal media. A $P < 0.05$ was considered statistically significant unless otherwise stated. However, for all multiple comparison tests the Bonferroni correction was applied for controlling Type I error.

## Results

In the present study, the frequency of MN and results obtained using MN-FISH were assessed in patients with FXS, their parents, and the control group. The general characteristics of the patients are presented in Table 1. The control group comprised five healthy boys between 10 and 13 years old. The mean age of the patients' mothers ($n = 5$) was 43 years (range, 35–50 years), and the mean age of the patients' fathers ($n = 5$) was 50 years (range, 41–73 years).

**Table 1: Characteristics of Fragile X syndrome patients**

| Patient | Age (years) | Gender | Cytogenetics (% fragility) | Methylation dependent PCR |
|---|---|---|---|---|
| 1 | 11 | Male | 13 | Methylation pattern concordant with Fragile X |
| 2 | 11 | Male | 24 | Methylation pattern concordant with Fragile X |
| 3 | 10 | Male | 14 | Methylation pattern concordant with Fragile X |
| 4 | 15 | Male | 9 | Methylation pattern concordant with Fragile X |
| 5 | 17 | Male | 4 | Methylation pattern concordant with Fragile X |

### Detection of micronuclei frequencies

In order to investigate whether folate deprivation might cause MN formation in cells containing fragility at Xq27.3 (FRAXA locus), cells were cultured in medium without folate for 72 h and then MN were scored in BN cells. The number of BN cells as well as MN in culture medium with or without folic acid is presented in Table 2. The frequency of MN in BN cells, number of T(+) MN in 100 BN cells, number of T(+) C(+) MN in 100 BN cells, and number of C(+) MN in 100 BN cells is represented in Figure 1. The frequency of BN cells, MN, T(+) MN and T(+) C(+) MN in both culture media were higher in patients than the control group [Figure 1 and Table 2]. MN was more frequent in patient mothers than patient fathers. The number of MN per cell in both culture media was higher in patients than in their parents or the control group in both culture media [Table 3].

### Detection of micronuclei contents with X chromosome-specific centromeric and telomeric probes

In order to investigate whether MN of fragile X patients includes X chromosome segments due to X chromosome fragility, we analyzed cytokinesis-blocked cells using FISH to define the X chromosome content of MN. The number of T (+) MN and T (+) C (+) MN in culture medium with or without folic acid is presented in Table 2. Figure 1 summarizes MN frequency as well as the frequency of T (+) or T (+) C (+) MN. Representative examples of MN following FISH analysis are presented in Figure 2.

The number of T (+) C (+) MN in both culture media was higher in patients than in their parents or the control group. The frequency of MN cells in both culture media was higher in

**Table 2: Pooled data of the frequency of micronucleus (in a total number of binucleated cells) from every group, namely, patients, mothers, fathers and control obtained from folate-deficient or normal culture medium**

| Variables | Patients | Mothers | Fathers | Controls | $P^{a,b}$ |
|---|---|---|---|---|---|
| BN | | | | | |
| Folate-deficient medium | 3845 | 1570 | 921 | 2075 | 0.365 |
| Normal medium | 3290 | 1752 | 1643 | 1564 | 0.140 |
| MN | | | | | |
| Folate-deficient medium | 103 | 42 | 14 | 53 | 0.030 |
| Normal medium | 65 | 64 | 23 | 24 | 0.223 |
| $T^+$ MN | | | | | |
| Folate-deficient medium | 19 | 7 | 4 | 6 | 0.242 |
| Normal medium | 9 | 8 | 5 | 3 | 0.792 |
| $C^+$ MN | | | | | |
| Folate-deficient medium | 0 | 3 | 0 | 2 | 0.276 |
| Normal medium | 2 | 4 | 0 | 0 | 0.073 |
| $T^+ C^+$ MN | | | | | |
| Folate-deficient medium | 7 | 5 | 1 | 4 | 0.761 |
| Normal medium | 4 | 6 | 1 | 2 | 0.508 |

Total number of MN containing T (+), C (+) or T (+) plus C (+) in every group (total number of BN cells analyzed). [a]Kruskal-Wallis test; [b]The results were considered significant according to the Bonferroni correction ($P<0.025$). BN: Binucleated cell, MN: Micronucleus, $T^+$: Telomere-positive, $C^+$: Centromere-positive (X chromosome-positive)

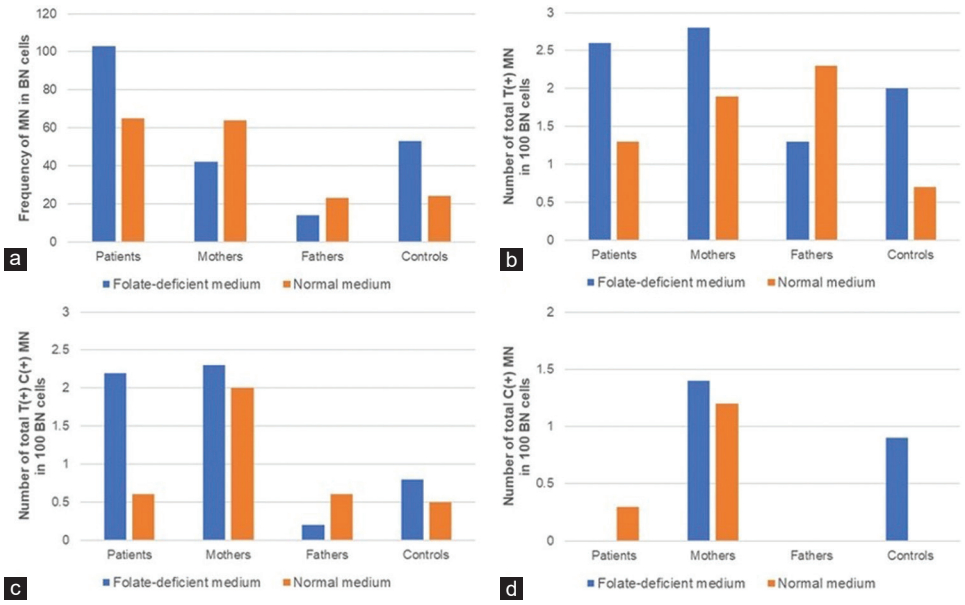


**Figure 1:** Graphic representation of (a) Frequency of micronuclei in binucleated cells (b) number of T (+) micronuclei in 100 binucleated cells, (c) number of T (+) C (+) micronuclei in 100 binucleated cells, (d) number of C (+) micronuclei in 100 binucleated cells

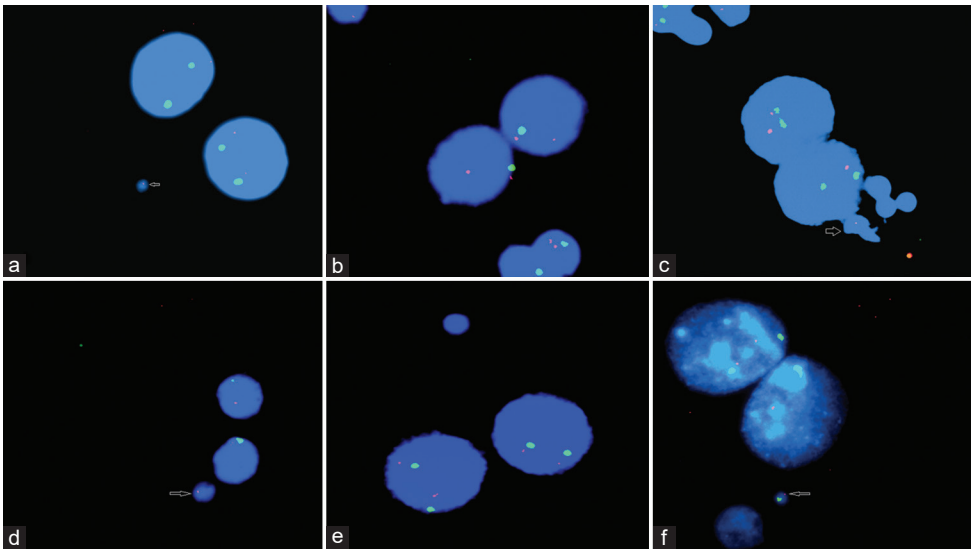


**Figure 2:** Examples of micronuclei observed in binucleated cells by micronuclei-fluorescence *in situ* hybridization analysis. Red signal represents the telomeric (T) probe and the green signal represents the centromeric (c) probe. (a) binucleated cell with T (–) and C (–) micronuclei (patient mother 2), (b) micronuclei- fluorescence *in situ* hybridization image of binucleated cell (patient 3), (c) binucleated cell with T (–) and C (+) micronuclei (patient mother 5), (d) binucleated cell with T (+) micronuclei (patient 1), (e) binucleated cell with T (+) micronuclei (patient mother 3), (f) binucleated cell with T (+) micronuclei (patient mother 2)

patients than their parents or the control group. MN was more frequent in patient mothers than patient fathers.

## Discussion

MN test has been used to evaluate genotoxic effects and chromosome instability. Several exogenous factors and endogenous factors affect genetic material and cause the formation of MN.[18] Although these factors are related to MN formation *in vivo* and *in vitro*, the chromosomal origin of MN was not completely identified until recently because of the limitations of traditional experimental methods used.[19]

Repeat expansion in fragile X patients causes DNA damage and failure to resolve or repair them appropriately can lead to chromosome fragility at FRAXA fragile site. FRAXA fragile site is induced by agents such as fluorodeoxyuridine (FdU). Wang *et al.*[20] compared human lymphoblastoid cells derived from normal donors and patients with FXS in terms of hypersensitivity to DNA damage and DNA repair capacity using the comet assay. As a result, comet assay revealed that Fragile X cells were not more sensitive to DNA damage induced by oxidative agents (hydrogen peroxide and bleomycin), an alkylating agent (ethyl methanesulfonate), an

**Table 3: Frequency of micronucleus per cell obtained in every group obtained from folate-deficient or normal culture medium**

| Variables | Folate-deficient medium | Normal medium | $P^{a,b}$ | Change |
|---|---|---|---|---|
| MN/Cell | | | | |
| Patients | 2.8 (1.9-6.9) | 2.8 (0.5-3.1) | 0.223 | −1.4 (−4-0.3) |
| Mothers | 2.5 (1.4-7.1) | 3.4 (0.9-5.7) | 0.500 | 0.6 (−3-3.1) |
| Fathers | 1.3 (1.2-1.7) | 2.0 (0.4-5.2) | 1.000 | −0.9 (−1.1-4) |
| Controls | 2.3 (1.6-6.8) | 1.6 (0.7-2.2) | 0.223 | −0.7 (−6.1-0.1) |
| $p^{c,d}$ | 0.089 | 0.223 | | 0.465 |
| $T^+$ MN | | | | |
| Patients | 2 (1-9) | 1 (0-6) | 0.343 | −2 (−7-5) |
| Mothers | 1 (1-2) | 1 (0-4) | 0.854 | 0 (−1-3) |
| Fathers | 1 (1-2) | 1 (0-3) | 1.000 | −1 (−1-2) |
| Controls | 1 (0-2) | 0 (0-2) | 0.334 | −1 (−2-1) |
| $p^{c,d}$ | 0.242 | 0.792 | | 0.268 |
| $C^+$ MN | | | | |
| Patients | 0 (0-0) | 0 (0-1) | 0.157 | 0 (0-1) |
| Mothers | 0 (0-2) | 1 (0-2) | 0.317 | 0 (0-1) |
| Fathers | 0 (0-0) | 0 (0-0) | 1.000 | 0 (0-0) |
| Controls | 0 (0-1) | 0 (0-0) | 0.157 | 0 (−1-0) |
| $p^{c,d}$ | 0.276 | 0.073 | | 0.115 |
| $T^+$ $C^+$ MN | | | | |
| Patient | 1 (0-4) | 0 (0-3) | 0.414 | 0 (−3-1) |
| Mother | 1 (0-3) | 1 (0-3) | 0.317 | 0 (0-1) |
| Father | 0 (0-1) | 0 (0-1) | 1.000 | 0 (−1-1) |
| Control | 0 (0-2) | 0 (0-1) | 0.157 | 0 (−1-0) |
| $P^{c,d}$ | 0.76179 | 0.508 | | 0.544 |

[a]Wilcoxon signed-rank test, [b]Intra-group comparisons between the folate-deficient and normal media (the results was considered significant according to the Bonferroni correction, $P<0.0125$), [c]Kruskal Wallis test, [d]Inter-group comparisons between the folate-deficient and normal media (the results were considered significant according to the Bonferroni correction, $P<0.025$). For evaluating differences between normal medium and folate-deficient medium, $P<0.05$ was considered statistically significant). BN: Binucleated cell, MN: Micronucleus, $T^+$: Telomere-positive, $C^+$: Centromere-positive (X chromosome-positive)

ultraviolet-mimicking agent (4-nitroquinoline N-oxide), a DNA cross-linking agent (mitomycin C), or a DNA topoisomerase II inhibitor (etoposide).[16] In Wang's study, it was postulated that DNA repair status is different in meiotic and mitotic stages.[16] Although DNA repair capacity in somatic lymphoblastoid cells from FXS is normal, DNA repair capacity in the germline is unknown.[16] We have found that MN frequency in Fragile X patients is higher than in healthy individuals. Besides, MN frequency observed in BN cells obtained from both culture media was higher in patients than their parents or the control group in the present study. Fenech and Crott[21] evaluated pooled data from *in vitro* studies in which cells were cultured for 9 days in medium containing 12, 24, and 120 nM folic acid showed that 120 nM and >120 nM folic acid provided a strong protective effect against genome damage and that the frequencies of MN, nuclear buds (NBUDs) and nucleoplasmic bridges were minimal at these concentrations.[2] Another study reported that frequencies of MN in cells cultured in folate-deficient medium were higher than in cells cultured in normal medium.[22] In our study, cultured cells from patients with FXS grown in folate-deficient medium exhibited a higher frequency of MN than in cells cultured in normal medium.

Górski also compared the MN frequency in patients with FXS ($n = 5$) with healthy controls ($n = 11$) using two different media (normal medium and hydroxyurea medium).[23] It was reported that fragility and MN formation were more frequent in cells cultured in hydroxyurea medium than in those grown in normal medium. Cultured cells in normal medium, demonstrated no statistically significant difference in terms of MN frequency between patients and controls. These results agree with those of the present study which demonstrated no statistically significant difference between patient and control groups in terms of MN frequency in cells cultured in normal medium. We found higher MN frequency in patients than the control group in the present study.

It is interesting to study the chromosome content of MN. In this respect, Lindberg *et al.*[5] evaluated the content of MN in human lymphocytes obtained from healthy volunteers and cultured in normal and RPMI medium containing 12–120 nM folic acid (12, 24, 60, and 120 nM). FISH was performed using pantelomeric and pancentromeric DNA probes to assess the frequencies of MN harboring telomeric and centromeric DNA sequences as well as frequencies of NBUDs harboring telomeric and centromeric DNA sequences. The frequency of MN increased in medium containing <120 nM folic acid and 62% of MN were T+ and 22% of MN were T+ and C+ .[22] Besides, interstitial DNA without C or T labels was more prevalent in NBUDS (43%) than in MN (%13) while DNA with only T labels or C and T labels was more frequent in MN (62% and 22%, respectively) than in NBUDs (44% and %10, respectively). Folate deprivation increased the frequency of NBUDs and MN harboring telomeric DNA sequences, NBUDs harboring interstitial DNA and NBUDs and MN with centromeric and telomeric DNA sequences. Moreover, these results demonstrated that MNs in BN lymphocytes were primarily derived from lagging chromosomes and terminal acentric fragments during mitosis. Bjerregaard *et al.* studied MN-FISH to investigate the effect of folate deprivation and for this purpose they cultured cells without folate for 3 days and then analyzed cytokinesis blocked twin-daughter G1 cells. The results of the study showed that folate deprivation led to only a modest increase in the frequency of MN containing FRAXA when compared to other chemical exposures of cells such as FdU treatment. They hypothesized that any FRAXA-containing MN would be lost from the population during the extended growth period required to deprive cells of folate.[24] We detected a modest increase in our study and this result may be due to a loss of FRAXA-containing MN related to the same mechanism which has been proposed in Bjerregaard's study.[24] The present study demonstrated that cultured BN cells contained T signal-positive MN in the control and patient groups. The number of T (+) C (+) MN of BN cells in both culture media was higher in patients than their parents or the control group. Wojda *et al.* while studying the chromosomal content of spontaneous MN in human lymphocytes have found that whole chromosome and telomeric fragments are frequently detected

but centric fragments are rarely noted in MN.[25] Previous studies utilizing FISH have demonstrated that the X chromosome tends to lag during lymphocyte anaphase, being micronucleated more efficiently than autosomes. In our study, the presence of the X chromosome in the micronucleus was similar to normal individuals and fragile X patients, which might be explained by the X chromosome's susceptibility of being most micronucleated chromosome. Hando *et al*.[26] reported that X+ signals were present in 72.2% of micronucleated cells. Therefore, it was considered that the X chromosome is nonrandomly involved in MN formation. MN-FISH demonstrated that X-chromosome was over-represented in MN especially in women.[17] Regarding this information, the nonsignificant results of T(+) MN and T(+) MN, C(+) MN between patients and mothers observed in our work can be explained. Although the T(+) MN in the patient group is not higher than in the mother group. However, when it is compared with the control group, T(+) MN in the patient group was higher. Age and gender have a great impact on the frequency of MN and micronucleation of the sex chromosomes will increase with advanced age.[5] The frequencies of MN and MN with telomeric fragments are changed due to the concentration of folic acid and the duration of folate deprivation. Low folic acid levels and prolonged exposure to folic acid deprivation may increase the frequency of MN.[5,24] The changes in folic acid concentration and the exposure time to low folic acid deficiency may lead to an increase in the MN frequency and the percentage of telomeric fragments in MN. The percentage of T(+) MN in the patient group was greater than their fathers regardless of culture media used (folate-deficient and normal medium). Women have a higher frequency of X+ MN than men and the present study is consistent with previous studies.[27-29] MN frequency was higher in patient mothers than their fathers. Previous reports have established a positive correlation between the frequency of micronucleated cells and advanced age.[16,30] The rate of micronucleus formation increases with age, particularly in women.[26,31] Mothers had the highest frequency of MN. This result can be explained by the rate of MN formation in women being higher than in males.[25,32] Several studies were conducted to evaluate the frequencies of MN containing X and Y chromosomes in males.[33] Carere *et al*.[33] evaluated the inclusion of a Y chromosome in MN of cultured lymphocytes from 10 males representing two age groups (20–29 and 51–55 years) and reported that the frequency of Y+ MN increased with age (51–55 years, 1.1/1000; 21–29 years, 0.1/1000). Another study reported that the Y chromosome in MN and Y chromosome loss increased in males over 45 years old.[28] Therefore, it was concluded that the high frequency of T+ MN in fathers could be attributed to the increased frequency of Y chromosome MN in older individuals. The age-dependent shortening of telomeres of the Y chromosome, resulting in instability of the chromosome ends, either in the short arm or in the long arm, may explain the increased frequencies of T+ MN's in fathers.[33]

In this study, it was aimed to measure chromosome damage by analyzing MN frequency in PBLs and the MN were hybridized with X chromosome centromeric and telomeric probes to evaluate the presence of X chromosome in MN of FXS patients. It is demonstrated that folate deprivation led only a modest increase in the frequency of MN in Fragile X patients. The number of T (+) C (+) MN in both culture media was higher in patients than their parents or the control group. This study's results will be helpful for future studies focused on DNA damage in Fragile X patients and the chromosomal origin of MN. In addition, the identification of the chromosome content of MN can contribute to understand the origin of MN formation.

## Acknowledgments

We would like to thank the volunteers who participated in the study.

## Compliance with ethical standards

The approval of the Ethics Committee (Ankara University Ethic Committee) was obtained.

## Financial support and sponsorship

This research was supported by Ankara University Management of Scientific Research Projects (Project No: 2006-08-09-238).

## Conflicts of interest

There are no conflicts of interest.

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