The Effects of Static Magnetic Fields and X-rays on Instability of Microsatellite Repetitive Sequences

TAKAHITO OKUDA¹,³, KIMIKO NISHIZAWA¹, YOSUKE EJIMA², SHIGEKAZU NAKATSUGAWA³, TAKEO ISHIGAKI³ and KANJI ISHIZAKI¹

¹Laboratory of Experimental Radiology, Aichi Cancer Center, Kanokoden 1–1, Chikusa-ku, Nagoya 464–8681, Japan
²Radiation Biology Center, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606–8501, Japan
³Department of Radiology, Nagoya University School of Medicine, Turumai-cho, Showa-ku, Nagoya 466–8550, Japan

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To determine the genetic effect of static magnetic fields (SMF), which are not supposed to produce any significant DNA damage, we took advantage of DNA mismatch repair (MMR) deficient cells, in which all the errors produced during DNA replication are left uncorrected. We first established a simple and less labor-intensive method to analyze genetic changes in microsatellite repetitive sequences in the MMR-deficient cells. After exposure to a strong SMF (6.34T) for 24h, both MMR deficient HCT116 cells and proficient HeLa S3 cells did not exhibit any significant effect on microsatellite changes. Moreover, when HCT116 cells were synchronized at the G1/S boundary by aphidicolin and exposed to SMF during the whole S-phase, no increase in microsatellite changes was either observed. In contrast, irradiation by a low dose X-ray (2Gy) significantly increased microsatellite changes in HCT116 cells. This suggested that exposure to strong SMF may not induce any significant level of genetic changes in microsatellite sequences.

INTRODUCTION

Genetic changes in human cells induced by any physical or chemical agents have so far been mainly analyzed through mutations of some selectable marker genes, such as the HPRT, TK and APRT genes¹,². Since the occurrence rate of spontaneous mutations of these genes in human cells is rather low (less than one in 10⁵–⁶), laborious work with more than 10⁷ cells is required to obtain significant data by analyzing these genetic markers. Furthermore, the type of mutation detectable with these markers is limited to the loss-of-function type. Recently, so-called
microsatellite sequences which include repeats of one to a few base units and which distribute across all human chromosomes have been extensively studied as genetic markers in human \(^3\). They are easily detected with PCR, and so far more than 10^4 microsatellite loci have been identified. These microsatellite sequences may be good targets to analyze the genetic effects of any agents because no selection is required to detect genetic changes, and because even a small number of cells may be sufficient to obtain significant results by simultaneously analyzing multiple loci. Furthermore, recent studies on the mismatch repair (MMR) mechanism in human cells revealed that in MMR deficient cells most of the errors produced in microsatellite sequences during DNA replication are not corrected, resulting in changes in microsatellite sequences\(^5,6\). Actually, highly frequent spontaneous microsatellite changes (MC) of as much as 10^2 or more are observed in MMR-deficient human cells, suggesting that the genetic effects induced by any agent may be sensitively detected in these cells by analyzing MC.

Electromagnetic fields (EMF) are produced by various sources, and we may have increasing chance of exposure to EMF in our daily lives. Such EMF include static magnetic fields (SMF) produced by magnetic resonance imaging (MRI) machines used for diagnosis in the clinical field, extremely low-frequency magnetic fields (ELFMF) mainly produced by commercial power lines, and high-frequency magnetic fields produced by cellular phones and the like, which have been issues of public concern in terms of overall health and genetic hazard to humans\(^8\). There are numerous reports on the biological effects of ELFMF\(^9\), and mutation induction by high density 50 Hz ELFMF in cells at a DNA synthesis phase has been recently reported in the HPRT gene, suggesting that EMF can induce errors during DNA replication\(^10\). In contrast, there are few reports on the biological effects of SMF\(^9\) although a recent work with *Drosophila melanogaster* reported that SMF could induce mitotic recombinations\(^11\). However, we run increased risks of exposure to strong SMF in the clinical field since MRI is now frequently used for diagnosis of various kinds of diseases. Therefore, we examined whether MC in MMR-deficient and proficient cells is affected by SMF, and compared those results with the effects of X-ray irradiation. We also synchronized cells to S phase so that we can especially detect the effects of SMF during DNA replication.

**MATERIALS AND METHODS**

An MMR-proficient human cell line, HeLa S3, and an MMR-deficient human cell line, HCT116\(^12\), were used in this study. Both cells were maintained in Dulbecco’s modified minimum essential medium supplemented with 10% fetal calf serum.

For X-ray irradiation, 10^2 cells were inoculated into a 10-cm dish and incubated at 37°C. On the next day, the medium was removed and cells were irradiated with 2 Gy X-ray (50 kV, 10 mA, dose rate 5.5 Gy/min) by using a soft-X-ray machine, LMBW-2 (Softex, Tokyo, Japan). Then a fresh medium was introduced and cells were further incubated for colony formation. After 10 to 14 days, colonies about 3 mm in diameter were cloned separately and each colony was transferred into a well of a 48-well plate. The number of colonies in a dish was also scored to determine the killing effect of X-ray irradiation. When cells in each well became confluent, the
medium was removed, cells were washed with phosphate buffered saline, and the plates were kept at -20°C until cell lysis.

For SMF exposure, approximately $10^4$ cells were inoculated into a slant-type tube and incubated at 37°C so that cells attached to a flat surface of the tube. Next day, cells in the tube were exposed to a magnetic field of a nuclear magnetic resonance analysis machine, GSX-270W (Nippon Denshi, Tokyo, Japan) with a peak strength of 6.34T, no gradient, and a horizontal orientation. The cells in SMF were kept at 37°C for 24 h. For sham-exposure, cells inoculated in the slant-type tube was kept at 37°C for 24 h in a conventional incubator set in a room next door to the machine.

For synchronization, HCT116 cells in a slant tube were treated with aphidicolin (5 µg/ml) for 22 h at 37°C[13] and then washed with a fresh medium followed by exposure to SMF for 6 h. After exposure to SMF, both synchronized and randomly growing cells were replated into a 10-cm dish at low density for colony formation. After 10 to 14 days, colonies were treated in the same way as X-ray irradiated cells.

To determine the effectiveness of synchronization, aphidicolin-treated cells were incubated in a medium containing 10 mM BUdR for 30 min at each time point after removal of the aphidicolin and fixed with Carnoy solution. Fixed cells were stained with an FITC-labeled anti-BUdR antibody (Neo Markers, Fremont, CA, USA), and the rate of labeled nuclei was determined under a fluorescent microscope.

In this study we analyzed the 5 microsatellite-loci shown in Table 1 with the primer sequences used for PCR. To prepare cell lyzate in situ in a 48-well plate, 100 µl of cell-lysis solution (10 mM Tris-Cl pH 7.5, 1 mM EDTA and 5 µg/ml Proteinase K) was added to each well and heated at 65°C for 2 h and then at 95°C for 15 min. After being cooled down to room temperature, the cell lyzate was directly used in PCR as temperate DNA. PCR was performed in 5 µl 1x Taq buffer containing 1 µl cell lyzate, 0.25 pmol of each primer, 2 mM MgCl₂ and 0.1 U Taq polymerase (Perkin Elmer Japan, Urayasu, Japan). One of primers was end-labeled with $^{32}$P using polynucleotide kinase (Toyobo, Osaka, Japan). After heating at 95°C for 4 min, PCR was performed for 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Then, samples

| Locus   | Repeat Unit | Primer sequence            |
|---------|-------------|-----------------------------|
| D2S123  | CA          | F  5'  AAACAGGATGCCCTGCCTTTA |
|         |             | R  5'  GGACTTCTCCACCTATGGGA |
| D2S119  | CA          | F  5'  CTTGGGGGACACAGGTCATT |
|         |             | R  5'  GAGAAATCCCCTAATTCCTGGA |
| D10S197 | CA          | F  5'  ACCAGCTGCACCATCGGTAC |
|         |             | R  5'  GTGATACCTGTCCTCGCTCC |
| D7S1794 | CTT         | F  5'  TTGTGACAGAAACATCGGCC |
|         |             | R  5'  TGCACAGAAATACTGCCCTA |
| BAT-26  | A           | F  5'  TGACTTTTGTACACGAGC |
|         |             | R  5'  AACCATTCAAATTTTAAACC |

Table 1. Microsatellite loci analyzed and their primer sequences
were denatured at 95°C for 5 min after mixing with the same volume of loading buffer (98% formamide and 10 mM EDTA) and loaded onto a 6% polyacrylamide denaturing gel. After electrophoresis, amplified DNA fragments of each microsatellite locus were detected by autoradiography.

To determine the sensitivity for detecting MC, we mixed HeLa and HCT116 cells, which exhibit different lengths at the D7S1794 locus, at ratios of 1:0, 10³:1, 10²:1, 10 : 1, 4 : 1, 1 : 1, 1 : 4 and 0 : 1. Then, 10⁴ cells of each mixture were inoculated into each well of a 48-well plate. On the following day, cells were lysed, and microsatellite sequences at the D7S1794 locus were analyzed as described above.

Statistical significance of difference between each group was determined by the $\chi^2$ test with Yates's correction.

RESULTS AND DISCUSSION

In this study, we first attempted to establish a method by which genetic changes in human cells can be detected sensitively but without intensive labor. Since microsatellite sequences are widely distributed on a whole human chromosome and known as more changeable than other unique sequences⁴, they would seem to be sensitive and reliable markers to detect both spontaneous genetic changes and those induced by any agent. Recent studies have revealed that MMR-deficient cells showed increased microsatellite instability because MMR is involved not only in the repair of mismatched base pairs but also in the correction of errors in repetitive sequences which occur during DNA replication⁵. This suggests that, in MMR-deficient cells, genetic changes may be sensitively detected by analyzing MC at multiple microsatellite loci.

With the methods described here, effective amplification and detection of microsatellite sequences were possible in both HeLa and HCT116 cells. Fig. 1 shows examples of microsatellite

![D7S1794](image)

**Fig. 1.** Examples of microsatellite analysis. The D7S1794 locus in non-irradiated HeLa and HCT116 cells is shown. Arrowheads indicate MC observed in HCT116 cells.
analysis in both cells, where MCs were indicated by arrowheads. We then determined the sensitivity for detecting the fragments with altered length by mixing cells with different lengths of PCR products. As shown in Fig. 2, even a fragment of HCT116 cells in a 1:100 mixture was detected by autoradiography. This suggests that MC which occurred in more than 1% of cells in a colony is detectable by our method.

Next, we analyzed the effects of 2 Gy X-ray irradiation on MC. After X-ray irradiation both HeLa and HCT116 cells showed survival rates as low as 54% and 45%, respectively. The numbers of changes at each microsatellite locus in both HeLa and HCT116 cells with and without X-ray irradiation are summarized in Table 2. In HeLa cells, few MC were observed in both X-ray-irradiated and non-irradiated cells. In contrast, in HCT116 cells, frequent MC were observed. Furthermore, when di- and tri-nucleotide repeat loci were combined, MC were observed in 68

![Image](image-url)

Table 2. MC induced by X-ray irradiation in HCT116 and HeLa cells

| Cell  | Dose | 0 Gy | 1:1000 | 1:100 | 1:4 | 1:1 | 4:1 | 1:0 |
|-------|------|------|--------|-------|-----|-----|-----|-----|
| HeLa  | 0 Gy | 0.95 | 0.05   | 0.88  | 0.95| 1.96| 0.92|     |
|       | 2 Gy | 0.95 | 0.05   | 0.86  | 0.89| 2.90| 0.88|     |
| HCT116| 0 Gy | 15/87| 14/84  | 11/93 | 8/96| 7/95|     |     |
|       | 2 Gy | 24/84| 20/84  | 13/84 | 11/96| 3/86|     |     |
out of 348 (19.5%) and 48 out of 360 (13.3%) clones after 2 and 0 Gy irradiation, respectively, and the difference was statistically significant (p = 0.032). These results suggest that our method is applicable for detection of genetic changes induced by radiation or other sources.

To determine the genetic effects of SMF, we analyzed both log-phase and S-phase cultures because Miyakoshi et al. reported that ELF-MF induced mutations in S-phase human cells\(^9\). We first determined the efficiency of synchronization of HCT116 cells by aphidicolin. Fig. 3 shows the ratio of S-phase cells which incorporated BUdR at various time points up to 12h after aphidicolin removal. The result shows that the HCT116 cells were well-synchronized at the G\(_1\)/S boundary after aphidicolin treatment and proceeded into the S-phase without delay when aphidicolin was removed. Since by 6 h after aphidicolin removal most of cells exited from S-phase, we decided to expose synchronized cells to SMF for 6 h.

![Fig. 3. The rate of S-phase cells after aphidicolin treatment. HCT116 cells were treated with aphidicolin for 22 h and then labeled with BUdR for 30 min at each time point after aphidicolin removal. Cells in S-phase incorporated BUdR and were detected by using the FITC-labeled anti-BUdR antibody.](image)

The colony-forming efficiencies of the cells exposed to SMF in random growing and in S-phase were 88% and 105% of non-exposed cells, respectively, suggesting that the exposure to SMF had no or very little, if any, effect on cellular viability. Numbers of MC observed after exposure to SMF were shown for each locus in Table 3. Similar to X-ray irradiation, HeLa cells showed only a few MC in both SMF-exposed and non-exposed cells. In HCT116 cells exposed to SMF frequencies of MC were varied in each locus. Although we have not any clear explanation for this variation at this time, small number of clones analyzed is the most possible. To know a general tendency, the results of di- and tri-nucleotide repeat loci were combined (Table 4). Synchronization by aphidicolin treatment induced a significant increase in MC in HCT116 cells. Since MC are mainly induced during DNA replication in MMR deficient cells\(^5\), and since aphidicolin is well known to inhibit DNA polymerase involved in the replication of mammalian...
DNA\cite{14}, some residual effects after the removal of aphidicolin might induce MC. Nevertheless, no significant change in MC frequencies was observed after exposure to SMF in either cells synchronized to S-phase or randomly growing. This shows that, in contrast to X-ray irradiation, exposure to SMF did not induce any significant amounts of MC. This is consistent with previous reports that SMF did not induce significant amounts of sister chromatid exchanges or chromosome aberrations\cite{15,16}. However, Koana et al. reported that SMF induced mitotic recombination in wing spot tests of Drosophila melanogaster\cite{1}. It is still possible that SMF exhibits any genetic effects on mammalian cells, which could be detected with a method other than used here and before. Furthermore, Miyakoshi et al. reported that exposure to strong ELFMF induced mutations of the HPRT gene specifically in S-phase cells\cite{9}. A difference in genetic effects between ELFMF and SMF may be the most plausible factor. Although both are supposed to induce no DNA damage, ELFMF can induce an electric current in and around cells exposed to it\cite{17}. In addition, there was a difference in the DNA sequences analyzed to detect genetic changes. We analyzed microsatellite repetitive sequences, whereas Miyakoshi et al. analyzed the unique HPRT

\begin{table}[h]
\centering
\caption{MC after exposure to SMF}
\begin{tabular}{lcccccc}
\hline
Cell & SMF exposure & Microsatellite locus \\
\hline
HeLa & None & D2S123 & D2S119 & D10S197 & D7S1794 & BAT-26 \\
 & Log phase 24 h & 0/34 & 0/32 & 1/37 & 0/47 & 0/47 \\
 & None & 12/96 & 8/95 & 13/89 & 8/96 & 13/93 \\
HCT116 & Log phase 24 h & 13/95 & 8/93 & 14/88 & 17/96 & 10/96 \\
 & None\textsuperscript{a} (S phase) & 21/96 & 6/90 & 19/95 & 16/79 & 15/86 \\
 & S phase 6 h & 14/96 & 12/96 & 22/94 & 13/96 & 10/96 \\
\hline
\end{tabular}
\textsuperscript{a}Cells were treated with aphidicolin for 24 h and then kept at 37\textdegree{}C for 6 h before replating for colony formation.
\end{table}

\begin{table}[h]
\centering
\caption{MC in HCT116 cells after SMF exposure: combined results with di- and trinucleotide repeat loci.}
\begin{tabular}{lcccc}
\hline
Culture condition & SMF exposure & MC \\
\hline
Log phase & None & 41/376 (10.9\%)\textsuperscript{a} \\
 & 24 h & 52/372 (14.0\%) \\
S phase & None & 62/360 (17.2\%)\textsuperscript{a} \\
 & 6 h & 61/382 (16.5\%) \\
\hline
\end{tabular}
\textsuperscript{a}Difference is statistically significant only between these two groups (p = 0.02)
gene, which might explain the difference in results.

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