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# Increase in X-Ray-Induced Mutations by Exposure to Magnetic Field (60 Hz, 5 mT) in NF-κB-Inhibited Cells

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**It is established that extremely low frequency magnetic fields (ELFMF) at the flux densities, i.e., 5 mT and less, are not mutagenic. However, exposure to ELFMF enhances mutations induced by X-rays. In this study, we examined the effects of long-term exposure to 5 mT ELFMF on mutation induction and X-ray-induced mutations in human malignant glioma cells (MO54) with different mutant IκB-α (a critical inhibitor of NF-κB) genes. Cells were exposed or sham-exposed to 5 mT ELFMF for up to 8 days with or without initial X-rays (4 Gy), and the mutant frequency of hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene was analyzed. An obvious increase in X-ray-induced mutations was observed after treatment with ELFMF in combination with X-irradiation in MO54 cells with tyrosine mutant IκB-α gene other than with serine mutant IκB-α gene or vector alone. Exposure to ELFMF alone increased mutations significantly in MO54 cells with tyrosine mutant IκB-α gene. In addition, X-ray-induced apoptoic cells were increased in MO54-V cells after exposure to ELFMF, while an anti-apoptotic effect of magnetic field was found in MO54-SY4 cells. Our data suggest that exposure to 5 mT ELFMF may induce mutations and enhance X-ray-induced mutations, resulting from the inactivation of NF-κB through the inhibition of tyrosine phosphorylation.**



*Key Words:* **extremely low frequency magnetic field (ELFMF); MO54 cells; NF-κB; hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene; mutant frequency.**

Because of the possible carcinogenic effects of extremely low frequency magnetic fields (ELFMF) on human health, ELFMF has received considerable research attention. Although many studies have been done concerning the genotoxic potential of ELFMF, conclusions are inconsistent among these reports. Recently, our research has shown that long-term exposures (from 1 to 6 weeks) to 5 mT ELFMF increase X-ray-induced 6-TG mutations significantly in HPRT gene using Chinese hamster ovary K1 (CHO-K1) cells (1). Almost at the same time, Walleczek *et al.* (2) reported the similar results using CHO cells, in which the exposure time and the magnetic-flux density were 12 h and 0.7 mT, respectively.

Several studies reported that exposure to electromagnetic fields affected the activities of PKC, PTKs and phospholipase-Cγ (3–5). It is well known that the activities of PKC and PTK play an important role in the regulation of NF-κB (6, 7). NF-κB is an important transcription factor, which mediates the expression of numerous genes involved in diverse functions such as inflammation, immune response, apoptosis, and cell proliferation. Ordinarily, NF-κB is sequestered in the cytoplasm by the inhibitory protein IκB-α. For the activation of NF-κB, two different pathways for the phosphorylation of IκB-α are demonstrated. One is serine (at residues 32 and 36) phosphorylation, which induces subsequent ubiquitin-dependent degradation of IκB-α (8–10). The other is tyrosine (at residue 42) phosphorylation, which has the potential to directly couple NF-κB to surface receptor associated tyrosine kinases, but not induces the degradation of IκB-α (7). It has been suggested that NF-κB is a crucial transcription factor for glial and neuronal cell function (11). Inhibition of NF-κB activity has also been reported to enhance the radiosensitivity of human fibrosarcoma cells (12) and human malignant glioma cells (13, 14). It has been reported that the biological state of the cells may be important for effects of ELFMF (15). We used MO54 human malignant glioma cells transfected with an expression vector that contains a gene encoding a form of IκB-α that cannot be phosphorylated. This form of IκB-α remains bound to NF-κB, thus, NF-κB cannot be activated. In the present study, MO54 cells with dif-

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ferent mutant IκB-α genes were treated with X-rays, and then exposed or sham-exposed to 5 mT ELFMF for 8 days. The effect of ELFMF with or without X-irradiation on mutant frequency of the HPRT gene was evaluated. We chose X-rays as a mutagen, since it has been shown that NF-κB is activated by X-irradiation (16).

## MATERIALS AND METHODS

*Cell culture and transfections.* MO54 cells, derived from a human malignant glioma, were kindly supplied from Dr. Rufus S. Day III (Cross Cancer Institute, Edmonton, Alberta, Canada). Cells were cultured in Dulbecco's modifiled Eagle's medium (Nikkon Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum (GIBCO, BRL) at 37°C in an atmosphere of 95% air and 5% $CO_2$. IκB-α cDNA (provided from Dr. Jun-ichiro Inoue, The institute of Medical Science, University of Tokyo, Tokyo, Japan) were used to generate mutant IκB-α genes by standard PCR mutagenesis using the appropriate primers. The methods of mutant constructions were reported peviously (13). In brief, the PCR products were subcloned into pcDNA3 vectors (Invitrogen, Leek, The Netherlands) for bacteria expression. Then the pcDNA3 plasmids containing different mutant IκB-α genes, such as S-IκB-α containing Ser-to-Arg (AGC-CGC) mutations at residues 32 and 36, Y-IκB-α containing Tyr-to-Phe (TAC-TTC) mutation at residue 42, and SY-IκB-α containing all these mutations, were introduced into human malignant glioma MO54 cells. A plasmind pcDNA3, without IκB-α cDNA, was used as a negative control. The methods of transfection were reported previously (17, 18). Approximately $10^6$ cells were transfected with 10 μg of pcDNA3-S-IκB, pcDNA3-Y-IκB, pcDNA3-SY-IκB or pcDNA3 by the electroporation with the unit operated at 800 V. The cells were then selected in normal medium containing antibiotic G418 (400–800 μg/ml, Nacalai Tesque, Inc., Kyoto, Japan), and isolated 2 or 3 weeks later. For each experiment, a new vial of frozen cells was thawed, and the cells were maintained at exponentially growing phase in 75-$cm^2$ flasks, and subcultured at 2- or 3-day intervals.

*ELFMF exposure system and X-ray irradiation.* The exposure apparatus for 5 mT ELFMF is described elsewhere (19). Environmental 60 Hz ELFMF during the sham exposure was $<0.5$ μT. Static magnetic fields other than geomagnetism were undetectable ($<0.1$ μT) for all experiments and residual geomagnetism was $<1$ μT. The atmosphere in the incubator for both units was saturated with humidified 95% air plus 5% $CO_2$. In this experiment, we used 15 ϕ annular culture dishes, in which the mean induced current intensity in the outer-ringed well was 115 mA/$m^2$ (1). The conditions of X-irradiation has been described previously (20).

*Western immunoblots.* For preparation of nuclear extracts, previously reported paper was followed (21). Protein concentration was determined using the SmertSpec 3000 system (BIORAD, Tokyo, Japan). Details of Western immunoblottings were described elsewhere (17). The antibodies used in this experiment were as follows: anti-IκB-α antibody (C-21, Santa Cruz Biotechnology), anti-NFκB/p65 antibody (C-20, Santa Cruz Biotechnology), and anti-β-actin antibody (Sigma).

*Assay for mutant frequency.* Details of the assay system for 6-TG mutant frequency are described in previous reports (22, 23). Briefly, cells cultured in the annular dishes were exposed or sham-exposed to 5 mT ELFMF for 8 days with or without initial X-irradiation (4 Gy). Cells were subcultured at 2- to 4-day intervals. Mutation assay was done by plating $2 \times 10^5$ cells in 10-cm dishes containing medium with 15 μM of 6-TG (Nacalai Tesque Inc., Kyoto, Japan). Twenty dishes were used for each mutation assay. About 100 viable cells per dish were cultured to determine the cloning efficiency. After 2 weeks, the number of colonies was scored. Each experiment was replicated at least two times. For the statistical evaluation of the mutant frequency, we used an analysis of variance (ANOVA) test for intergroup differences. When a significant $F$ value was found ($P < 0.05$), the Fisher's PLSD test was used for multiple comparison.

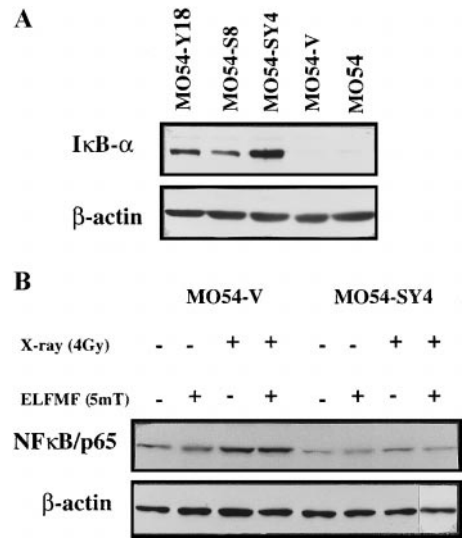


**FIG. 1.** Expression of mutant IκB-α proteins and the effects of exposure to X-rays and/or ELFMF on NF-κB nuclear translocation. (A) Expression of S-IκB, Y-IκB and SY-IκB proteins. IκB-α proteins were detected using anti-IκB-α monoclonal antibody. After removing the antibody, the same blot was reprobed with the anti-β-actin monoclonal antibody. (B) NF-κB protein levels in the nucleus of MO54-V and MO54-SY4 cells after exposure to X-rays and/or ELFMF. Cells were exposed to an initial dose of 4 Gy X-ray followed by exposure or sham-exposure to magnetic field (60 Hz, 5 mT) for 3 h. Then NF-κB protein levels were determined. Nuclear extract preparation and Western blotting analysis were performed as described under Materials and Methods. NF-κB proteins were detected using anti-NFκB/p65 monoclonal antibody. After removing the antibody, the same blot was reprobed with the anti-β-actin monoclonal antibody.

*Detection of apoptotic cells.* Cells cultured on the cover slides were exposed or sham-exposed to 5 mT ELFMF for 2 days with or without initial X-irradiation (4 Gy). Then cells were fixed in 10% formaldehyde. DeadEnd Colorimetric Apoptosis Detection System (Promega) was used to detect apoptotic cells. For the quantification of apoptotic cells, at least 500 cells were scored in randomly selected several fields.

## RESULTS

*Expression of mutant IκB-α proteins and the effects of exposure to X-rays and/or ELFMF on NF-κB nuclear translocation.* The expression of IκB-α in the parental MO54 cells and in the S-IκB (MO54-S8)-, Y-IκB (MO54-Y18)-, SY-IκB (MO54-SY4)-introduced clones are shown in Fig. 1A. The clones, MO54-S8, MO54-Y18 and MO54-SY4, were overexpressing S-IκB, Y-IκB, and SY-IκB proteins, respectively, in which those proteins were non-phosphorylated type. NF-κB protein levels in the nucleus of MO54-V and MO54-SY4 cells after exposures to X-rays and/or ELFMF are shown in Fig. 1B. Exposure to ELFMF alone for 3 h had no effect on NF-κB nuclear translocation in both MO54-V and

**TABLE 1**

Mutagenicity Data in MO54 Cells (with Different Mutant IκB-α Genes) Treated with ELFMF, X-Rays, and X-Rays + ELFMF

| Cell line[a] | Treatment | 6-TG$^r$ colonies observed/20 dishes | Cloning efficiency at the time of selection (%) | Mean number of mutants per $10^6$ surviving cells |
|---|---|---|---|---|
| MO54 | sham | 26 | 69.1 | 9.41 |
| | 5mT | 16 | 71.1 | 5.63 |
| | 4Gy | 44 | 38.1 | 28.87 |
| | 4Gy + 5mT | 23 | 42.0 | 13.69**[b] |
| MO54-V | sham | 24 | 88.7 | 6.76 |
| | 5mT | 12 | 56.1 | 5.35 |
| | 4Gy | 73 | 37.2 | 48.72 |
| | 4Gy + 5mT | 56 | 40.1 | 34.91**[b] |
| MO54-Y18 | sham | 39 | 83.3 | 11.70❖❖[c] |
| | 5mT | 49 | 66.6 | 18.39◇[d] |
| | 4Gy | 47 | 36.8 | 31.92 |
| | 4Gy + 5mT | 82 | 46.2 | 44.37*[b] |
| MO54-S8 | sham | 1 | 35.2 | 0.71❖[c] |
| | 5mT | 1 | 40.2 | 0.62 |
| | 4Gy | 4 | 20.0 | 5.00 |
| | 4Gy + 5mT | 5 | 33.2 | 3.77**[b] |
| MO54-SY4 | sham | 3 | 40.2 | 1.87❖[c] |
| | 5mT | 2 | 34.0 | 1.47 |
| | 4Gy | 11 | 18.8 | 14.63 |
| | 4Gy + 5mT | 95 | 36.0 | 62.5**[b] |

[a] MO54-V, MO54 cells transfected with vector alone; MO54-Y18, MO54 cells transfected with tyrosine mutant IκB-α gene; MO54-S8, MO54 cells transfected with serine mutant IκB-α gene; MO54-SY4, MO54 cells transfected with both serine and tyrosine mutant IκB-α genes.

[b] $*P < 0.05$ $**P < 0.01$, compared with 4Gy cells in respective group.

[c] ❖$P < 0.05$ ❖❖$P < 0.01$, compared with MO54-V sham cells.

[d] ◇$P < 0.05$, compared with MO54-Y18 sham cells.

MO54-SY4 cells. However, nuclear NF-κB protein level was increased 3 h later after 4 Gy X-irradiation, and exposure to ELFMF did not alter the extent of X-ray-induced NF-κB nuclear translocation in MO54-V cells. NF-κB nuclear translocation was not affected by exposure to X-rays and/or ELFMF in MO54-SY4 cells.

*Effect of magnetic field on mutation induction.* The mutagenicity data in MO54 cells and cells with different mutant IκB-α genes treated with ELFMF, X-rays and X-rays + ELFMF were summarized in Table 1. For the frequency of spontaneous HPRT mutation, there was no obvious difference between parental MO54 cells and cells with vector alone (MO54-V). However, it varied among the MO54 cells with different mutant IκB-α genes. Spontaneous mutations were decreased in MO54-S8 and MO54-SY4 ($P < 0.05$) but increased in MO54-Y18 cells ($P < 0.01$) compared with MO54-V cells. Exposure to 5 mT magnetic field increased HPRT mutations significantly in MO54-Y18 cells ($P < 0.05$); nevertheless, there was no obvious effect of the magnetic field on mutations in other cells.

*Effect of magnetic field on X-ray-induced HPRT mutant frequency.* The X-ray-induced mutations decreased significantly ($P < 0.01$) in MO54 and MO54-V cells after exposure to ELFMF (Table 1). The ability of the magnetic field to alleviate the X-ray-induced mutations in MO54 cells and MO54-V was found ($P < 0.01$). On the contrary, exposure to the ELFMF enhanced X-ray-induced mutations significantly in MO54-Y18 cells ($P < 0.05$), and particularly in MO54-SY4 cells ($P < 0.01$) (Table 1). The mutant frequency ratio T/S (Treatment/Sham) is shown in Fig. 2.

*Effect of magnetic field on X-ray-induced apoptosis.* Exposure to ELFMF followed by X-irradiation altered the extent of apoptosis in MO54-V and MO54-SY4 cells. X-ray-induced apoptoic cells were increased in MO54-V cells after exposure to ELFMF for 2 days, while an anti-apoptotic effect of magnetic field was found in MO54-SY4 cells (Fig. 3).

## DISCUSSION

IκB-α is a strong inhibitor of NF-κB among IκB family members such as IκB-α, IκB-β, and IκB-ε (24, 25). In this study, we obtained human MO54 cell lines expressing dominant-negative IκB-α phosphorylation site mutants (Fig. 1A). Studies in several laboratories have shown that mutations in specific phosphorylation sites of IκB-α can inhibit the signal-dependent activation of NF-κB to a variety of stimuli (8–10). The nuclear fraction of NF-κB was not affected in MO54-SY4 cells, but increased obviously in MO54-V cells after

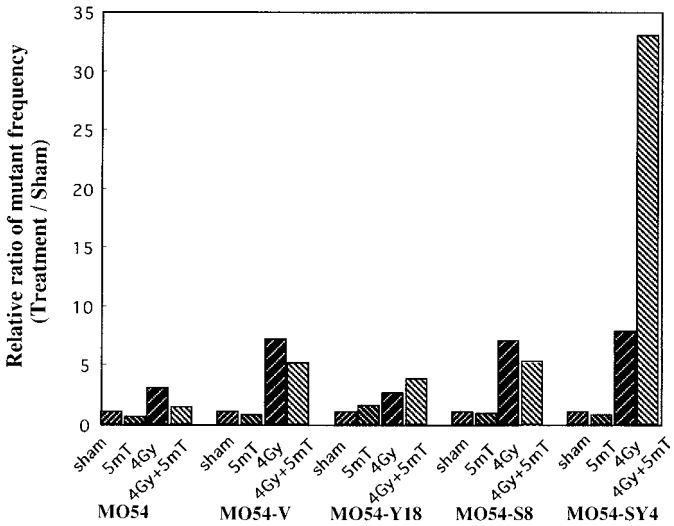


**FIG. 2.** Mutations induced in MO54 cells (with different mutant IκB-α genes) exposed to ELFMF, X-rays, or X-rays + ELFMF. Approximately $10^6$ cells cultured in an out-ringed well of 15-cm $\phi$ annular dishes were irradiated with X-rays (4 Gy), then exposed to ELFMF (60 Hz, 5 mT) for 8 days. Twenty plates were used for each mutation assay, which was replicated at least two times. MO54-V, MO54 cells with vector alone; MO54-Y18, MO54 cells with tyrosine mutant IκB-α gene; MO54-S8, MO54 cells with serine mutant IκB-α gene; MO54-SY4, MO54 cells with both serine and tyrosine mutant IκB-α genes.

irradiation (Fig. 1B). These results suggest that overexpression of IκB-α mutant gene in MO54 cells could prevent NF-κB from translocation to nucleus and activation by X-ray irradiation.

Previously we found that exposure to ELFMF (5 mT, 60 Hz) could not affect cell growth and gene expression (19). Miller *et al.* (26) also reported that exposure to ELFMF alone (0.08, 0.1, 1.0, or 1.3 mT) had no effect on the NF-κB signaling pathway in the human promonocytic U937 leukemia cell line. These results are consistent with our data that the nuclear amount of NF-κB was not altered after exposure to ELFMF (60 Hz, 5 mT) for 3 h in MO54-V and MO54-SY4 cells (Fig. 1B). In addition, X-ray-induced NF-κB nuclear translocation also did not change in both clones after ELFMF exposure. Therefore, the exposure to ELFMF may have no or very little effect on X-ray-induced NF-κB translocation.

ELFMF at the flux densities, i.e., 5 mT and less, may not be mutagenic (1, 27), which is consistent with the present results that exposure to ELFMF (5 mT, 60 Hz) did not affect the spontaneous mutations in MO54 parental cells (Table 1). However, exposure to ELFMF decreased X-ray-induced mutations significantly in MO54 and MO54-V cells (Table 1), although ELFMF seemed to have no effect on X-ray-induced NF-κB activation. The mutant frequencies among NF-κB-inhibited cells are different by exposure to X-rays and/or ELFMF (Fig. 1B and Table 1). These results clearly demonstrated that exposure to 5 mT ELFMF increased HPRT mutations significantly in MO54-Y18 cells ($P < 0.05$) and the ability of the ELFMF to alleviate the X-ray-induced mutations in MO54 and MO54-V cells was found ($P < 0.01$). On the contrary, exposure to the ELFMF enhanced X-ray-induced mutations significantly in MO54-Y18 cells ($P < 0.05$) and particularly in MO54-SY4 cells ($P < 0.01$) (Table 1 and Fig. 2). These results suggest that although ELFMF at 5 mT could not be mutagenic per se it may modify the action of known mutagens. The present data indicate that different responses of MO54 cells to ELFMF in mutation induction may be attribute to the different statuses of NF-κB. For example, MO54 cells with IκB-α tyrosine mutant gene are more sensitive to ELFMF. It should be noted that there were obvious differences in spontaneous HPRT mutations among NF-κB-inhibited cells. Spontaneous HPRT mutation was evidently increased in MO54-Y18 cells but decreased in MO54-S8 and MO54-SY4 cells compared with MO54-V cells. Probably such difference is related to the introduction of tyrosine and/or serine mutant IκB-α genes.

In order to explore the mechanism that why X-ray-induced mutations were different among various IκB-α mutant gene clones after exposure to magnetic field, effects of exposure to ELFMF with or without X-irradiation on apoptosis were examined in this study. Apoptosis is a cell-intrinsic mechanism that

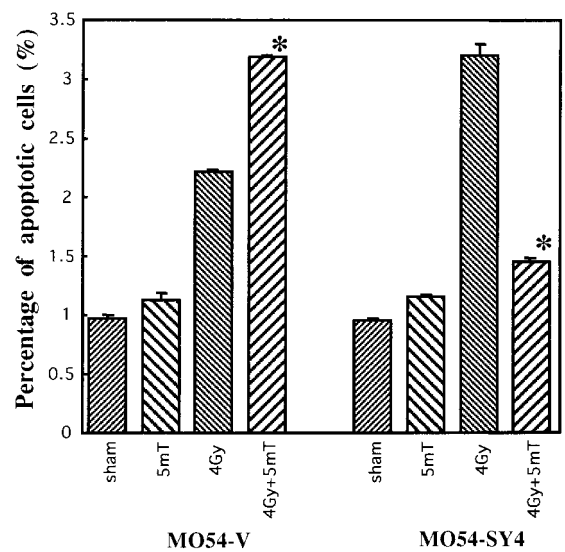


**FIG. 3.** Quantification of apoptosis after exposure or sham-exposure to magnetic field (60 Hz, 5 mT) for 48 h with or without X-irradiation (4 Gy). Induction of apoptosis is shown as a percentage of the attached cell population. MO54-V, MO54 cells with vector alone; MO54-SY4, MO54 cells with both serine and tyrosine mutant IκB-α genes. Each column represents the mean value with SD. * $P <$ 0.05 compared with X-irradiation (4 Gy) alone in respective group.

leads healthy cells to choose self-elimination. In some conditions, it might free the organism from retention of potentially mutated or transformed cells through apoptosis. If the cell's ability to respond to mild DNA damage by apoptosis is impaired, it will result in a higher rate of survival of the damaged cells and then increase mutation (28). It was reported that dexamethasone-induced apoptosis but not spontaneous apoptosis was substantially increased in thymocytes from 0.4 to 1.0 mT (60 Hz) magnetic field-exposed animals (29). However, it is also suggested that magnetic field increases cell survival by inhibiting apoptosis via modulation of $Ca^{2+}$ influx in some cell lines, for example, U937 and CEM cell lines (30). Therefore, different human cell types maybe respond differently to ELFMF. In this study, X-ray-induced apoptoic cells were increased in MO54-V cells after exposure to magnetic field for 2 days (Fig. 3), which is consistent with our mutation data that X-ray-induced mutations were decreased in MO54 and MO54-V cells after exposure to ELFMF (Table 1).

Recently, it has been reported that radiation-induced NF-$\kappa$B activation proceeds in the absence of I$\kappa$B-$\alpha$ degradation and requires tyrosine phosphorylation (31). In addition, it is supposed that activation of NF-$\kappa$B plays a key role in the anti-apoptotic pathway of radiation-induced apoptosis (32). It is consistent with our results that there were more apoptotic cells in MO54-SY4 cells than in MO54-V cells after X-ray irradiation (Fig. 3), therefore, one can understand that X-ray-induced mutations in MO54-Y18 cells were lower than those in MO54-V cells. However, it is notable that exposure to ELFMF increased X-ray-induced mutations significantly in MO54-Y18 and MO54-SY4 cells, while decreased those mutations in MO54 cells and MO54–V cells (Table 1). In addition, X-ray-induced apoptoic cells were increased in MO54-V cells after exposure to ELFMF for 2 days, while an anti-apoptotic effect of magnetic field was found in MO54-SY4 cells (Fig. 3). Therefore, we suppose such reverse response of cells to ELFMF may be resulting from the different status of NF-$\kappa$B after X-irradiation between MO54-V cells (activated NF-$\kappa$B) and MO54-SY4 cells (inactivated NF-$\kappa$B). Increase in X-ray-induced apoptosis by exposure to ELFMF may be due to the activation of NF-$\kappa$B.

Exposure to ELFMF increased HPRT mutations in MO54-Y18 cells ($P < 0.05$), but not in MO54-SY4 cells (Table 1). It may be related to serine mutant I$\kappa$B-$\alpha$ gene since spontaneous mutations are obviously decreased in MO54-S8 and MO54-SY4 cells. Additionally, we observed very clear increase in X-ray-induced mutations by exposure to ELFMF in MO54-SY4 cells (Fig. 2). Our data suggest that exposure to 5 mT ELFMF may enhance spontaneous and X-ray-induced mutations, resulting from the inactivation of NF-$\kappa$B through inhibiting tyrosine phosphorylation.

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