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Microsatellite analysis for determination of the mutagenicity of extremely low-frequency electromagnetic fields and ionising radiation *in vitro*

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Abstract

Extremely low-frequency electromagnetic fields (ELF-EMF) have been reported to induce lesions in DNA and to enhance the mutagenicity of ionising radiation. However, the significance of these findings is uncertain because the determination of the carcinogenic potential of EMFs has largely been based on investigations of large chromosomal aberrations.

Using a more sensitive method of detecting DNA damage involving microsatellite sequences, we observed that exposure of UVW human glioma cells to ELF-EMF alone at a field strength of 1 mT (50 Hz) for 12 h gave rise to 0.011 mutations/locus/cell. This was equivalent to a 3.75-fold increase in mutation induction compared with unexposed controls. Furthermore, ELF-EMF increased the mutagenic capacity of 0.3 and 3 Gy γ-irradiation by factors of 2.6 and 2.75, respectively. These results suggest not only that ELF-EMF is mutagenic as a single agent but also that it can potentiate the mutagenicity of ionising radiation.

Treatment with 0.3 Gy induced more than 10 times more mutations per unit dose than irradiation with 3 Gy, indicating hypermutability at low dose.
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Keywords: ELF-EMF; Gamma radiation; Microsatellite mutations

1. Introduction

Most people are exposed to extremely low-frequency electromagnetic fields (ELF-EMF) produced by power lines and electrical appliances. While some epidemiological studies have related exposure to ELF-EMF to an increased risk of certain types of adult and childhood cancer including leukaemia, cancer of the central ner-

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vous system and lymphoma [1–4], others have failed to find such an association [5–8]. However, interpretation of these results is difficult because unreliable methods of assessment of exposure were employed and contact with other mutagens, such as cigarette smoke, was not taken into account.

In vitro studies with well-defined exposure conditions and end points may provide a more reliable means of estimating the possible carcinogenic potential of ELF-EMF than investigations based upon cancer risk estimates in human populations. Many such studies have been conducted, but contradictory results regarding the genotoxic potential of ELF-EMF, at exposure levels ranging from 1 μT to 10 mT, have been reported [9,10] using end points such as chromosomal abnormalities, micronucleus formation and DNA strand breaks. In general, the conclusion from these studies has been that EMF exposure alone is not genotoxic [11,12]. However, these methods cannot reveal molecular alterations, which may be subtle—perhaps involving modifications to one or a few nucleotide bases. Therefore, it is not surprising that the majority of such studies did not show ELF-EMF-related genotoxic effects. Nonetheless, genetic aberrations have been demonstrated in some studies [13–18] after exposure ELF-EMF according to various delivery schedules.

Vijayalaxmi and Obe [10] examined all reports between 1990 and 2003 of ELF-EMF-induced genotoxicity and concluded that 46% of studies did not identify increased genetic damage; 22% of such investigations did indicate a genotoxic effect of EMF exposure and 32% of the studies were inconclusive. The International Agency for Research on Cancer (IARC) has recently classified ELF-EMF as possibly carcinogenic—a categorization that necessarily implies that weak ELF electromagnetic fields may promote DNA damage and hence may be genotoxic [19].

It has also been reported that EMF exposure in combination with tumour-promoting agents can increase tumour incidence in animals [20]. Moreover, exposure of cultured melanoma cells to ELF-EMF alone (400 mT at 50 Hz) caused mutations at the hypoxanthine phosphoribosyl transferase (*HPRT*) locus and EMF enhanced the mutation rate induced by X-irradiation [21,22]. Although exposure to a 60-Hz, 0.7-mT magnetic field (about 560-fold lower flux density) enhanced the mutagenicity of ionising radiation to the *HPRT* gene, the treatment was not mutagenic at this locus in the absence of ionising radiation [23]. On the other hand, most investigations have found no evidence of enhanced mutagenicity of ionising radiation by EMF [24]. Again, the significance of these findings is uncertain, because the determination of carcinogenic potential has largely been based on investigations that cannot reveal subtle molecular alterations.

We propose that part of the reason for the controversy associated with the mutagenicity of ELF-EMF is that the techniques used, while capable of identifying extensive and severe cellular DNA damage, are not sensitive enough to detect small-scale but potentially harmful genetic damage. Therefore, we have developed a more sensitive method of detecting DNA damage involving examination of microsatellite DNA sequences.

Microsatellites are non-coding DNA sequences distributed throughout the genome. They comprise variable numbers of short repeats of one to five base pair units. The role of microsatellites is unclear. While they may exert subtle influences on the regulation of gene expression, most human microsatellites probably have no biological role [reviewed in 25,26]. Higher spontaneous mutation frequencies in normal tissues have been reported in microsatellite sequences [27] and mutations occur 100 times more often in microsatellites than in coding genes [28]. Several studies have shown that such repeat sequences are hypermutable compared with the *HPRT* locus [29] and are hypermutable by radiation in somatic [30,31] and germ-line cells [32–36].

We have found the utilisation of microsatellite sequences for determination of radiation-induced mutations to be at least 1000 times more sensitive than analysis of coding genes such as the *HPRT* gene—the current gold standard method for detection of DNA mutation [31]. We now report on the refinement of this methodology using an automated, non-radioactive and high-throughput system for detecting and analysing DNA fragments after exposure of cultured cells to EMF, ionising radiation or a combination of the two.

2. Materials and methods

2.1. Cells and γ-irradiation procedure

Analyses were conducted using the glioma cell line UVW (passage 7), which has been established and characterised in our laboratory in terms of growth properties and sensitivity to low-dose γ-irradiation [31]. The doubling time of this cell line is approximately 18 h. The cells were cultured in Eagle's minimum essential medium supplemented with 10% foetal bovine serum and 2 mM glutamine, at 37 °C in an atmosphere of 5% CO_2. Media and supplements were obtained from Invitrogen, UK. Plastic-ware was obtained from McQuilken, UK.

Cells were seeded into T25 flasks and grown until they were 70% confluent (in exponential growth phase). They were treated with 0, 0.3 or 3 Gy using a ^{60}Co source at a dose-rate of 0.3 Gy/min, and transferred immediately to an EMF generator.

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The EMF irradiator comprised two identical solenoids, each 300 mm long by 120 mm diameter. Each coil was constructed from 400 turns of 1.5-mm diameter copper wire, with an additional 52 turns of wire at either end that could be energised independently from the main coil. These 'shim' coils were used to improve the field uniformity within the sample region, and it was possible to achieve a measured variation in field strength of less than 1% within a 200 mm by 100 mm diameter cylindrical volume. Each coil was housed within its own double-skinned mu-metal enclosure, supported horizontally in a temperature-controlled Hereaus CO_2 incubator [37]. The field attenuation between coils was >2000-fold. Additional temperature stabilisation was provided by pumping water from a reservoir (within the incubator) through tubing wound around each coil. Six thermocouples sited within each solenoid, and within the incubator were used to monitor the temperature. The coils were independently energised, using two 50-Hz ac power-supplies. These were computer-controlled and a versatile computer package was developed (using 'Labview') to control and log each experiment. The software allowed 50-Hz ac fields to be generated in either one or both of the coils.

Six T25 flasks were placed inside each coil. One coil was energised and the other used as a sham control under blinded conditions with both coils maintained at 36.5 °C. Cells in the exposed coil were subjected to a field strength of 1 mT (50 Hz) for 12 h. Field strength and temperature in both coils were monitored throughout the procedure by computer. The following treatments were applied: 0 Gy external beam irradiation ± 1 mT (50 Hz) EMF irradiation; 0.3 Gy external beam irradiation ± 1 mT (50 Hz) EMF irradiation; and 3 Gy external beam irradiation ± 1 mT (50 Hz) EMF irradiation. Subsequent manipulations were conducted by technical staff who were blinded to the irradiation treatments.

2.2. Post-irradiation procedure

Immediately after irradiation, the cell density was adjusted to enable the deposition, on average, of one cell per well of 96-well culture dishes. This procedure was adopted to ensure that cells were separated as soon as possible after treatment, before they started to divide, so that each clone represented one irradiated cell. Microscopic examination was performed after 3–5 days. Wells containing more than one colony (hence derived from more than one cell) were discarded.

The cells were incubated at 37 °C and 5% CO_2 for approximately 2 weeks. Each colony was transferred to a single well of a six-well plate and again cultivated for 2 weeks. Once confluent, these cells were transferred to T25 flasks and grown for approximately 10 days, yielding enough cells to produce sufficient DNA for mutation analysis. DNA was then extracted from each colony of cells [38], yielding a DNA sample derived from a single irradiated cell. One hundred clones from each treatment group were analysed for the presence of mutations.

2.3. Fluorescent PCR

PCR reactions were carried out in a final volume of 12.5 μl consisting of 10 ng DNA, 0.3 μM forward primer, 0.3 μM reverse primer, 2.5 mM $MgCl_2$, 200 μM dNTPs (Larova Biochemie GmbH, Germany), 1.25 μl NH_4-based reaction buffer (BioTaq, Bioline, London, UK) and 2 U of Taq polymerase (BioTaq, Bioline, London, UK). The forward primer was conjugated at its 5′ end to one of three fluorescent dyes, enabling automated fragment analysis, and purified by HPLC (Proligo, Paris). Sequences were amplified in 30 cycles at 94 °C for 30 s, at 60 °C for 30 s, at 72 °C for 30 s and at 72 °C for 20 min using a DNA Engine Peltier Thermal Cycler (GRI, Essex, UK). Thirteen different microsatellite loci were analysed. These are distributed randomly throughout the genome. The loci analysed and the corresponding primers are shown in Table 1.

2.4. Analysis of fluorescent DNA fragments

PCR reactions incorporating all three dyes were analysed simultaneously by adding 0.5 μl of each PCR product to the same well of a 96-well plate with 0.2 μl DNA size standards

Table 1
Loci with corresponding PCR primers used for determination of mutations

Locus	Forward primer sequence	Reverse primer sequence
D1S199	AGCCATGATCATGACACTACA	GACCATGTGCTCCGTAAATA
D4S414	TTGCACAAAGCATCAGCC	TCAGGAACCTCAGCCCAT
D5S2088	AGCTGGAATCTGTGTGTAACA	GCTGACCTTTGCTGTGTG
D7S500	CCAGAATTGAAAACTCAGCA	ATTGATTGAGGAACTGAACTTACCT
D9S257	ACAGGTAAATACATTCTACCCTACA	GTTTGAAGTGCTCTCCAGTG
D10S1765	ACACTTACATAGTGCTTTCTGCG	CAGCCTCCCAAAGTTGC
D12S89	ATTTGAGAGCAGCGTGTTTT	CCATTATGGGGAGTAGGGGT
D12S98	GGAAGGGAGATGAAACACACAC	GTTGGTGACACCTAAATCACA
D13S1323	AGGCCCCCTGGAGAATGTT	TGCCACAGGTTTAAGCAGTAAAGAC
D17S791	GTTTTCTCCAGTTATTCCCC	GCTCGTCCTTTGGAAGAGTT
D18S61	ATTTCTAAGAGGACTCCCAAACT	ATATTTTGAAACTCAGGAGCAT
D20S186	GCACAAAGCAGACAGGAATA	CCAAGTTTGCAAGTAAGTAAGG
D22S683	AACAAAACAAAACAAAACAAACA	GGTGGAAATGCCTCATGTAG

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(Beckman Coulter, UK) and 40 μl deionised formamide. Samples were overlaid with a drop of mineral oil. The remainder of the procedure, which was automated, was carried out in a Beckman Coulter CEQ 8000 apparatus. Samples were separated by capillary gel electrophoresis followed by detection of fluorescent fragments by excitation with a diode laser.

Fragment analysis was then carried out with the CEQ 8000 FRAG-3 software. This allowed the calculation of mean peak-height ratio for the alleles at the selected loci. Mutations were classified as changes in allele size, loss of heterozygosity (LOH), or allelic imbalance (AI). LOH was scored in samples that exhibited a 99% decrease in peak-height ratio. Allelic imbalance (AI) was assigned to the loci that had undergone a 50% increase or decrease in allelic ratio compared with the mean value [39,40] and alterations in allele size were identified as additional peaks in chromatograms. Potential mutations identified in this way were confirmed or refuted by replicate analyses starting at the PCR reaction stage of the process.

2.5. Statistical analysis

Mutation rates were compared between treatment groups using Fisher's exact test. Confidence intervals for mutation rates were calculated using an exact Poisson method [41].

3. Results and discussion

Three types of mutation were identified: change in allele size, LOH and AI. Alterations in size of alleles are due to acquisition or loss of repeat units. LOH is the result of a deletion in one cell at the time of irradiation, whereas, AI is caused by allelic loss occurring a few or several cells divisions after the radiation insult, as a consequence of genomic instability. In the case of AI, a proportion of cells from which DNA was extracted still harbour the normal allele and a proportion have the allele deleted. This is observed as a decrease in the quantity of one allele. Examples of mutations identified in clones derived from irradiated cells are shown in Fig. 1. Total mutations, comprising altered allelic size, AI and LOH resulting from the various radiation treatments are presented in Table 2. All types of mutation were observed in every treatment group.

Using autoradiography of radiolabelled PCR products separated on denaturing polyacrylamide gels, we previously reported a microsatellite mutation rate, following γ-irrradiation of UVW cells, of 8.7×10^{-4} mutations/locus/Gy/cell [31]. In the present study, employing fluor-tagging of amplified sequences and capillary gel electrophoresis, we observed 3.0×10^{-3} mutations/locus/Gy/cell after a 3-Gy treatment, suggesting that the automated method enabled the detection of 3.4 times more mutations than the manual method. This apparent heightened sensitivity may be due to our ability to score with confidence AI mutations revealed by fluorescent fragment analysis. Such genetic aberrations could not be identified unambiguously on autoradiograms of electrophoresed microsatellite sequences.

In the absence of ionising radiation, cells exposed to 1 mT ELF-EMF alone experienced a greater rate of mutation induction, by a factor of 3.75 ($P = 0.019$), than cells that were sham-exposed. This was unexpected because the majority of previous observations of genotoxicity by ELF-EMF occurred when ELF-EMF was applied either at much higher doses (10–400 mT) or in tandem with other genotoxic or mutagenic agents such as γ-irradiation [10]. This also suggests that this phenomenon may not have been revealed previously utilising less sensitive methodology.

A second aspect of the study was the examination of the effect of the combination of ELF-EMF with the well-characterised mutagen γ-radiation. This allowed comparison with previous findings suggesting that EMF alone is not genotoxic but acts as a promoter of DNA damage by ionising radiation [42–44]. Our results indicate that ELF-EMF increased the mutagenic capacity of 0.3 and 3 Gy γ-irradiation by factors of 2.62 ($P = 0.002$) and 2.75 ($P = 0.002$), respectively (Table 2). Therefore, these results suggest not only that EMF may increase the DNA damage induced by ionising radiation but also that EMF is mutagenic as a single agent.

An important finding was that, in the absence of EMF, 0.3 Gy was 11.0 times more efficient than 3 Gy, per unit dose, in inducing mutations. When applied in combination with 1 mT EMF, 0.3 Gy was 10.2 times more mutagenic than 3 Gy. We have previously reported that UVW cells, in common with most tumour-derived cell lines, showed evidence of increased sensitivity (measured as clonogenic survival) per unit dose to radiation in the range 5–50 cGy [45]. This feature, known as low-dose hyper-radiosensitivity (HRS) [46], may be exploited to enhance the efficacy of radiotherapy of resistant tumours by ultra-fractionation—the administration of multiple fractions of <0.5 Gy [47]. However, it is not known whether HRS could also increase the severity of adverse effects on normal tissues exposed to low-dose radiation.

HRS may be a manifestation of incomplete activation of repair mechanisms in response to low radiation dosage [48]. Failure to restore damaged DNA, associated with inefficiency of cell kill at low dose, suggests that a consequence could be increased frequency of mutations. If our finding that 0.3 Gy treatment induced more mutations per unit dose than 3 Gy, were to be corroborated in other models that also display low-dose HRS, the use

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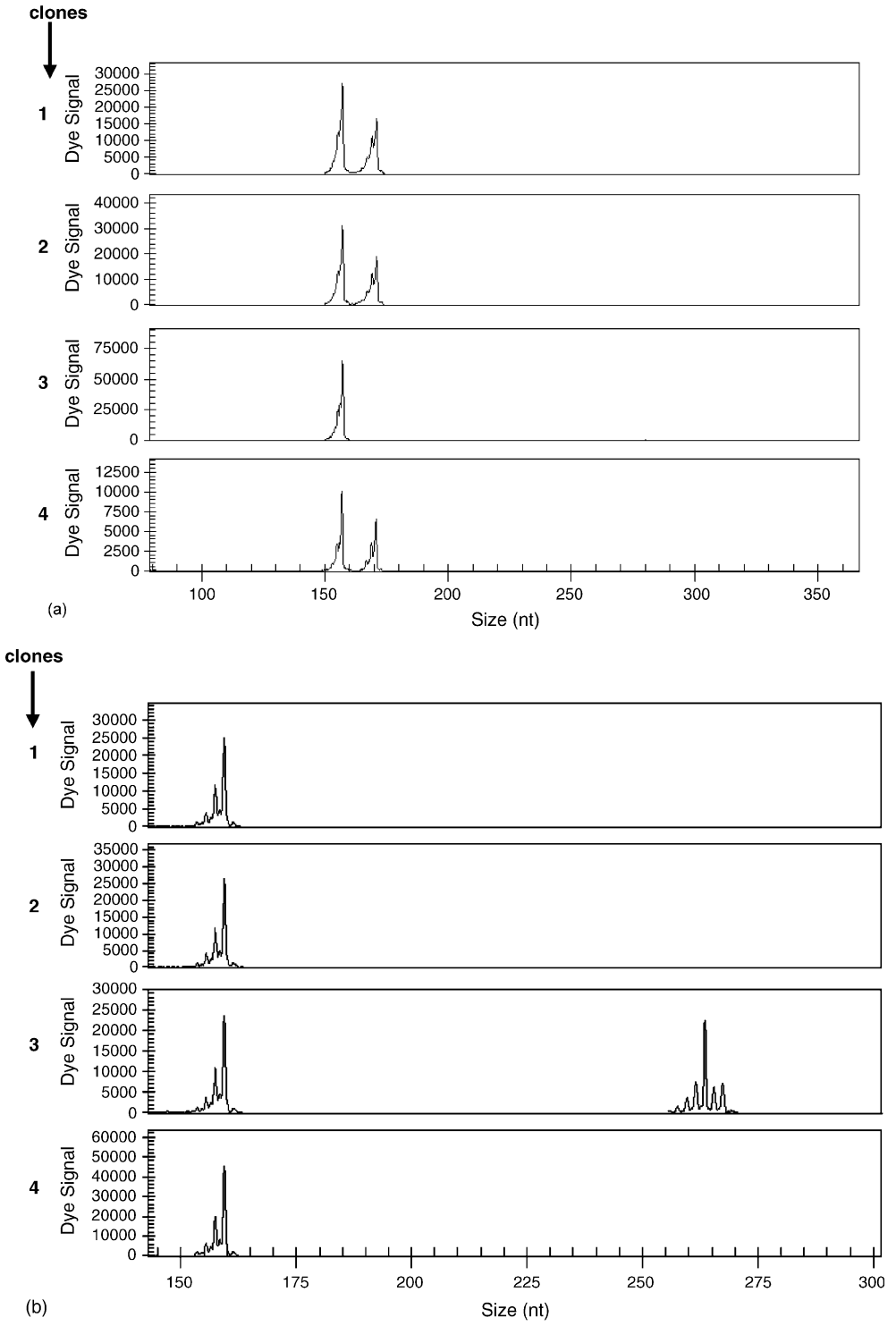

Fig. 1. Microsatellite mutation detection by electrophoresis of fluorescently labelled PCR products. The chromatograms depict abundance of product (fluorescence intensity) against size (nucleotides) of alleles at loci D18S61 (a), D5S2088 (b) and D7S500 (c) for four of the 100 clones examined per locus. (a) Loss of heterozygosity: at this locus, clones 1, 2, and 4 are heterozygous. Clone 3 exhibits loss of one allele. (b) Change in allele size: at this locus, clones 1, 2, and 4 are homozygous. Clone 3 has acquired a larger allele, in addition to the normal allele. (c) Allelic imbalance: in clone 3, some of the cells in the population have lost one allele. This is manifest by the reduced height of one allelic peak.

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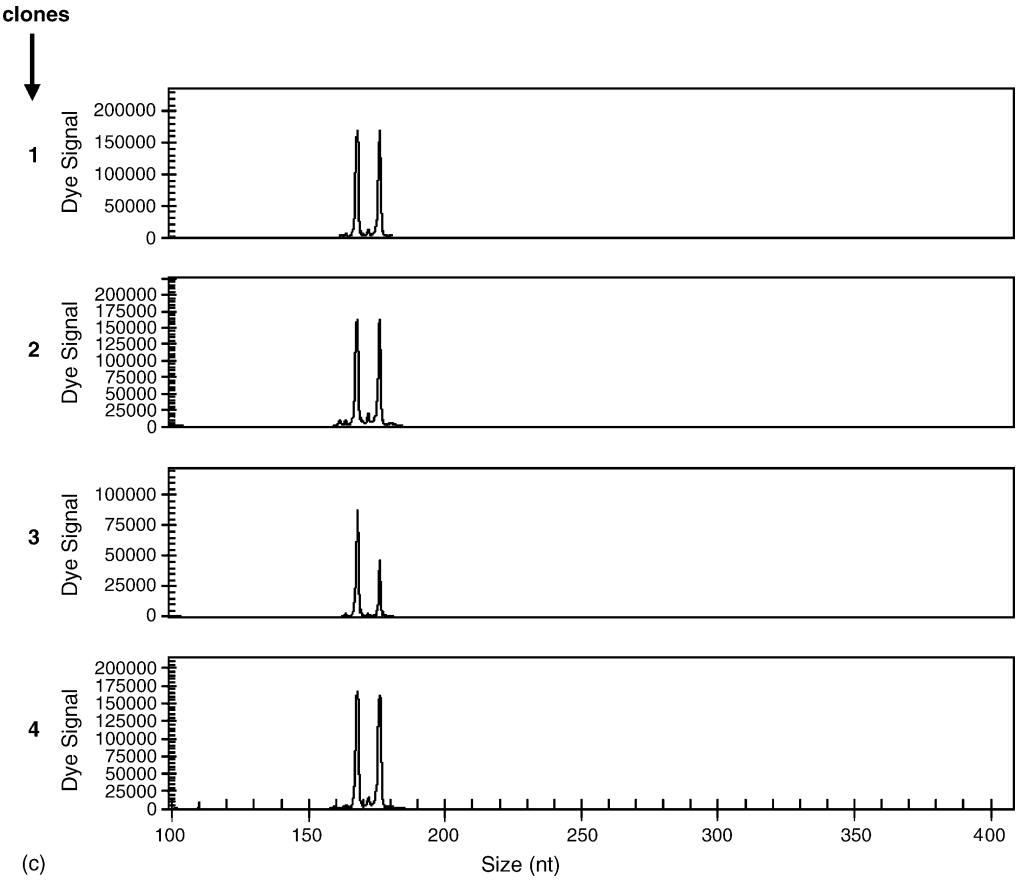

Fig. 1. (*Continued*).

of ultra-fractionated radiotherapy, designed to exploit HRS at sub-Gy doses of radiation, would be contraindicated. The increased frequency of mutations produced by combining EMF with γ-radiation treatment suggests that EMF exposure may inhibit the repair of DNA lesions.

We have demonstrated in this and previous studies, that microsatellite loci are suitable for use as reporter sequences for DNA-damage induction. Instability of microsatellite and minisatellite sequences are associated with at least 35 human diseases, including tumour-associated microsatellite instability and neurological disorders including fragile X syndrome, myotonic dystrophy and Huntington's disease [49]. Whether the changes in repetitive sequences of DNA demonstrated in this

Table 2
Frequency of mutations at 1300 microsatellite loci (13 loci/cell × 100 cells) in each of six treatment groups exposed to gamma and/or EMF radiation

Gamma	EMF	No. of mutations				Mutation rate[a] (/locus/cell × 10^{-3})	Ratio (EMF exposed/not exposed)
		AI	LOH	Δ size	Total		
0 Gy	0 mT	1	2	1	4	3.1 (0.9–7.3)	3.75
	1 mT	7	7	1	15	11.5 (6.6–18.4)	
0 3 Gy	0 mT	6	2	5	13	10.0 (5.5–16.7)	2.62
	1 mT	24	6	4	34	26.2 (18.1–36.5)	
3 Gy	0 mT	5	3	4	12	9.2 (4.9–15.5)	2.75
	1 mT	18	8	7	33	25.4 (17.5–35.6)	

AI: allelic imbalance; LOH: loss of heterozygosity; Δ size: shorter or longer alleles, suggestive of alteration of repeat number.
[a] 95% confidence intervals are shown in parentheses.

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study reflect changes in coding sequences and whether there is a physiological consequence of these alterations (e.g. cancer induction or promotion) are topics of intense debate, which require further study [26].

4. Conclusions

The treatment of cells with ELF-EMF of field strength 1 mT, in the absence of ionising radiation, induced more mutations than were observed in untreated controls. It is necessary to determine the mutation rate in response to lower magnetic flux density (at the μT level) to assess the significance of the ELF-EMF exposure that is of concern to most of the population.

Exposure of cells to both γ-radiation and ELF-EMF radiation resulted in an increased mutation frequency compared with the effect of ionising radiation alone. These results suggest not only that ELF-EMF is mutagenic as a single agent but also that it can potentiate the mutagenicity of ionising radiation.

Finally, the observation of higher mutation rate per unit dose at 0.3 Gy compared to 3 Gy indicates that further study of the potential adverse effects of low-dose ionising radiation is warranted.

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