

SHORT REPORT

Biallelic mutation of *MSH2* in primary human cells is associated with sensitivity to irradiation and altered RAD51 foci kinetics

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Background: Reports of differential mutagen sensitivity conferred by a defect in the mismatch repair (MMR) pathway are inconsistent in their conclusions. Previous studies have investigated cells established from immortalised human colorectal tumour lines or cells from animal models.

Methods: We examined primary human *MSH2*-deficient neonatal cells, bearing a biallelic truncating mutation in *MSH2*, for viability and chromosomal damage after exposure to DNA-damaging agents.

Results: *MSH2*-deficient cells exhibit no response to interstrand DNA cross-linking agents but do show reduced viability in response to irradiation. They also show increased chromosome damage and exhibit altered RAD51 foci kinetics after irradiation exposure, indicating defective homologous recombinational repair.

Discussion: The cellular features and sensitivity of *MSH2*-deficient primary human cells are broadly in agreement with observations of primary murine cells lacking the same gene. The data therefore support the view that the murine model recapitulates early features of MMR deficiency in humans, and implies that the variable data reported for MMR-deficient immortalised human cells may be due to further genetic or epigenetic lesions. We suggest caution in the use of radiotherapy for treatment of malignancies in individuals with functional loss of *MSH2*.

The DNA mismatch repair (MMR) system reduces spontaneous mutations by correcting the misinsertion errors of replicative DNA polymerases. In addition to the recognition and processing of replication errors, MMR proteins have been implicated in the response to several other forms of DNA damage (reviewed by Stojic *et al*¹). However, evidence of sensitivity to various DNA damaging agents is contradictory.^{2–9} Frequently cited explanations for the discrepancies between reports are the differences between the cell clones, the methods used, and the repair and death-promoting pathways in mouse and human cell types and in tissue culture adaptation. These pathways are particularly problematic in MMR-deficient cells, which acquire more mutations per division than MMR-proficient cells. Key to understanding the likely effect of MMR deficiency is the examination of non-adapted primary cells. These have been studied since the development of an *Msh2*-deficient mouse; however, until now it was not known to what extent the murine

Abbreviations: DEB, diepoxybutane; IR, ionising radiation; MMC, mitomycin C; MMR, mismatch repair; MNU, *N*-methyl-*N*'-nitrosourea; MSI, microsatellite instability; NHL, non-Hodgkin lymphoma; PBL, peripheral blood lymphocyte; si, small interfering

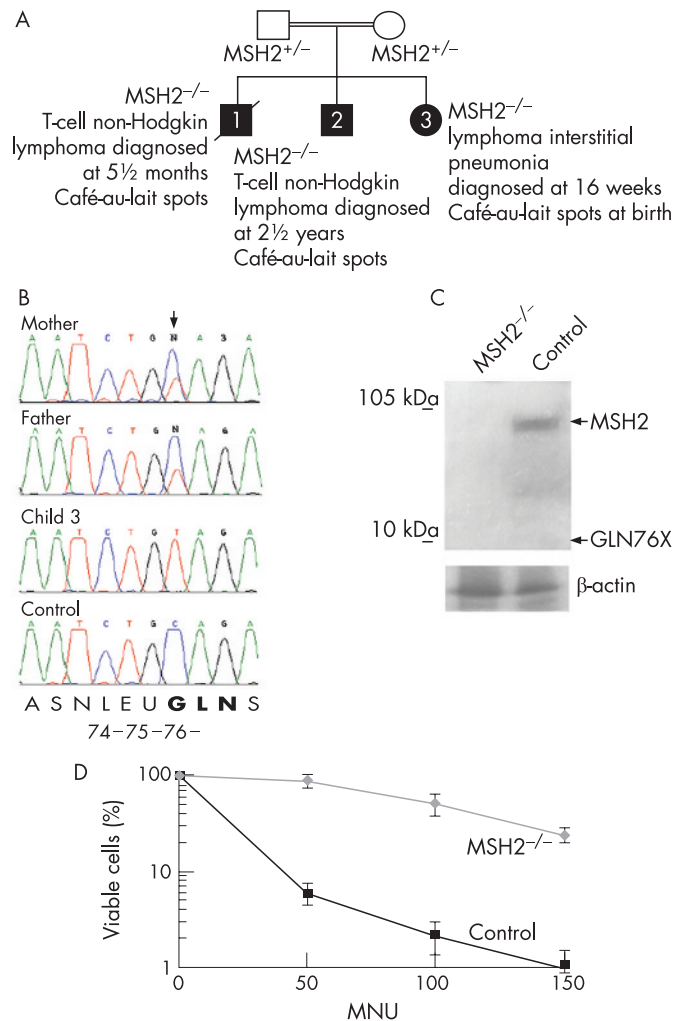


Figure 1 Homozygous *MSH2* 226C→T cells are *MSH2*-deficient and tolerant to *N*-methyl-*N*'-nitrosourea (MNU). (A) Partial pedigree of the *MSH2* family. Genotypes and ages at diagnosis are shown. The extended family had numerous affected members with colon cancer, and members with cancer of the oesophagus, bowel, stomach and optic glioma. (B) Partial sequence of *MSH2* exon 2 from genomic DNA isolated from peripheral blood lymphocytes (PBLs) of child 3, the child's parents and a normal control (sequence identifier: NM_000251). (C) Immunoblot of *MSH2* protein (PC57, Ab-3; Oncogene Research Products, Calbiochem, Darmstadt, Germany) from fibroblasts derived from a biopsy of child 3 and age-matched control fibroblasts (PBLs) from fibroblasts. β -actin loading control is shown (ab8227; Abcam, Cambridge, UK). Expected sizes of full-length *MSH2* and the truncated Gln76X protein are indicated by arrows. (D) Tolerance of *MSH2* 226C→T homozygous fibroblasts from child 3, (diamonds) and age-matched control fibroblasts (squares) to MNU at micromolar concentrations shown. In this analysis, as throughout this report, the primary fibroblast cells were examined at passage 10 or before (<30 doublings). Experiments were performed in triplicate, and the standard deviation about the mean is shown.

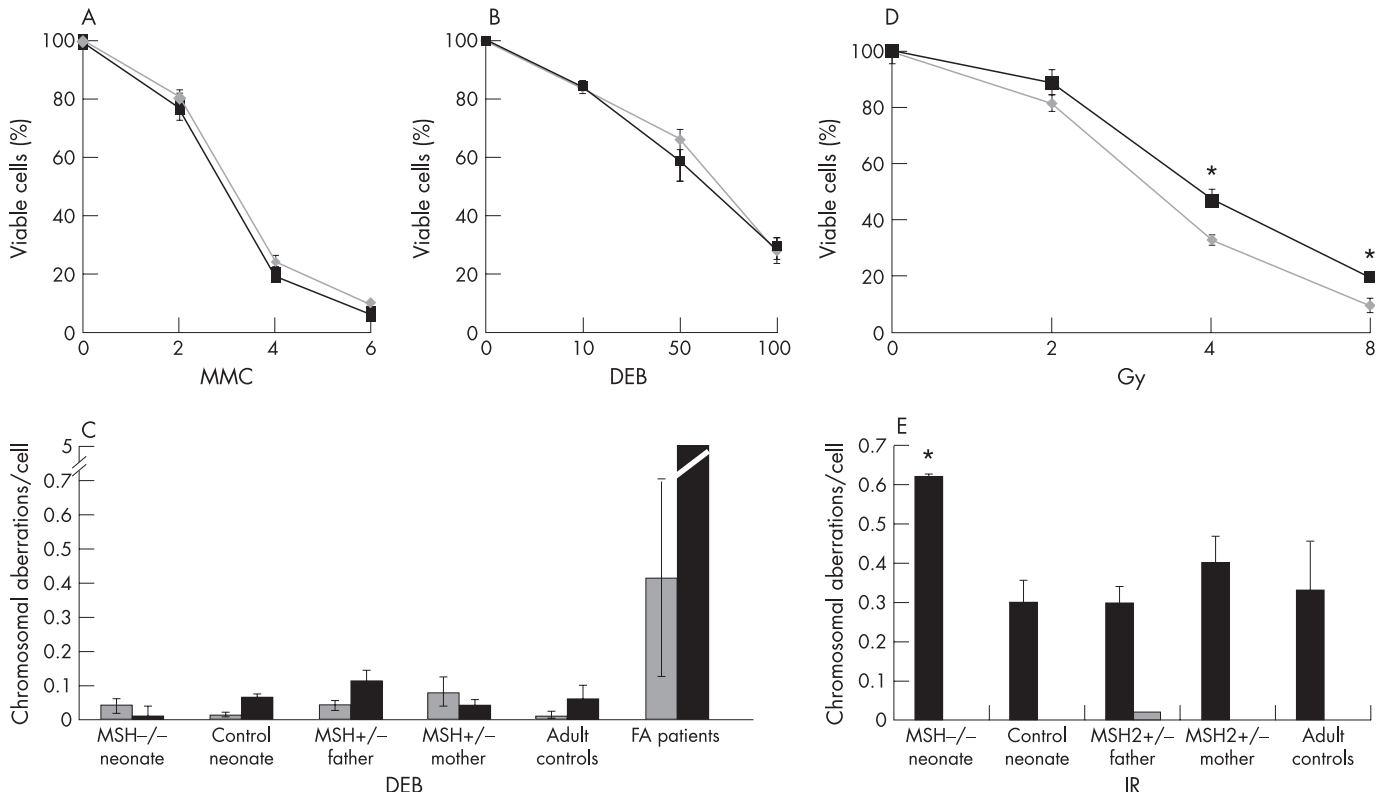


Figure 2 Sensitivity and DNA damage after exposure to interstrand cross-linking agents and ionising radiation. Survival, expressed relative to untreated cells, of homozygous *MSH2* 226C→T fibroblasts (grey lines and diamonds) and control neonate fibroblasts (black squares and lines) after exposure to micromolar concentrations shown of (A) mitomycin C (MMC), (B) 1,2,3,4-diepoxybutane (DEB) or (D) ionising radiation. Each experiment was performed in triplicate and the standard deviation about the mean for three experiments is shown. *Significant difference for cell viability between groups ($p < 0.001$, t test). The average number of chromosome aberrations per cell in peripheral blood lymphocytes (PBLs) from family members and controls is shown in the absence of treatment (grey) or after exposure to DNA-damaging agents (black). (C) 0.1 μg/ml DEB, (E) 3 Gy ionising radiation: 25 metaphase spreads were examined for each case and standard error about the mean for two experiments is shown for family member samples. The average and standard error is shown for aberrations/cell from PBLs of (C) 38 normal adult and 28 Fanconi anaemia patients, and (E) 10 normal adult controls. *Significant difference in chromosome aberrations after treatment ($p < 0.05$, unpaired Mann-Whitney test).

system reflected the human cellular phenotype. Here we report, for the first time to our knowledge, the examination of the DNA damage and cell viability response of primary non-tumour cells from a family with members homozygous and heterozygous for a mutation in the MMR gene, *MSH2*.

METHODS AND RESULTS

We identified a biallelic *MSH2* 226C→T mutation in a male child of Arab ethnicity (figure 1A, child 2) with T-cell non-Hodgkin lymphoma (NHL) whose parents were both heterozygous for the mutation (figure 1A,B). His brother had died from a similar T-cell NHL (figure 1A, child 1).

During the mother's third pregnancy the fetus showed evidence at 20 weeks of a mediastinal mass and at 16 weeks after birth, a thoracotomy was carried out. No discrete lesion was identified, and histopathological analysis of biopsied material showed lymphoid interstitial pneumonia, a diffuse pulmonary disorder characterised by an interstitial infiltrate of mature lymphocytes, including T-cells.¹⁰ Sequencing of peripheral blood lymphocyte (PBL) DNA from child 3 showed homozygosity for the *MSH2* mutation 226C→T (figure 1B). The transition introduces a stop codon in glutamine 76 of exon 2 and is predicted to result in nonsense-mediated decay of the transcript.¹¹ Any escaping transcript would produce a severely truncated protein (deletion of amino acids 76–934), lacking all known functional domains of *MSH2*.

Characterisation of *MSH2*-deficient cells

Biopsied tissue from child 3 was cut into small explants and cultured in Ham's F10 culture medium containing 15% fetal calf serum. As controls, neonate fibroblasts were derived from a skin biopsy of a 3-month-old boy with a suspected mitochondrial metabolic disorder (although none was found on investigation).

We examined protein expression in fibroblasts cultured from the biopsy and age-matched skin fibroblast controls (*MSH2* is reported to be expressed in all tissues including the lung and skin^{12–13}). No *MSH2* protein, full-length or truncated, was detected in lysate from *MSH2* 226C→T homozygous fibroblasts, whereas full-length *MSH2* expression was evident in the control cells (figure 1C).

To establish whether the *MSH2*-deficient cells exhibited features of a MMR defect, we examined them for resistance to the DNA-methylating agent *N*-methyl-*N'*-nitrosourea (MNU). Viable cells were identified using trypan blue (Sigma Chemical Co., Minneapolis, Minnesota, USA) exclusion and counted. Cells were exposed for 1 hour to MNU at 50, 100 and 150 μmol/l before further culture in complete medium for 14 days. These steps were performed in the presence of *O*⁶-benzylguanine (20 μmol/l) to inhibit endogenous methyltransferase activity that might otherwise remove the methyl groups added by MNU. MMR-deficient cells are resistant to the toxic effects of DNA methylating agents such as MNU, whereas MMR-proficient cells undergo replication arrest and cell death (thought to occur either via 'futile MMR',

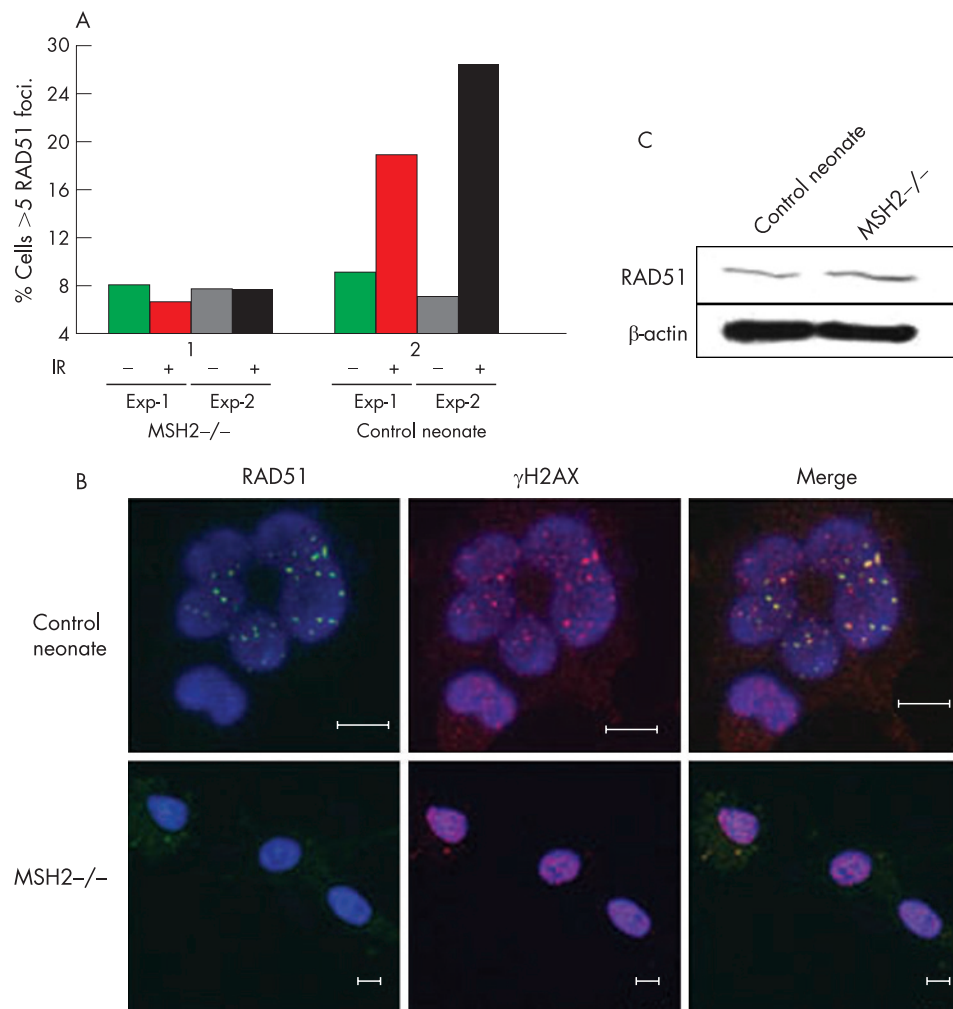


Figure 3 Reduced DNA repair intermediates in MSH2-deficient cells. (A) Percentage of untreated (grey) and irradiated (10 Gy) (black) MSH2-deficient and age-matched control fibroblasts exhibiting >5 RAD51 foci; 100 γ H2AX positive nuclei per experiment were evaluated for the presence of RAD51 foci. (B) Representative immunohistochemical staining of MSH2-deficient and control cells. The presence of γ H2AX foci (red) was used as a marker of double-stranded DNA damage. Immunostaining of RAD51 (ab213, Abcam) is shown in green and nuclei labelled with Hoechst stain are blue. Bar: 10 μ m. (C) Immunoblot of RAD51 protein expression in MSH2-deficient and age-matched control fibroblasts. β -actin loading control is shown.

and/or signalling to the apoptotic machinery).¹⁴ Control neonatal fibroblasts were sensitive to MNU, whereas resistance was evident in MSH2-deficient cells (figure 1D), indicating a defect in processing DNA-methylation products, consistent with a fault in MMR.

We also examined the DNA of MSH2 226C→T cells (passage 8) for evidence of microsatellite instability (MSI) (using Bat-25, Bat26 and D5S346 as markers). Although MSI is often associated with MMR deficiency in solid tumours arising in adults, it is not detected in non-tumour tissue of biallelic MSH2 mutation carriers in humans or mouse.¹⁵⁻¹⁷ DNA from a colon tumour, negative for MLH1 expression, showed MSI positivity, whereas this was not detected in the DNA from MSH2 226C→T homozygous fibroblasts (supplementary figure 1 (available online at <http://jmg.bmj.com/> supplemental) and data not shown), consistent with their non-tumour and non-clonal status.

Response of primary MSH2-deficient cells to DNA-damaging agents

We assessed the survival of neonatal fibroblasts after treatment with ionising radiation (IR) and interstrand cross-linking agents. PBLs from family members and controls were examined for chromosomal aberrations after exposure to IR. The survival of MSH2-deficient fibroblasts in the presence of interstrand cross-linking agents, diepoxybutane (DEB) and mitomycin C (MMC), did not differ significantly from controls (figure 2A,B). Furthermore, when PBLs were examined for chromosome abnormalities after exposure, no significant differences were seen between MSH2-deficient cells and normal controls. Cells

from individuals with Fanconi anaemia are highly sensitive to cross-linking agents (reviewed by Kennedy and d'Andrea¹⁸) and in the same assay, PBLs from these patients showed increased DNA damage in response to DEB (figure 2C). We similarly assessed the effect of IR on cell survival and DNA integrity. A modest, but significant, loss of viability was evident in MSH2-deficient fibroblasts (figure 2D) compared with control cells ($p < 0.001$, t -test). Consistent with this, an increase in chromosome damage after IR was also apparent in PBLs of the neonate homozygous for the MSH2 mutation (child 3), but not in PBLs from heterozygous family members, an age-matched control or adult controls (figure 2E).

To establish whether DNA repair factors were perturbed in primary human MSH2-deficient cells, we examined BRCA1 and RAD51 "foci" by microscopy. These areas are thought to represent sites of DNA-damage repair. An hour after exposure to 10 Gy IR, the expected increased localisation of RAD51 into foci was evident in control neonatal fibroblasts, but was not in the MSH2-deficient cells (figure 3A and B). These observations were not due to loss of RAD51 expression, which was equally present in MSH2-deficient and control cells (figure 3C), suggesting altered RAD51 formation in focal sites. No difference in recruitment of BRCA1 to foci was observed (data not shown).

To test that the effect was not due to another gene mutation in the family under study, we next undertook MSH2 small interfering (si)RNA knock-down in primary fibroblasts from two healthy neonates. In addition we examined RAD51 foci formation at various times after exposure to IR. In cells treated with control siRNA RAD51 foci were evident 10 minutes after

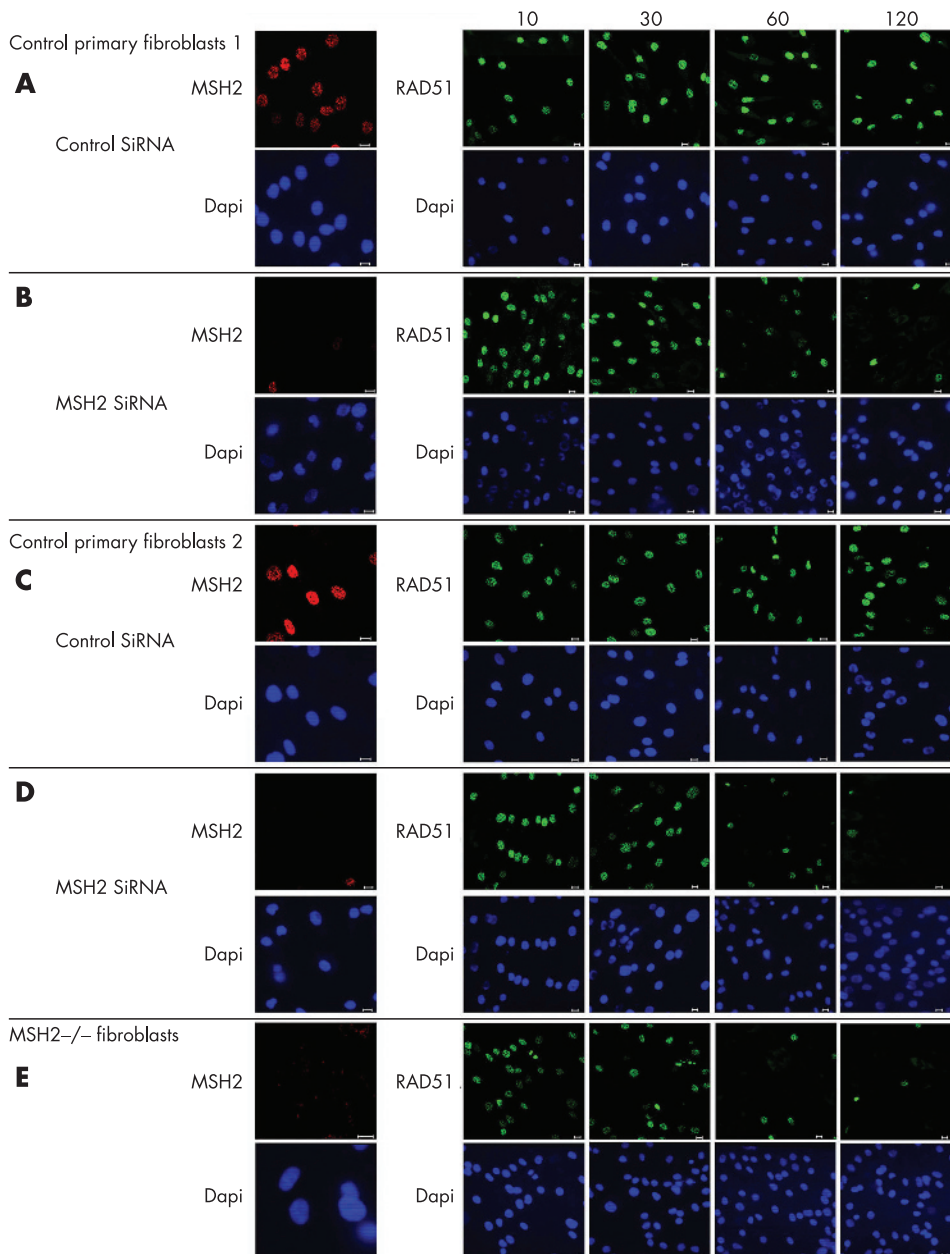


Figure 4 Altered RAD51 foci kinetics in *MSH2*-deficient primary fibroblasts after irradiation. Immunohistochemical examination of RAD51. Primary skin fibroblasts from an 18-month-old boy (A,B) and 16-month-old girl (C,D), were transfected for 96 hours with *MSH2* or control siRNA (OnTarget; Dharmacon, Lafayette, Colorado, USA), according to the manufacturers' instructions. These cells, together with fibroblasts from child 3 (E), were then exposed to irradiation (10 Gy) and recovered for the time shown. They were then fixed in 4% paraformaldehyde, permeabilised in 0.1% Triton-X and incubated with anti-*MSH2* or anti-RAD51 antibodies, appropriate secondary fluorophore-conjugated antibodies and stained for DNA with Hoechst-33342 (shown in blue). All bars are 10 μ m.

irradiation and remained detectable after 2 hours (figure 4 A,C). In cells transfected with the *MSH2* siRNA and in fibroblasts from child 3, RAD51 foci were also evident 10–30 minutes post-irradiation. However after 1 hour, few cells still exhibited foci (figure 4 B,D,E). These data indicate that the kinetics of RAD51 foci formation after irradiation are regulated at least in part by *MSH2*.

DISCUSSION

The clinical features of the family in this study are consistent with previous case reports of human germline homozygosity or compound heterozygosity for *MMR* gene mutations. These features include café-au-lait spots and early onset of haematological malignancy or brain tumours, as reviewed by Ostergaard *et al.*,¹⁹ who suggested that colorectal neoplasia might be the fully extended manifestation of biallelic *MMR* gene loss of function. The frequent occurrence of lymphoma in this group of patients is in agreement with observations of *Msh2* knockout mice, which also develop early-onset lymphomas.^{20–21}

By examining primary cells from individuals of a rare pedigree, our data provide a new perspective in the controversy of differential mutagen sensitivity attributed to MMR deficiency. In our analysis of primary human material, passage numbers were kept at <10 to ensure tissue-culture adaptation was restricted. We found that human *MSH2* deficiency is not associated with overt sensitivity to DNA interstrand cross-linking agents but is involved in the DNA damage response to IR. These data are also in agreement with observations from the cells of *Msh2*-knockout mice, which exhibit sensitivity to DNA damage in response to IR, perturbed RAD51 foci formation⁸ and have aberrant homologous recombination.^{20–22} Together, these data suggest that lack of functional *MSH2* results in constitutional IR sensitivity and deregulated homologous recombinational repair in mammalian cells. Hence the variability of response to DNA damaging agents in previous reports using cells of tumour origin or immortalised cell lines may be a result of further genetic or epigenetic lesions that lead to various levels of resistance or sensitivity to further agents.

Cells from individuals bearing mutations in specific DNA repair genes, for example in the Fanconi pathway and ATM, exhibit sensitivity to particular therapeutic agents (DNA interstrand cross-linking agents and IR respectively). Here we have shown that normal primary cells from an individual lacking functional MSH2, exhibit increased sensitivity to IR. These findings suggest caution in the use of radiotherapy for treatment of malignancies arising in people with functional loss of MSH2 and possibly other MMR gene-mutation backgrounds.

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The supplementary figure can be viewed on the JMG website at <http://jmg.bmj.com/supplemental>

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