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A human cancer-associated truncation of MBD4 causes dominant negative impairment of DNA repair in colon cancer cells

SA Bader*,1, M Walker1 and DJ Harrison1

1Department of Pathology, Edinburgh Cancer Research Centre, University of Edinburgh, Crewe Road, Edinburgh EH4 2XR, UK

MBD4 binds to methylated DNA and acts as a thymine DNA glycosylase in base excision repair. Deficiency of MBD4 in mice enhances mutation at CpG sites and alters apoptosis in response to DNA damage, but does not increase tumorigenesis in mismatch repair-deficient mice. However, in humans, frameshift mutation of MBD4, rather than deletion, is what occurs in up to 43% of microsatellite unstable colon cancers. There is no murine equivalent of this mutation. We now show that recombinant truncated MBD4 (MBD4tru) inhibits glycosylase activities of normal MBD4 or Uracil DNA glycosylase in cell-free assays as a dominant negative effect. Furthermore, overexpression of MBD4tru in Big Blue (lacI)-transfected, MSI human colorectal carcinoma cells doubled mutation frequency, indicating that the modest dominant negative effect on DNA repair can occur in living cells in short-term experiments. Intriguingly, the whole mutation spectrum was increased, not only at CpG sites, suggesting that truncated MBD4 has a more widespread effect on genomic stability. This demonstration of a dominant negative effect may be of significance in tumour progression and acquisition of drug resistance.

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Keywords: MBD4; colorectal cancer; genomic instability; Big Blue; mutation frequency

MBD4 is a methyl-CpG-binding DNA glycosylase involved especially in the repair of mismatches arising from deamination of methyl-C in mammalian cells. MBD4 has been shown in vitro to excise mismatched thymine (T) bases from oligo templates (Hendrich et al., 1999; Petronzelli et al., 2000a,b), and mouse knockout models have found that in the absence of Mbd4, mutation frequency (MF) in vivo increases, mainly at methyl-CpG sites (Millar et al., 2002; Wong et al., 2002). MBD4 can also bind to MLH1 and Fas-associated death domain (FADD) proteins (Bellacosa et al., 1999; Screaton et al., 2003), and the small intestine of Mbd4−/− mice shows reduced apoptosis in response to a variety of DNA-damaging agents (Cortellino et al., 2003; Sansom et al., 2003; Screaton et al., 2003). Absence of Mbd4 in mice also increases tumorigenicity in the tumour-susceptible APCmin background (Millar et al., 2002; Wong et al., 2002). The tumorigenic effect may be due to an increase in MF, decreased apoptosis or a combination of both. However, absence of Mbd4 in an Msh2-null background does not increase tumorigenicity above that seen owing to the MMR-deficiency (Sansom et al., 2004).

In humans, a naturally occurring frameshift mutation of a polynucleotide tract in MBD4 has been found in microsatellite unstable (MSI) colon and other cancers (Bader et al., 1999; Riccio et al., 1999; Menoyo et al., 2001; Yamada et al., 2002) that leads to a premature stop in translation of the mRNA. The mutation occurs in 20–43% of cases (mixed tissue samples), whereas micro-dissected samples (enriched for tumour cells) of 89% sporadic MSI colon tumours had truncated MBD4 mutations in at least one focus tested (Bader et al., 2000). Crucial in this study in human cells, for comparison with prior studies in mice, the equivalent mutation of MBD4 is not present in MSI mouse models as the murine gene does not contain a coding polyA microsatellite. The Mbd4-null phenotype modeled in mice is an experimental condition only, as in humans mutation or loss of MBD4 only occurs in the presence of the frame-shifted allele. Although mutation in MBD4 is thought to occur in MSI tumours as a consequence of the microsatellite instability caused by mismatch repair (MMR) protein inactivation, we have postulated that MBD4 mutation may still affect tumour progression in humans. Bi-allelic inactivation of MBD4 (by frameshift mutation of both alleles, or of one allele with chromosomal loss of the second) has been seen in a small number of cases or samples (Bellacosa et al., 1999; Bader et al., 2000). However, by far the most common condition appears to be frameshift of one allele in the presence of a remaining normal allele, and there has been no documented evidence of the null genotype as studied in the mouse models. A recombinant N-terminal portion of MBD4 containing amino acids (aa) 1–165 including the MBD has been shown to be still able to bind to methyl-CpG oligos including also those with T·G mismatches (Hendrich et al., 1999). It is therefore possible that the naturally-occurring MBD4 mutation exerts a dominant negative effect, where the truncated protein is not only defective in glycosylase activity, but also can inhibit normal MBD4 function via competitive binding of mismatch sites. In this study, we describe how truncated MBD4 can inhibit normal glycosylase activity in a cell-free system, and increase mutation frequency across a wide spectrum of mutation changes in living colon cancer cells even on a background of pre-existing MSI.
MATERIALS AND METHODS

Recombinant proteins, in vitro transcription/translation and Western immunobLOTS

For MBD4\textsuperscript{tru}, random primed cDNA was made from HCT116 cells and PCR amplified with forward primer 4NcoMet and MBD4.12A-Bam. PCR product was double digested with NcoI and BamHI and cloned into NcoI + BamHI digested bacterial expression vector pET6H. Recombinant proteins were expressed in *Escherichia coli* BLR (DE3)/pLysS and purified over nickel-agarose columns (Qiagen, West Sussex, UK) followed by Fractogel EMD SO\textsubscript{4}\textsuperscript{2−}-650(M) columns (Merck) as described (Free et al, 2001). MBD4 proteins were also made by in vitro transcription/translation using linearised MBD4 cDNA-containing pET6H plasmids in T\textsubscript{7}-T<sub>T</sub>coupled reticulocyte lysate (Promega, Hampshire, UK) in the presence of \([\text{35}^S]\) Methionine.

Nuclear extracts from cell lines were made using the Proteo Extract subcellular proteome extraction kit (Calbiochem, Nottinghamshire, UK) according to the manufacturer’s instructions.

For Western immunoblotting, proteins were subjected to SDS-PAGE on 10% BisTris NuPage gels (Invitrogen, Paisley, UK) in MOPS buffer, blotted onto Hybond P membrane (Amersham, Buckinghamshire, UK) and probed with antibodies using standard protocols. Antibodies used were monoclonal anti-His tag (Novagen, Nottinghamshire, UK), polyclonal anti-MBD4 (H-300, raised against amino acids 281–580 and capable of binding to truncated MBG4 with normal sequence up to residue 313, Santa Cruz) and monoclonal anti-lamin B (Calbiochem).

Glycosylase assays

Assays were performed essentially as described before (Hendrich et al, 1999), with modification of the piperdine cleavage assay involving the inclusion not only of the active wt recombinant glycosylase (MBD4 or UDG) but also a range of 0–100 ng MBG4\textsuperscript{tru} as competitor in a constant volume. Briefly, 10 ng recombinant wtMBD4 or 0.2 U uracil DNA glycosylase (New England Biolabs, Hertfordshire, UK) protein was incubated with annealed oligo-nucleotides (the reverse strand of each pair having been end-labelled with \([\text{32}^P]\) ATP) with or without recombinant MBG4\textsuperscript{tru} in a constant total volume of 40 \(\mu\)l. Primer combinations (Hendrich and Bird, 1998; Hendrich et al, 1999) used were BH:BHrev, BH:BHrevU, MM2:MM3revU.

DNA for transfection

A portion of 2 \(\mu\)g of \(\lambda\)LIZ (Big Blue shuttle vector, Stratagene, Cambridgehire, UK) DNA was methylated at 37°C for 30 min in vitro using excess SssI methylase according to the manufacturer’s protocol (New England Biolabs), and then concentrated for 1 h at room temperature using DNA ligase (New England Biolabs) before transfection. MBG4\textsuperscript{tru} cDNA was made by cloning the PCR product from HCT116 cDNA (primers MBG4.1a and MBG4.14) into pGEMT (Promega) and subsequently it was transferred into pcDNA3 (Invitrogen). cDNA insert was sequenced by cycle sequencing to confirm identity and presence of mutant \(A\_n\) polynucleotide stretch as well as lack of any other introduced mutations.

Cell lines and culture, including transfection

All cell lines except HCA7 were grown in RPMI1640 + 5% foetal calf serum with selective agents used as described. HCA7 was grown in DMEM + 10% foetal calf serum. DLD1 cells were cotransfected with 2 \(\mu\)g of \(\lambda\)LIZ (prepared as above) and 500 ng pcDNA3.1 Hygro (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol for 24 wells. After transfection, cells were seeded into 10 cm dishes and colonies were selected using 300 \(\mu\)g ml\textsuperscript{−1} hygromycin B (Invitrogen). Colonies were screened for intact \(\lambda\)LIZ by PCR amplification using primer pairs spanning lacI and at the end of each arm of \(\lambda\)LIZ. Successful packaging of positive clones was checked using protocols described below. Cell lines with \(\lambda\)LIZ (T54c4, T54c10) were transfected with PvuI-linearised MBG4\textsuperscript{tru}-pcDNA3 expression plasmid or PvuI-linearised empty vector, using Lipofectamine 2000 as described above, and colonies were selected using 600 \(\mu\)g ml\textsuperscript{−1} Geneticin (Invitrogen).

Methylation check of transfected \(\lambda\)LIZ

A portion of 1 \(\mu\)g genomic DNA of cell lines was set up for overnight digestion with no enzyme, HpaII or MspI. Samples were then PCR amplified using primer pairs for \(~450\) bp fragments of either lacI (2A and 13A, spanning two HpaII/MspI sites) or the CpG island of MBG1 (59 and 60, spanning three HpaII/MspI sites). Where the CpG sites were methylated, HpaII did not cut and PCR amplification could occur, whereas MspI cuts independent of methylation. Controls for efficacy of digestion of DNA by HpaII of unmethylated DNA were provided by the MBG1 samples (MBG1 being a constitutively expressed gene with unmethylated CpG island).

Big Blue plaque assays and sequencing

Cell pellets were frozen at \(-70°C\) and then used for DNA extraction by the phenol–chloroform protocol as described in the Big Blue Instruction Manual (Stratagene). The \(\lambda\) shuttle vector containing the lacI target gene was recovered from genomic DNAs as described in the Big Blue Instruction Manual and packaged with \(\lambda\) packaging extract (Transpack, Stratagene). After initial dilution plating of packaging reactions, a density of 10,000 PFU per plate was targeted on 24.5 x 24.5 cm assay dishes, but each plating session was accompanied by a 1/40th dilution plating and all plaques counted for accurate assessment of the number of plaques screened. Blue mutant plaques were identified against a red background on a lightbox, and re-plated to verify as true mutants. Mutant frequency was calculated by dividing the number of blue plaques by the total number plated. Assays were performed for DNAs packaged from at least four independent pellets of cells per cell line, and usually between 250,000 and 400,000 PFU screened per packaged DNA. At least 30 well-separated mutant plaques per cell line were picked from plates for sequencing, starting from one corner of the plate and working inwards to the centre with each blue plaque encountered taken, so as to avoid any colour/mutation bias. Cored plaques were placed in SM buffer, from which an aliquot of buffer/phage suspension was taken and used as template in PCR amplification of the entire lacI gene (primers 1A and 8 as in the Stratagene manual). PCR products were then sequenced using \([\text{35}^P]\)dATP and Thermosequenase cycle sequencing kits (Amersham) from primers recommended in the Big Blue Instruction Manual (Stratagene), the products run on 6% acrylamide : urea gels and then exposed to autoradiographic film. To aid in visual screening for mutant bases, samples were run in batches of 10, with all G’s together, all C’s together and so on. For analysis of mutation spectra, enough plaques were sequenced to identify about 50 independent mutations (defined as unique base changes at unique nucleotides, that is, disregarding multiple incidences in different plaques) for each cell line.

Statistical analyses

Comparison of mutation frequencies was carried out by two-tailed \(t\)-test. Statistical comparisons of mutation spectra were carried out using the Monte Carlo approximation to Fisher’s exact test of Adams and Skopek (1987), with 1700 iterations in a...
programme available on the web (Cariello et al., 1994) and the significance level set at 0.05. The test conducts pairwise comparisons using the 11 mutational classes shown in Figure 4B and Supplementary Table 2), including the subset of G:C to A:T mutations that occurred at CpG sites.

RESULTS

Preparation of recombinant proteins

We prepared recombinant His-tagged wild-type/full-length (FL-MBD4) and truncated (MBD4\textsuperscript{tru}) human MBD4 proteins for use in glycosylase assays. MBD4\textsuperscript{tru} comprises wild-type protein sequence from aa 1–313 with three further novel residues 314–316, and includes the MBD (aa 80–146) with 167 aa of intervening sequence and the complete C termini including the MBD (aa 1–316) and the complete C termini including the MBD (aa 1–313 after which there are three novel residues (KDH) owing to the frameshift. (B) Diagram of MBD4 proteins; the MBD4\textsuperscript{tru} sequence is the same as FL up to aa 313 after which there are three novel residues (KDH) owing to the frameshift. (B) Diagram of MBD4 proteins; the MBD4\textsuperscript{tru} sequence is the same as FL up to aa 313 after which there are three novel residues (KDH) owing to the frameshift. (C) MBD4 in nuclear fractions of colorectal carcinoma cell lines 1: HCT116 2: T54c4 3: T72con1, detected by Western blot hybridisation with antibody H-300. Arrows indicate endogenous FL (upper) and tru (lower) MBD4. (D) Loading control Western blot using anti-lamin B.

Dominant negative inhibition by mutant MBD4

Our assays also showed that MBD4\textsuperscript{tru} could inhibit T·G glycosylase activity of another DNA repair enzyme that
Hpa amplification of DNA digested with Icarried intact, concatenated. Two clones, T54c10 and T54c4 were generated, and they that the total MF increased in all clones transfected with MBD4tru. The MBD4tru used for MF assays and further transfected with presence of þ

likely a provenance as offspring from the same parental cell that or seen in two or more of 30 sequenced plaque samples) as shown in Table 2 and Figure 4A. These identical changes represent more likely a provenance as offspring from the same parental cell that

High Blue cell lines
The Big Blue LIZ reporter shuttle vector containing part of the lac-lacI operon (Stratagene) was transfected into DLD1 cells that are wild-type for MBD4. Although cellular methylation of transfected LIZ was expected to occur as such foreign, silent DNA usually undergoes this modification (as was the case Big Blue mice; You et al., 1998), we wished to guarantee methylation of LIZ CpG sites at the outset and so pretreated LIZ with SssI methylase before transfection. Because LIZ does not have a selectable marker, it was cotransfected with pCNA3Hygro1 and clones were selected with hygromycin B. Clones were screened by PCR for presence of LIZ and tested for packaging ability, before being used for MF assays and further transfected with MBD4tru DNA. Two clones, T54c10 and T54c4 were generated, and they that carried intact, concatenated LIZ and packaged efficiently. Methylation of the integrated LIZ was confirmed by PCR amplification of DNA digested with HpaII or MspII, spanning a region of lac containing two HpaII/MspII sites (Figure 3). Efficacy of HpaII digestion was confirmed by PCR amplification of part of the CpG island of an expressed, unmethylated gene (MBD1) from the same digested DNA samples (Figure 3). The endogenous MBD4 genes of both T54c4 and T54c10 were also PCR amplified and sequenced to confirm their polya tract wild-type status. These two clones were transfected with pCNA3Hygro1, expression plasmid or empty vector alone, and genetin-resistant clones were isolated. MBD4tru DNA-containing clones were confirmed by PCR, and expression of the exogenous gene was verified by Western blot hybridisation (Supplementary Figure 1).

MBD4tru increases mutation frequency
We next determined the mutation frequency (MF) of a human MSI colon cancer cell line, DLD1, with/without MBD4tru. We found that the total MF increased in all clones transfected with MBD4tru. The background spontaneous Big Blue mutation frequencies of the two parental clones of DLD1 were 79 × 10⁻⁵ (T54c10) and 178 × 10⁻⁵ (T54c10) (Table 1). Expression of exogenous MBD4tru increased the MF of T54c4 to 292–310 × 10⁻⁵ (two clones tested), and for T54c10 to 306–440 × 10⁻⁵ (three clones tested) (all P-values <0.05) (Table 1 and Supplementary Table 1). This is an increase in total MF of about 2–4 fold above that for parental cells and indicates increased genomic instability beyond that due to pre-existing MSI. Furthermore, sequencing of mutant plaques revealed that a significant proportion of blues for each clone tested carried one of several identical nucleotide changes (a ‘jackpot’ (Heddle 1999) mutation, that is, the same base change at the same base-pair location and defined in our experiments as accounting for >6%, or seen in two or more of 30 sequenced plaque samples) as shown in Table 2 and Figure 4A. These identical changes represent more likely a provenance as offspring from the same parental cell that

| Cell line | MBD4tru | mean MF × 10⁻⁵ | s.d. | P | Relative MF increasea |
|-----------|---------|----------------|------|---|------------------------|
| T54c10    | –       | 178            | 47   |   | –                      |
| T57c12    | +       | 327            | 28   | 2.27 × 10⁻⁴ | 1.8                     |
| T57c32    | +       | 440            | 19   | 8.64 × 10⁻⁶ | 2.5                     |
| T57c45    | +       | 306            | 28   | 3.38 × 10⁻³ | 1.7                     |
| T54c4     | –       | 79             | 6    |   | –                      |
| T72c2     | +       | 310            | 37   | 8.27 × 10⁻⁸ | 3.9                     |
| T73c13    | +       | 242            | 131  | 1.34 × 10⁻³ | 3.1                     |
| T72con1   | –       | 157            | 30   | 4.83 × 10⁻⁴ | 2.0                     |
| T72con2   | –       | 81             | 41   | 0.64              | 1.0                     |
| T72con3   | –       | 83             | 9    | 0.45              | 1.0                     |
| T72con4   | –       | 60             | 5    | 7.23 × 10⁻⁴ | 0.8                     |

Table 2
Jackpot mutations

| Cell line | No. plaques | No. jackpots | Total % of MF owing to jackpots | Base changes |
|-----------|-------------|--------------|---------------------------------|-------------|
| T54c10    | 76          | 2            | 25                              | A to G, T to G |
| T57c12    | +           | 97           | 1                               | T to A      |
| T57c32    | +           | 124          | 2                               | A to G, T to C |
| T57c45    | +           | 30           | 4                               | C to T, A to G, G to A (in C2G) |
| T54c4     | –           | 30           | 2                               | C to T, T to C |
| T72c2     | +           | 30           | 3                               | G to A      |
| T73c13    | +           | 30           | 3                               | C to T, G to A, A to T |
| T72con1   | –           | 30           | 2                               | T to C      |

Figure 3 Methylation of transfected LIZ shuttle vector. Genomic DNA of cell lines was digested with HpaII (H), MspI (M) or treated with no enzyme (–), and then PCR amplified using primer pairs to amplify a CpG-rich part of (A) transfected LIZ or (B) endogenous MBD1 gene. Cell lines shown are T54c10 (clone of DLD1 +LIZ) and T54c12, c32, 45 (all clones of T54c10 +MBD4tru).

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Figure 4  Big Blue mutation frequencies and spectra of cell lines +MBD4tru. (A) The left panel shows T54c10 and transfected (+MBD4tru) derivatives as indicated, whereas the right panel shows T54c10 and transfected (+MBD4tru) derivatives. The Y-axis shows the mutation frequency of each cell line, ×10^−5 with bars showing the standard deviation. Within each column the colours represent subsets of mutations, according to the proportions of plaques sequenced: black – independent mutations (frequency ≤ 6% those sequenced); grey – not sequenced; other colours – jackpot mutations (frequency > 6%) (NB: the same colour in different columns does not necessarily indicate the same mutation base change). (B) Mutation spectra of T54c10 and two clones +MBD4tru. The heights of columns indicate the percentage incidence of each type of base change.

an MF that is itself higher than that for normal, genomically stable somatic cells. It also highlights that the sensitivity of this assay system to detect small changes in mutation frequency is limited. In addition, our data demonstrate that genomic instability, as evinced by overall MF and the proportion of MF owing to an increased number of jackpots, increases further in the presence of MBD4tru.

MBD4tru affects a wide mutation spectrum

The mutation spectra of three cell lines (parental T54c10 and two +MBD4tru clones T57c12 and T57c32) were determined by sequencing about 50 independent mutations. These spectra showed that the mutations occurring in the presence of MBD4tru spanned the spectrum of possible changes with no bias towards G:C to A:T transitions at CpG sites and no significant difference from the parental cell line containing only wild-type MBD4. T54c10 vs T57c12 P = 0.48, T54c10 vs T57c32 P = 0.52 (Figure 4B and Supplementary Table 2). In addition, the base changes found in the jackpots included examples of several types of base change (Table 2). This is in contrast to the shift of mutation spectra in the absence of Mbd4 in the mouse model, where the predominant change seen is an increase in C to T transitions at methyl-CpG sites.

DISCUSSION

The role of MBD4 was initially thought to be limited to the detection and repair of T→G mismatches that occur at methyl-CpG dinucleotides as a result of deamination of the methyl-C. This was demonstrated by the behaviour of recombinant protein in cell-free gel-shift and glycosylase systems. Some other base-pair mismatches affecting CpG dinucleotides were also shown to be lower affinity substrates for MBD4 activity (Petronzelli et al, 2000a,b) and as time has progressed more substrates have been documented, at least in vitro (Liu et al, 2002; Yoon et al, 2003). The role of MBD4 as a methyl-CpG-focused DNA mismatch glycosylase was subsequently supported by increases in mutation frequency at CpG sites in mouse models lacking Mbd4 (Millar et al, 2002; Wong et al, 2002). However, we and others have found an association of truncating mutations of MBD4 in human MSI cancers, especially colon, and in most cases these mutations occur in the presence of a remaining intact MBD4 allele (Bader et al, 1999, 2000; Riccio et al, 1999), that is, the null genotype is not seen naturally. As the MBD of MBD4 was reported to be able to bind methylated oligos with a T→G mismatch, we have hypothesised that a dominant negative activity could be present in cells heterozygous for an MBD4tru mutation that has occurred as a result of prior MMR gene abnormality. In the absence of any known dimerisation of MBD4 molecules for activity, we envision the inhibition to occur via steric recognition and competition for binding sites. In this study, we have shown that MBD4tru can indeed inhibit glycosylase activity of wtMBD4 and at least one other glycosylase (UDG) in cell-free assays.

We went on to determine the mutation frequencies of living cells using the ΔLIZ shuttle vector as an indicator. This is the first time that the Big Blue system has been reported using human cells so there are no prior reports of MF of the lacI gene in human cells or tissues for comparison. However, the values of the parental clones T54c10 and T54c4 are between 16× and 90× the spontaneous MF of ΔLIZ in somatic tissues of untreated Big Blue mice (lacI reporter system) and a similar lacZ transgenic mouse reporter system, ranging from 2 to 5×10^−5 in several reports (Morrison and Ashby, 1994). The high MF of these clones of DLD1 is due to the defect in the MSH6 MMR gene of this cell line, and similarly, high MF of DLD1 or other MSI colon cancer cells compared with
normal diploid fibroblasts has been reported elsewhere using the HPRT or ouabain® endogenously expressed gene systems (Bhattacharyya et al., 1994, 1995). We found that expression of MBĐ4™ roughly doubles mutation frequency in cells even on the background of MMR defect, on agreement with the dominant negative hypothesis. The increase in genomic instability was indicated not only by an increase in overall MF, but also the relative effect of jackpot mutations. The latter observation was also a novel interpretation of Big Blue data, useful in this instance of cell culture rather than the usual animal experiments.

The first report of substrates of MBĐ4 showed preference for T·G products of methyl-C deamination within methyl-CpG sites (Hendrich et al., 1999). Later reports demonstrated a larger set of in vitro CpG mismatches for MBĐ4 recognition, such as U·G (Petronzelli et al., 2000a,b), 5-flourouracil (5-FU)·G (Petronzelli et al., 2000b), ethenecytosine (εC)·G (Petronzelli et al., 2000a), 5-formyluracil (5-FOU)·G (Liu et al., 2002), T·O6-methyl-guanine (O6-meG) (Cortellino et al., 2003) and thymine glycol (Tg)·G (Yoon et al., 2003). Total absence of MBĐ4 in mice caused an increase in MF especially at CpG sites (Millar et al., 2002; Wong et al., 2002). This was as predicted from the originally reported range of substrates and by the fact that the other main T·G mismatch enzyme, Thymine DNA glycosylase (TDG), does not have an MBĐ and so does not focus on methyl-CpG sites. Repair of T·G and U·G mismatches at non-methylated CpG sites, an MBĐ function lost in MBĐ4-null cells, would be covered by redundant glycosylases TDG and UDG. We had predicted that dominant negative action of MBĐ4™ would likewise increase C to T transitions, especially at methyl-CpG sites in human cells, owing to inhibition of wtMBĐ4 with some gain-of-function inhibition of TDG and UDG. Our observation that the mutation spectrum in the presence of MBĐ4™ does not specifically show an increase of C to T transitions at methyl-CpG sites, but instead increases mutation frequency across the entire spectrum, implies that MBĐ4™ not only inhibits wtMBĐ4 activity at methyl-CpG sites, but also activities of the other glycosylases at least, and perhaps also other DNA repair pathways. This conclusion is consistent with the data implicating MBĐ4 in a wider role of DNA damage substrate recognition and repair leading to apoptosis in MBĐ4-null mice (Sansom et al., 2003; Screeton et al., 2003) treated with for example the methylating agent temozolomide and the crosslinking chemical cisplatin. The important difference between the mouse studies and this is that the effects presented here occurred owing to expression of a naturally occurring truncation mutant of MBĐ4 found in authentic human tumours. The agreed sequence of events in human tumours involves a mutational event that leads to an MMR gene abnormality thus causing the MSI phenotype that then by chance hits an MBĐ4 allele. The dominant negative activities of MBĐ4™ in our experiments suggest that wtMBĐ4 is involved directly in more than just methyl-CpG repair or that MBĐ4™ is able to interfere nonspecifically in repair systems, or both. The former is consistent with the data from MBĐ4 knockout experiments. The increase in MF seen here following relatively short-term culture with a high level of expression of truncated MBĐ4 is demonstration in principle of the dominant negative effect of this mutant protein. In the physiological situation with expression of endogenous truncated MBĐ4 at a relatively lower level because of NMD, the increase in MF is also expected to be less. In this way, NMD has an important role in protecting cells from damaging activities of frameshifting mutations. However, in view of the stochastic nature of mutations arising as a result of perturbed DNA repair, over the extended period of time of 5–20 years that it takes to get malignant colon tumours, even in patients with MSI owing to HNPCC, it is feasible for even the low level of MBĐ4™ to compound the genomic instability owing to MMR defects. Such dominant negative interference with a tumour cell’s biology may also have relevance to therapeutic intervention. We are in the process of using the same cell lines to test for changes in sensitivity to DNA-damaging agents with respect to signalling to apoptosis, and it is possible that cells carrying MBĐ4™ mutations will also be more tolerant of the types of damage tested in MBĐ4-null systems. It will also be important to study the effects of MBĐ4™ at more physiological levels, for example, by creating a mouse knock-in model whereby a truncated MBĐ4 allele is expressed under the control of the endogenous promoter.

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Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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