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The role of mismatch repair in small-cell lung cancer cells

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Abstract

The role of mismatch repair (MMR) in small-cell lung cancer (SCLC) is controversial, as the phenotype of a MMR-deficiency, microsatellite instability (MSI), has been reported to range from 0 to 76%. We studied the MMR pathway in a panel of 21 SCLC cell lines and observed a highly heterogeneous pattern of MMR gene expression. A significant correlation between the mRNA and protein levels was found. We demonstrate that low *hMLH1* gene expression was not linked to promoter CpG methylation. One cell line (86MI) was found to be deficient in MMR and exhibited resistance to the alkylating agent MNNG. Surprisingly, MSI was not detected in 86MI and it appears to express all the major MMR components hMSH2, hMSH6, hMLH1, hPMS2, hMSH3, hMLH3, MBD4 (MED1) and hExo1. These data are consistent with at least two possibilities: (1) A missense mutation in one of the MMR genes, which dissociates MSI from drug resistance, or (2) inactivation of a second pathway that leads to MMR-deficiency and MNNG resistance, but induces negligible levels of MSI. We conclude that MMR deficiency is largely not associated with the pathogenesis of SCLC.

Keywords: Mismatch repair; Small-cell lung cancer; Microsatellite instability; Methylation; Methylnitronitrosoguanidine

1. Introduction

Defects in the human mismatch repair (MMR) pathway are involved in most hereditary nonpolyposis colorectal cancer (HNPCC) cases (for a review see Ref. [1]). It has been shown that HNPCC patients carry germ-line mutations primarily in the MMR genes *hMSH2* and *hMLH1* and to a lesser extent in *hMSH6* and *hPMS2* [1]. Moreover, it has been shown that tumour cells with mutations in these MMR genes are also deficient in MMR *in vitro* [1]. A high proportion of sporadic colon cancers that lack MMR gene mutations have inactivated the *hMLH1* gene by promoter CpG methylation [2]. In addition, loss of MMR results in resistance to alkylating agents, e.g. 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) [3–6]. Several studies have suggested a connection between lesion recognition by the MMR machinery and the induction of apoptosis [6,7].

HNPCC tumours and a proportion of sporadic colon cancers display a widespread specific type of genomic instability that is recognised by examining short repeated sequences, i.e. microsatellites [1,8,9]. Microsatellite instability (MSI) has also been reported in a number of non-colonic sporadic cancers, e.g. endometrial, gastric and lung cancers [9].

MSI has been examined in small-cell lung cancers (SCLC) [10–13]. However, there are conflicting data on MSI frequencies: MSI of 0–76% have been reported [10–13]. These results underline the uncertain nature of MSI in the pathogenesis of SCLC. It has been suggested that the large discrepancy in the reported MSI frequencies among lung cancer studies partly reflects differences in number

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and types of microsatellite loci analysed, as well as in the definition of MSI [14].

Human homologues of the bacterial *MutS* (*hMSH2, hMSH3, hMSH4, hMSH5, hMSH6*) and *MutL* (*hMLH1, hMLH3, hPMS1, hPMS2*) genes have been described [6,15]. Bacterial MutS and MutL function as homodimers in the repair of mismatch nucleotides generated by replication misincorporation errors, recombination between divergent sequences, and chemical or physical damage to nucleotides (for a review see Ref. [16]). The human proteins have similar functions, but appear to have evolved specific, enhanced and redundant roles as heterodimers in MMR and lesion recognition (hMSH2–hMSH3, hMSH2–hMSH6, hMLH1–hPMS2) as well as meiosis (hMSH4–hMSH5) [6,17]. In the absence of MMR, polymerase replication errors in simple repeat sequences result in the MSI phenotype [1].

To examine the MMR pathway in SCLC, we have studied a panel of 21 SCLC cell lines.

2. Materials and methods

2.1. Cell lines and growth conditions

Twenty-one SCLC, two colorectal (HCT116 and DLD-1), and one lymphoblastoid (TK6) cell lines were studied. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in medium containing 10% fetal calf serum (FCS) (v/v), no antibiotics, and mycoplasm-free. Eleven SCLC cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 (GLC2, GLC3, GLC14, GLC16, GLC19, GLC26, GLC28, 24H, H69, 86MI and N417), eight were grown in Waymouth (DMS53, DMS79, DMS92, DMS114, DMS153, DMS273, DMS406 and DMS456), and two were grown in Eagle's minimum essential medium (54A and 54B). The establishment of the SCLC cell lines has been previously described in Ref. [18]. HCT116 and DLD-1 (ATCC, Manassas, VA, USA) were grown in McCoy and TK6 (ATCC, Manassas, VA, USA) in RPMI 1640. Both HCT116 and DLD-1 are MMR-deficient as a result of altered *hMLH1* and *hMSH6*, respectively [19]. HCT116 and DLD1 are resistant to alkylating agents [20], whereas TK6 is MMR-proficient, and sensitive to alkylating agents [3,19]. In all experiments, cells were harvested in the exponential growth phase.

2.2. Northern analysis

Northern analysis was performed in two independent experiments as described in Ref. [21]. Briefly, total RNA or mRNA was extracted from cultures of 10^7 cells and either 10 μg total RNA or 3 μg mRNA was separated on formaldehyde agarose gels and blotted onto nylon membranes (NEN, Boston, MA, USA). Membranes were separately hybridised with ^{32}P-labelled (Amersham, Denmark) full-length cDNA probes for *hMSH2*, *hMSH6* [22], *hMLH1* and *hPMS2* [23] and *hExo1* [24]. 28S and *GAPDH* probes (Clontech, Denmark) were used as loading standards. Moreover, wild-type human *MBD4* (*MED1*) cDNA (272 bp) was amplified from normal human foreskin fibroblasts (ATCC, Manassas, VA, USA). Polymerase chain reaction (PCR) was carried out with a cloned *Pfu* polymerase (Stratagene, Denmark) using the primers: forward 5′-AGAGGTTT CACATCTTACTCCG-3′ and reverse 5′-AGCAAGG GATTACATTCACTGC-3′. The *MED1* cDNA was sequenced on an ABI 310 sequencer (Applied Biosystems, Denmark) and the wild-type status was verified. The mRNA expression was visualised on a phosphorimager (STORM 840, Molecular Dynamics, Denmark), and quantified with Image Quant 4.2 (Molecular Dynamics, Denmark). Expression of MMR genes was adjusted to either the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or the 28S expression. In order to compare data from different membranes, RNA from DMS273 was loaded on all gels and expression values from all cell lines were normalised to the expression level in this cell line.

2.3. Western analysis

Western analysis was performed in two independent experiments as described in Ref. [21]. Briefly, cultures of 10^7 cells were sonicated and 22.5 μg of protein extract was separated on 1.0 mm 8% Tris–glycine gels (Invitrogen, Denmark) and blotted onto polyvinylidene fluoride (PVDF) membranes (Invitrogen, Denmark). The following antibodies and concentrations were used: Monoclonal mouse-anti-hMSH2 (0.2 μg/ml, NA27, Calbiochem, Germany) and polyclonal rabbit-anti-hMLH1 (1 μg/ml, PC56, Calbiochem, Germany), polyclonal rabbit–anti-hMSH6 (1:2000) [25], polyclonal rabbit–anti-hPMS2 (1:2000) [26], polyclonal rabbit–anti-hMLH3 (1:500), polyclonal rabbit–anti-hMSH3 (1:250, clone 52, BD Biosciences, Erembodegem, Belgium), monoclonal mouse–anti-MGMT (1:4000, Neomarkers, Fremont, CA, USA), polyclonal rabbit–anti-hExoI (1:2000) [26], monoclonal mouse–anti-α-tubulin (1:10.000, DM 1A, Sigma, Denmark), horse radish peroxidase (HRP) conjugated polyclonal goat–anti-mouse-immunoglobulin (1:5000, DAKO, Denmark), and HRP conjugated polyclonal goat–anti-rabbit-immunoglobulin (1:2000, DAKO, Denmark). The protein expression was visualised with enhanced chemiluminescence (ECL) Plus (Amersham, Denmark) and quantified by chemifluorescence scanning (STORM 840, Molecular Dynamics, Denmark). Expression of MMR proteins was adjusted to the α-tubulin expression and as for Northern analysis expression values from all cell lines were normalised to the expression level in DMS273.

2.4. hMLH1 *promoter methylation*

DNA from H69 and DMS114 was extracted by a proteinase-K digestion followed by a 2-propanol extraction. Fully methylated human DNA (Intergen, Oxford, UK) was used as a positive control. One microgram DNA was treated with sodium bisulphite according to the manufacturer's protocol (Intergen, Oxford, UK) [2]. After bisulphite treatment, a region of the *hMLH1* promoter was amplified with PCR primers that amplify bisulphite-modified DNA, but not methylated or unmethylated DNA. The primers used were 5′-GAGTAGTTTTTTTTTAGGAGTGAAG-3′ (forward) and 5′-AAAAACTATAAAACCCTATACCTAATC TA-3′ (reverse) [27]. The PCR products were sequenced on an ABI 310 sequencer (Applied Biosystems, Denmark) and the number of methylated CpG sites in the amplified region was determined.

2.5. *MMR activity*

The MMR activity of 17 SCLC cell lines, HCT116, and TK6 was examined in cellular protein extracts as previously described in Refs. [28,29]. Prior to MMR activity assays, the extracts (100 μg) were tested positive for DNA replication activity using a SV40 Large T-antigen Replication Assay Kit according to the manufacturer's protocol (CHIMERx, Milwaukee, WI, USA). The SCLC cell line 86MI and lymphoblastoid cell line, TK6, were examined in two independent experiments. *In vitro* MMR was determined by a previously described heteroduplex repair assay and measures the strand-specific repair of mismatches in M13mp2 DNA [28]. The DNA substrate employed contain a strand incision in the minus strand to direct the repair of a $(TT)_2$ loop mismatch in the lacZ-complementation reporter gene. Repair values between 0 and 10% represent experimental fluctuation in this assay.

2.6. *Cellular sensitivity to MNNG*

The cellular sensitivity to MNNG (kindly provided by Dr Thomas Helleday) was analysed by a proliferation assay (Roche, Denmark). In this assay, MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is converted, by living cells only, into purple formazan that is subsequently dissolved. Absorbance of converted dye is measured at a wavelength of 570 nm with a background subtraction at 690 nm. Three independent experiments were performed with the MMR-deficient 86MI SCLC cell line, the MMR-proficient GLC19 and DMS153 SCLC cell lines, and the TK6, HCT116 and DLD-1 control cell lines. Briefly, cells were plated in 96-well plates at a density of 5×10^3 cells/well and allowed to attach overnight. To avoid removal of MNNG-induced DNA adducts by O_6-methylguanine–DNA methyltransferase (MGMT), cells were treated for 1 h with 25 μM O_6-benzylguanine (Sigma, Denmark) in serum-free media prior to the addition of MNNG. This pretreatment has previously been shown to completely deplete human cells of cellular MGMT enzyme activity [30]. MNNG was dissolved in dimethyl sulphoxide (DMSO) (Merck, Denmark) and treatments were carried out at different doses (0–40 μM) in serum-free medium with 0.1% DMSO (v/v) for 1 h. After treatment, serum was added to a final concentration of 10% and cells were incubated for an additional 48 h, without removing the MNNG. Then 20 μl (5 mg/ml) MTT (Sigma, Denmark) in PBS was added to each well and incubated for 4 h. Subsequently, 100 μl 10% sodium dodecyl sulphate (SDS) (w/v)/0.01 M HCL solution was added and incubated over night. Absorbance (570–690 nm) was determined in each well using a MR5000 microplate reader (Dynatech, Switzerland) and the IC_{50} dose was calculated. The IC_{50} dose is defined as the MNNG concentration (μM) required for a 50% inhibition of cell growth.

2.7. *Microsatellite analysis*

Microsatellite analysis of the monomorphic BAT26 locus was performed in DNA samples from all 21 SCLC cell lines and HCT116 and DLD-1 as control cell lines. Single cell clones were not isolated for this analysis. Approximately 50 ng of DNA was used in PCR reactions using a fluorescence (HEX) labelled forward primer (5′-TGACTACTTTTGACTTCAGCC-3′) and unlabelled reverse primer (5′-AACCATTCAA CATTTTTAACCC-3′). PCR products were run on a urea containing polyacrylamide gel (4.75% (w/v)) on an ABI 377 sequence analyser using GeneScan 3.0 (Applied Biosystems, Denmark). To confirm the initial microsatellite analysis of 86MI cells, single cell sub clone analysis was performed as previously described in Ref. [31]. Briefly, cells were trypsinised into a single cell suspension and seeded into 96-well plates at a density of 1 cell/well. After 2 weeks, 23 single-cell clones were identified and expanded for an additional 4 weeks. From each clone, genomic DNA was isolated using isopropanol precipitation of proteinase K-digested cells. Microsatellite analysis of the 23 single cell clones was performed using the HNPCC Microsatellite Instability Test kit according to the manufacturer's instructions (Roche, Denmark) [8]. The microsatellite loci analysed were D5S346 (APC), BAT25, BAT26, D17S250 (Mfd15CA) and D2S123. As starting material, we used 100 ng genomic DNA. The PCR products were analysed on an ABI 377 sequence analyser and GeneScan 3.0 (Applied Biosystems, Denmark).

2.8. DNA sequencing of hExo1

DNA from the 86MI SCLC cell line was extracted by a proteinase-K digestion followed by a 2-propanol extraction. The 14 exons of the *hExo1* gene were sequenced as previously described in Ref. [24].

2.9. Statistical analysis

For determining correlations between the expression of the MMR genes and proteins, Pearson's product–moment correlation on the logarithmic data were employed. Differences in MNNG sensitivity among the cell lines were analysed by two-sided *t*-test.

3. Results

3.1. MMR gene expression

The expression of hMSH2, hMSH6, hMLH1 and hPMS2 was determined in 21 SCLC cell lines by both northern and western blotting analyses. In all cell lines, studied transcripts and proteins of the expected sizes were detected (Figs. 1c and 2c). No gross alteration in predicted transcript or protein size was found in any of the SCLC cell lines analysed. Among the SCLC cell lines, a highly heterogeneous expression of *hMSH2* and *hMSH6* mRNA was observed (Fig. 1a). For example, the difference in mRNA expression between cell lines (DMS153 and 54A) was 36-fold for *hMSH2* and 20-fold for *hMSH6*. A more moderate heterogeneity (DMS114 and H69) between 8- and 7-fold was observed for the expression of *hMLH1* and *hPMS2* mRNA (Fig. 1b). The expression heterogeneity was also reflected on the protein level. For example, the hMSH2 protein varied 3-fold and hMSH6 20-fold when the DMS153 and 54A cell lines were compared (Fig. 2a). The hMLH1 and hPMS2 protein expression varied 4- to 3-fold (DMS114 and H69) (Fig. 2b).

Other reports have indicated that the hMSH2 protein is co-ordinately expressed with its heterodimeric partner hMSH6 [32]. We also found a strong and positive correlation between the expression of the hMSH2 and hMSH6 proteins ($r=0.75$, $P<0.001$) (Fig. 3b). In addition, we observed a very strong correlation in the expression of *hMSH2* and *hMSH6* mRNA ($r=0.89$, $P<0.001$) (Fig. 3a). These results are consistent with a coordinated transcriptional regulation of these genes. Additionally, a strong correlation between the protein levels of the heterodimeric partners hMLH1 and hPMS2 was observed ($r=0.79$, $P<0.001$) (Fig. 3d). This correlation was also evident in the expression of *hMLH1* and *hPMS2* mRNAs ($r=0.59$, $P<0.01$) (Fig. 3c). Correlations between components from different heterodimers, e.g. hMSH6 and hPMS2 were tested for both protein and mRNA (data not shown). No correlations was found, with the exception of a weak correlation between *hMSH2* and *hMLH1* mRNA ($r=0.49$, $P<0.05$). Significant correlations between mRNA and protein expression of hMSH2, hMSH6 and hMLH1 were observed (hMSH2, $r=0.59$, $P<0.01$, hMSH6, $r=0.65$, $P<0.01$, hMLH1, $r=0.61$, $P<0.01$). No significant correlation between hPMS2 mRNA and protein was apparent ($r=0.34$, $P>0.05$) (data not shown).

3.2. hMLH1 promoter methylation

Methylation of the *hMLH1* promoter and consequent lack of hMLH1 protein expression has been found to be a major cause of MMR deficiency in sporadic colorectal tumours [2]. It was formally possible that the heterogeneous expression of *hMLH1* mRNA in the SCLC cells was a result of altered promoter methylation. Genomic DNA from two SCLC cell lines (H69 and DMS114) which displayed an 8-fold difference in *hMLH1* mRNA expression (Fig. 1b) was examined for *hMLH1* promoter methylation using the sodium bisulphite method [2]. A 199 bp region of the *hMLH1* promoter containing 25 CpG sites was amplified, sequenced, and the number of methylated CpG sites was determined. Neither H69 nor DMS114 contained CpG methylated sites whereas all 25 CpG sites were methylated in the positive control DNA (data not shown). Thus, it is unlikely that the lowered level of *hMLH1* mRNA in H69 was the result of CpG methylation of the *hMLH1* promoter.

3.3. Mismatch repair activity

MMR activity in 17 of the 21 SCLC cell lines, HCT116, and TK6 was examined using a previously published well-defined system (see Materials and methods) [28,29]. All but one of the SCLC cell lines was found to be MMR-proficient. The 86MI cell line was found to be MMR-deficient in two independent experiments (Fig. 4). The MMR defect in 86MI was unexpected since all of the core MMR proteins appear to be expressed normally in this cell line (Fig. 2).

3.4. Cellular sensitivity to MNNG

A strong correlation has been found between cellular resistance to MNNG and MMR defects [3,6,20]. We examined the 86MI cell line for its cellular sensitivity to the alkylating agent MNNG compared with two MMR-proficient SCLC cell lines (GLC19 and DMS153), and the two well-described MMR-deficient and MNNG-resistant cell lines, HCT116 and DLD-1 [20], as well as the MMR-proficient and MNNG-sensitive TK6 cell line

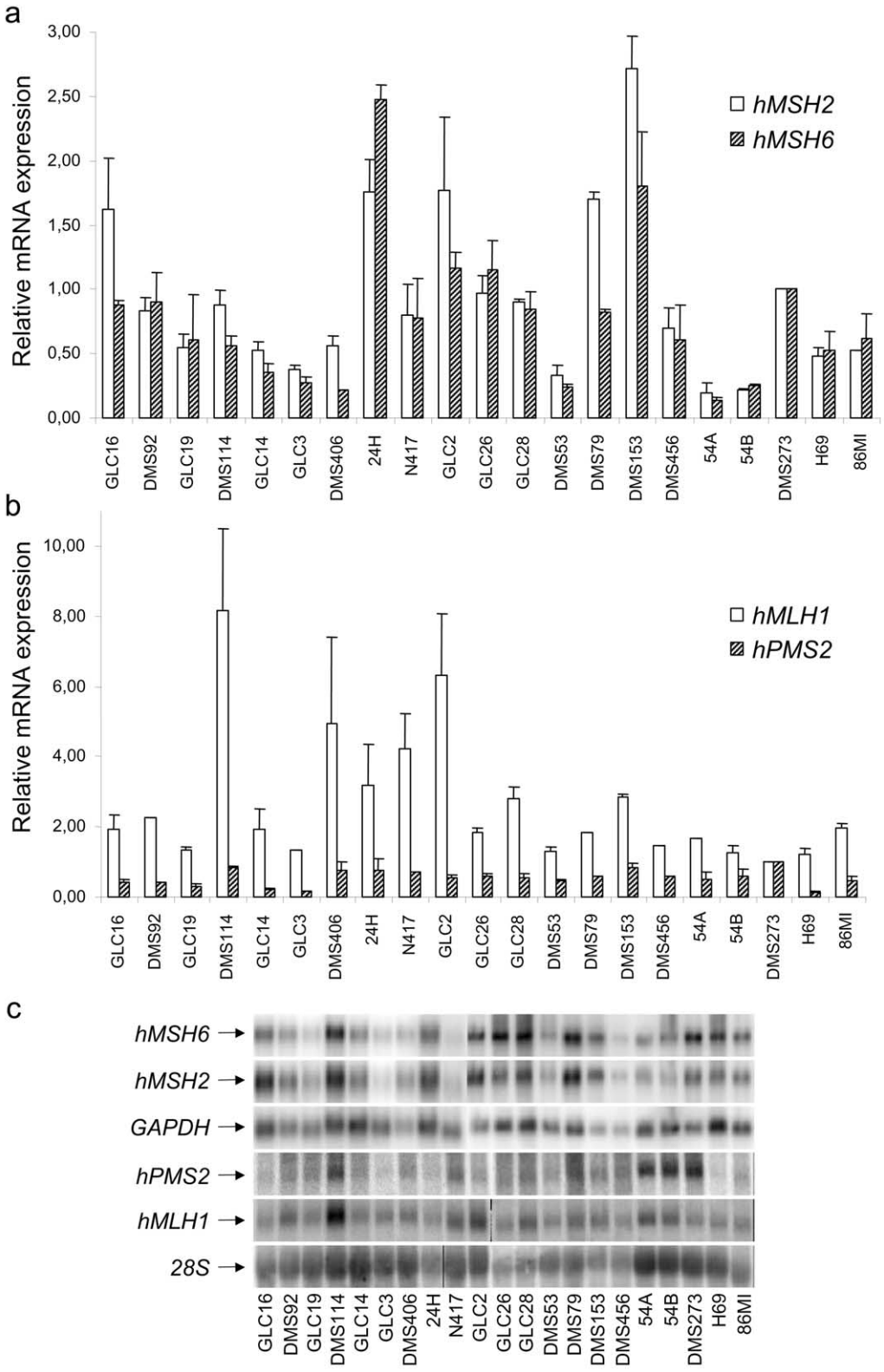

Fig. 1. Heterogeneous expression of MMR genes in SCLC cell line: (a) quantified expression of *hMSH2* and *hMSH6* mRNA; (b) quantified expression of *hMLH1* and *hPMS2* mRNA. Histograms represent mean values of two independent experiments; bars, standard error of mean (SEM). (c) Northern blots of SCLC cell line mRNAs probed with *hMSH2*, *hMSH6, hMLH1, hPMS2, GAPDH* and 28S.

[3,19] (Fig. 5a). The IC_{50} doses for each cell line were calculated. In all experiments, the 86MI cell line was significantly more resistant to MNNG than GLC19, DMS153 and TK6 (Table 1). Moreover, the cellular resistance of 86MI to MNNG was comparable to HCT116 and DLD-1 (Table 1). By Western analysis, the MGMT protein level was measured in the 86MI, DMS153 and GLC19 cell lines (Fig. 5b). No relationship between MNNG sensitivity and MGMT expression levels was observed.

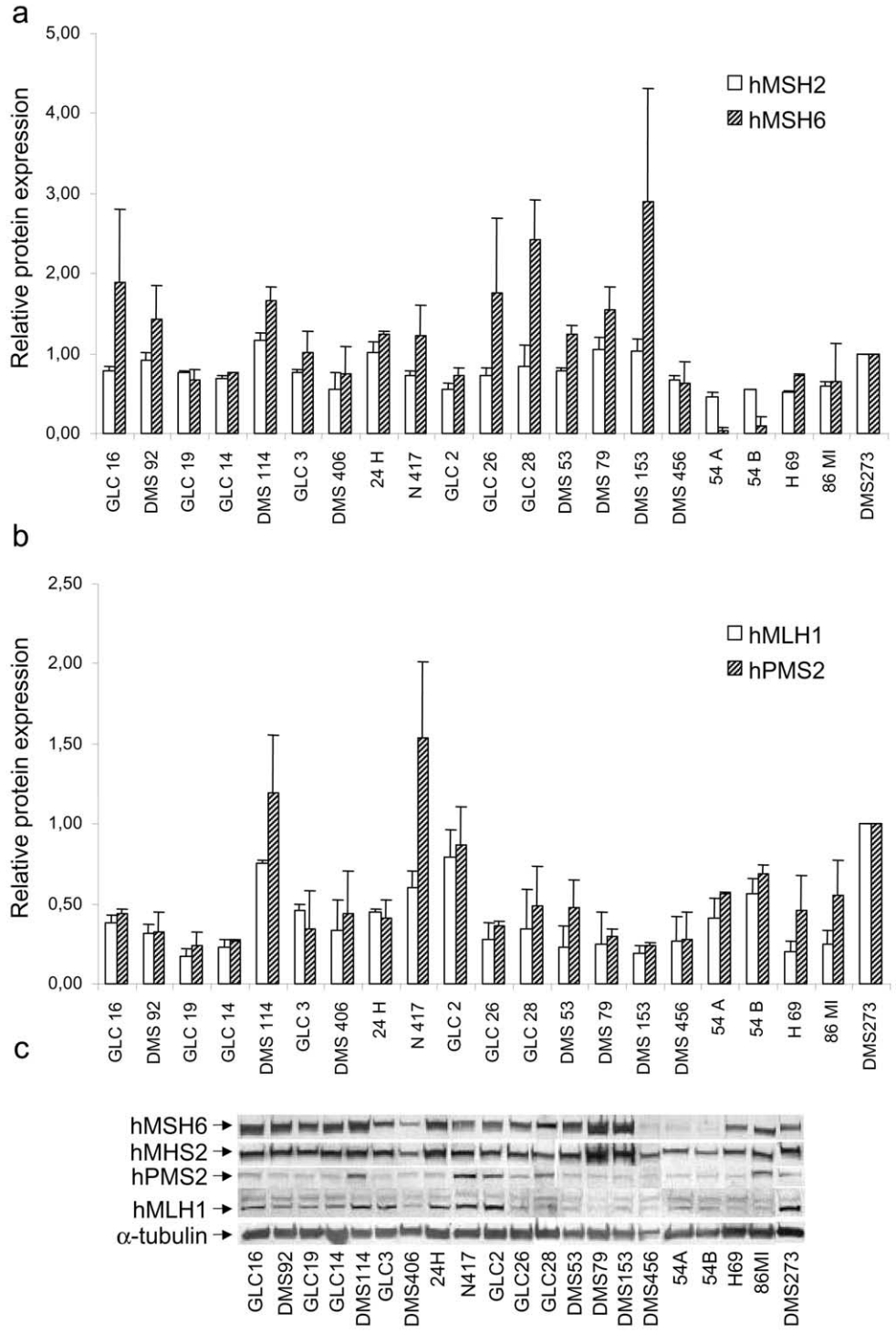

Fig. 2. Heterogeneous expression of MMR proteins in SCLC cell lines: (a) Quantified expression of hMSH2 and hMSH6 proteins; (b) quantified expression of hMLH1 and hPMS2 proteins. Histograms represent mean values of two independent experiments; bars, S.E.M. (c) Western blots of SCLC cell line proteins stained with anti-hMSH2, -hMSH6, -hMLH1, -hPMS2 and anti-α-tubulin.

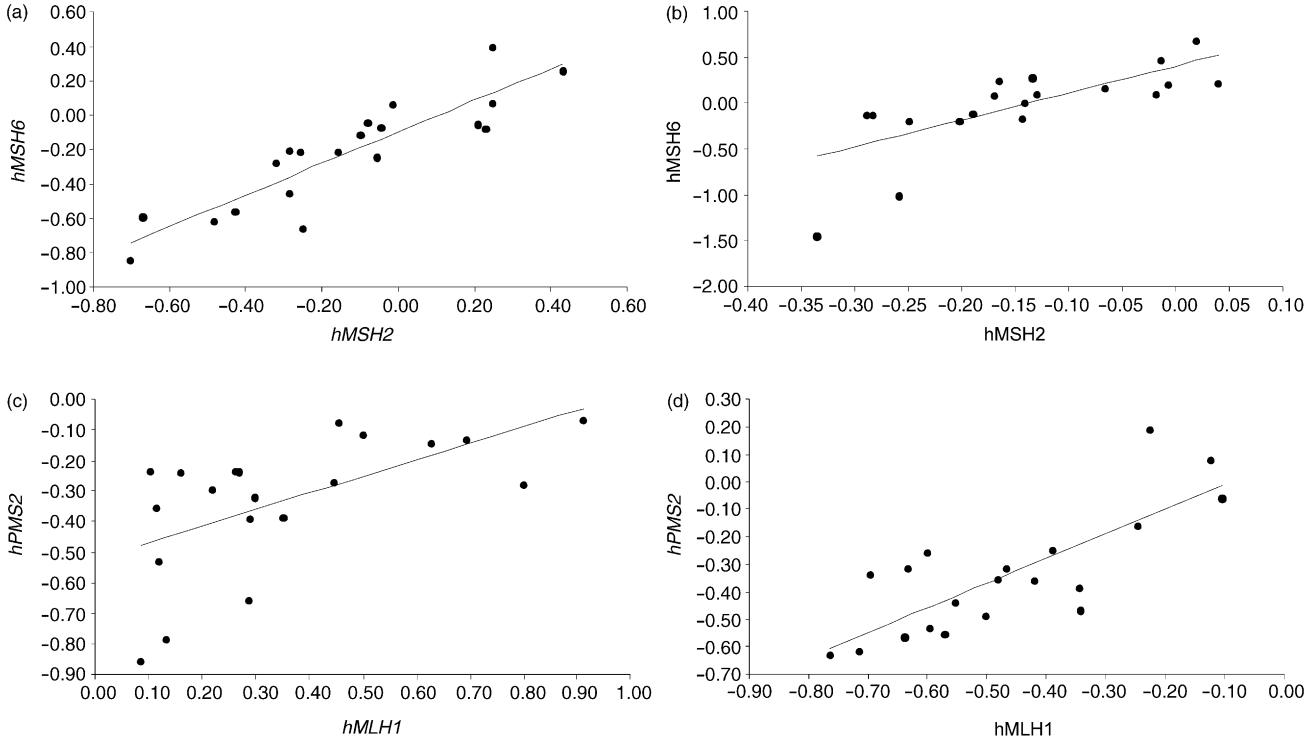

Fig. 3. Coordinated expression of heterodimeric MMR partners: (a) plot of *hMSH2* and *hMSH6* mRNA levels; $r=0.89$, $P<0.001$; (b) plot of hMSH2 and hMSH6 protein levels; $r=0.75$, $P<0.001$; (c) plot of *hMLH1* and *hPMS2* mRNA levels; $r=0.59$, $P<0.01$; (d) plot of hMLH1 and hPMS2 protein levels; $r=0.79$, $P<0.001$. Each data point represents logarithmic mean values of two independent experiments. Correlation coefficients (r) are determined by the Pearson's product moment correlation.

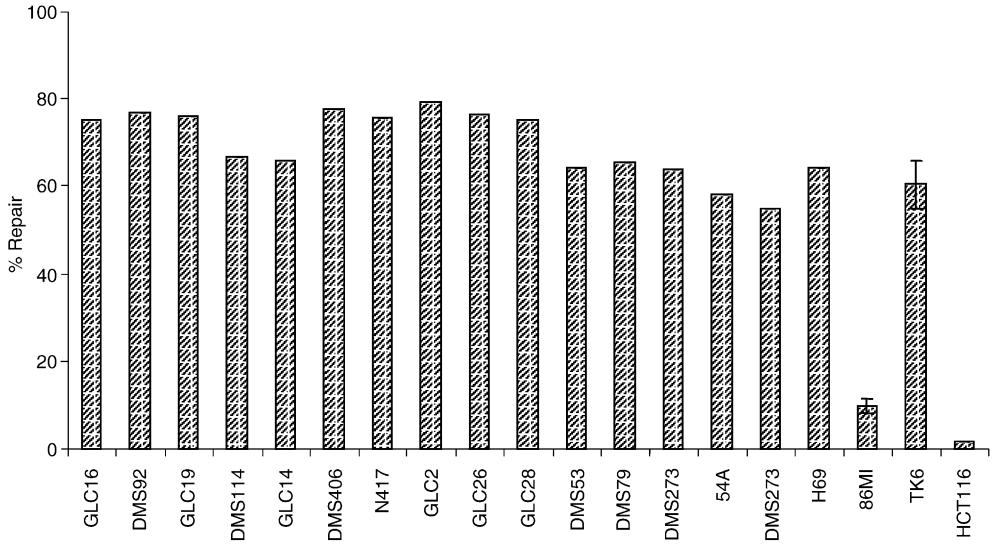

Fig. 4. MMR activity of SCLC cell lines. Repair values are expressed as percentage of mock-treated controls. HCT116 and TK6 served as controls. For 86MI and TK6, histograms represent mean values of two independent experiments; bars, S.D. Data on TK6 originates from Ref. [29].

3.5. Microsatellite instability

The largely monomorphic microsatellite marker, BAT26, was examined in DNA samples from all SCLC cell lines, as well as the control cell lines, HCT116 and DLD-1. None of the 21 SCLC cell lines appeared to display aberrations at the BAT26 locus, whereas HCT116 and DLD-1 exhibited MSI (Fig. 6a). Since up to 15% of colorectal tumours that display MSI do not show alteration of the BAT26 locus, we examined MSI in the 86MI cell using the NCI/ICG-HNPCC recommended panel of microsatellite markers [8]. Single cell

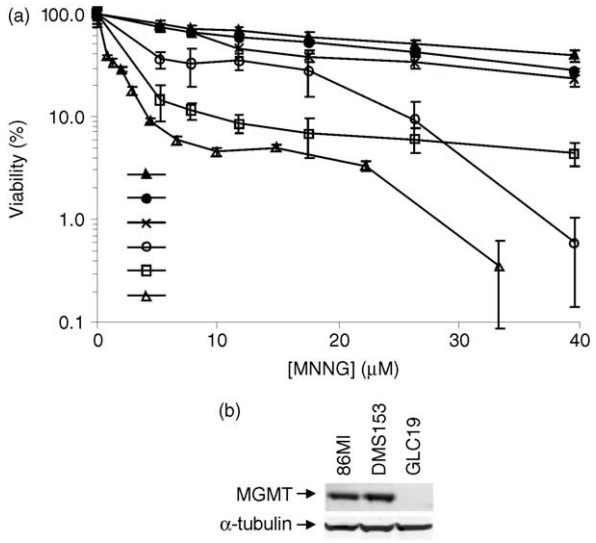

Fig. 5. MNNG resistance of the MMR-deficient 86MI SCLC cell line: (a) cellular MNNG sensitivity of MMR-deficient (86MI, HCT116 and DLD-1) and MMR-proficient (TK6, GLC19 and DMS153) cell lines measured by the MTT assay. Each data point represents the mean value of eight wells; bars, S.D. (b) Western blots of 86MI, DMS153 and GLC19 protein extracts stained with anti-MGMT and anti-α-tubulin.

Table 1
The MNNG concentration that results in a 50% inhibition of growth (IC_{50}) in 86MI compared with cell lines deficient or proficient in MMR

Cell line	IC_{50} (μM MNNG)	*P* value
86MI	22.74±1.21[b]	–
HCT116	20.20±1.90[b]	>0.05[a]
DLD-1	19.58±1.04[c]	>0.05[a]
DMS153	4.96±1.21[b]	<0.001[a]
GLC19	3.87±0.68[b]	<0.001[a]
TK6	1.15±0.19[b]	<0.001[a]

S.D., standard deviation; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; MMR, mismatch repair.
[a] Two-sided *t*-test.
[b] Median±S.D., $n=3$.
[c] Mean±S.D., $n=2$.

clones were isolated from the 86MI cell line and five MSI loci were analysed (BAT25, BAT26, D5S346, D2S123 and D17S250). None of the 23 single-cell clones contained microsatellite alterations at any of the five loci (Fig. 6b).

3.6. *Investigation of the* hExo1 *gene and other MMR components in 86MI*

Other MMR genes, i.e. *hExo1, hMSH3, hMLH3*, and *MBD4* (*MED1*) have also been implicated in human MMR and carcinogenesis [1,22,24,26,33–37]. When compared with the MMR-proficient DMS273 SCLC cell line, the MMR-deficient 86MI cell line showed a comparable expression of the *MED1* mRNA, as well as the hMSH3 and hMLH3 proteins (Fig. 7a and b). For *hExo1*, a very low mRNA expression was observed in the 86MI cells (Fig. 7a). However, by a subsequent Western analysis, equal hExoI protein levels were observed in the DMS273 and 86MI cell lines (Fig. 7b). Moreover, a sequence analysis revealed a wild-type *hExo1* gene in the 86MI cell line (data not shown).

4. Discussion

In the present study, we have examined the MMR pathway in a panel of 21 SCLC cell lines by combining protein and mRNA expression analysis, functional MMR activity studies and examination of MSI. In addition, we studied *hMLH1* promoter methylation and cellular sensitivity to MNNG in selected SCLC cell lines. We found that all SCLC cell lines express the four MMR genes and proteins, hMSH2, hMSH6, hMLH1 and hPMS2 (Figs. 1 and 2). We observed highly heterogeneous expression levels of both mRNA and protein among the cell lines (Figs. 1 and 2). The reason for this heterogeneity is unknown. Loss of heterozygosity at MMR gene loci as reported for the *hMLH1* and *hMSH3* loci in non-small cell lung cancers (NSCLCs) may be a possible explanation for this phenomenon [38].

We detected a significant correlation in expression between components of the same MMR heterodimer for both hMSH2–hMSH6 and hMLH1–hPMS2 (Fig. 3b and d). Such a correlation has been previously reported for hMSH2–hMSH6 [32]. We also detected a significant correlation in mRNA expression between *hMSH2* and *hMSH6*, as well as *hMLH1* and *hPMS2* (Fig. 3a and c). These data indicate coordination in the expression of functionally related MMR genes.

Absent or low *hMLH1* expression as a consequence of promoter CpG methylation has previously been shown in colorectal cancers [2]. We examined two cell lines that displayed an 8-fold difference in *hMLH1* mRNA expression (H69 and DMS114) for altered *hMLH1* promoter methylation. No methylated CpG sites were observed. This result is consistent with the notion that heterogeneous levels of *hMLH1* mRNA are not the result of altered CpG methylation in these SCLC cell lines. Interestingly, CpG methylation of the *hMLH1* promoter and consequent loss-of-expression of the hMLH1 protein has been observed in at least one SCLC cell line (R. Brown, University of Glasgow, Glasgow, UK). However, our results would appear to suggest that this is a rare occurrence in SCLC cell lines and perhaps SCLC tumours.

We found that 16 out of 17 SCLC cell lines were fully proficient for MMR of a $(TT)_2$ loop mismatch substrate (Fig. 4). It is interesting to note that equivalent wild-type MMR proficiency was observed regardless of the

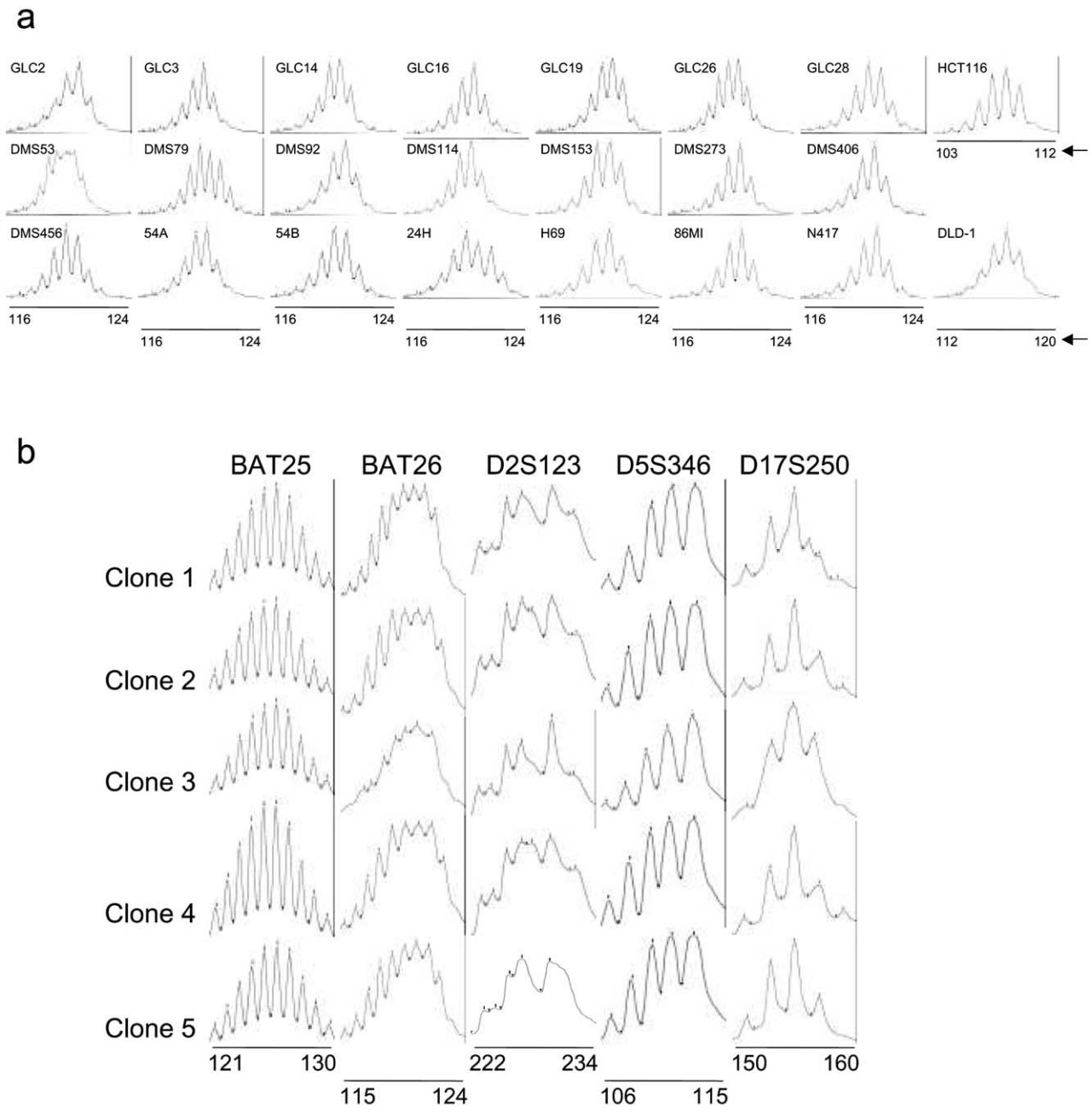

Fig. 6. No evidence of MSI in SCLC cells: (a) analysis of the BAT26 microsatellite locus in DNA from 21 SCLC cell lines and two MMR-deficient control cell lines, HCT116 and DLD-1; (b) analysis of the NCI/ICG-HNPCC microsatellite markers in single cell clones of the 86MI cell line. Shown are five representative single cell clones; bars indicate migration positions of each sample compared with an internal marker. Arrows indicate the different migration position of BAT26 in the HCT116 and DLD-1 cells.

relative levels MMR proteins. One cell line, 86MI, showed a pronounced reduction in MMR activity of the $(TT)_2$ loop mismatch substrate that was similar to the well-defined MMR-deficient HCT116 cell line (Fig. 4). The 86MI cell line displayed an average expression level of hMSH2, hMSH6, hMLH1, and hPMS2 at both the mRNA and protein level (Figs. 1 and 2). This result suggests that the MMR deficiency is either caused by a missense mutation that does not alter the mRNA or protein or that the deficiency resides within another known or unknown MMR gene. Since the MMR assay examines the repair of a 2-bp insertion–deletion loop type (IDL) substrate, we consider it likely that one of the other MMR proteins is altered. For example, both hMSH3 and hMLH3 have been shown to be specifically associated with IDL repair in yeast [39]. Moreover, both hMSH3- and hMLH3-deficient cells display a modest MSI phenotype that is largely confined to di-, tri- and tetra-nucleotide repeats [35]. However, the 86MI cell line expressed both the hMSH3 and hMLH3 proteins at

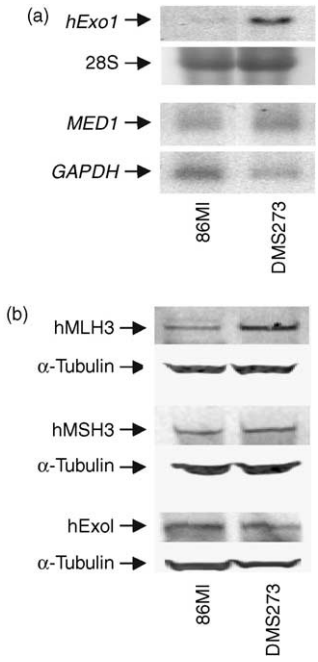

Fig. 7. Analysis of the *hExo1* gene and other MMR components in the 86MI cell line: (a) northern blots of mRNAs from the MMR-deficient 86MI and MMR-proficient DMS273 SCLC cell lines probed with *hExo1*, 28S, *MED1* and *GAPDH*; (b) western blots of 86MI and DMS273 protein extracts stained with anti-hMLH3, -hMSH3, -hExoI and anti-α-tubulin.

levels comparable to the MMR-proficient SCLC cell line (DMS273) (Fig. 7b). The *MBD4* (*MED1*) gene has previously been implicated in the repair of G:T mismatches and binding to hMLH1, and is mutated in MSI-positive cancers of various histological subtypes [36,37]. We constructed a *MED1* gene probe and observed a comparable *MED1* mRNA expression between the 86MI and DMS273 cell lines (Fig. 7a). Finally, we examined the *hExo1* gene expression of the 86MI line and observed almost undetectable levels of *hExo1* mRNA compared with the DMS273 cell line (Fig. 7a). The 5′→3′ exonuclease, hExo1 has recently been shown to bind to hMSH2, hMSH3 and hMLH1 suggesting a role for hExo1 in MMR [22,24,26,33]. However, in the 86MI cell line a sequence and western analysis revealed a wild-type *hExo1* gene and a hExoI protein level equivalent to the DMS273 cell line (Fig. 7b). Hence, expression analysis of a large number of MMR genes and proteins implicated in human carcinogenesis provided no direct explanation of the MMR-deficient phenotype of the 86MI cell line.

None of the SCLC cell lines displayed MSI at the BAT26 largely monomorphic locus (Fig. 6a). However, in at least one study the BAT26 locus was reported unaltered in 15% colorectal tumour samples that were defined as high MSI tumours by using the NCI/ICG-HNPCC recommended panel of microsatellite markers [8]. Thus, in the 21 SCLC cell lines, we cannot exclude that a MSI-positive phenotype in a few cell lines is missed by our analysis of the BAT26 locus. Nevertheless, when the panel of NCI/ICG-HNPCC microsatellite markers was examined in 23 single cell clones of the 86MI cell line, no MSI was detected (Fig. 6b). In a previous study, MSI analysis of only 14 single-cell clones of the MMR-deficient HCT116 cell line were shown to be sufficient to detect allelic shifts at the D5S346 (APC) locus in more than half of the subclones [31]. In contrast, we observed no alterations at the D5S346 (APC) locus in 23 subclones of the 86MI cell line (Fig. 6b). These results are consistent with at least three possibilities. One is that the panel of five MSI markers employed is too small to detect MMR-dependent MSI in SCLC tumours that is suggested to be less than 2% [9]. Secondly, previous studies that demonstrated high MSI frequencies (45–76%) in SCLC have employed other markers [10–12]. Thus, it is formally possible that another panel of validated markers will be required to examine MSI in SCLC. Finally, it is possible that the 86MI cell line harbours a mutation(s) in a yet unknown MMR gene that does not significantly contribute to MSI or a different pattern of MSI. In support of this possibility, a previously described Werner syndrome fibroblastoid cell line, PSV811, has been shown to display a modest MMR-deficient phenotype with no detectable MSI [40].

The 86MI cell line was also found to be significantly resistant to MNNG (Fig. 5). Such resistance has only been observed in cells containing a mutation of *hMSH2*, *hMSH6*, *hMLH1* or *hPMS2* [6,7,20]. Mutation of *MSH3* does not result in resistance to MNNG [6,41]. In order to test if the MNNG-resistant phenotype of the 86MI cell line was caused by MGMT overexpression, the MGMT protein level was measured in 86MI and the MNNG-sensitive DMS153 and GLC19 cell lines (Fig. 5b). In 86MI and DMS153, comparable MGMT levels were observed. This indicates, that the MNNG-resistant phenotype of the 86MI cell line is unrelated to MGMT. In the GLC19 cell line, the MGMT protein was undetectable. However, compared with DMS153 the GLC19 cell line was equally sensitive to MNNG (Table 1), indicating that the O_6-benzylguanine pretreatment has completely depleted the DMS153 cells of cellular MGMT activity.

The phenotype of 86MI is consistent with at least two possibilities. A mutation in one of the four MMR genes, which dissociates MSI from drug resistance, or inactivation of a secondary known or unknown MMR gene that produces negligible levels of MSI.

On the basis of our findings, we suggest that a heterogeneous expression pattern of MMR genes, as observed in our panel of SCLC cell lines, does not necessarily reflect the functional MMR phenotype of the specific cell type analysed. Finally, MMR deficiency and consequent MSI and resistance to alkylating agents appear to be rare in human SCLC tumours.

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