Mismatch Repair Defects and O⁶-Methylguanine-DNA Methyltransferase Expression in Acquired Resistance to Methylating Agents in Human Cells*

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† The abbreviations used are: O⁶-meGua, O⁶-methylguanine; MGMT, O⁶-meGua-DNA methyltransferase; MNU, N-methyl-N-nitrosourea; PCR, polymerase chain reaction; (k)bp, (kilo)base pair(s); CMV, cytomegalovirus; TBS, Tris-buffered saline; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

Fifteen variants with ≥30-fold resistance to N-methyl-N-nitrosourea were isolated from the Burkitt’s lymphoma Raji cell line. Eight had received a single treatment with a highly cytotoxic dose. The remainder, including the previously described RajiF12 cell line, arose following multiple exposures to initially moderate but escalating doses. Surprisingly, methylation resistance arose in three clones by reactivation of a previously silent O⁶-methylguanine-DNA methyltransferase gene. Five clones, including RajiF12, displayed the microsatellite instability and increased spontaneous mutation rates at the hypoxanthine-guanine phosphoribosyltransferase locus, consistent with deficiencies in mismatch repair. Defects in either the hMutSα or hMutLα mismatch repair complexes were identified in extracts of these resistant clones by in vitro complementation using extracts from colorectal carcinoma cell lines. Defects in hMutLα were confirmed by Western blot analysis. Remarkably, five methylation-resistant clones in which mismatch repair defects were demonstrated by biochemical assays did not exhibit significant microsatellite instability.

The ability to remove altered bases from DNA is central to cellular protection against DNA damage by cytotoxic drugs. Removal can be effected by excision repair, which may involve the replacement of relatively long or short stretches of DNA or by direct reversal of the damage. An example of the former is provided by the excision of cisplatin-DNA adducts by the long-patch nucleotide excision repair pathway. Loss of the nucleotide excision repair pathway in the genetic disorder xeroderma pigmentosum is associated with sensitivity to cisplatin (for review see Ref. 1). The in situ demethylation of DNA O⁶-methylguanine (O⁶-meGua)³ by the O⁶-meGua-DNA methyltransferase (MGMT) provides an example of the latter strategy, and loss of MGMT expression in the Mex⁻ (or Mer⁻) phenotype confers sensitivity to methylating agents. This selective sensitivity of Mex⁻cells directly implicates persistent DNA O⁶-meGua lesions in cell death following exposure to methylating agents (for review see Ref. 2).

As an alternative to DNA lesion removal, tolerance mechanisms also provide escape from cytotoxic DNA damage. One known mechanism of DNA damage tolerance in human cells is loss of DNA mismatch repair. The DNA mismatch correction pathway normally corrects replication errors and prevents recombinational exchanges between nonidentical DNA sequences (for review see Ref. 3). The usual substrates for mismatch correction are mispaired or unpaired normal DNA bases. There is increasing evidence that mismatch repair proteins play a significant part in processing diverse types of drug-induced DNA lesions (for review see Ref. 4). Mismatch repair interacts with DNA damage including O⁶-meGua, 6-thioguanine, and as yet undefined alterations introduced by cisplatin and doxorubicin. It contributes directly to the cytotoxicity of these lesions, and mismatch repair-competent cells may be sensitive to their lethal effects, although other mediators of cell death such as the p53 and p21 proteins are sometimes important for cytotoxic manifestations (5). Cells that acquire resistance to prolonged drug exposure are found to have defects in mismatch repair functions (6–10). The phenomenon of cellular resistance acquired through mismatch repair defects is known as tolerance because the DNA lesions persist but, in the absence of mismatch repair, they are not processed into lethal intermediates and are unable to exert their potential cytotoxic effects. Resistance to these kinds of drugs is a significant therapeutic problem, and DNA damage tolerance may be of clinical importance.

The association of the human cancer syndrome Hereditary Non Polyposis Colorectal Cancer (HNPPC) with defective DNA mismatch repair has provided the genetic framework by which to define the human pathway (11, 12). Complementary biochemical studies have implicated five mismatch repair proteins in the early steps of the correction process. These proteins are encoded by the hMSH2, hMLH1, hMSH6/GTBP, hPMS2, and hMSH3 (also known as DUP1 or MRPI (13)) genes (14–19). The initial correction steps involve the interactions of a number of heterodimers formed by these proteins. Thus, current models of mismatch correction suggest that the initial mismatch recognition is likely to be carried out by one of two complexes designated hMutSα and hMutSβ (16, 20, 21), which bind to the mismatched DNA segment. The hMutSα heterodimer is composed of hMSH2 and hMSH6/GTBP. In the hMutSβ complex, hMSH2 is partnered by hMSH3. The α and β recognition factors have different, but partly overlapping, specificities for mismatch binding that depend on the mismatch itself and perhaps also on the context in which it appears (21). hMutSα preferentially recognizes single-base mismatches (transition/transversion intermediates) and single-base loops (frameshift intermediates) that arise by DNA slippage during replication of tracts of repeated mononucleotides. The preferred substrates of
hMutSβ are two to four base loops. Although this simple model is compatible with much of the experimental evidence, the properties of the only reported hMSH3-defective human cell line suggest that mismatch recognition by the α and β complexes may be governed by more complex factors than these simple numerical rules (19). After recognition of the mismatch, a second heterodimer, hMutLa, is recruited (17). This complex comprises the hMLH1 and hPMS2 proteins and probably serves to assemble the components necessary for the excision of the mismatched DNA segment.

Deficiencies in both the hMutSo and hMutLo complexes have been found in drug-resistant cells (reviewed in Ref. 22). To investigate the relative frequencies of defective hMutSo and hMutLo complexes in tolerance to a methylating agent, we isolated and characterized a number of N-methyl-N-nitrosourea (MNU)-resistant variants of the Raji cell line. We evaluated two different protocols that were designed to mimic different therapeutic regimes. In the first, cells were exposed to a single high dose of the methylating agent. The second involved chronic exposure to escalating MNU doses. Several methylation-tolerant clones were isolated using both treatment regimes. Three independent clones had acquired MNU resistance through reexpression of their silent MGMT gene. We examined several phenotypic characteristics and defined the mismatch repair defect in a number of the remaining methylation-tolerant clones. These defects included hMutLo but were predominately in hMutSo, most likely hMSH6/GTBP. In several resistant clones, a demonstrable defect in mismatch repair was not accompanied by a detectable mutator phenotype.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from Sigma except where stated otherwise. Formamide (Fluka) was deionized with AG501-X8 resin (Bio-Rad). Recrystallized MNU was a gift from Dr. Peter Swann, Department of Biochemistry, University College London, UK. Antibodies against hMLH1 and hPMS2 were obtained from Pharmingen and against hMSH2 from Santa Cruz Biotechnologies.

Cell Lines and Culture—The TK−Mex Raji cell line is routinely maintained in our laboratory. At the start of this study, three cultures of Raji cells were expanded from a small inoculum (106 cells), and a single clone was isolated from each population by dilution and seeding into 96-well plates. These three clones were used to generate MNU-resistant derivatives. The LoVo, DLD-1 and HCT116 colorectal carcinoma cell lines were obtained from C. Dixon, Cancer Genetics Laboratory, Imperial Cancer Research Fund and cultured as described previously (23). Isolation of MNU-resistant Cells—Multiple cultures of exponentially growing cells, 105–106 cells in 10 ml RPMI medium supplemented with 10% fetal calf serum, were treated with 500 μg/ml MNU for 28 days of culture to allow the outgrowth of survivors, surviving cells were then expanded to 106 cells and cloned by single cell plating in 96-well plates. These three clones were used to generate MNU-resistant variants of the Raji cell line. We evaluated two different protocols that were designed to mimic different therapeutic regimes. In the first, cells were exposed to a single high dose of the methylating agent. The second involved chronic exposure to escalating MNU doses. Several methylation-tolerant clones were isolated using both treatment regimes. Three independent clones had acquired MNU resistance through reexpression of their silent MGMT gene. We examined several phenotypic characteristics and defined the mismatch repair defect in a number of the remaining methylation-tolerant clones. These defects included hMutLo but were predominately in hMutSo, most likely hMSH6/GTBP. In several resistant clones, a demonstrable defect in mismatch repair was not accompanied by a detectable mutator phenotype.

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Isolation of MNU-resistant Cells—Multiple cultures of exponentially growing cells, 105–106 cells in 10 ml RPMI medium supplemented with 10% fetal calf serum, were treated with 500 μg/ml MNU. After a period of 28 days of culture to allow the outgrowth of survivors, surviving cells were then expanded to 106 cells and cloned by single cell plating in 96-well plates. Chaonically treated cells received 0.01 μM MNU. When exponential growth resumed, they were treated with 0.02 μM MNU. The procedure was repeated using successive treatments with 0.03, 0.04, 0.05, 0.1, 0.32, and 0.5 μM MNU. Clones were isolated by single-cell plating. A single representative of each treated culture was chosen for further characterization.

O6-Methylguanine-DNA Methyltransferase Measurements—O6-Methylguanine-DNA methyltransferase activity in cell extracts was measured using heat-depurinated [H3]MNU-treated DNA as described previously (23).

Microsatellite Instability—Analysis was performed on subclones derived from each resistant cell clone. Colonies that had undergone approx- imately 20 cell doublings were lysed in situ in 96-well plates, and aliquots were removed for PCR. Dinucleotide repeat microsatellites were amplified using fluorescent-labeled primers. Lengths were determined on an ABI automatic DNA sequencer as described previously (23). Four loci were analyzed: D10S197, D11S904, D13S175, and D17S941. Mononucleotide repeat microsatellites, Bat25, Bat26, and Bat40 were amplified, separated on sequencing gels, and analyzed by Southern blotting using one of the radiolabeled PCR primers as a probe.

Mutation Rate Analysis— Cultures (25 for each clone tested) were initiated using small (100 cell) inocula and expanded to 106 cells, which were distributed in 96-well plates (approximately 105 cells/well) in medium supplemented with 5 μg/ml 6-thioguanine. After 28 days, the frequency of positive wells containing 6-thioguanine-resistant cells was determined. Mutation rates were calculated from the equation, M = ln (1 - P). In 2, where P is the frequency of mutants, and C is the total number of cell plaques in a well. In some cases, mutation frequencies were estimated by plating growing cultures in 96-well plates under the same selective conditions.

O6-Methylguanine Processing Assay—Cell extracts were prepared as described (24). Plasmid pSVori methylated with 0.48 mM MNU for 30 min at 37 °C was incubated with extract in the presence of [3H]dATP, and incorporation of radioactivity into material adhering to DE81 paper (Whatman) was determined as described (24).

Mismatch Binding Assay—The preparation of cell extracts and details of the bandshift assay for mismatch binding have been described previously (25). The substrates were 34-mer-duplex oligonucleotides containing a single GT mispair (25) or an unpaired CA dinucleotide (duplex C in Ref. 26).

Mismatch Repair Substrate—The substrate for in vitro mismatch correction was constructed from molecules derived by subcloning a 211-bp PvuI/DraI fragment of the previously described HK7 M13 (27) into the pBR-CMV phagemid (Stratagene). The inserted region contained the heteroduplex cassette sequence that can be used to generate specific mismatches within restriction endonuclease sites that are diagnostic for mismatch correction.

For the experiments described in this paper, C-containing viral strands were purified by standard techniques (28). Closed circular duplexes that contained T in the complementary position were purified by banding on CsCl gradients. After ethanol precipitation, duplex circular DNA was linearized by digestion with NotI. After phenol extraction and ethanol precipitation, linear DNA (150–250 μg) was mixed with a 2-fold excess of single-stranded DNA, and the mixture was adjusted to 50% formamide, 10 mM EDTA, pH 8.0, in a total volume of 2–3 ml. The mixture was dialyzed sequentially against 95% formamide, 10 mM EDTA, pH 8.0, for 2 h; 50% formamide, 200 mM Tris–HCl, 10 mM EDTA, pH 8.0, for 2 h; 100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA for 2 h; and finally against 10 mM Tris–HCl, 1 mM EDTA, pH 7.5, for 2 h. Nicked circular molecules were purified by agarose gel electrophoresis and electroelution. The purified 4470-bp molecules contain a unique CT mispair that inactivates an MluI restriction site. The mispaired T is 580-5 bp of a single nick (see Fig. 1). Small amounts of renatured matched linear molecules were present in all preparations. These and their MluI digestion products could be easily resolved from the diagnostic products and did not detectably affect the correction assay.

In Vitro Mismatch Correction—Cell extracts were prepared from 1–5 × 109 cells as described previously (24). Mismatch correction was assayed in 25 μl of 30 mM Hepes–KOH, pH 8.0, 7 mM MgCl2, 0.5 mM dithiothreitol, 0.1 mM each dNTP, 4 mM ATP, 40 mM phosphocreatine, 1 mM of creatine phosphokinase (rabbit muscle-type I), 90 ng of DNA substrate, and up to 200 μg of cell extract. Mixtures were incubated for 80 min at 37 °C. The reaction was terminated by addition of 10 mM EDTA, 0.5% SDS. Samples were freed of protein by proteinase K digestion (1 mg/ml, 15 min) followed by phenol extraction. DNA was ethanol-precipitated, dissolved in buffer, and digested with MluI, which is diagnostic for removal of the mismatch. Digestion products were separated on 0.8% agarose gels in 40 mM Tris-acetate, pH 8.0, 1 mM EDTA buffer containing ethidium bromide and visualized under short wave-length ultraviolet light. The mismatched substrate is shown schematically in Fig. 1. An MluI site is located at position 463, and digestion of the uncorrected substrate generated unit-length linear 4470-bp molecules. Digestion of molecules that have undergone nick-directed correction (TC to GC) to generate the second MluI site produces two fragments of 3.9 kbp and 567 bp (see Fig. 4, fragments A and D). Digestion of the small amount of contaminating matched linear molecules generated during the annealing reaction produces traces of fragments of 3.3 and 1.17 kbp (see Fig. 4, fragments B and C) that are resolved from the products of mismatch correction. Thus, digestion with MluI of DNA recovered after incubation with repair-proficient cell extracts generated a mixture of unit-length linear molecules and fragments A–D. The smaller fragments (C and D) were not generally visible. In the products recovered from repair-deficient cell extracts, only a single DNA fragment was visible together with a small amount of fragment B from contaminating linear molecules. Thus, the presence of fragment A (3.9 kbp) that is resolved from unit-length 4.47-kbp molecules, is diagnostic for mismatch correction.

hMSH2 Sequencing—Cytoplasmic RNA was extracted from parental Raji cells and RajiF12 variant and used to generate hMSH2 cDNA.
Mlu generates a second restriction site that is diagnostic for repair. Using Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Schwalbach/Taunus, Germany). PCR was used to amplify the hMSH2 cDNA in five overlapping fragments. The following primers, designed to introduce BamHI and EcoRI recognition sequences, were used: fragment 1, CGGGATCCCAACAGGAGGTTGAGAGG and CGGAATTCTGGGCAACTCGGACAT; fragment 2, CGGGATCCGAGTCCTCTTTGTGGTC and CGGAATTCGCGCTGAT; fragment 3, CGGGATCCCTAGGTACG and CGGAATTCGACAATAGCTTATCAATATTACC. The sequences of the primers used to amplify fragments 2, 3, 4, and 5 were taken from Ref. 29. The PCR reactions were carried out in 100 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl2, 250 μM each dNTP, 0.15 μM each primer, and 10 units/μl AmpliTaq DNA polymerase (Perkin-Elmer). 30 cycles of 93 °C for 1 min, 60 °C for 2 min, 72 °C for 3 min, followed by 1 cycle of 72 °C for 10 min were performed. Fragments were purified from agarose gels and ligated into M13mp18 or M13mp19 vectors (Life Technologies, Inc., Paisley, UK). After bacterial transformation, individual clones were sequenced using M13 primer.

**RESULTS**

Resistance to MNU—Eighteen individual cultures of the Mex Burkitt’s lymphoma cell line Raji were treated with MNU. Nine cultures received a single treatment with 0.5 mM MNU that resulted in an estimated survival of 10^-7. The remainder were treated with an initial dose of 0.01 mM; surviving cells were allowed to recover and were retreated with 0.02 mM MNU. This regime of repeated treatments was continued with escalating doses up to a maximum of 0.5 mM. Individual colonies were isolated from each treated culture by cloning in 96-well plates. To ensure independence, only one MNU-resistant clone from each culture was characterized. Fifteen clones were analyzed further.

Fourteen clones exhibited an increase in MNU resistance of at least 30-fold as measured by the drug concentration required to arrest cell growth. Proliferation of the parental Raji cells was inhibited by treatment with 0.01 mM MNU (Fig. 2). Following treatment, these sensitive cells underwent one cell division during the first 24 h, but thereafter there was no further increase in cell number. In contrast, clones isolated after acute or chronic MNU treatment withstood exposure to ≥0.3 mM MNU and continued to proliferate at rates closely similar to that of the untreated controls. Two examples are shown in Fig. 2. The extent of MNU resistance in the isolated clones was comparable to that previously reported for the methylation-tolerant RajiF12 cells (6). The effect of higher MNU concentrations was not systematically evaluated for all of the clones, but some, for example Raji10, were resistant up to at least 1 mM MNU. In all, 14 of 15 clones, 8 from acute exposure and 6 from chronic exposure to escalating drug doses, exhibited resistance to 0.3 mM MNU. The clones isolated after acute treatment were given a single number designation: Raji 3, Raji 7, etc. Those derived by chronic exposure were numbered Raji 101, Raji 102, etc. One
Table I
Summary of properties of MNU-resistant clones

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>1</th>
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<th>3</th>
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<th>5</th>
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<tr>
<td>Mex status</td>
<td>Mex+</td>
<td>Mex-</td>
<td>Mex-</td>
<td>Mex-</td>
<td>Mex-</td>
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<tr>
<td>(A)n repeats</td>
<td>Stable</td>
<td>Unstable</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>(CA)n repeats</td>
<td>Stable</td>
<td>Unstable</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>Relative HPRT mutation rate(a)</td>
<td>x1</td>
<td>x4</td>
<td>x3-4</td>
<td>x1</td>
<td>x1</td>
</tr>
<tr>
<td>Mismatch binding</td>
<td>Proficient</td>
<td>Proficient</td>
<td>Proficient/Deficient</td>
<td>Deficient</td>
<td>Proficient</td>
</tr>
<tr>
<td>Mismatch repair</td>
<td>Proficient</td>
<td>Proficient</td>
<td>Proficient</td>
<td>Proficient</td>
<td>Proficient</td>
</tr>
<tr>
<td>Representative clones</td>
<td>Raji 101</td>
<td>Raji 105</td>
<td>Raji 102</td>
<td>Raji 103</td>
<td>Raji 104</td>
</tr>
<tr>
<td></td>
<td>Raji 106</td>
<td>Raji 10</td>
<td>Raji 9</td>
<td>Raji 12</td>
<td>Raji 7</td>
</tr>
</tbody>
</table>

\(a\) Significant changes in phenotype are shown in bold type.

Table II
MGMT activity in MNU-resistant clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>MGMT activity units/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raji</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Raji Mex+</td>
<td>0.31</td>
</tr>
<tr>
<td>LoVo</td>
<td>0.7</td>
</tr>
<tr>
<td>Raji 101</td>
<td>0.32</td>
</tr>
<tr>
<td>Raji 105</td>
<td>0.33</td>
</tr>
<tr>
<td>Raji 106</td>
<td>0.20</td>
</tr>
<tr>
<td>Raji 102</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Raji 103</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Raji 104</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

clone, Raji 107, was not significantly more resistant to MNU than Raji and was not characterized in detail, although it served as a control in some experiments. The RajiF12 cell line, isolated following chronic MNU treatment, has been described previously (6).

Resistance to the unrelated DNA cross-linking agent, mitomycin C, was not significantly increased (<2-fold) in any of the the MNU-resistant clones (data not shown). This indicates that the loss of MNU sensitivity in these cells is not a consequence of a generalized resistance to DNA damaging agents arising, for example, through loss of a common apoptosis pathway.

We investigated MGMT expression, microsatellite stability, and spontaneous mutation at the HPRT locus in the resistant clones. These properties together with mismatch binding by cell extracts, were used to assign the resistant clones to five different but overlapping phenotypes. The ability to carry out mismatch correction and to process DNA \(O^6\)-meGua in vitro were also analyzed. The findings are summarized in Table I. Complementation with extracts prepared from colorectal carcinoma cells and Western blot analysis was used to define defective mismatch repair functions in the resistant clones.

**Phenotype 1. Increased MGMT Expression**—The TK\(^+\) variant Raji cells are Mex\(^-\) and cell extracts containing undetectable levels of MGMT (<0.05 units/mg of protein). In contrast, extracts of three of the resistant cell lines, Raji 101, Raji 105, and Raji 106, contained approximately 0.3 units of MGMT/mg of protein. The colorectal carcinoma cell line LoVo included for comparison was also Mex\(^-\) and expressed 0.7 units of MGMT/mg of protein. These values lie in the normal range for Mex\(^-\) cell lines, including the Mex\(^-\) Raji variant (Table II). The remaining clones isolated by the escalating dose regime and all of the clones derived by acute treatment did not contain detectable MGMT activity (Table II and data not shown). Raji 101 and Raji 106 did not exhibit microsatellite instability at either (A)\(_n\) or (CA)\(_n\) repeats (Table III). The HPRT\(^-\) mutation frequency in cultures of these three clones was comparable to that of the parental Raji cells, consistent with the absence of a mutator phenotype. Bandshift assays with cell extracts indicated that all were proficient in the recognition of a GT mismatch and a two-base loop in the standard substrates (Table I, data not shown; see Fig. 6). Extracts of Raji 101, Raji 105, and Raji 106 were also able to correct a single CT mismatch to regenerate an MluI restriction site in the nicked circular DNA duplex (Table I, data not shown). Since Mex\(^-\) Raji cells exhibit comparable 30–50-fold resistance to MNU and there is no evidence of mismatch repair defects in Raji 101, Raji 105, and Raji 106 cells, it seems likely that the resistance of these three clones is a direct consequence of their expression of MGMT. The phenotype of Raji 101, Raji 105, and Raji 106 was designated Phenotype 1 (Table I).

**Phenotype 2. Generalized Microsatellite Instability**—One clone, Raji 10, exhibited considerable instability at both mono- and dinucleotide repeat microsatellites. Examples are shown in Fig. 3, and the data are summarized in Table III. The rate of mutation at (CA)\(_n\) repeats was 5 \(\times\) 10\(^{-3}\) mutations/cell/generation, an increase of >60-fold over the rate in the parental Raji cells. (A)\(_n\) microsatellites were also unstable in Raji 10, and the rate of mutation was increased 20-fold compared with parental Raji cells (80 cf. 4 \(\times\) 10\(^{-6}\) mutations/cell/generation). The rate of mutation at the HPRT locus in Raji 10 measured by fluctuation analysis was 10\(^{-6}\) mutations/cell/generation, approximately 4-fold higher that of the parental Raji cells (2.7 \(\times\) 10\(^{-7}\)cell/generation). Extracts of Raji 10 cells were proficient in binding to both a single GT mispair and a CA loop in the bandshift assay (data not shown) but, unlike extracts of the parental Raji cells, were unable to correct a CT mismatch in the nicked circular heteroduplex (Fig. 4a). In addition, in comparison to the parental Raji cells, Raji 10 cell extracts carried out little mismatch repair-related DNA synthesis on an MNU-methylated plasmid (Fig. 5). These data indicate that Raji 10 cells are also defective in processing \(O^6\)-meGua by the mismatch repair pathway. Raji 10 was the single representative of Phenotype 2. The microsatellite instability, mismatch binding proficiency, and general properties of Raji 10 are consistent with a mismatch repair defect involving the hMutL\(\alpha\) complex.

**Phenotype 3. Selective Microsatellite Instability**—Three clones (Raji 8, Raji 9, Raji 19) together with the previously described RajiF12 cell line, exhibited selective microsatellite instability at (A)\(_n\) microsatellites (Fig. 3; Table III). The (A)\(_n\) microsatellite mutation rate in the four clones was between 3 \(\times\) 10\(^{-3}\) and 10\(^{-2}\) mutations/cell/generation, an increase of 7–25-
fold over the parental Raji cells. There was no evidence of instability at four (CA)ₙ microsatellites (Table III). The spontaneous mutation rate at the HPRT locus in RajiF12 was previously estimated to be 3.5-fold higher than the parental Raji cells (6). This value was confirmed by fluctuation analysis (Raji = 2.7 × 10⁻⁷; RajiF12 = 10⁻⁶ mutations/cell/generation). The HPRT⁻ mutation frequencies in cultures of Raji 8, Raji 9, and Raji 19 were also increased between 3- and 4-fold over the parental level (Table I), compatible with a moderate mutator phenotype at this locus. Extracts of Raji 8, Raji 9, and Raji 19 were all proficient in binding to a GT mispair and to a CA loop in the bandshift assay (data not shown). A GT mismatch binding and single-base mismatch correction defect in RajiF12 have been reported previously (6, 31) and were confirmed in this study (data not shown). All four clones were also unable to correct a CT mismatch in the in vitro assay (Fig. 4 and data not shown). Extracts of Raji 9, Raji 8, Raji 10, and Raji 19 were deficient in processing the MNU-treated plasmid substrate. Despite their CT mismatch correction defect, Raji 8 extracts were competent in O⁶-methylguanine (O⁶-meGua) processing (Fig. 5b) and incorporated similar levels of dAMP to the parental Raji extracts.

Raji 8, Raji 9, Raji 19, and RajiF12 were assigned to Phenotype 3 on the basis of their shared selective microsatellite instability at mononucleotide repeats, evidence of a mutator phenotype at HPRT, and mismatch correction defect. This phenotype is heterogeneous, however, and of the four clones, only RajiF12 is detectably defective in mismatch binding; Raji 8 retains some ability to process O⁶-meGua. Direct sequencing of all 16 exons of the RajiF12 hMSH2 gene did not reveal any mutations. The mismatch binding deficiency of RajiF12 is, therefore, most likely due to a defect in hMSH6/6GTBP.

Phenotype 4. No Extensive Microsatellite Instability—Extracts of two other clones, Raji 102 and Raji 103, exhibited an impaired mismatch binding similar to that previously reported for RajiF12 (Fig. 6). They did not, however, display a significant mutator phenotype at microsatellites or at HPRT as measured by fluctuation analysis (Fig. 3; Tables 1 and 3). Because of this absence of a significant mutator effect, they were assigned to a separate group, Phenotype 4 (Table I). The phenotype of Raji 103 was consistent with a partial defect. A single alteration was observed in one mononucleotide microsatellite in Raji 103 (Table III), but we did not observe enough changes to conclude that Raji 103 exhibited instability. The HPRT mutation rate was also not significantly different from that of the Raji parental cells (3 versus 2.7 × 10⁻⁷). Raji 103 cell extracts were able to perform limited mismatch correction (Fig. 4b) and retained a corresponding detectable, but reduced, level of binding to either a GT mispair or a CA loop (Fig. 6). Binding to an AC mismatch, which is independent of both hMSH2 and hMSH6/6GTBP, by extracts of Raji 103 and the parental Raji cells, was indistinguishable (Fig. 6). This serves as a positive control for the extracts. Extracts of Raji 103 were defective in processing plasmid DNA containing O⁶-meGua (Fig. 5). Incorporation of dAMP into the methylated substrate by Raji 103 extracts was somewhat higher than repair-defective cell extracts, consistent with the partial nature of the mismatch correction and binding defects in these cells.

Raji 102 cells also showed no evidence of microsatellite instability at either (A)ₙ or (CA)ₙ microsatellite loci (Table III), and the spontaneous HPRT mutation rate in Raji 102 was similar to that of the parental Raji cells (1.5 × 10⁻⁷ mutations/cell/generation). Extracts of Raji 102 were not, however, detectably correct the CT mismatch (Fig. 4c) or bind to a GT mispair or to a CA loop (Fig. 6). Raji 102 extracts were also deficient in processing plasmid DNA containing O⁶-meGua (Fig. 5b). The DNA mismatch binding and correction defects were directly implicated in the methylation resistance of Raji 102. A spontaneous revertant to MNU sensitivity, Raji 102B, which arose during normal culture, was fully correction-proficient (Fig. 4c). Extracts of Raji 102B had also regained mismatch binding activity (Fig. 6).

Phenotype 5. Resistance Without Additional Phenotype—The remaining five clones, Raji 3, Raji 7, Raji 12, Raji 17, and Raji 104, were assigned to Phenotype 5 (Table I). These clones remained Mex⁻ as determined by direct assay of MGMT in cell extracts. Representative members, Raji 7, Raji 12, and Raji 104, showed no detectable microsatellite instability at either (A)ₙ or (CA)ₙ microsatellite loci (Table III). Within the limits of detection of the assay, all were proficient at mismatch binding. Notwithstanding the absence of detectable microsatellite instability or deficiency in mismatch recognition, Raji 7, Raji 12, and Raji 104 were found to be defective in CT mismatch correction in vitro (see Fig. 8d). Processing of MNU-treated plasmid DNA was investigated in Raji 12 and Raji 104. Raji 12 extracts did not process the MNU-treated plasmid. Despite the mismatch repair defect, the level of dAMP incorporation by Raji 104 extracts approached that of the repair-competent Raji and Raji 107 extracts (Fig. 5b). The mismatch repair defect in Raji 104 that is associated with methylation tolerance is not, therefore, detectable by this assay.

Identification of Mismatch Repair Defects in Phenotypes 2–5—The CT mismatch correction assay was used to define the
Fig. 3. Examples of microsatellite analysis in MNU-resistant clones. 

a, stability of dinucleotide repeats in Raji 17. CA repeat microsatellite D17S941 was amplified by PCR using fluorescent-labeled primers. Products were separated on DNA sequencing gels and detected by fluorometry. Seven subclones of Raji 17 that had been grown for at least 20 generations are illustrated. Both alleles of this locus appear to be the same length.

b, instability in dinucleotide repeats in Raji 10. Two loci D13S175 (left panel) and D10S197 (right panel) were amplified by PCR and fluorescent primers. Products were separated and detected as above. Eleven subclones for each locus are illustrated. Deviations from the wild-type (wt) pattern are labeled (±2bp).

c–e, instability at poly(A) microsatellites. Poly(A) microsatellites Bat 25 (c) or Bat 40 (d and e) were amplified by PCR. Products were separated on sequencing gels, transferred by Southern transfer, and analyzed by probing with a radioactively labeled primer. c, no instability is apparent in the 10 subclones of Raji 103 illustrated; d, examples of variation in subclones of RajiF12. e, examples of variation in Raji 19. Arrow(s), altered allele(s). A full summary of these analyses is presented in Table III.
biochemical defects in representatives of phenotypes 2–5. Extracts of wild-type HeLa (Fig. 7a) or Raji (Fig. 4) cells carried out efficient repair of the mismatched substrate. The extent of correction increased with increasing extract concentration up to 200 μg of protein/assay, at which point >50% of the mismatched molecules were repaired (Fig. 7a). In contrast, extracts of the colorectal carcinoma cells DLD-1, HCT116, or LoVo, which are known to be defective in mismatch correction, were unable to carry out detectable repair of the mismatched substrate (Fig. 7b). Full repair was restored when 100 μg of HCT116 and a similar amount of DLD-1 extract were combined (Fig. 7b). Partial complementation was also observed when LoVo and HCT116 extracts were mixed.

Colorectal carcinoma cell extracts were used to complement the mismatch repair deficiencies in extracts of the MNU-tolerant clones. Mixing Raji 10 and Raji 9 extracts complemented their defects and restored wild-type levels of CT mismatch repair (Fig. 5a). Wild-type levels of repair were restored to phenotype 2 Raji 10 cells by the addition of DLD-1, but only a very minor increase in correction was achieved by the addition of HCT116 extract (Fig. 5a). Conversely, phenotype 3 Raji 9 cell extracts were fully complemented by HCT116 but not by DLD-1 extracts. All these data are consistent with a defective hMutLo complex in Raji 10 and a defective hMutSo complex in Raji 9 cells. Using a similar approach, repair activity was restored to RajiF12 and Raji 102 by HCT116 but not DLD-1 cell extracts (Fig. 8b). The methylation-tolerant phenotype, the absence of detectable mismatch binding, and the correction defects of these clones are therefore also most likely the result of defective hMutSa. Their selective mononucleotide microsatellite instability indicates that a defect in hMSH6/GTBP is more probable. This was confirmed in the case of RajiF12 by the absence of hMSH2 gene mutations. The partial repair activity of Raji 103 was augmented by the addition of extracts of HCT116 but not of DLD-1 (Fig. 8c). This observation, together with the partial mismatch binding activity of Raji 103 extracts, is consistent with an impaired hMutSa function in Raji 103 cells. We conclude that the methylation tolerance of members of both phenotypes 3 and 4 is associated with defects in hMutSa, most probably in the hMSH6/GTBP subunit.

The same analysis was performed on two of the members of phenotype 5 that were mismatch correction-defective. Combining extracts of Raji 12 and Raji 104 complemented their CT mismatch repair defects and restored wild-type levels of correction. Correction was also restored to Raji 12 cell extracts by the addition of Raji 9 or DLD-1 extracts. In contrast, Raji 10 and HCT116, but not Raji 9 or DLD-1 extracts, complemented Raji 104 (Fig. 8d). Thus, Raji 12 and Raji 104 belong to different biochemical complementation groups. Despite their apparent lack of mutator phenotype, they are defective in hMutLo and hMutSa, respectively.
MGMT and Mismatch Repair in Methylation-resistant Human Cells

**DISCUSSION**

**MGMT Reactivation**—The development of resistant disease bedevils most chemotherapy regimes, and the level of expression of MGMT is a known factor in resistance to anticancer methylating agents such as dacarbazine and temozolomide (32, 33). Among our in vitro derived MNU-resistant cell lines, three clones (Phenotype 1) had gained resistance as a consequence of a stable increase in MGMT expression. The parental Raji cells have retained their Mex<sup>−</sup> phenotype for many years in our laboratory, and these experiments were initiated from clonal Mex<sup>−</sup> populations. This makes it unlikely that the altered Mex status is the result of outgrowth of a subpopulation of preexisting Mex<sup>−</sup> cells. It is more probable that MNU treatment both initiated and selected for the change to a Mex<sup>−</sup> phenotype. MGMT expression is regulated by an epigenetic mechanism in which the usual relationship between cytosine methylation and gene expression is apparently reversed (34, 35). Thus, the silent MGMT gene in Mex<sup>−</sup> cells, including the parental Raji cells used in this study, is hypomethylated at CpG sites compared with its expressed counterpart in Mex<sup>+</sup> cells. Reduction of cytosine methylation by treatment of Mex<sup>−</sup> cells with azacytidine can reduce MGMT gene expression (34), although the relationship between methylation status and MGMT expression is complex (36). It seems probable that reactivation of the previously silent MGMT gene by altered methylation underlies the MNU resistance of the Raji 101, Raji 105, and Raji 106 clones. There is some evidence that chronic MNU treatment of cells can effect increases in DNA cytosine methylation (37) and, although our sample size is too small for statistical significance, it is interesting that all three revertants arose during the escalating dose regime. Although selection for a Mex<sup>−</sup> phenotype has not previously been reported after MNU treatment, chronic exposure of hamster cells to the DNA cross-linking chloroethylnitrosourea, mitozolomide, which kills cells by inducing DNA cross-links that MGMT can prevent, is known to increase MGMT expression without apparent MGMT gene amplification (38). Drug-induced reactivation of the silent MGMT gene may be a more common phenomenon than has been suspected to date.

**Epigenetic Regulation of Mismatch Repair Genes?**—The widespread changes in methylation that are likely to accompany MNU-induced reactivation of MGMT will also tend to alter the expression of other genes. It is possible, therefore, that the high frequency of mismatch repair defects that are commonly observed among methylation-tolerant cell lines

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**FIG. 6.** Mismatch binding by Raji 102 and Raji 103 extracts. Cell extracts (15 μg protein) of Raji 102, Raji 103, the Mex<sup>−</sup> revertant Raji 101 or the parental Raji cells as indicated were incubated with radiolabeled duplexes containing a single GT mispair (left panel), AC (right panel), or CA loop (center). The extreme right panel shows GT mispair binding by 15 μg of Raji 102B, the MNU-sensitive revertant of Raji 102. Bound and free oligonucleotides were separated on 6% polyacrylamide gels as described under “Experimental Procedures.”

**FIG. 7.** CT mismatch correction by HeLa and colorectal carcinoma cell extracts. a, HeLa cell extracts (0–200 μg of protein as shown) were incubated with CT mismatch-containing pBK-MluI DNA as described under “Experimental Procedures.” An extract of DLD-1 cells (200 μg of protein) was included as a control. DNA was recovered and digested with MluI, and the products were analyzed by agarose gel electrophoresis. b, colorectal carcinoma cell extracts. HeLa, HCT116, DLD-1, or LoVo cell extracts (100 μg of protein) were incubated with CT mismatch-containing pBK-MluI DNA as described under “Experimental Procedures.” DLD-1/HCT116 and LoVo/HCT116 were assayed in combination (100 μg of protein from each extract) as indicated. DNA was recovered and digested with MluI, and the products were analyzed by agarose gel electrophoresis.
partly reflects a sensitivity of mismatch repair gene expression to changes in DNA cytosine methylation. Indeed, the frequency at which mismatch repair-defective methylation-tolerant cells arise in our and others’ experiments is probably too high to be accounted for by the mutational inactivation of two mismatch repair alleles, even taking into account the use of potent mutagens in their selection. The properties of Raji 102 cells in particular are consistent with epigenetic changes. Their resistance was correlated with a loss of mismatch binding and repair as a consequence of a defect in the mismatch recognition protein hMSH6/GTBP. Recovery of mismatch recognition and repair ability in Raji 102B coincided with the cells’ spontaneous reversion to normal MNU sensitivity. Similar spontaneous loss of methylation tolerance has been observed in Chinese hamster (6) and HeLa cell lines.2 In the former case, reversion to MNU sensitivity was also accompanied by the reappearance of mismatch recognition activity. Phenotypic reversal of this nature could result from spontaneous reversion of MNU-induced mutations or subsequent spontaneous compensatory mutations. It is perhaps more plausible, however, that alterations in cytosine methylation underlie the reversible loss of hMSH6/GTBP function in some tolerant cells, including Raji 102. Widespread changes in DNA cytosine methylation are common in colon carcinogenesis (39). A susceptibility to silencing might contribute to the loss of expression of critical mismatch repair genes during the development of mismatch repair-defective colon tumors. Loss of repair capacity by gene silencing would not be associated with mismatch repair gene mutations. The recent demonstrations that loss of hMLH1 expression is frequently observed in tumors and tumor cell lines that do not have mutations in the hMLH1 gene and that the absence of expression is correlated with increased cytosine methylation in the hMLH1 promoter (40) are consistent with this suggestion. There are also indications that exogenously supplied genes are more frequently inactivated by cytosine methylation in mismatch repair defective cells (41).

Mismatch Repair Defects Associated with Known Phenotypes—In Raji 10, the only representative of Phenotype 2, instability at both mono- and dinucleotide microsatellites and complementation of repair in cell extracts suggested that the hMutLa complex was defective. Western analysis confirmed this inference. The phenotype of Raji 10 was similar to hMLH1-defective methylation-tolerant colorectal carcinoma cell lines HCT116 (15, 42) and SW48 (23). Human tumor cell lines with deficient hPMS2 also exhibit a methylation-tolerant phenotype (18), suggesting that the hMutLa complex is involved in processing O6-methylguanine into a lethal lesion from which tolerance offers an escape. hMutLa defects are also associated with mismatch repair-related tolerance to methylation damage in HeLa cells (9) and tolerance to cisplatin and doxorubicin DNA damage in ovarian carcinoma cell lines (8). The latter resistant lines do not produce detectable hMLH1 mRNA and provide evidence that the hMLH1 protein is required to stabilize its hPMS2 partner (43). Thus, hPMS2 deficiency can occur as a secondary consequence of hMLH1 defects. Normal amounts of hMLH1 are present in the endometrial carcinoma cell line HeC-1A (8), which is mutated in hPMS2 (18), indicating that hMLH1 may not require hPMS2 for stability. The absence from Raji 10 of detectable levels of either component of hMutLa most

2 P. Karran, unpublished data.

HCT116 or DLD-1 as shown were used to assay CT mismatch correction. d Raji 104 and Raji 12. CT mismatch correction by extracts of Raji 104 or Raji 12 (100 µg of protein) combined with other cell extracts as shown was determined.
likely reflects a primary defect in hMLH1 in these cells. An apparently reduced level of both hMLH1 and hPMS2 in Raji 12 cell extracts is also compatible with a defective hMLH1.

Six resistant clones were assigned, somewhat arbitrarily, to two separate phenotypes, 3 and 4, on the basis of a mild mutator effect and/or a mismatch binding defect. Complementation analysis indicated that despite the heterogeneity in cellular phenotype, members of Phenotypes 3 and 4 were most likely defective in a single gene product, the hMSH6/GTBP component of hMutSα. Inactivation of the hMSH6/GTBP gene has been reported in other methylation-tolerant cell lines. Both alleles are mutated in the methylation-tolerant MT1 derivative of the TK6 lymphoblast cell line (44). In addition, chromosome transfer experiments suggest that hMSH6/GTBP variants make up the larger of two genetically defined complementation groups of methylation-tolerant HeLa cells (Ref. 45). Neither MT1 nor the HeLa variants exhibited any detectable defect in mismatch binding in the bandshift assay. Raji 8, Raji 9, and Raji 19 were similar in this regard. These repair-defective cells apparently produce a partially functional hMSH6/GTBP that is able to interact with hMLH2 and mismatched DNA as determined by the bandshift assay. In the case of Raji 8, the defective hMSH6/GTBP also permits considerable processing of DNA O⁶-methylguanine as measured by nondenaturing DNA synthesis. Other hMSH6/GTBP defects, such as those in the colorectal carcinoma line DLD-1 (16, 23) or RajiF12 (6), abolish the interaction of the recognition complex with mismatched DNA. In the particular case of RajiF12, the presence of an apparently normal-length hMSH6/GTBP protein in Western blots and the absence of protein-truncating mutations in RajiF12 hMSH6/GTBP cDNA indicate that abolition of mismatch recognition by the RajiF12 hMutSα complex is most probably the result of inactivation of hMSH6/GTBP by missense mutation. This presumed mutation might disrupt the RajiF12 hMSH6/GTBP protein interaction with hMLH2, mismatched DNA, or both. Thus, hMSH6/GTBP variants, some of which have impaired mismatch binding, whereas others bind apparently normally and may even allow some mismatch processing, can be readily isolated by selection for methylation tolerance. Detailed characterization of these defective proteins offers a way to define regions of the hMSH6/GTBP gene product that interact with mismatched DNA or with the other proteins involved in the initial steps of mismatch correction.

The reason for the predominance of hMSH6/GTBP defects among our methylation-tolerant cells is not clear. It may reflect the preferential recognition of O⁶-methylguanine-containing base pairs by the hMutSα complex, although analogous hMSH2 variants, which are common among mismatch repair-defective human tumors, appear to be rather rare among methylation-tolerant cells. Alternatively, the preponderance of hMSH6/GTBP variants may have a genetic rather than a biochemical basis. Heterozygosity for hMSH6/GTBP in Raji cells would facilitate the emergence of tolerant cells with hMSH6/GTBP defects. Heterozygosity would not necessarily compromise mismatch repair capacity in these parental cells because, in general, expression of a single copy of the known mismatch repair genes, by chromosome transfer into defective cells (10, 19, 42) or in cells of heterozygous individuals (15, 46, 47), provides sufficient mismatch repair activity. It is also possible that the hMSH6/GTBP gene might be particularly susceptible to epigenetic silencing of the type outlined above.

**Mismatch Repair Defects Without Precedented Phenotypes**—About a third of the resistant clones did not display a detectable increase in MGMT expression. There was no loss of mismatch binding activity or evidence of a microsatellite instability, two features of mismatch repair-defective cells. Nevertheless, Raji 3, 7, 12, 17, and 104 all exhibited full resistance to the cytotoxic action of MNU. Mismatch repair appeared normal in Raji 3 and Raji 17. We have not characterized these clones in detail, and in particular, we have not determined the extent of formation and persistence of DNA methylation adducts. It is possible that their resistance is a consequence of a reduced load of O⁶-methylguanine in DNA which might come about through a metabolic change that protects the DNA from damage or by an increased O⁶-methylguanine repair that is independent of MGMT. Alternatively, the cells may exhibit a methylation-tolerant phenotype that does not involve loss of the mismatch repair pathway or results from mismatch repair defects that selectively impair recognition of DNA O⁶-methylguanine and escape detection by the correction assays we used.

The most striking observation among Phenotype 5 clones was the mismatch repair deficiency of Raji 7, Raji 12, and Raji 104. Despite the absence of detectable microsatellite instability, they were unable to correct the CT mismatch in vitro, and complementation analysis identified defects in hMutSα in Raji 104 and hMutLα in Raji 12. Western blotting indicated a possible reduced expression of hMutLα in Raji 12. It appears that the in vitro mismatch correction assay but not the microsatellite assay is sensitive to the alteration in hMutLα expression in Raji 12. Together with Raji 102 (and possibly also Raji 103),

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3 G. Aquilina and M. Bignami, unpublished data.
4 J. Jiricny and F. Palombo, personal communication.
5 M. Bignami, personal communication.
6 R. Hampson and P. Karran, unpublished data.
which have mismatch recognition defects that can be detected by in vitro assay but display no significant cellular manifestations of compromised mismatch repair. Raji 7, Raji 12, and Raji 104 comprise a group of phenotypically silent mismatch correction-deficient cell strains. Methylation tolerance resulting from a loss of mismatch repair is often accompanied by only moderate increases in spontaneous mutation rate (6, 45, 48). It appears that some mismatch repair defects do not confer dramatic microsatellite instability on tolerant cells. It has also been pointed out that microsatellite instability and an extreme phenotype at HPRT are not necessarily associated in tumor cell lines (49). It is interesting in this regard that heryozygous dominant negative mutations in mismatch correction genes do not confer a significantly more dismal prognosis as measured by age of onset and overall tumor incidence (46). In addition, if there are mismatch repair-defective tumors that do not display microsatellite instability, the frequency of mismatch repair defects among human tumors is likely to have been underestimated.

In summary, we have isolated several methylation-tolerant cell lines in which we have demonstrated mismatch repair defects by a biochemical assay. Three additional clones gained their MNU resistance through reactivation of a previously silent MGMT gene. This observation and the reported hypermethylation of the promoter region of the hMLH1 gene in some tumor cells that do not express the hMLH1 protein (40), suggests that mismatch repair genes might be susceptible to silencing by cytosine methylation. Together with previous studies of methylation-tolerant cells (6, 7, 9, 45) and human colorectal carcinoma lines (18, 23, 42), our data indicate that both the hMutSα and hMutLo mismatch repair complexes participate in processing DNA O6-methylGua into a lethal intermediate. The majority of our mismatch-tolerant clones were defective in the hMutSα mismatch recognition complex, most probably the hMSH6/GTBP protein, and the considerable phenotypic heterogeneity among hMSH6/GTBP-defective variant cells may reflect different inactivating mutations. More than a third (5 of 12) of methylation-tolerant clones were demonstrably deficient in mismatch correction but did not display detectable mutator effects such as microsatellite instability. These included cells in which either hMutSα or hMutLo appeared to be defective. Thus, microsatellite instability may not be diagnostic for all mismatch repair deficiencies, even those involving known proteins.

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Mismatch Repair Defects and $\textit{O}^6$-Methylguanine-DNA Methyltransferase Expression in Acquired Resistance to Methylating Agents in Human Cells

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