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Human variants of O⁶-alkylguanine-DNA alkyltransferase

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Abstract

This article summarizes the current understanding of known variant forms of the *MGMT* gene that encode an altered protein. Epidemiological studies have been carried out to test whether these alterations are associated with altered cancer risk. Laboratory studies using recombinant proteins and cells expressing the known variants have investigated the possible effects of these sequence alterations on the ability of the encoded O⁶-alkylguanine-DNA alkyltransferase protein to protect cells from alkylation damage and to respond to therapeutic inactivators currently undergoing trials for cancer chemotherapy.

1. Introduction

There is an increasing realization that polymorphisms in genes involved in either the metabolism of carcinogens/toxins or the responses to damage caused by these agents including DNA repair may have a profound effect on sensitivity to these agents and thus on human health. Identification of polymorphic variants in such genes and understanding the mechanistic implications of such alterations is now a major research area. This review summarizes information on the known variant forms of the *MGMT* gene that affect the amino acid composition of the protein. Several well-documented functions of the O⁶-alkylguanine-DNA alkyl transferase protein encoded by this gene (*MGMT*) might be affected by these changes. They include: (a) the ability of *MGMT* to protect cells from carcinogenic and mutagenic alkylating agents; (b) the ability of *MGMT* to alter the sensitivity of tumors to therapeutic methylating and chloroethylating agents; (c) the susceptibility of the *MGMT* protein to inactivation by drugs which are currently being evaluated to improve therapy with such therapeutic agents; (d) the ability of *MGMT* to render cells more sensitive to the genotoxic effects of dihaloalkanes and other bifunctional agents. This article provides an outline of studies describing *MGMT* protein variants, investigations of the alterations in the properties of these variant proteins that may produce phenotypic effects, and epidemiological correlations between the genes encoding these variants and cancer incidence or response to therapy.

2. *MGMT* gene and its protein product

Human *MGMT* protein consists of 207 amino acids. The protein contains a bound zinc atom, which is coordinated with 4 residues in the N-terminal domain of the protein, Cys5, Cys24, His29 and His85 [1]. Although the protein is active when stripped of zinc, the zinc form shows a higher rate of repair of methylated adducts [2]. This is presumably due to a change in the conformation of the protein, which activates the Cys145 acceptor site. This site and the

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surrounding substrate binding pocket and the DNA binding domain are all located in the C-terminal domain which begins at residue 86 [3]. This domain is not active alone but activity is restored when it is mixed with the N-terminal domain under conditions allowing their association [4]. Therefore, it appears that the N-terminal domain fulfills a structural role and maintains the C-terminal domain in an active configuration.

High resolution crystal structures have been obtained for active human MGMT protein [1,5], the Cys¹⁴⁵-S-methyl and Cys¹⁴⁵-S-benzyl forms of this protein produced after alkyl transfer [1], for a C145S mutant bound to a double stranded DNA containing an *O*⁶-methylguanine substrate [3] and for wild type protein covalently bound via Cys145 to a double stranded DNA containing a *N*¹-ethanoxanthine derivative [3]. These structures together with experimental data on the effects of site-directed mutagenesis at key residues and repair kinetics provide useful insight into the repair reaction. A crystal structure of the protein bound to a double stranded DNA with a chemically modified cytosine base is also published [6]. This structure shows an alternative means of DNA binding across a sticky-ended DNA junction to a second MGMT molecule [6] that might explain the highly cooperative nature of binding seen in solution [7]. This cooperative binding could aid in the rapid recognition of lesions but details are not yet clear. A model of the structure of some of the known genetic variants is also available [8].

The *MGMT* gene spans >170kb and is localized on chromosome 10 at 10q26 [9,10]. It contains 5 exons only four of which are coding and the mRNA transcribed from it is <1 kb. The four introns are very large with three each exceeding 40kb [9,10]. The promoter region, which lacks TATA and CAAT boxes, involves a 1.2kb sequence, which includes the first exon and part of the first intron. This region contains a minimal promoter and an enhancer element.

3. Existing variants

Table 1 shows the five reported genetic variants of the coding region of the *MGMT* gene, four of which lead to alterations in the amino acid sequence of the protein. Other possible variants that have not been confirmed in multiple studies and mutants that were reported to occur in tumor tissue but were not established to be present in normal tissues of the patients [11] are not included.

The currently known variants affecting the primary structure of MGMT are: **W65C** [12,13]; **L84F** [12-29]; **I143V/K178R** [14-21,24,26-32] and **G160R** [30,33]. The SNPs leading to the I143V and K178R changes are in almost perfect disequilibrium [14,15,21,31] and it can be assumed that both changes occur in the protein derived from this gene but in some cases only one of the alterations was tested for. There is also a silent mutation with a C to T variation at codon 53 encoding Leu [12,16,21,23,25].

The wide range of frequencies of the alleles shown in Table 1 in published studies reflects the very small sample size of some studies and possibly the gene pool from which they were identified. The most common alterations are L84F and I143V/K178R, which have been detected in c. 20-25% of the population studied. The L84F polymorphism may be slightly more prevalent in Caucasians and the I143V/K178R was significantly more common in Caucasians than Chinese [14]. Alterations G160R and W65C are clearly very rare (at most 1-2%). Although the original paper reporting the G160R form described a sample set of 40 individuals with a frequency of about 15% [33], there are obvious technical problems in the analysis and subsequent studies found none or only a very few examples of this alteration [14,30,31,34, 35].

4. Correlation studies

Multiple reports have appeared in which *MGMT* variants have been investigated in various patient populations and controls with the aim of determining whether any of the variants may affect cancer risk or the efficacy of treatment. Overall, although there are some intriguing preliminary indications, there is no clear and consistent picture from these studies. This is probably because the sample sizes were too small to detect anything other than a very strong effect. A summary of these studies is given below listed by the variant.

L84F

The L84F alteration was linked to an increased incidence of glioblastoma [36], bladder [23], breast [27] and prostate cancer [19] and to an increased occurrence of tumors with p53 overexpression or mutation [22]. However, other evidence suggests it is associated with a decreased incidence of head and neck cancer [37] and endometrial cancer [26]. No effect of L84F was observed on colon cancer risk [28,38] but it was associated with a better prognosis [38]. There was also no effect of L84F in small studies of breast, lung, pancreatic, melanoma or gastric cancer risk [16,17,20,29,37]. When only heavy smokers were considered, the L84F variant did appear to increase breast cancer risk [20] and it appeared to increase pancreatic cancer risk when combined with a polymorphism in *XRCC1* [29]. The L84F form was associated with a better prognosis in patients with colorectal cancer [38].

I143V/K178R

This form has been linked to a poor response to chemotherapy [16]. The I143V/K178R *MGMT* was also weakly associated with increased risk of lung cancer development in some studies [30,39]. However, no association was seen with lung cancer in Caucasians [32] and in a report from Poland [17,37]. There was no association with breast, pancreatic, prostate or gastric cancer or melanoma [16,17,19,20,27,29,37] and the risk of head and neck cancer [37] and of colorectal cancer was apparently decreased in women but not men [28]. A decreased risk was also reported in endometrial cancer among heavy smokers with the I143V/K178R alteration [26].

G160R

This rare variant clearly imparts resistance to *O*⁶-benzylguanine (BG) [40] as described below but screening of lymphocytes from a pool of 56 subjects showed no individual who had a resistance to inactivation that was similar to that expected from this mutant [34]. This is consistent with later studies [14,30,31,35], which found a much lower frequency of the G160R variant than that reported in the initial paper [33].

5. Studies with purified proteins or with cells expressing variant forms of *MGMT*

Several parameters could be affected by alterations in the amino acid sequence. These include: the rate of repair of *O*⁶-methylguanine and the sequence specificity of its repair; the ability to repair more bulky adducts at the *O*⁶-position of guanine with respect to rate and sequence specificity; the ability to repair other DNA base adducts such as *O*⁴-methylthymine; the amount of *MGMT* protein present in the cell; the degradation of the alkylated form of the protein; and the ability to be inactivated by low molecular weight inhibitors such as BG and related compounds. Only a few of these parameters have been investigated. These are listed below for each variant along with some discussion based on the crystal structure and mechanistic studies of wild type human *MGMT* as to how the encoded amino acid side chain alterations could produce the observed effects.

W65C

Expression of recombinant human W65C protein using a pQE-vector gave a poor yield and the purified protein was less stable than wild type MGMT when incubated at 37°C *in vitro* [41]. This is in agreement with early studies in which this variant was expressed in either bacteria or a mammalian cell line and is probably due to a lack of stability of this protein *in vivo* [42]. Such expression provided considerably less protection from N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) than expression of a wild type MGMT or L84F MGMT from the same vector. This reduced level of protein and activity occurred even though the mRNA was produced at the same level as the wild type and is presumably due to the rapid degradation of the protein [42]. It is possible that the side chain of Trp65, which is located in the first helix in the N-terminal domain, aids in the interaction between the domains as suggested in a modelling study [8] and that without this interaction the protein is rapidly degraded.

L84F

The purified L84F recombinant protein did not differ in activity from wild type MGMT in the repair of *O*⁶-methylguanine *in vitro* [41,42]. Expression of this variant in either bacteria or a mammalian cell line protected the cells from MNNG toxicity to the same extent as expression of wild type [42]. Also, as described below, the L84F variant did not differ from wild type in the relative repair *in vitro* of *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine, an adduct formed by the tobacco specific nitrosamines 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosornicotine [43]. The lack of alterations in DNA repair by L84F is perhaps not surprising since this is a conservative change of one hydrophobic amino acid side-chain for another at a position in the N-domain of MGMT, a significant distance away from the active site. However, Leu84 is adjacent to His85, which is one of the residues coordinated with the zinc ion in MGMT and the effect of the L84F substitution on zinc binding and on the rate constant for repair, which is known to be enhanced by zinc [2], has not been determined.

Another possibility is that the L84F alteration interferes with the claimed ability of the alkylated form of MGMT to bind the estrogen receptor [44]. It was suggested that this interaction may occur via a LXXLL motive located at residues 98-102 in MGMT [44]. It was further postulated that the alteration to phenylalanine at position 84 prevents this interaction with the estrogen receptor accounting for an inability to suppress breast cancer growth leading to an increased breast cancer risk and other linkages between the L84F variant and metabolic/behavioral changes influencing cancer risk [27,28]. Direct support for this ingenious hypothesis is lacking.

Lymphocytes from individuals with the L84F MGMT were examined for the response to NNK by measuring chromosome aberrations [24]. A difference was found between those with two wild type alleles and those with one or two variant alleles. There was a 1.7-fold increase in NNK-induced aberrations in the L84F variant cells. This effect was greater in some groups such as smokers, males and older individuals. Lymphocytes from individuals with the I143V/K178R form of MGMT were also examined and there was a smaller increase in NNK-induced aberrations that was not statistically significant. However, when the results from nine individuals who had variant alleles at both loci were compared to those with two wild type alleles, the effect was much greater in all groups [24]. It is hard to explain these results based on the known properties of MGMT. NNK produces both *O*⁶-methylguanine and *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine, which are repaired by MGMT, and other DNA adducts all of which may contribute to the formation of chromosome aberrations. It is possible that the polymorphism leads to a lower level of MGMT protein and activity is reduced in this way but unfortunately, this was not measured.

I143V/K178R

This form has attracted the most attention since the Ile143 is located in the active site pocket and is very close to the Cys145 acceptor site. However, this protein appears not to differ from wild type in the repair of methylated DNA [21,41,45]. Similarly, the I143V/K178R form did not differ from wild type in the repair of *O*⁶-*n*-butylguanine or BG when these were contained in oligodeoxynucleotides [45]. Expression of I143V/K178R in *E. coli* GWR111, which lacks endogenous activity, protected the cells from killing by MNNG and gave an equal protection to cells expressing wild type MGMT [16]. The level of MGMT protein expression was similar and extracts from these cells were able to repair *O*⁶-methylguanine in an oligodeoxynucleotide substrate.

All of the studies described above confirmed that the I143V/K178R MGMT is active and this is consistent with structural and comparative studies. It is noteworthy that the position of the I143V change in MGMT is not invariant when sequences from a wide range of organisms, which are now available, are compared. The most common alteration at this position is the presence of Val rather than Ile. A significant number of these MGMT sequences including those from *S. cerevisiae* and *B. subtilis* have this change. The position of the K178R change is not in a conserved part of the protein and indeed human MGMT can be truncated to end at position Leu176 without loss of activity [46]. It is therefore unlikely that the conservative change of the side chain in this non-essential residue would affect the activity.

The wild type MGMT and the I143V/K178R form were able to repair of *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine but preferred *O*⁶-methylguanine with at least a 2-fold increase in rate [45]. However, when competitive assays of repair of these two adducts were carried out, the relative repair of *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine depended quite strongly on the sequence context [43]. The I143V/K178R form was less sensitive to sequence context than wild type or the L84F form in its ability to repair this bulky adduct [43]. This effect was also seen with the MGMT mutant I143V indicating that the change at Lys178 in the naturally occurring variant does not influence this difference from wild type. It was suggested that the I143V substitution alters the geometry of the MGMT binding pocket to permit efficient repair of bulky *O*⁶-[4-oxo-4-(3-pyridyl)butyl]guanine even when it is in conformations that may be poorly repaired by other human MGMTs.

A slight indication that the I143V/K178R form might be associated with an increased steady state level of MGMT protein was obtained in a study in which peripheral blood mononuclear cells from 131 individuals were assayed [21]. There were only 3 individuals with two I143V/K178R alleles but these had an MGMT value of 15.5 ± 2.1 fmol/ μ g DNA compared to 8.4 ± 3.6 for 94 individuals with two wild type alleles. It is therefore possible that increased synthesis, decreased inactivation or degradation of the polymorphic form may lead to an increased capacity to repair DNA. It should be cautioned that MGMT activity in such cells may also be strongly influenced by environmental factors. With such a small sample size and single measurements it is unclear if this difference is reliable.

The I143V/K178R variant showed only a very slight alteration in sensitivity to inactivation by BG (c. 1.6-fold increase in ED₅₀) but it had a lesser sensitivity than wild type MGMT to inactivation by *O*⁴-benzylfolate (BF) [41]. BF is a potent MGMT inhibitor (ED₅₀ of 0.01 μ M) *in vitro*, which is potentially very useful since it shows selectivity to cell types that express the folate receptor, which include a number of tumors but not normal cells [47]. The ED₅₀ for inactivation by BF of the I143V/K178R MGMT was increased by about 3-fold [41]. A similar change was seen with mutant I143V and no change with mutant K178R MGMT indicating that only the alteration at Ile143 was responsible [41].

There was also a small difference in the sensitivity to inactivation by O^6 -(4-bromophenyl) guanine (Patrin-2) when I143V/K178R or the I143V mutant was compared to wild type [21]. These experiments used comparisons during a 1 h incubation at only a single (10 μ M) concentration of Patrin-2 which gave c. 76% loss of activity in wild type and c. 67% loss with I143V/K178R so it is not possible to calculate a difference in the ED_{50} . It is not clear why the wild type MGMT in these experiments appears to show a considerably lower inactivation by Patrin-2 than that previously reported where the ED_{50} was estimated at 0.005 μ M [48].

The altered reaction with BG, Patrin-2 and BF when the side chain of residue 143 is changed by replacing Ile with Val is likely to be due to a small alteration in the interaction of these inhibitors at the active site. This change is very small with BG and Patrin-2 but larger with BF. This suggests that it may affect the interaction with parts of BF other than the benzyl group. At present, it is not known why BF binds more tightly to the active site of MGMT than BG but this question is being addressed by crystallographic and modeling studies. It is also not clear to what extent this change would affect sensitivity to the drug in therapeutic trials and this question can really only be answered definitively by analysis of the *MGMT* genotype of patients treated with these drugs.

G160R

This replacement has only a small effect on the rate constant for repair of methylated DNA and the G160R form is as effective as wild type in protecting cells from MNNG and BCNU [49]. However, the G160R MGMT was strongly resistant to inactivation by BG with an ED_{50} value 40 times that of wild type [40]. CHO cells expressing the G160R form were not killed as readily as those expressing wild type MGMT by the combination of BCNU with BG or its metabolite O^6 -benzyl-8-oxo-guanine [49]. Therefore, any patients with this form of MGMT would definitely respond much less well to therapy with BG plus an alkylating agent. This would probably apply even to individuals with one allele since there is a very strong selection pressure during such treatment.

The G160R variant was much less effective than wild type MGMT in the repair of DNA containing O^6 -[4-oxo-4-(3-pyridyl)butyl]guanine or BG [43,45]. As described above, the wild type MGMT repaired the O^6 -[4-oxo-4-(3-pyridyl)butyl]guanine adduct relatively well in some sequence contexts but not in others. The G160R variant was consistently poor in the repair of this bulky adduct and when presented with substrates containing both O^6 -methylguanine and O^6 -[4-oxo-4-(3-pyridyl)butyl]guanine did not repair the bulky adduct until all of the O^6 -methylguanine was removed. This is consistent with the explanation that the presence of the relatively large and highly charged side chain of the arginine in the substrate binding pocket discriminates against larger adducts such as benzyl- and 4-oxo-4-(3-pyridyl)butyl-.

Although clearly functioning as a protective protein against alkylating agents forming O^6 -guanine adducts, MGMT has a paradoxical effect in increasing the mutagenic and toxic effects of dihaloalkanes, such as 1,2-dibromoethane [50] and some related bifunctional agents such as 1,3-butadiene diepoxide [51]. This effect is due to reaction at the active site of MGMT to generate a reactive intermediate at Cys145, which then reacts to form a covalent DNA adduct. Several mutants in MGMT that impart resistance to BG have been found to be lacking in the ability to activate $\alpha\omega$ -dihaloalkanes in this way [52,53]. Fortunately, the P140K mutant, which is currently undergoing human gene therapy trials, is among this group whereas the G160R variant differed only slightly from wild type [54].

6. Other *MGMT* variants and possible effects on MGMT expression

In addition to the variations in the *MGMT* gene described above which alter the encoded protein, there are known SNPs corresponding to polymorphisms in the 5' UTR, the 3' UTR and

in introns. These changes could affect expression of the protein and depending on the extent of linkage disequilibrium with other loci, this could contribute to the apparent effects of protein variants described above. With the notable exception of the recent paper by Margison and colleagues [21], the significance of such changes has not been investigated extensively. Their studies indicate that linkage disequilibrium at the 5' end of the gene can be detected for markers separated by >180 kb and that the marker with the strongest association with activity is located in the 3' end of the first intron.

There are a number of known variations in the promoter and enhancer region of the gene. These include G135T, G290A, C485A, C575A, G666A, C777A, G795C, A1034G and C1099T (numbered according to the defined promoter) [15,17,21,25]. The significance of these changes is not yet established but they may, in some cases, alter the expression level of the protein, which shows considerable inter-individual variation [21]. Such studies are difficult to control since MGMT expression may also be altered by exogenous factors and by epigenetic alterations. The 1099C to T change, which occurs in the enhancer region in exon 1 (frequency c. 4-22%), increased transcription of a luciferase reporter gene [17]. This alteration was not associated with a statistically significant change in lung cancer incidence [17] but the sample population was very small and a larger study would be of interest. A link between the 485C to A promoter sequence alteration (frequency c. 29-37%) and an increased susceptibility to lung cancer was seen [25]. However, this change did not affect the promoter activity suggesting it must be in linkage disequilibrium with another gene imparting susceptibility.

7. Conclusions

With the exception of the very rare G160R and W65C, there are no very convincing studies showing that the known human variants of MGMT protein have any striking differences from wild type protein in *in vitro* experiments with purified protein. Subtler changes such as alterations in sequence specificity for repair of bulky adducts and modest alterations in sensitivity to some therapeutic agents do seem to exist for I143V/K178R. The extent to which these alterations might impact human health is currently unclear.

Despite a large number of reports, current epidemiological studies do not provide a clear picture of whether human variants in the *MGMT* gene alter susceptibility to cancer. Investigations claiming such associations are preliminary at best, and it is clear that studies with larger numbers of individuals will be needed to confirm some of the trends reported in these investigations. In order to provide a mechanistic link with altered properties of MGMT, some association with common mutations in proto-oncogenes or tumor suppressor genes will be needed. Similarly, it is not yet established that *MGMT* variants affect the response to chemotherapy. If inactivators of MGMT activity such as BG, Patrin-2 or BF have success in clinical trials and are administered to large numbers of patients, it will be possible to assess whether the *MGMT* genotype affects response to these drugs.

At present, apart from the sequence specificity in the ability to repair bulky adducts, there are no reported changes in the activity of MGMT proteins that could explain the alterations in tumor susceptibility described in the published epidemiological studies. The possibility that the polymorphisms alter MGMT expression levels either directly or via linkage to other loci cannot be ruled out. However, it should be noted that several potentially relevant parameters that may be affected by variations in MGMT primary sequence have not been investigated. One of these possibilities is the ability to repair minor alkylation adducts such as *O*⁴-methylthymine. Wild type human MGMT is very poor at this repair but alkyltransferases from some other species such as the *E. coli* *Ogt* gene product are quite proficient [10,55]. Therefore, only variants with increased ability to repair *O*⁴-methylthymine would be significant. This is

feasible since alkyltransferases from some other species such as the *E. coli Ogt* gene product are quite proficient in *O*⁴-methylthymine repair.

A second possibility is that changes in primary sequence affect the degradation of the alkylated form of MGMT. The rapid removal of this inactive protein may be necessary to allow continued repair by other molecules of MGMT or the replacement of the molecules used up in the repair reaction. Rapid degradation of alkylated MGMT occurs via the proteasome after polyubiquitination [56,57]. Alkylation causes a structural change via a steric clash of the alkyl group with the carbonyl group of residue Met134 opening the hinge formed by Asp139 [1]. This causes a significant alteration in the structure but it is not known where the lysine that is linked to ubiquitin is located or how this structural alteration causes recognition by the E2/E3 ligase needed for attachment. It is possible that variants could affect this process at anyone of these steps. Although an alteration in ubiquitination was an attractive hypothesis to explain effects of the I143V/K178R variant, Lys178 has been ruled out as the site of ubiquitin attachment and direct examination of the turnover of the methylated or benzylated K178R form did not show any difference [57].

Finally, it has been reported that mammalian MGMT is phosphorylated at multiple sites [58, 59]. The functional significance of these alterations is unclear but phosphorylation may affect any of the properties discussed above as well as interactions with other proteins which could in turn affect activity *in vivo* or sub-cellular distribution.

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Table 1*MGMT* variants in the coding region

Variant nucleotide	SnplD	Exon	Amino acid change	Approx allele frequency [#]
C2740123T	rs1803965	3	None Leu53	c. 17% (12-22%)
G2740159C	rs2282164	3	Trp65⇒Cys	c. 1% (0-2%)
C2740214T	rs12917	3	Leu84⇒Phe	c. 21% (12-27%)
G2799046A	rs2308318	5	Gly160⇒Arg	< 1% (0-15%)
A2798995G	rs2308321	5	Ile143⇒Val [*]	c. 24% (11-28%)
A2799101G	rs2308327	5	Lys178⇒Arg [*]	

* These alterations are in perfect linkage disequilibrium and always occur together.

[#] Published values from very small studies with major differences from other studies within the same racial population are not included.