The alleles of the DNA repair gene $O^6$-alkylguanine-DNA alkyltransferase are expressed at different levels in normal human lung tissue

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$O^6$-Alkylguanine-DNA alkyltransferase (MGMT) confers resistance to many of the mutagenic and toxic effects of certain classes of alkylating agents by repairing the DNA lesions responsible. The levels of expression of this protein are of interest in relation to the prevention and treatment of cancer in man. They vary widely between individuals, and the basis of this variation is not understood. RT–PCR–RFLP analysis of mRNA from normal human lung tissue reveals that the two MGMT alleles are frequently expressed at different levels, indicating that there is a genetic component to inter-individual variation of MGMT levels and that at least some of this variation maps close to or within the MGMT locus.

Introduction

The DNA in cells is under constant attack from an enormous variety of endogenous and exogenous agents that introduce a vast array of different types of damage into the genome. In order to maintain functional integrity, an equally impressive array of DNA damage detection and processing mechanisms has evolved. One of these systems, relevant to both carcinogenesis and cancer chemotherapy, is the DNA repair protein $O^6$-alkylguanine-DNA alkyltransferase (MGMT). This protein operates by the stoichiometric transfer of the $O^6$-alkyl group to a cysteine residue in its own active site (for recent reviews see refs 1–4). As has been clearly shown in normal, transgenic and knockout rodent models, MGMT constitutes a major line of cellular defence against the toxic and carcinogenic effects of DNA alkylation at the $O^6$ position of guanine. Mice deficient in MGMT are more susceptible to tumour induction by alkylating carcinogens (5–7), while in transgenic models, mice expressing increased levels of MGMT are more resistant to alkylating carcinogens (8,9) and also have a lower frequency of spontaneous tumours (10,11). In humans, MGMT is expressed at different levels in different tissues and there is considerable inter-individual variation in the activity levels in particular tissues (reviewed in ref. 3). However, the causes of inter-individual differences in expression levels are unclear, in particular, whether there is a genetic component for this variability.

In chronic smokers, the bronchial epithelium is repeatedly exposed to carcinogenic compounds present in tobacco smoke. Among these carcinogens, alkylating agents such as 4-(methylamino)-1-(3-pyridyl)-1-butanone (NNK) and $N$-nitrosonornicotine (NNN) are thought to play an important role in the damage events that can ultimately result in neoplastic transformation (12). The metabolic systems that activate and inactivate such molecules and the pathways processing the induced genetic damage are considered to be major determinants of cancer risk in smokers (13). Since NNN and NNK generate lesions in DNA that are recognized by MGMT (14), differences in MGMT activity in normal tissues may be another factor that influences inter-individual differences in susceptibility to lung cancer.

Unequal expression of the alleles of autosomal genes (allelic expression imbalance; AEI) has long been known in tumours (e.g. refs 15,16) where it reflects somatic alterations at the DNA level, such as amplification, deletions, duplications or translocations, and the deregulation of epigenetic control mechanisms. These processes are hallmarks of tumour development (17). Until recently, AEI in normal human tissues has been mainly of interest in relation to imprinting, a process in which $CpG$ methylation is thought to play an important role but that affects comparatively few genes in humans (18). However, it is now becoming apparent that AEI in normal tissues is not a rare phenomenon: Yan et al. (19) reported AEI in half of 12 human genes they examined. They also showed that the segregation of expression patterns in pedigrees is consistent with Mendelian inheritance of alleles with low or high expression, thus indicating that AEI results from sequence variation in $cis$ acting elements affecting expression. In the present report, we have investigated in a panel of normal human lung tissues whether or not MGMT alleles are expressed at different levels.

Materials and methods

Sample selection

DNA and total RNA were extracted from the histologically normal lung tissue of resectable-staged NSCLC patients as described previously (20). The use of previously collected, archived, patient-derived material for the analysis of lung cancer-related gene expression was approved by the Liverpool Research Ethics Committee (2K/007). Initially the DNA samples were screened to identify previously collected, archived, patient-derived material for the analysis of lung cancer-related gene expression was approved by the Liverpool Research Ethics Committee (2K/007). Initial screening on a panel of 42 samples identified 12 with $AEI$ in MGMT and 7 with $AEI$ in CBR91. Patients with $AEI$ in MGMT had stage 1 and 2 NSCLC, while patients with $AEI$ in CBR91 had stage 2 NSCLC. The DNA was extracted using the Puregene Blood kit (Gentra Systems) and the RNA was extracted using the TRIzol reagent (Invitrogen). Microdissection was performed on the histologically normal lung tissue of each patient prior to DNA and RNA extraction. A total of 30 NSCLC patients were selected for analysis. The use of resectable-staged NSCLC patients, as well as the use of archival material, was approved by the Liverpool Research Ethics Committee. The selection of samples was based on two main criteria: first, the samples had to be from patients in whom the primary tumour was resectable, and second, the samples had to be of histologically normal lung tissue. The patients were not selected based on their stage of lung cancer.

$RT$–$PCR$

Normal tissue cDNA was synthesised from 1 μl aliquot of total RNA (~35 ng) in a 20 μl poly T-primed reaction using a Promega Reverse Transcription System (as directed by the manufacturer) and was stored at $-20\,{}^\circ\text{C}$. An aliquot (0.5 μl) of this target cDNA was used in $RT$–$PCR$ assays. Primers for

Abbreviations: AEI, allelic expression imbalance; MGMT, $O^6$-alkylguanine-DNA alkyltransferase; NNK, 4-(methylamino)-1-(3-pyridyl)-1-butanone, NNN, $N$-nitrosonornicotine.
MGMT (HypF: 5′ gagcgtgcctgtgtgacag 3′ and HpyR: 5′ ggctctgtggaaaatggcatt 3′) and for KRAS (KRASF: 5′ gtccttaattgttcacacc 3′ and KRASR: 5′ aagctgtctggttgtgagc 3′) were positioned such that the product obtained was cDNA specific. This was verified experimentally by test amplifications of cDNA and genomic DNA targets. A stock reaction mix was prepared according to the number of tubes + 1 consisting of, per tube: 42 μl distilled water, 5 μl of each primer, 0.5 μl of a 250 mM dNTP mix and 1.25 U of Roche Taq polymerase. The stock mix was vortexed after Taq addition and 49 μl added to each tube. Reactions were immediately transferred to a Perkin Elmer 9600 thermocycler and heated at 94°C for 2 min, followed by 32 cycles of 58°C for 1 min, 74°C for 1 min, and 94°C for 1 min, and finally 58°C for 2 min and 74°C for 10 min. PCR products were visualised by UV illumination of 2.5% TBE agarose gels containing 0.5 μg/ml ethidium bromide.

PCR
PCR set-up and cycling conditions (30 cycles) were identical to those used in the RT–PCR assays with the exception of the particular target (in this case genomic DNA) and primer positioning. Amplification of MGMT exon 3 was carried out using primers HpyDF (5′ agacgtgtgcccatgaagt 3′; located in intron 2) and HpyR (5′ ggctctgtggaaaatggcatt 3′).

RFLP–RT–PCR and RFLP–PCR analyses
Ten microlitres of each PCR product (~0.5 μg) was digested with the 3 U of the particular restriction endonuclease (NEB) necessary to visualize the cSNP under analysis, in 40 μl reactions (manufacturer’s 1× buffer) at 37°C for 2 h. Digests were visualized by gel electrophoresis and relative allelic expression quantified by running 1 μl aliquots of the digest on an Agilent 2100 bioanalyser (Agilent Technologies Wokingham, UK) across a DNA 1000 LabChip®.

Results
Fifteen of the 83 individuals included in the analysis were found to be heterozygous. For 12 of these normal tissue samples, the corresponding total RNA was also available and was used to determine the relative transcript levels of the two MGMT alleles.

Marked differences in the relative abundance of the MGMT transcripts encoded by the two MGMT alleles were observed in seven of 12 patients (Figure 1) as exemplified by samples 267, 279 and 282 (Figure 1A). The extent of the differences were up to >4-fold (Figure 2) and this is considerably higher than the average reported by Yan et al. (19) for the genes that they examined.

In order to assess if the imbalance was in any way a technical artifact, a consequence of the materials and/or methods used in our study, we examined the expression of the two alleles of the unrelated gene KRAS2. In contrast to the observation with MGMT, the KRAS2 gene demonstrated no allelic imbalance of expression (Figure 1B), in 13 informative normal pulmonary tissue samples. This observation emphasizes the large differences seen in the MGMT alleles.

Discussion
In the present report, marked differences in the expression levels of the two MGMT alleles have been found in the normal lung. To our knowledge, this is the first report of AEI in MGMT normal human tissues.

The basis of allelic expression differences could be the clonal expansion of a cell carrying a mutation in one allele. However, this seems unlikely as in each case the clone would need to have given rise to histologically normal lung structures and moreover, a similar phenomenon would need to have occurred at high frequency in the patients examined. Another possibility is that the observed AEI is exclusively due to epigenetic modifications of cis acting elements, as observed in imprinted genes (18). This cannot be excluded without examining the segregation of allelic expression levels in normal human tissues.

![Figure 1](https://example.com/figure1.png)
families. However, imprinting has not been described for genes in 10q26, the chromosomal region where MGMT is located, nor in the syntenic mouse region. Therefore, the most probable cause for the differences in allelic expression are (germ-line) sequence differences between alleles.

As MGMT expression in tumours can differ substantially from that of surrounding normal tissue (3), it would be interesting to know whether AEI occurs in tumours and how it relates to the patterns observed in normal tissue. However, since, as pointed out in the Introduction, allelic expression patterns in tumours reflect genetic and epigenetic aberrations occurring during tumour development, differences in expression pattern between normal tissues and matching malignant lesions might be anticipated. Analysis of allelic imbalance in tumours is thus unlikely to shed additional light on the processes governing inter-individual differences in normal tissues.

There are several mechanisms by which sequence variation within a gene can influence its transcript levels. These include variations in sequence motifs that directly affect transcription factor binding and/or message processing. There is extensive evidence, mainly from malignant tissues and immortalized cell lines, that CpG methylation of the promoter and of sequences outside the promoter region can affect MGMT expression (24–26; reviewed in refs 1.4). However, there is no evidence that this occurs in an allele-specific manner or for the general relevance of such mechanisms in normal tissues. The polymorphism used to show AEI does not affect coding and is thus unable to influence the functional activity of the protein (22), although it is possible that it might affect the efficiency of MGMT mRNA transcription and or processing. However, it seems more probable that the marker reflects the presence of another polymorphism that does affect mRNA expression or processing and therefore the functional activity of the MGMT gene. The fact that the same marker allele was over- and under-represented in different samples indicates that such determinants of allele specific differences in expression are not in strong linkage disequilibrium with the marker used.

Our results indicate, for the first time, that there is genetic component to inter-individual variation of MGMT levels in normal human tissues. It is probable that at least some of the sequence variation involved is located close to or within the MGMT locus and that it acts in cis. Since MGMT protects cells against the toxic and mutagenic effects of DNA guanine O\(^6\)-alkylation damage, differences in expression levels in normal tissues may significantly contribute to cancer risk. They may also play a role in the toxicities in normal tissues that determine the success or failure of anticancer drug therapies that involve O\(^6\)-alkylating agents.

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References


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