Pergamon

European Journal of Cancer Vol. 32A, No. 13, pp. 2319–2326, 1996

Printed in Great Britain
0959-8049/96 $15.00 + 0.00

PII: S0959-8049(96)00304-8

Original Paper

Analysis of O^6-Methylguanine-DNA Methyltransferase mRNA in Fine Needle Biopsies from Human Melanoma Metastases by Reverse Transcription and Polymerase Chain Reaction

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O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein, which removes alkyl groups from the O6 atom of guanine residues. Tumour cells which lack MGMT are sensitive to cytostatic drugs such as dacarbazine (DTIC), whose active species bind to this site. To explore whether analyses of MGMT expression can be used as a predictive test for clinical sensitivity to DTIC in melanomas, we developed a method to assay MGMT mRNA levels in cells obtained by fine needle aspiration biopsies of metastases. cDNA was synthesised from mRNA prepared from biopsy material. Polymerase chain reaction was performed using primers complementary to MGMT cDNA and to β-actin, which served as an internal control. Analyses of 44 biopsies from 35 patients showed a considerable variation in MGMT mRNA, with 15 samples (34%) lacking detectable mRNA. In 6 out of 8 patients in whom more than one tumour was analysed, separate metastases had different levels of MGMT mRNA. There was no correlation between MGTM activity studied by a biochemical assay and MGMT mRNA levels when these were compared in 10 surgical biopsies. Copyright © 1996 Elsevier Science Ltd

Key words: DNA repair, human melanoma, messenger RNA, O^6-methylguanine-DNA methyltransferase, polymerase chain reaction

Eur J Cancer, Vol. 32A, No. 13, pp. 2319–2326, 1996

INTRODUCTION

THE DOMINATING clinical problem in chemotherapy of metastatic malignant melanoma is the high frequency of drug-resistant tumours. The most commonly used drug in melanoma is the monofunctional alkylating agent 5-(3′,3′-dimethyl-1-triazeno)imidazole-4-carboxamide (dacarbazine, DTIC). Single-agent therapy with DTIC results in objective tumour remissions in approximately 20% of patients, while DTIC-based combination chemotherapy gives remission rates of 30–40% [1]. Moreover, most responders only obtain partial remissions with median durations of only a few months. The majority of patients are thus resistant to chemotherapy, and there is a need for a predictive test capable of identifying a group of patients with an increased chance of responding to treatment with DTIC.

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Received 8 Nov. 1995; revised 28 May 1996; accepted 3 Jun. 1996.

DTIC is a prodrug, which is demethylated in the liver to 5-(3′-methyl-1-triazeno)imidazole-4-carboxamide (MTIC). MTIC is further decomposed to a methyldiazonium ion which methylates guanine base residues in DNA at both the O6 and N7 positions [2]. O^6-methylguanine is an important cytotoxic and mutagenic lesion [3]. Methyl and other short alkyl adducts at the O6 atom of guanine are removed by specialised repair proteins, O^6-methylguanine-DNA methyltransferase (MGMT, EC 2.1.1.63), present in both prokaryotic and eukaryotic cells [3]. In a single-step suicide reaction, the alkyl adduct is transferred from the oxygen atom to a cysteine moiety in the active site of the protein, which is thereby inactivated. The repair capacity is therefore directly related to the number of MGMT molecules present in the cell, and continuous *de novo* synthesis of MGMT is required to maintain repair activity. Human MGMT is a 22 kDa protein encoded by a gene [4] on chromosome 10q26 [5]. MGMT is present in varying amounts in all normal human tissues [6–9], and the MGMT activity of a particular

tissue or cell type also varies between individuals [6, 9, 10]. Interestingly, approximately 20% of human tumour cell lines and many *in vitro* transformed cell lines express the so-called Mex^- or Mer^- phenotype and lack MGMT activity [11–13]. These cells are sensitive to methylating agents [11–13], including MTIC [14] and chloroethylnitrosoureas [15]. Xenografts of Mex^- human tumour cells are sensitive to treatment with chloroethylnitrosourea [16]. In recent years, important insights into the molecular mechanisms responsible for the absence of MGMT protein in Mex^- cells have been gained. While deletions of MGMT genes are rarely seen in tumour cell lines [4], the Mex^- phenotype is frequently a result of downregulation of gene expression at the level of transcription [4, 17]. It has been reported that in human tumour cell lines and xenografts, MGMT expression is inversely correlated with methylation of CpG dinucleotides in the promoter region of the gene [18, 19].

The observation that a fraction of tumour cell lines are Mex^- raises the question of whether some patients have Mex^- tumours, and whether such tumours are responsive to chemotherapy with chloroethylnitrosoureas or methylating drugs such as DTIC. If so, analysis of tumour cell MGMT expression might be a useful predictive test for drug sensitivity. The possibility that elevated levels of MGMT may cause resistance to DTIC is supported by the finding that melanoma cells with *in vitro*-induced resistance to MTIC exhibit increased repair of methylation damage in DNA [14]. Several studies of MGMT activities in human tumour biopsies have been reported, including more than 100 biopsies of brain tumours [20–24], several colorectal carcinomas [7, 25], cancers of the stomach [7, 26], lung [7, 22], urinary bladder [7, 27], breast [28], ovary [22, 29] and melanomas [30, 31]. All investigators have found considerable variation in MGMT activity between tumours in different patients. Although some glioma biopsies [21, 22, 24] and occasionally other tumours [22, 30] completely lack detectable MGMT activity, this is a rare finding. However, in many studies, a significant proportion of tumour biopsies show low MGMT activities which might be associated with sensitivity to drugs that induce adducts at the O6 atom of guanine. Although these investigations have shown a considerable variation in MGMT activity in tumours, there are, so far, no reports on meaningful comparisons of tumour MGMT levels and the response to chemotherapy. Thus, it is still unknown whether MGMT is of importance for the outcome of clinical chemotherapy with agents such as DTIC or chloroethyl nitrosoureas.

We have previously shown that human melanoma metastases differ in MGMT activity [31]. A disadvantage of biochemical assays of MGMT activity is the requirement for surgical excision of metastases. We, therefore, decided to develop a more sensitive method that would allow us to study MGMT in cells obtained by fine needle aspiration (FNA) biopsies of tumours. Since there is evidence that MGMT expression is regulated at the level of transcription [4, 17], it may be possible to estimate MGMT activity by measurements of mRNA. We have now established a sensitive reverse transcription–polymerase chain reaction (RT–PCR) technique for measurements of MGMT mRNA. Since sufficient material can be obtained by FNA biopsy without excision of the tumour, this method makes it possible to analyse the effect of chemotherapy on the individual tumour that has been assayed, as well as to do repeated analyses of the same tumour. We now report initial results comparing analyses of MGMT mRNA by Northern blot and RT–PCR, a comparison of results of RT–PCR analyses of mRNA with MGMT activity assays in tumour biopsies, as well as results of analyses of MGMT mRNA by RT–PCR in material obtained by FNA biopsies of melanoma metastases.

MATERIALS AND METHODS

Cell lines

The two human lung cancer cell lines U1690 (small cell lung cancer, Mex^-) and U1810 (non-small cell lung cancer, Mex^+) were obtained from Dr Jonas Bergh [32]. The cell lines were cultured in Eagle's MEM with Earle's salts, supplemented with 2 mmol/l L-glutamine, 10% FCS, 125 IU/ml benzylpenicillin and 125 μg/ml streptomycin.

Patients and tumour biopsies

Tumour biopsies were obtained from patients with malignant melanoma who were followed at the Department of Oncology, Karolinska Hospital, Sweden. The investigation was approved by the Ethical Committee of the Karolinska Hospital.

In most cases, tumour cells were obtained by FNA biopsies performed according to the procedure described by Zajicek [33]. A total of 44 FNA samples were obtained from lymph node and subcutaneous metastases in 35 patients. The characteristics of patients and tumour biopsies are shown in Table 1. Approximately two-thirds of the patients had distant metastases at the time of biopsy and 13 of the patients had received chemotherapy, either as single-agent therapy with DTIC, or as combination regimens of DTIC with one or two of the following drugs: cisplatin, vindesine, bis-chloroethylnitrosourea and interferon alfa-2b.

One part of the fine needle aspirates was used to prepare smears, which were air-dried and stained by May-Grünwald-Giemsa for morphological analysis. The second

Table 1. *Characteristics of patients subjected to FNA biopsy* (n = 35)

Gender	
Male	26
Female	9
Median age	62 years
Range	27–88 years
Clinical stage*	
Stage III	12
Stage IV	23
Site of biopsied tumour	
Subcutaneous	24
Lymph node	20
Previous chemotherapy†	
Yes	13
—DTIC as single agent	8
—Combination regimen‡	5
No	22

*According to AJCC: Stage III: regional metastases only; Stage IV: distant metastases. †In all cases except two, biopsies were performed at least 17 days after last day of chemotherapy. One biopsy was taken during ongoing DTIC therapy and one was obtained 5 days after DTIC therapy. ‡DTIC was included in all combination chemotherapy regimens.

part of the aspirates was suspended in cold Eagle's MEM (Flow) supplemented with 5% foetal calf serum (FCS) for RNA extraction.

The morphological analysis consisted of diagnosis of melanoma metastasis, determination of the proportion of tumour cells when mixed with lymphoid cells from lymph nodes and the presence of necrosis. Results of RT–PCR analyses were used only when samples contained $\geqslant 90\%$ tumour cells of which $\geqslant 50\%$ were without signs of necrosis.

In 12 patients, metastases were excised by surgery and immediately transferred to ice-cold Eagle's MEM. The surrounding normal tissue was carefully dissected away and the samples were divided into small pieces which were frozen in liquid nitrogen and stored at −70°C until tissue extracts were made.

RT–PCR analysis of MGMT mRNA

Cells obtained by FNA biopsy were washed twice with phosphate-buffered saline on ice and lysed in the presence of 0.9% Nonidet P-40 and 8.7 mM ribonucleoside vanadyl complexes (Sigma) for 1 min, in 250 µl of 10 mM Tris–HCl pH 7.8 and 150 mM NaCl, followed by centrifugation at 14000 rpm for 2 min in an Eppendorf table-top centrifuge. After transfer of the supernatants to new tubes, 250 µl of 40 mM Tris–HCl pH 7.8, 40 mM EDTA, 700 mM NaCl, and 50 µl of 10% SDS (sodium dodecyl sulphate) were added and the samples were incubated for an additional 60 s. The samples were then extracted twice with phenol/chloroform (1:1) and the RNA was precipitated and washed with ethanol. The RNA samples were enriched for mRNA using poly-A^+ messenger affinity paper (Hybond mAP, Amersham, U.K.). Reverse transcription, primed by random hexamers (Promega), was carried out with 200 U of Superscript RNaseH-reverse transcriptase (Gibco BRL, Gaithersburg, Maryland, U.S.A.) per µg of mRNA, according to the manufacturer's instructions, in the presence of rRNasin ribonuclease inhibitor (Promega). Aliquots of 0.1–5 µl of the cDNA reaction mixture were incubated for PCR with 1 U of AmpliTaq DNA polymerase in 1 × AmpliTaq buffer (Perkin Elmer Cetus, Norwalk, Connecticut, U.S.A.) and 0.2 mM of each dNTP. The $MgCl_2$ concentration was adjusted to 2 mM. The PCR reactions were performed for 30 cycles using a two-step protocol (1 min at 95°C followed by 2 min at 60°C). The following primer pairs were used for simultaneous amplification of MGMT and β-actin sequences: MGMT(s): 5′-AGT GCA CAG CCT GGC TGA AT and MGMT(a): 5′-CTG CAG ACC ACT CTG TGG CA. β-actin(s): 5′-GAA ACT ACC TTC AAC TCC ATC and β-actin(a): 5′-CTA GAA GCA TTT GCG GTG GAC GAT GG. For each sample, a control PCR reaction was performed without previous reverse transcription, in order to detect non-specific PCR products. The PCR products of 271 bp (MGMT) and 303 bp (β-actin) were separated by agarose gel electrophoresis in the presence of 0.5 µg/ml of ethidium bromide and visualised by UV transillumination. Agarose gels were photographed using positive/negative Polaroid 665 film (Polaroid) and the intensity of each DNA band was measured by densitometry with an LKB UltroScan XL Enhanced Laser Densitometer (Pharmacia Biotech, Sollentuna, Sweden).

For each sample, PCR analyses were performed using at least three different amounts of template cDNA (in the range 0.1–5 µl) and the intensities of the DNA bands corresponding to MGMT and β-actin products were analysed as a function of the amounts of template cDNA used. The ratio of the slopes of regression lines was calculated in the

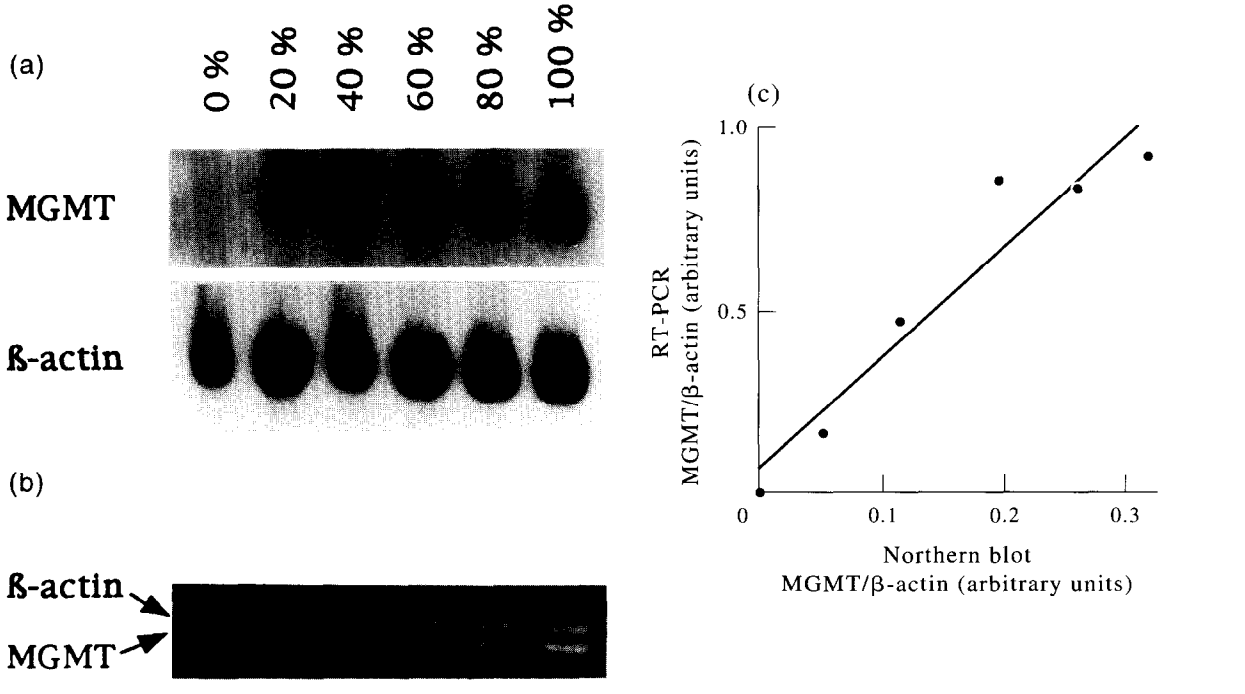

Figure 1. Comparison of measurements of MGMT and β-actin mRNA in U1810 (Mex^+) and U1690 (Mex^-) cell lines by Northern blot analysis (a) and RT–PCR (b). Percentages refer to the proportion of Mex^+ RNA present in the mixture. A negative of the gel photo with PCR products and the autoradiograph of the probed Northern filter were analysed by densitometry. Results are shown as the ratios of cDNA band intensity (RT–PCR) and signal strength (Northern blot) for MGMT over β-actin (c). Line: result of regression analysis (correlation coefficient 0.95, $P = 0.004$).

range where the yield of each PCR product was a linear function of the amount of cDNA template.

Northern blot analyses

Total RNA isolated from cell lines as described above was electrophoretically separated on 1% formaldehyde-agarose gels and transferred to nitrocellulose filters [34]. Hybridisation was performed at 44°C using ^{32}P-labelled DNA probes. The MGMT cDNA probe was prepared from plasmid pHM14 [35], obtained from Dr Peter Karran and the β-actin cDNA from plasmid pAc18.1 [36], obtained from Dr Stig Linder.

Measurements of MGMT activities

MGMT activities were measured in extracts of 10 frozen surgical tumour biopsies. Tumour samples of approximately 0.1 cm^3 were homogenised in a microdismembrator II (B. Braun, Melsungen, Germany) for 30 s. The dry powder was suspended in equal volumes of lysis buffer containing 300 mM KCl, 50 mM Tris–HCl (pH 7.5), 10 mM dithiothreitol, 1 mM EDTA and 0.5 mM phenylmethylsulphonylfluor-

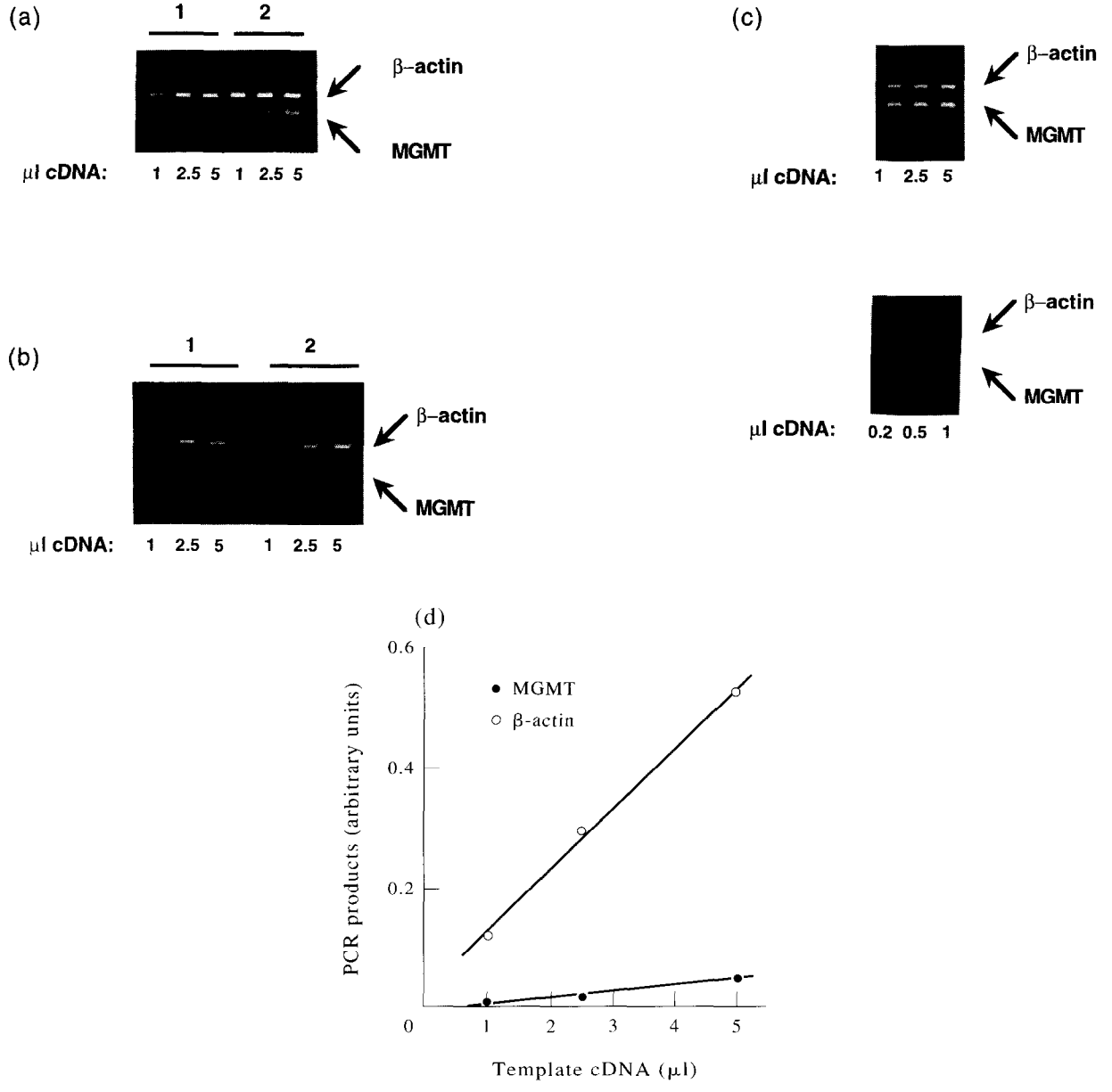

Figure 2. Agarose gel electrophoresis of RT–PCR products obtained from RNA extracted from FNA biopsies of melanoma metastases. (a) Two metastases showing intermediate levels of MGMT mRNA. (b) Two metastases which lack detectable levels of MGMT mRNA. (c) A metastasis with a high amount of MGMT mRNA. The upper panel shows that the maximum yield of both products was obtained when 1 μl of cDNA was used as template; with increasing amounts of cDNA, the PCR reactions reached a plateau phase. By reducing the amounts of cDNA template, PCR conditions were obtained where the yield of products was dependent on the amount of cDNA, lower panel. (d) Example of a result of densitometry of MGMT and β-actin products analysed with agarose gel electrophoresis as shown in Figure 2a–c. In this example, the MGMT/β-actin mRNA ratio was 0.087.

ide and left on ice for 30 min. Debris was then removed by centrifugation for 30 min at 13 000 rpm at 4°C. MGMT activities of cell extracts were measured by the removal of [^{3}H]methyl groups from the O6 atom of guanine residues in *M. luteus* DNA (Sigma), as previously described [32]. MGMT activities were expressed as pmol of methyl groups removed from the substrate per mg of extract protein.

RESULTS

Comparison of analyses of mRNA with Northern blot and RT–PCR

To investigate whether analysis of samples containing varing amounts of MGMT mRNA with RT–PCR would give results similar to those obtained using Northern blot analysis, RNA was prepared from two lung cancer cell lines: U1810, which is Mex$^+$ and expresses high levels of MGMT, and U1690, which is Mex$^-$ and lacks MGMT [32]. RNA from the two cell lines was then mixed in different proportions and assayed for MGMT and β-actin mRNA with both Northern blot and RT–PCR. We found that both Northern blots and RT–PCR analyses showed an approximately linear increase in the ratio of MGMT to β-actin mRNA as a function of the proportion of U1810 RNA in the mixture (Figure 1). The results obtained with each technique yielded results which were similar, with a correlation coefficient of 0.95 ($P = 0.004$). This indicates that RT–PCR gives an estimate of the amount of MGMT mRNA, which is comparable to that obtained by Northern blot analysis.

RT–PCR analyses of MGMT mRNA in biopsies of melanoma metastases

Figure 2 shows some examples of agarose gel electrophoresis of RT–PCR products of RNA from FNA biopsies of metastases showing high, intermediate and undetectable levels of MGMT mRNA. For each sample, PCR was performed using varying amounts of template cDNA in order to obtain results where the yield of PCR products is a function of the amount of template (Figure 2a). In some cases, the amount of template cDNA had to be reduced since the PCR reactions reached a plateau phase with the initial amounts of template that were used (Figure 2c). Results of densitometry of negative Polaroid photographs of ethidium bromide stained gels were used to obtain plots of the yield of each PCR product as a function of the amounts of template cDNA in the reactions (Figure 2d). The relative levels of MGMT mRNA in the samples were estimated by calculating the ratios between the slopes of regression lines for MGMT and β-actin products.

RT–PCR was performed on material obtained by FNA biopsies from 44 metastases: 24 subcutaneous and 20 lymph node metastases, in 35 melanoma patients. Analyses were also performed on RNA extracted from 12 tumours excised by surgery (nine lymph node and three subcutaneous metastases). There was a marked variability in MGMT mRNA levels between individual tumours in both classes of metastases (Figure 3). When biopsy material obtained from separate metastases in the same patient was analysed, there was sometimes a considerable variation between MGMT mRNA levels in the metastases (Figure 4, patients 2, 3 and 5–8). Biopsies obtained by FNA lacked detectable MGMT mRNA in 15 cases (34%). Average levels of MGMT mRNA were not notably different in biopsies from 20 patients who had received chemotherapy compared to samples from 24 patients who had no received chemotherapy (data not shown). Due to the limited number of patients who have received chemotherapy following analysis of MGMT mRNA in tumour biopsies, it was not possible to analyse whether there was a relationship between treatment result and mRNA levels.

Comparison between determinations of MGMT mRNA by RT–PCR and MGMT activity by a biochemical assay

In 10 cases, it was possible to compare results of MGMT activity measured by the biochemical method and mRNA assays by RT–PCR in the same extracts made from surgical tumour biopsies (Figure 5). While the variation in mRNA levels was larger than in MGMT activities, the majority of tumours in this analysis showed intermediate to high levels

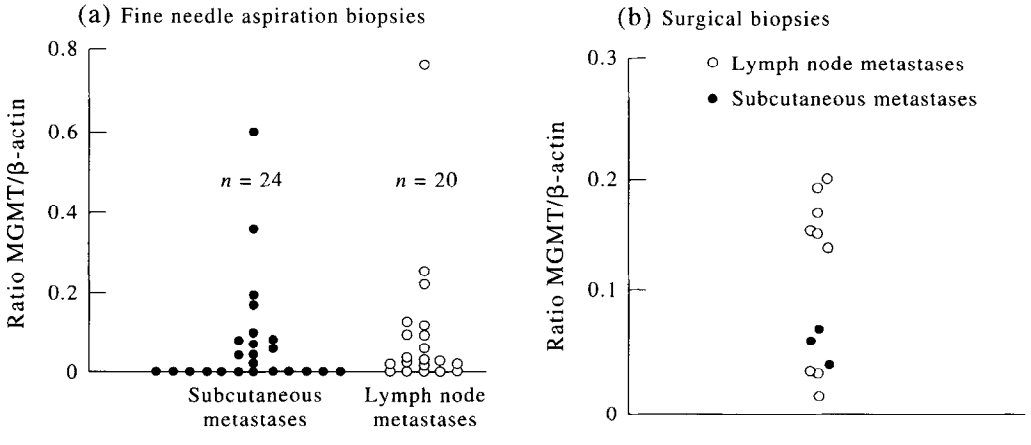

Figure 3. Analyses of MGMT mRNA levels were performed in material from FNA biopsies of 44 metastases in 35 patients with malignant melanoma (a). In addition, mRNA was analysed by the same procedure in 12 surgically removed melanoma metastases (b).

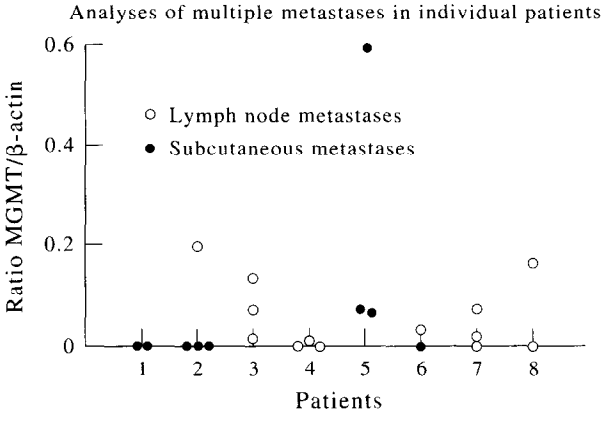

Figure 4. Heterogeneity of MGMT mRNA in separate metastases in the same patient. When MGMT mRNA was analysed in different metastases in the same individuals, 6 out of 8 patients (patients 2, 3 and 5–8) showed considerable variation in MGMT mRNA between tumours.

of both MGMT mRNA and MGMT activity, and none of the extracts lacked MGMT activity or mRNA. There was no significant correlation between MGMT activity and mRNA levels.

DISCUSSION

The aim of this work was to develop a method that is sufficiently sensitive to analyse MGMT mRNA in material obtained from FNA biopsies of human tumours. Since initial attempts to use Northern blot analysis on such material showed that in most cases the yield of RNA was too small for this purpose, we chose to develop a RT–PCR assay. This assay is sensitive enough to allow analysis of MGMT mRNA in the small amounts of cells typically obtained by FNA biopsies, although the yield of material is still sometimes too sparse, particularly when very small metastases are biopsied.

A practical problem encountered when clinical tumour biopsies are analysed is the presence of varying amounts of non-tumour cells (blood cells and stroma). In this respect, the use of FNA biopsy is superior to surgical biopsy, since the FNA technique frequently enriches the proportion of tumour cells in the sample by preferential aspiration of tumour cells compared to stroma cells. To ensure that measurements are made largely on tumour cell RNA, it is essential to examine the composition of the cell suspensions by microscopy. We found that, in the majority of biopsies, the proportion of tumour cells was $\geq 90\%$. Results of analyses were disregarded in cases where the amount of non-tumour cells exceeded 10%.

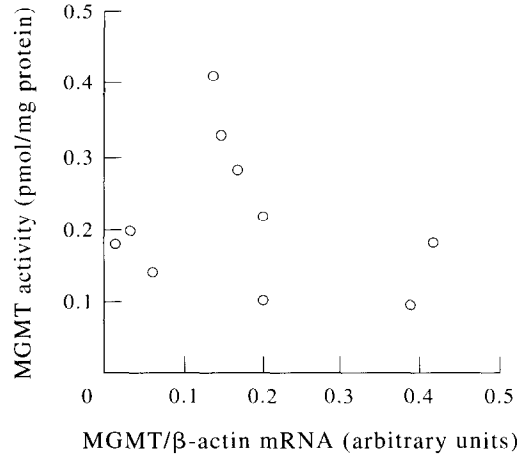

Figure 5. Comparison of results of measurements of MGMT activity and MGMT mRNA in 10 surgically excised melanoma metastases.

Initial experiments comparing analyses of RNA mixtures from Mex^+ and Mex^- cells by Northern blot and RT–PCR gave comparable results. This validates the results obtained on biopsy material and also suggests that, if an appropriate internal standard is used, the RT–PCR technique may be an alternative to Northern blot analysis in experimental work on cultured cells.

The considerable variability in MGMT mRNA between tumours, even in the same individual, is in agreement with our earlier measurements of MGMT activity in melanoma metastases [31]. The variable levels of MGMT mRNA in separate metastases in the same patient is consistent with the not infrequent clinical observation that, in patients with multiple metastases, some tumours respond to DTIC chemotherapy while others do not. A practical consequence of this is that, if assays of MGMT mRNA are to be used to predict DTIC sensitivity, it will not be enough to analyse a single tumour in patients with multiple metastases. Rather, it should be important to sample multiple tumours in such patients.

Recently, results of RT–PCR analyses of MGMT mRNA in 10 human brain tumour biopsies were reported [37]. A wide variation in MGMT mRNA levels was observed. MGMT mRNA has also been analysed in human normal and tumour tissues using *in situ* hybridisation [38, 39]. With this technique, mRNA levels of individual cells are visualised, but the method requires surgical biopsy material and has, to our knowledge, not been adapted to analyse samples obtained by FNA biopsy. Using this technique, a large interindividual variation in MGMT mRNA levels in human breast carcinomas and brain tumours has been demonstrated [38], similar to the findings reported here.

In 10 cases, it was possible to compare results of MGMT activity measured by the biochemical method and mRNA assays by RT–PCR in the same extracts made from surgical tumour biopsies. There was no significant correlation between MGMT activities and mRNA levels. The reason for this is unclear, but similar findings have been made by others. In human mammary carcinomas and brain tumours, MGMT mRNA analysed by *in situ* hybridisation was compared to MGMT activity measurements. Although these authors reported some correlation between these parameters, a several fold variation in MGMT activity was frequently seen in biopsies with similar mRNA levels [38]. Taken together, these results indicate that the relationship between MGMT mRNA and protein activity may be more complex than expected. This is supported by a recent investigation of MGMT activity, protein levels and mRNA during different phases of the cell cycle (Egyházi S, Skog S, Margison GP, Hansson J, Ringborg U. Regulation of O^6-methylguanine-DNA methyltransferase during the cell cycle in a human non-small cell lung cancer cell line in relation to

1,3-bis(2-chloroethyl)-1-nitrosourea cytotoxicity. Submitted for publication). We found that, whereas MGMT protein levels remain relatively constant throughout the cell cycle, MGMT activity declines during early S-phase, whereas MGMT mRNA levels increase from the G1 to the G2/M phase of the cycle. Thus, the relative distribution of cells in the phases of the cell cycle may affect the relationship between MGMT activity and mRNA levels.

When the present results are compared to our previous investigations of MGMT activity in melanoma metastases [31], we find that mRNA assays with RT–PCR more frequently give negative results than MGMT activity measurements. There are several possible explanations for this. The biochemical assay of MGMT activity measures the disappearance of [^{3}H]-labelled methyl groups from the O6 guanine position in DNA, and may not be specific for MGMT. The activity of any other repair mechanism which removes such methyl groups would be indistinguishable from MGMT, and might cause an overestimation of MGMT activity. O^6-methylguanine is a substrate for nucleotide excision repair in bacteria [40], and involvement of this pathway in the repair of O^6-alkylguanine in mammalian cells has also been suggested [41]. Tumour extracts with high excision repair activity might thus exhibit removal of methyl groups from the substrate even if they lack MGMT activity. Technical limitations of the RT–PCR assay could cause a false low yield of MGMT products. It is well known that mRNA is susceptible to degradation in damaged cells undergoing necrosis or during preparation of samples for analysis. We consider this to be an unlikely explanation for the increased frequency of negative RT–PCR assays, since we discarded biopsies in rare instances where a $\geqslant 50\%$ of cells showed signs of necrosis. Moreover, extensive degradation of mRNA would most likely be detected by a low level of β-actin RT–PCR products. RT–PCR reactions give a linear yield of products only over a limited range of template mRNA. It is, therefore, not unlikely that some biopsies contain low levels of MGMT mRNA, which will result in detectable MGMT activity in a biochemical assay, but fall below the threshold level required to obtain detectable PCR products. This is most likely to be a problem when small tumours are examined, where the yield of biopsy material is minimal. We are currently attempting to improve the sensitivity of the RT–PCR assay by using more sensitive methods to detect PCR products.

Immunohistochemical studies have shown that melanoma tumours frequently exhibit considerable heterogeneity regarding the MGMT expression of tumour cells [42] (S. Egyházi, Karolinska Hospital, Sweden). This heterogeneity could have important clinical implications, since a small proportion of cells expressing high levels of MGMT could expand due to the selective killing of sensitive cells during chemotherapy, and lead to the development of a drug-resistant tumour. By combining immunohistochemistry or immunocytochemistry on cells obtained by FNA biopsies with RT–PCR, it should be possible to obtain a semiquantitative estimate of the average MGMT mRNA levels as well as information on tumour cell heterogeneity regarding MGMT expression.

An important question is whether the tumour cells in some human malignancies exhibit the Mex$^-$ phenotype. Other investigators have reported that a small proportion of gliomas [21, 22, 24] and some other tumours [22, 30] lack MGMT activity. We have previously found low levels of MGMT activity, which might be attributed to the presence of non-tumour cells in the biopsied tumour, in some melanoma biopsies, while none completely lacked activity [31]. In a more recent immunohistochemical investigation, using polyclonal antibodies reactive to MGMT, we identified a small proportion of melanoma metastases apparently lacking MGMT in the tumour cells (S. Egyházi, Karolinska Hospital, Sweden). The present RT–PCR results are consistent with the possibility that Mex$^-$ tumours exist in melanoma patients.

These results, as well as our previous studies of MGMT activity and immunohistochemistry, indicate that some human melanoma metastases have high MGMT expression. In these cases, it is likely that MGMT may contribute to DTIC resistance. Patients with such tumours would be appropriate candidates for attempts to overcome drug resistance by depletion of tumour cell MGMT activity with O^6-benzylguanine or similar agents, which could sensitize tumours to DTIC.

1. Houghton AN, Legha S, Bajorin DF. Chemotherapy for metastatic melanoma. In CM Balch, AN Houghton, GW Milton, AJ Sober, S-j Soong, eds. *Cutaneous Melanoma.* Philadelphia, J.B. Lippincott, 1992, 498–508.
2. Meer L, Janzer RC, Kleihues P, Kolar GF. *In vivo* metabolism and reaction with DNA of the cytostatic agent 5-(3′,3′-dimethyl-1-triazeno)imidazole-4-carboxamide. *Biochem Pharmacol* 1986, **35**, 3243–3247.
3. Pegg AE. Mammalian O6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res* 1990, **50**, 6119–6129.
4. Tano K, Shiota S, Collier J, Foote RS, Mitra S. Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O6-alkylguanine. *Proc Natl Acad Sci USA* 1990, **87**, 686–690.
5. Natarajan AT, Vermeulen S, Darroudi F, *et al.* Chromosomal localization of human O6-methylguanine-DNA methyltransferase (MGMT) gene by *in situ* hybridization. *Mutagenesis* 1992, 7, 83–85.
6. Myrnes B, Giercksky KE, Krokan H. Interindividual variation in the activity of O6-methylguanine-DNA methyltransferase and uracil-DNA glycosylase in human organs. *Carcinogenesis* 1983, **4**, 1565–1568.
7. Myrnes B, Norstrand K, Giercksky K-E, Sjunneskog C, Krokan H. A simplified assay for O6-methylguanine-DNA methyltransferase activity and its application to human neoplastic and non-neoplastic tissues. *Carcinogenesis* 1984, **5**, 1061–1064.
8. Grafstrom RC, Pegg AE, Trump BF, Harris CC. O6-alkylguanine-DNA alkyltransferase activity in normal human tissues and cells. *Cancer Res* 1984, **44**, 2855–2857.
9. Gerson SL, Miller K, Berger NA. O6-alkylguanine-DNA alkyltransferase activity in human myeloid cells. *J Clin Invest* 1985, **76**, 2106–2114.
10. Sagher D, Karrison T, Schwartz JL, Larson RA, Strauss B. Heterogeneity of O6-alkylguanine DNA alkyltransferase activity in peripheral blood lymphocytes: relationship between this activity in lymphocytes and in lymphoblastoid lines from normal controls and from patients with Hodgkin's disease or non-Hodgkin's lymphoma. *Cancer Res* 1989, **49**, 5339–5344.
11. Day III RS, Ziolkowski CHJ, Scudiero DA, *et al.* Defective repair of alkylated DNA by human tumor and SV40-transformed human cell strains. *Nature* 1980, **288**, 724–727.
12. Sklar R, Strauss B. Removal of O6-methylguanine from DNA of normal and xeroderma pigmentosum-derived lymphoblastoid cells. *Nature* 1981, **289**, 417–420.

13. Yarosh DB, Foote RS, Mitra S, Day III RS. Repair of O6-methylguanine in DNA by demethylation is lacking in mer human tumor cell strains. *Carcinogenesis* 1983, **4**, 199–205.
14. Maynard K, Parsons PG, Cerny T, Margison GP. Relationships among cell survival, O6-alkylguanine-DNA alkyltransferase activity and reactivation of methylated adenovirus 5 and herpes simplex virus type 1 in human melanoma cell lines. *Cancer Res* 1989, **49**, 4813–4817.
15. Erickson LC, Bradley MO, Ducore JM, Ewig RAG, Kohn KW. DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. *Proc Natl Acad Sci USA* 1980, 77, 467–471.
16. Fujio C, Chang HR, Tsujimura T, Ishizaki K, Kitamura H, Ikenaga M. Hypersensitivity of human tumor xenografts lacking O6-alkylguanine-DNA alkyltransferase to the anti-tumor agent 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea. *Carcinogenesis* 1989, **10**, 351–356.
17. Pieper RO, Futscher BW, Dong Q, Ellis TM, Erickson LC. Comparison of O-6-methylguanine DNA methyltransferase (MGMT) mRNA levels in mer^+ and mer^- human tumor cell lines containing the MGMT gene by the polymerase chain reaction. *Cancer Commun* 1990, 2, 13–20.
18. Costello JF, Futscher BW, Tano K, Graunke DM, Pieper RO. Graded methylation in the promoter and body of the O6-methylguanine-DNA methyltransferase gene (MGMT) correlates with MGMT expression in human glioma cells. *J Biol Chem* 1994, **269**, 17228–17237.
19. Qian X, von Wronski MA, Brent TP. Localization of methylation sites in the human O6-methylguanine-DNA methyltransferase promoter: correlation with gene suppression. *Carcinogenesis* 1995, **16**, 1385–1390.
20. Wiestler O, Kleihues P, Pegg AE. O6-alkylguanine-DNA alkyltransferase activity in human brain and brain tumors. *Carcinogenesis* 1984, **5**, 121–124.
21. Frosina G, Rossi O, Arena G, Gentile SL, Bruzzone E, Abbondandolo A. O6-alkylguanine-DNA alkyltransferase activity in human brain tumors. *Cancer Lett* 1990, **55**, 153–158.
22. Citron M, Decker R, Chen S, *et al.* O6-methylguanine-DNA methyltransferase in human normal and tumor tissue from brain, lung and ovary. *Cancer Res* 1991, **51**, 4131–4134.
23. Chen JM, Zhang YP, Wang C, Sun Y, Fujimoto J, Ikenaga M. O6-methylguanine-DNA methyltransferase activity in human tumors. *Carcinogenesis* 1992, **13**, 1503–1507.
24. Mineura K, Izumi I, Watanabe K, Kowada M. Influence of O6-methylguanine-DNA methyltransferase activity on chloroethylnitrosourea chemotherapy in brain tumors. *Int J Cancer* 1993, **55**, 76–81.
25. Redmond SMS, Joncourt F, Buser K, *et al.* Assessment of P-glycoprotein, glutathione-based detoxifying enzymes and O6-alkylguanine-DNA alkyltransferase as potential indicators of constitutive drug resistance in human colorectal tumors. *Cancer Res* 1991, **51**, 2092–2097.
26. Kyrtopoulos SA, Vrotsou B, Golematis B, Bonatsos M, Lakiotis G. O6-methylguanine-DNA transmethylase activity in extracts of human gastric mucosa. *Carcinogenesis* 1984, **5**, 934–947.
27. Souliotis VL, Giannopoulos A, Koufakis I, Kaila S, Dimopoulos C, Kyrtopoulos SA. Development and validation of a new assay for O6-alkylguanine-DNA alkyltransferase based on the use of an oligonucleotide substrate, and its application to the measurement of DNA repair activity in extracts of biopsy samples of human urinary bladder mucosa. *Carcinogenesis* 1989, **10**, 1203–1208.
28. Cao E-H, Fan X-A, Yuan X-H, Xin S-M, Liu Y-Y, Yu H-T. Levels of O6-methylguanine acceptor protein in extracts of human breast tumor tissues. *Cancer Biochem Biophys* 1991, **12**, 53–58.
29. Chen SS, Citron M, Spiegel G, Yarosh D. O6-methylguanine-DNA methyltransferase in ovarian malignancy and its correlation with postoperative response to chemotherapy. *Gynecol Oncol* 1994, **52**, 172–174.
30. Moriwaki S, Nishigori C, Takebe H, Imamura S. O6-alkylguanine-DNA alkyltransferase activity in human malignant melanoma. *J Dermatol Sci* 1992, **4**, 6–10.
31. Egyházi S, Hansson J, Ringborg U. O6-methylguanine-DNA methyltransferase activities in biopsies of melanoma tumors. *Br J Cancer* 1995, **71**, 37–39.
32. Egyházi S, Bergh J, Hansson J, Karran P, Ringborg U. Carmustine-induced toxicity, DNA crosslinking and O6-methylguanine-DNA methyltransferase activity in two human lung cancer cell lines. *Eur J Cancer* 1991, **27**, 1658–1662.
33. Zajicek J. Aspiration biopsy cytology. I. Cytology of supradiaphragmatic organs. In GL Wied, ed. *Monographs in Clinical Cytology*. Basel, Karger, 1974, 1–5.
34. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1989.
35. Rydberg B, Spurr N, Karran P. cDNA cloning and chromosomal assignment of the human O6-methylguanine-DNA methyltransferase. *J Biol Chem* 1990, **265**, 9563–9569.
36. Nudel U, Zakut R, Shani M, Neuman S, Levy Z, Yaffe D. The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucleic Acids Res* 1983, **17**, 1759–1771.
37. Mincura K, Watanabe K, Yanagisawa T, Kowada M. Quantification of O6-methylguanine-DNA methyltransferase mRNA in human brain tumors. *Biochim Biophys Acta* 1996, **1289**, 105–109.
38. Eberhagen I, Bankmann M, Kaina B. Detection of O6-methylguanine-DNA methyltransferase expression in human tumors by means of *in situ* hybridization. *Anticancer Res* 1995, **15**, 761–768.
39. Wani G, D'Ambrosio SM. Differential expression of the O6-alkylguanine-DNA alkyltransferase gene in normal human breast and skin tissue: *in situ* mapping of cell type-specific expression. *Mol Carcinogen* 1995, **12**, 177–184.
40. Samson L, Thomale J, Rajewsky MR. Alternative pathways for the *in vivo* repair of O6-alkylguanine and O4-alkylthymine in *Escherichia coli:* the adaptive response and nucleotide excision repair. *EMBO J* 1988, 7, 2261–2267.
41. Bronstein SM, Skopek TR, Swenberg JA. Efficient repair of O6-ethylguanine, but not O4-ethylthymidine or O2-ethylthymidine, is dependent upon O6-alkylguanine-DNA alkyltransferase and nucleotide excision repair activities in human cells. *Cancer Res* 1992, **52**, 2008–2011.
42. Lee SM, Rafferty JA, Elder RH, *et al.* Immunohistological examination of the inter- and intracellular distribution of O6-alkylguanine DNA-alkyltransferase in human liver and melanoma. *Br J Cancer* 1992, **66**, 355–360.

Acknowledgements—We thank Helena Lööw and Åsa Staland for excellent technical assistance and Gun Sandell-Littborg for help with obtaining medical records of patients. This work was supported by the Swedish Cancer Society, Stockholm, Sweden.