Research Article

Expression of O^6-Alkylguanine-DNA Alkyltransferase in Normal and Malignant Bladder Tissue of Egyptian Patients

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Bladder tumour tissues and corresponding uninvolved mucosa (normal tissue) of Egyptian bladder cancer patients were assessed for O^6-alkylguanine-DNA-alkyltransferase (MGMT) activity by functional assay of tissue extracts (36 paired samples), and distribution by immunofluorescence (IF) microscopy of fixed material (24 paired samples). MGMT varied widely from 42–253 fmoles/mg protein and from 3.2–40 fmoles/μg DNA in normal and 58–468 fmoles/mg protein and 2.5–49.5 fmoles/mg protein, in the tumour tissues; only one tumour had undetectable activity. Pairwise comparison of MGMT activity in tumour and adjacent normal tissue showed no significant difference based on DNA content but was 1.75-fold higher in tumour ($P < .01$) based on protein. There was no effect of gender or bilharzia infection status. IF showed that in tumours, both the mean percentage of positive nuclei (57.3 ± 20.3%) and mean integrated IF (5.47 ± 3.66) were significantly higher than those in uninvolved tissues (42.8 ± 13.5% $P = .04$) and (1.89 ± 1.42; $P < .01$), respectively. These observations suggest that, overall, MGMT levels are increased during human bladder carcinogenesis and that MGMT downregulation is not a common feature of bladder cancers. Based on this, bladder cancers would be expected to be relatively resistant to chemotherapy which involved O^6-guanine alkylating antitumour agents.

1. Introduction

Carcinoma of the urinary bladder is the most common malignancy among Egyptian males, accounting for 16% of total cancer incidence while in females it ranks second to breast cancer [1, 2]. The high incidence of bladder cancer in Egypt is associated with chronic urinary infection with *Schistosoma haematobium* [2, 3] which is thought to lead to endogenous nitrosation and the formation of N-nitroso compounds [4–6]. Such compounds have been shown to alkylate DNA causing a range of DNA adducts with differing mutagenic, and carcinogenic potential [7]. O^6-alkylguanine is one such adduct which has been shown to be a toxic, mutagenic and carcinogenic lesion. This damage can be processed by the DNA repair protein, O^6-alkylguanine-DNA-alkyltransferase (MGMT), which transfers the alkyl group to a cysteine residue at its catalytic site in a process that leads to the inactivation of the protein. Since each MGMT molecule acts only once, cells have a limited capacity to repair O^6-alkylguanine lesions, which is determined by the steady state number of MGMT molecules at the time of alkylating agent exposure. Further repair following depletion of the pool requires *de novo* synthesis of the protein [8, 9].

While MGMT may be an important factor in the aetiology of bladder cancer, it may also play an important role in cellular defence against the toxic effects of certain types of alkylating agents used in cancer chemotherapy, usually referred to as O^6-alkylating agents [10, 11]. In this context, cytosine methylation of the MGMT promoter has generally been accepted to result in loss of MGMT expression [12, 13], but this is reported to be rare in bladder tumours occurring in 2–9% of those examined [14, 15]. Tumour

MGMT activity has also been reported to be higher [16] or lower [4] than normal bladder tissue. The reasons for these conflicting results are not clear but may reflect the underlying pathogenesis of bladder cancer or the use of normal bladder tissue from different patients as controls instead of the corresponding uninvolved mucosa from the same subject. Studies of tumour extracts also give no indication of possible regional heterogeneity or intracellular localisation of MGMT. We have therefore determined the distribution and levels of immunoreactive MGMT and MGMT activity in human bladder tumour tissues and corresponding uninvolved mucosa using immunohistochemistry and a functional assay for MGMT activity. The results were examined in relation to tumour type, gender, and schistosomiasis status. There was no significant difference in activity between samples from men and women and from those with or without evidence of bilharzia infection. But overall, tumour samples displayed higher levels of MGMT expression than associated normal tissues.

2. Materials and Methods

2.1. Human Tissue Specimens. Tissue samples were collected during radical cystectomy of 36 Egyptian bladder cancer patients attending the department of Urology, Faculty of Medicine, Alexandria University following taking informed consent and ensuring non compromise of pathologic diagnosis. All samples were taken from the urinary face of the bladder mucosa and were frozen immediately on dry ice and stored at $-70°$C. Samples were collected, in pairs, each pair comprised a sample of bladder tumour and a sample of bladder mucosa with no macroscopic sign of tumour invasion (referred to as uninvolved tissue). Schistosomiasis infection was established from a self-reported clinical history of schistosomiasis or schistosoma ova detected in histological specimens. Most (32) patients had evidence of bilharzia infection: 23 had a transitional cell carcinoma (TCC), 5 a squamous cell carcinoma (SCC), and 4 a TCC with SCC foci; of the four patients without infection, 2 had adenocarcinomas (Ad), 1 a SCC, and 1 a TCC with SCC foci.

2.2. Assay of Tissue Levels of MGMT. MGMT activity in bladder tissue extracts was assayed in all 36 paired samples by measuring the transfer of [³H] from a [³H]-methylated DNA substrate to protein in the tissue extract using the methods described by Watson and Margison [17]. MGMT activity was expressed as fmoles of methyl groups transferred per mg of protein and per μg of DNA in the tissue extract. MGMT activity in paired uninvolved and tumour tissue from the same patient were compared using a paired sample t-test.

2.3. Tissue Localisation of Human MGMT. Frozen bladder tissues were fixed in 4% neutral buffered formalin then processed to paraffin wax. Sections (3μm) were cut and mounted onto APES-subbed slides, dewaxed in xylene, and rehydrated in graded ethanol. Tissues were then microwaved (650 watts) in 10 mM citrate buffer (pH 6.0) to retrieve the immunoreactivity of the cross-linked proteins. Slides

were treated with 3% H_2O_2 in TBS to block endogenous peroxidase, washed in ddH$_2$O and TBS, and incubated with 10% normal swine serum. Sections were incubated at $4°$C with antihuman MGMT 1°Ab (diluted to 1 : 1000). Slides were then washed in TBS, followed by application of swine-antirabbit biotinylated IgG and ABC peroxidase. The sections were developed with DAB/H_2O_2, washed, dehydrated, and mounted. As a negative control, adjacent sections were incubated with preimmune serum instead of the MGMT antiserum.

2.4. Quantitative Immunohistochemistry. Quantitation of MGMT in tissue sections was performed using immunofluoresence (IF) in 24 paired samples. Sections were stained for MGMT as above except that the ABC complex and DAB visualisation steps were omitted and replaced by Cy3/DAPI fluorescent staining; samples were stained with Avidin-Cy3 (1 : 1000) followed by TBS wash then counterstained with DAPI (0.2 μg/ml). Samples were then washed with ddH$_2$O and mounted in PBS-buffered glycerol (9 : 1).

Fluorescent microscopy images of the mounted slides were generated using a Nikon microscope equipped with a mercury arc lamp and with red and blue filter sets (for Cy3 and DAPI fluorescence resp.). Images were captured using a Hitachi HVC20 camera and analysed with the Lucia G image analysis software package using a macro that automated data collection and performed calculations of the number of positive nuclei, the mean and integrated fluorescence, and mean area.

The macro operated the following steps: (i) highlighting the position of nuclei in tissue sections by the use of a DAPI filter, (ii) superimposing the nuclear outline on the Cy3 image (through TRITC filter), (iii) capturing this image and comparing it against negative control sections, and (iv) measuring the fluorescence intensity of MGMT-Cy3 staining and the area of each nucleus within the nuclear boundaries recorded from the DAPI image.

For each sample, data were averaged from 10 to 20 fields (depending on the size of the section). MGMT expression in the tissue sections was quantified using 3 parameters: (1) the percentage of immunoreactive MGMT positive nuclei which indicates the fraction of cells containing detectable levels of nuclear MGMT, (2) the mean fluorescence intensity of Cy3 MGMT staining which corresponds to the average amount of MGMT protein per unit nuclear area in the positively staining cells, and (3) the integrated fluorescence (area fraction of Cy3 positive nuclei x mean fluorescence) which represents the total amount of MGMT protein in the tissue. These fluorescent parameters were compared in paired uninvolved and tumour tissue from the same patient were compared using a paired sample t-test

3. Results

All uninvolved (normal) bladder tissue samples contained measurable MGMT activity which ranged from 3.2 to 39.8 fmoles/μg DNA (12.4-fold variation). MGMT activity was detected in 35/36 tumour samples with the activity
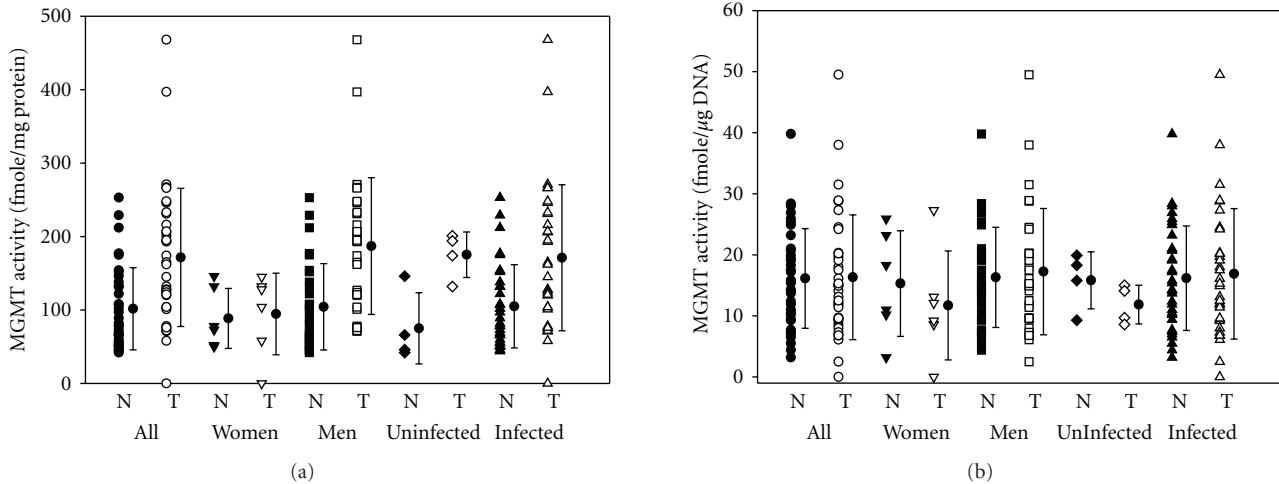
FIGURE 1: Relationships between MGMT activity (upper: fmole/mg protein, lower: fmole/ug DNA) in normal (N) and tumour (T) tissues of Egyptian bladder cancer patients. Data are also subdivided according to gender and bilharzia status. Statistical analyses are presented in Table 1.
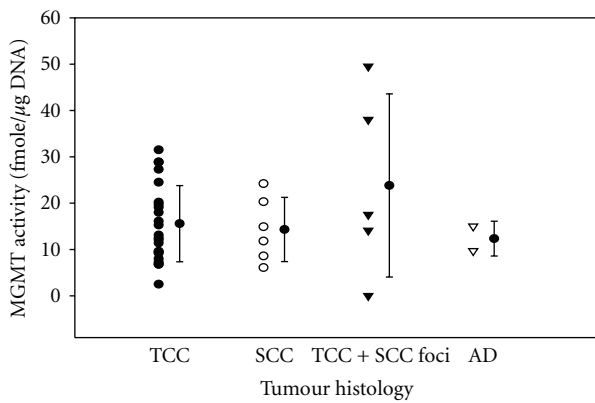

FIGURE 2: Relationship between MGMT activity (fmole/μg of cellular DNA: means indicated by asterisks, error bars indicate standard error) and tumour histology. TCC, transitional cell carcinoma; SCC, squamous cell carcinoma; AD, adenocarcinoma.

ranging up to 49.5 fmoles/μg DNA. There was no significant difference in activity between samples from men and women and from those with or without evidence of bilharzia infection (Table 1; Figure 1) or in activity from samples of different histological tumours (Figure 2). Tumour MGMT activity when expressed as fmoles/mg protein was significantly higher than that in uninvolved tissue from the same patient (Table 1). However, when MGMT was expressed as fmole/μg DNA there was, overall, no significant difference between tumour and normal tissue (Table 1). Breaking this down, tumour MGMT activity based on protein content was 1.5–5-fold higher in 22 sample pairs, 10 pairs of samples had similar levels of activities in tumour and normal tissue, while 3 tumour samples had 1.5-fold lower activity compared to uninvolved tissue. On the other hand, based on DNA content, the MGMT activities of 9 tumour samples were up to 3-fold higher than the corresponding controls; in

16 patients, there were similar levels, while in 10 patients tumour samples had 1.5- to 2-fold lower activities than control.

Representative examples of the inter- and intracellular distribution and intensity of MGMT staining in the bladder sections are illustrated in Figure 3. MGMT antiserum staining showed mild upregulation of MGMT expression in the metaplastic epithelium (2b) in comparison with the normal urothelium (2a). The intensity of tumour staining showed marked variation between patients' samples. One SCC bladder sample (2a) showed relatively homogenous pattern and intense nuclear staining as well as some cytoplasmic staining, whereas another SCC sample (moderately differentiated and keratinised) presented a heterogeneous pattern and less intense nuclear staining with some faint cytoplasmic staining (2b). The invasive TCC sample showed more intense but less uniform staining of the tumour nuclei (3a) than did the papillary TCC sample (3b). In all cases, no cellular staining was observed with preimmune serum and all nuclei acquired the blue colour of the haematoxylin counterstain. There was an obvious inconsistent pattern of staining in the tumour tissue relative to the uninvolved mucosa. In some of the samples, differences in the intensity of MGMT nuclear staining were observed between normal urothelium and the corresponding tumour tissue from the same patient (4 and 5). While in some cases the urothelial nuclei showed heavy staining compared to the tumour tissue (4a) and (4b), in other cases the bladder urothelium showed heterogeneous and generally reduced nuclear staining in comparison to its corresponding tumour (5a) and (5b).

Twenty four paired bladder samples from the previous series of tumour and uninvolved tissues were stained for MGMT using IF Cy3 staining with DAPI counterstain. In tumour tissues, there was no association between the % positive nuclei and either the mean fluorescence ($r = 0.10$; $P = .64$) or the integrated fluorescence ($r = 0.37$; $P = .21$), but there was an association between the mean and integrated
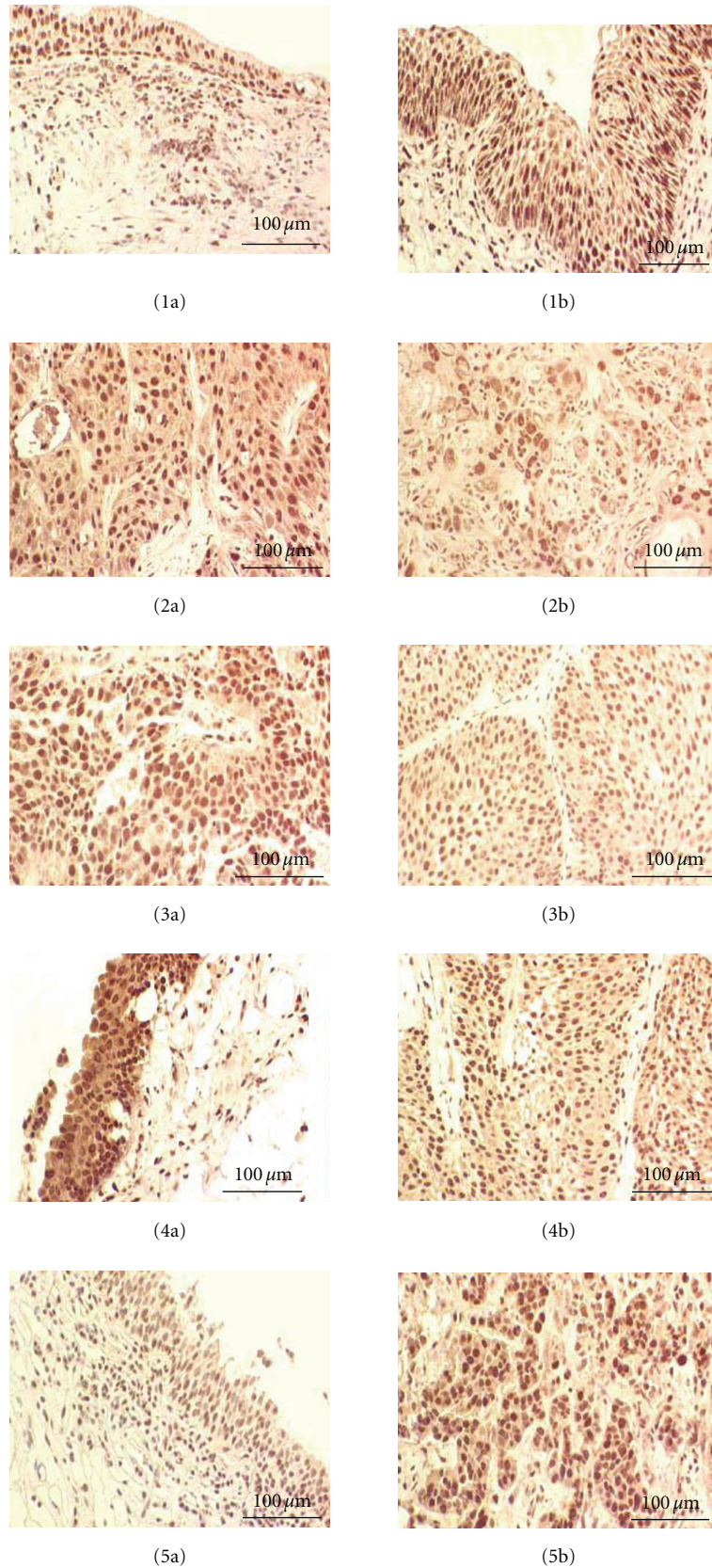
Figure 3: Immunohistochemical detection of MGMT in bladder tissue from patients with bladder cancer: normal urothelium (1a) and squamous metaplastic epithelium 1(b); SCC (2a) and moderately differentiated SCC (2b) sections of bilharzial bladder; invasive TCC (3a) and low grade papillary TCC (3b); non neoplastic urothelium with some dysplastic changes (4a) and grade II papillary TCC (4b); normal urothelium (5a) and invasive TCC (5b). Note that the intensity of staining is highest in the high grade section (5b). Scale bars = 100 μm.

TABLE 1: MGMT activity in extracts of bladder tumours and uninvolved tissue.

| | n | MGMT activity (mean ± SD) | | | | | |
| | | Fmoles/mg protein | | | Fmoles/µg DNA | | |
		Uninvolved tissue	Tumour tissue	Difference (tumour uninvolved)	Uninvolved tissue	Tumour tissue	Difference (tumour uninvolved)
Total	36	101.6 ± 55.9	171.6 ± 94.0	70.0 ± 75.8*	16.1 ± 8.2	16.3 ± 10.2	0.2 ± 8.5
Males	30	104.3 ± 58.7	187.0 ± 93.1	82.7 ± 73.6*	16.3 ± 8.2	17.3 ± 10.3	1.0 ± 8.9
Females	6	88.5 ± 40.8	94.5 ± 55.6	6.0 ± 54.1	15.3 ± 8.7	11.7 ± 8.9	−3.6 ± 5.2
Schistosomiasis infected	32	105.0 ± 56.6	171.1 ± 94.4	66.1 ± 75.6*	16.2 ± 8.6	16.9 ± 10.7	0.7 ± 8.6
Schistosomiasis negative	4	75.0 ± 48.5	175.3 ± 31.0	100.3 ± 79.3	15.8 ± 4.7	11.9 ± 3.2	−4.0 ± 7.3

*$P < .001$
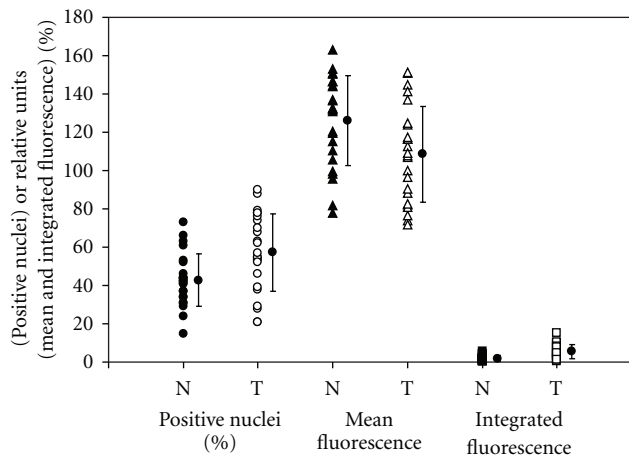
FIGURE 4: Ranges and means (asterisks, error bars indicate standard error) in immunofluorescence values for MGMT in normal (N) and tumour (T) tissue. Values for positive nuclei are shown as %, and for mean fluorescence and integrated fluorescence as relative units.

fluorescence ($r = 0.45$; $P = .03$). In contrast, in uninvolved tissues there were significant associations between the % positive nuclei and both the mean fluorescence ($r = 0.54$; $P = .006$) and the integrated fluorescence ($r = 0.90$; $P < .001$) and also between the mean and integrated fluorescence ($r = 0.54$; $P = .006$). This probably reflects a higher degree of heterogeneity in the tumour tissues.

The percentage of MGMT positive nuclei varied from 21 to 90% in tumour tissues, and from 15 to 73% in uninvolved bladder tissues. The mean percentage positive nuclei for tumours was significantly higher ($P = .002$) than that for the corresponding uninvolved tissues (Table 2; Figure 4). In contrast, the average of the mean fluorescence was significantly lower in tumours than in normal tissues ($P = .004$; Table 2; Figure 4). However, integrated MGMT fluorescence varied from 0.56 to 15.43 in bladder tumours (27.6-fold variation) and from 0.29 to 5.78 in uninvolved bladder tissue (19.9-fold variation). The mean of this parameter was again significantly higher in tumours than in the corresponding normal tissue ($P < .001$ Table 2; Figure 4). There were no

differences in these parameters in tumour tissues of different histology (data not shown).

In normal (uninvolved) bladder tissues, MGMT activity was correlated with the % positive nuclei and integrated fluorescence but not with the mean fluorescence (Figure 5) whereas in tumour tissue, MGMT activity was only correlated with the mean fluorescence (Figure 5).

MGMT activity in normal tissue was highly correlated with that found in tumour tissue (Figure 6) whereas there were no correlations between immunofluorescence parameters in normal tissue and the corresponding parameters in tumour tissue (Figure 6).

The marked variations in MGMT expression levels within and between patient tumour samples, along with the numbers of samples in the study, have precluded testing any correlation between MGMT expression and other factors such as gender and bilharzial infection.

4. Discussion

In this study, MGMT protein was measured in bladder tissues using quantitative IF and MGMT activity using a functional assay. A wide range in immunoreactive protein levels was detected; not all cells were stained and in positively staining cells intensity varied but was mainly confined within the nucleus. Only one tumour sample had no detectable MGMT activity; whether or not this may have been due to promoter methylation was not investigated. Also, we cannot exclude the possibility that the unstained or low MGMT-expressing cells in the examined population may be completely or partially MGMT-promotor methylated. The results presented here are consistent with those of other reports examining other tumour types and showing that staining with MGMT antibody is predominantly nuclear [18, 19]. MGMT staining also varied markedly from one patient to another. Whilst there was interindividual variation in the cellular distribution of reaction, staining was present in all cell types in most samples. In some TCC as well as SCC cases, most of the cells expressed the protein in a relatively homogenous pattern, whereas in others, marked intercellular heterogeneity was observed. In addition, cytoplasmic
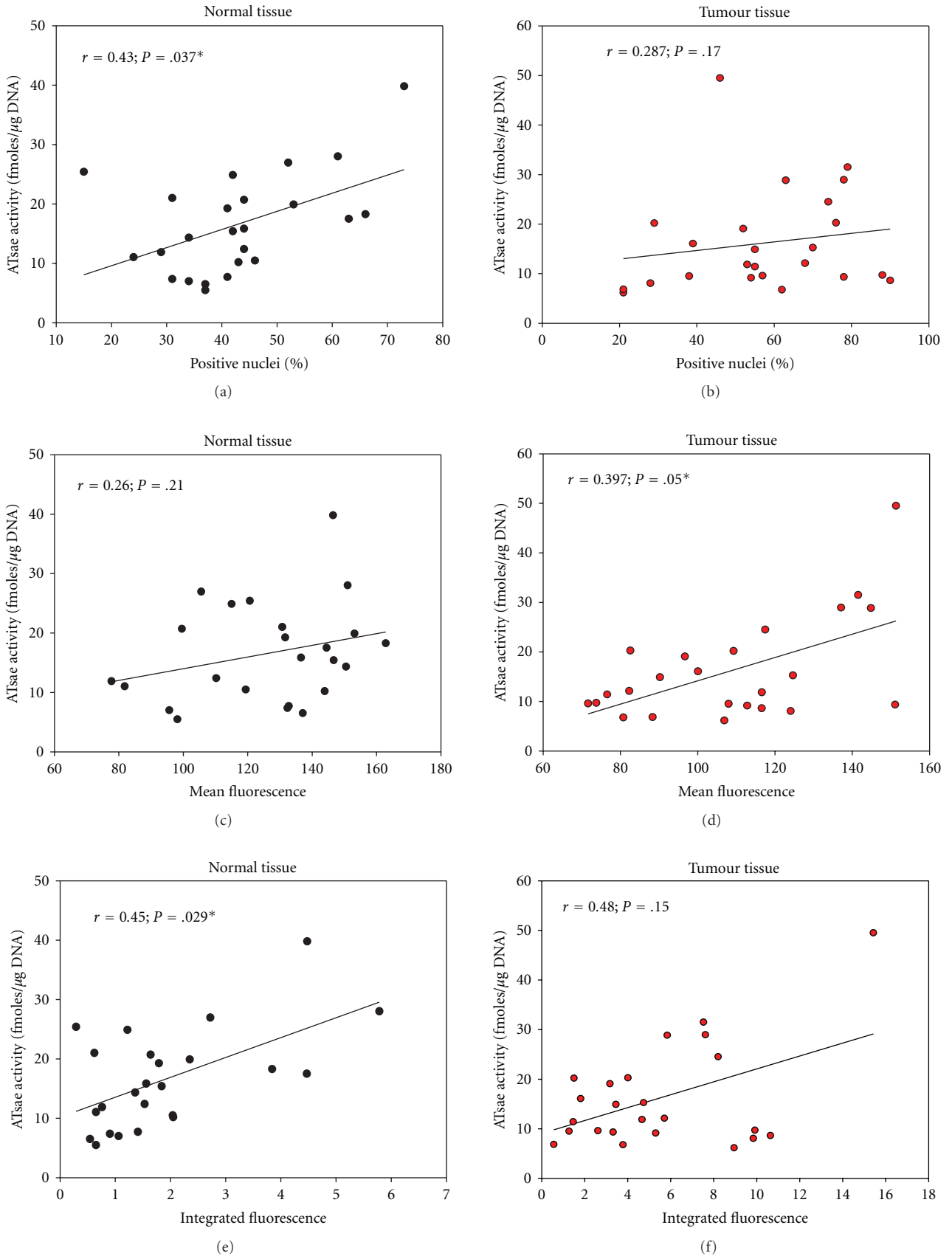
Figure 5: Relationship between MGMT activity (fmole/μg of cellular DNA) and immunofluorescence parameters of MGMT content (top: %positive nuclei, middle: mean fluorescence, bottom: integrated fluorescence) in normal and tumour tissue.
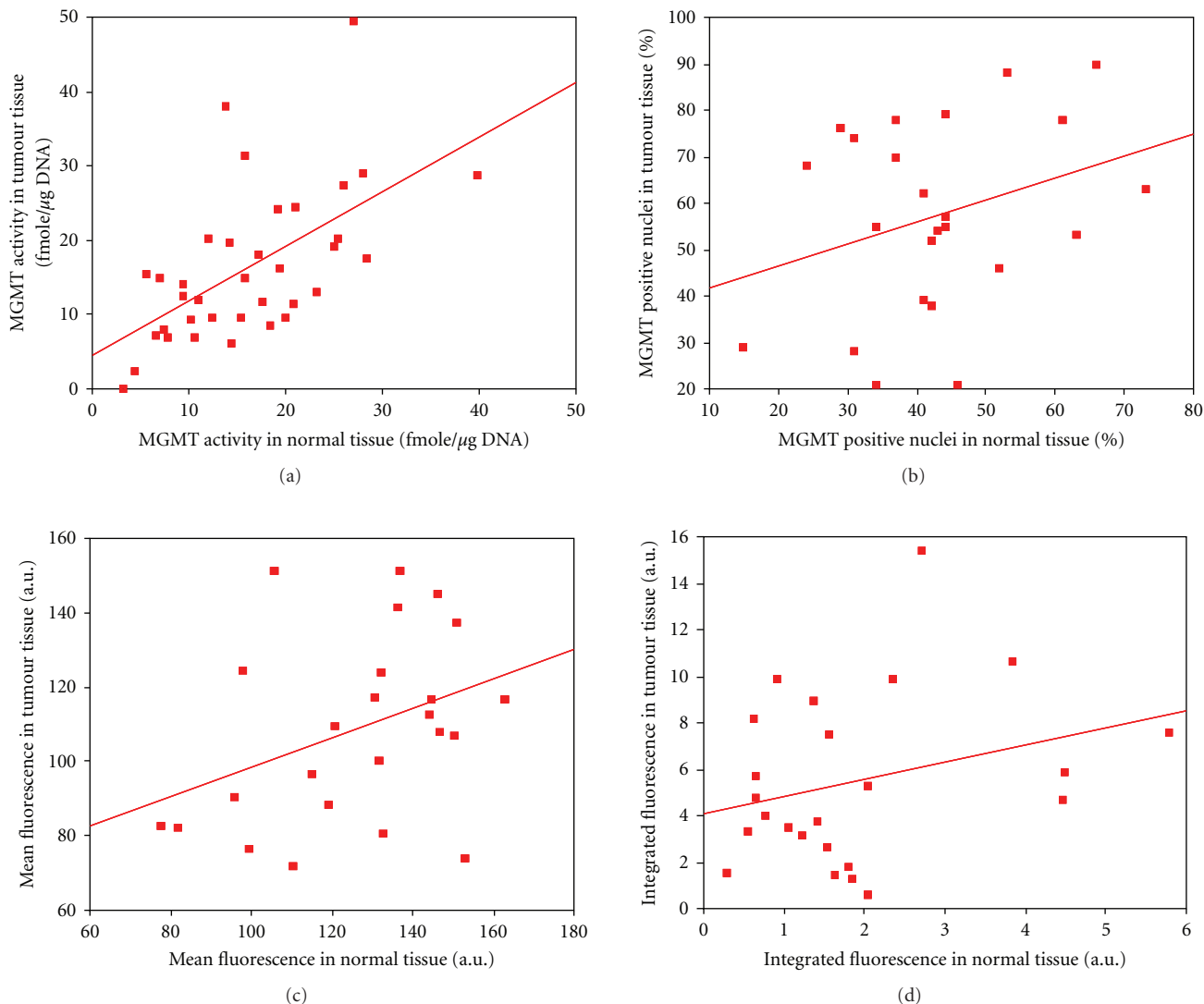
FIGURE 6: Relationship between tumour and normal tissue MGMT activity (fmoles/ug DNA, (a)), % positive nuclei (b), mean fluorescence (c) and integrated fluorescence (d), parameters of MGMT content.

TABLE 2: MGMT content in bladder tumours and uninvolved tissues measured by quantitative immunofluorescence.

Variable	Uninvolved tissue	Tumour tissue	Difference tumour uninvolved	P
% positive nuclei	42.8 ± 13.5	57.3 ± 20.3	14.5 ± 20.5	.002
Mean fluorescence	126.0 ± 23.5	108.5 ± 24.9	-17.5 ± 27.1	.004
Integrated fluorescence	1.9 ± 1.4	5.5 ± 3.7	3.6 ± 3.5	<.001

staining of MGMT was observed in many samples with variable intensities. Previous studies have reported a nuclear localisation for MGMT with only occasional mention of cytoplasmic staining [18, 20] while others reported the presence of high levels of cytoplasmic MGMT in cancer [21, 22]. The significance of cytoplasmic immunoreactive MGMT protein is unclear, but it may be that cytoplasmic staining reflects a relative inefficiency in the ubiquitin-mediated MGMT degradation pathway [23] giving rise to more persistent inactive cytoplasmic protein.

The wide range of MGMT activity observed in uninvolved tissues and bladder cancer tissues is similar to that previously reported for bladder tissue [24–27]. In the present study, although differences in functional activity were observed between tumour tissue and uninvolved bladder tissue from the same patient when expressed per unit protein, no differences were seen when expressed per unit DNA. Normalisation per unit DNA is generally considered to be more appropriate as cells vary greatly in protein content and because MGMT acts on O^6-alkylguanine lesions

in DNA; it also provides an indication of the number of MGMT molecules per cell. Differences between different sets of published data are thus likely to reflect the use of different normalisation procedures (per unit protein or per unit DNA). It is worth noting, however, that bladder tumours have a very high frequency (more than 50%; [28]) of polyploid cells, so that using DNA as a parameter, the number of MGMT molecules per cell may be grossly underestimated. Basing the results on either protein or DNA content, together with the % positive nuclei or integrated MGMT fluorescence, which are indications of the overall level of MGMT expression, would thus suggest that tumour cells contain substantially higher levels of MGMT than normal cells. The basis of this apparent MGMT upregulation remains to be established.

There is a large body of evidence to suggest that MGMT activity can be an important determinant in the efficacy of chemotherapy involving the "O^6-alkylating" agents [10, 29], and it is reasonable to consider if bladder cancer patients might benefit from such chemotherapy. There was marked heterogeneity of MGMT distribution with, in some cases, the majority of the tumour cells expressing relatively low levels of MGMT. If bladder tumour patients were to be treated with O^6-alkylating agents, this would likely lead to an initial beneficial response in terms of tumour reduction, but later, disease recurrence from those cells and areas of highest MGMT expression. Indeed, such alkylating agents are not used in bladder cancer treatment although a prospective study to investigate the pretreatment expression of MGMT and response to therapy employing appropriate alkylating agents is feasible. Bladder cancer treatment might, however, benefit from the use of MGMT inactivating agents such as BeG [30] or Lomeguatrib [31] which would potentially ablate MGMT activity in bladder cells, relatively increasing the sensitivity of the tumours cells to chemotherapy with Temozolomide or Dacarbazine. Clinical studies of these agents have established that MGMT activity can be depleted by systemic administration of such agents, but this increases the myelosuppressive effect of the chemotherapeutic agent [11]. Given our present results, intravesical administration of MGMT inactivators prior to systemic, or even local, chemotherapy using O^6-alkylating agents seems a feasible proposition.

Abbreviations

MGMT: O^6-alkylguanine-DNA-alkyltransferase
ABC complex: Avidin-biotin complex
APES: 3-aminopropyltriethoxysilane
DAB: 3′, 3′-diaminobenzidine
DAPI: 4,6-diamidino 2-phenyindole
PBS: Phosphate buffer saline
TBS: Tris-buffered saline.
IF: Immunofluorescence.

Acknowledgments

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