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# **Cytostatic Drug Treatment Causes Seeding of Gene Promoter Methylation**

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## Abstract

Epigenetic changes in multiple genes are emerging as an important mechanism for tumour cells to acquire resistance to chemotherapy. In the present work we test the hypothesis that epigenetic organisation in cancer cells can be affected by cytostatic drugs. Colorectal cancer cells were cultured for several weeks in the presence of 6-thioguanine. Bisulfite sequencing of the CpG-rich promoter regions of two expressed genes showed a significantly increased frequency of methylated CpG sites in drug-treated cells, as compared with controls: 4.7% and 1.7%, respectively, for the HPRT gene; and 11.1% and 8.2% for CDX1. Essentially all of the increase for the CDX1 gene was in a 4 CpG sub-region previously found to correlate with gene activity ( $P=0.006$ ). This pattern of sparse promoter methylation fits with a recently proposed “seeding” two-step mechanism leading up to gene inactivation in cancer cells. Taken together, our findings suggest activation in cancer cells of an epigenetic process enabling a tumour to generate drug-resistant variant cells.

## 1. Introduction

Resistance to cancer chemotherapy can be mediated by mutations in specific genes such as *MDR1* (P-glykoprotein) (1). This has been well documented in cells *in vitro* and in transgenic mice. Nevertheless in a clinical setting neither the status of *MDR1* or any other so far identified individual gene has served as a useful predictor of therapy response (reviewed in 2). The contribution of epigenetic changes to treatment failure has, however, been reported recently, including CpG methylation of the *hMLH1* promoter in ovarian cancer occurring during development of drug resistance. This has even been reported to become detectable as free tumour DNA circulating in patient sera (3). At very high drug levels (typically giving >95% cell kill and DNA synthesis inhibition) changes in overall DNA methylation have been documented, with some drug types causing hypermethylation, for example carboplatin. When a lung cancer cell line was exposed to 6-thioguanine (6TG) at 100  $\mu\text{M}$  there was a decrease in total DNA content of methylated bases, but there was no effect at 10  $\mu\text{M}$  (4). There are also data suggesting that an interplay between epigenetic changes in multiple genes is needed for a tumour to acquire resistance (5, 6). However, an unresolved issue is whether resistant cells are present as rare epigenetic variants already at the start of treatment, eventually to become enriched by selection, or whether in sensitive cells epigenetic regulatory mechanisms can be affected by a cytostatic drug stress. It has been known for some time that environmental stress factors are associated with epigenetic changes. In plants chemical mutagens and cold can lead to epigenetic modification (7, 8). Such observations have also been made in mammals. Tumours induced in rodents by exposure to chemical carcinogens have been found to contain not only oncogene mutations, but also epigenetically silenced tumour suppressor genes including p16 (9); moreover, cells or mice treated with nickel or DNA alkylating agents develop cancer-related epigenetic changes (10-12).

In the present work we attempt to answer the question as to whether tumour cells respond to clinically attained chemotherapy levels with epigenetic modifications. If that is the case then this

may help in designing strategies to combat an important source of therapy failure. Colorectal cancer (CRC) cell lines were exposed to relatively low levels of 6-thioguanine (6TG), a drug in clinical use, allowing some growth to continue for several weeks. Bisulfite sequencing of the promoter regions of two genes was then carried out. The first gene, *HPRT*, is involved in cellular resistance to this drug, as it is essential for 6TG to become incorporated into DNA in order to exert its cytostatic effect, and the second gene, *CDXI*, has no known functional relationship to the effects of 6TG.

## 2. Materials and Methods

### 2.1. Cells and 6TG treatment

Mononuclear cells were separated from freshly collected venous blood from healthy donors by Lymphoprep (Axis-Shield, Oslo, Norway) gradients and used directly. The T84 and SW620 CRC cell lines both originate from male patients. They have both been shown to be hMLH1-positive, suggesting normal DNA mismatch repair function. The CRC lines were cultured under standard conditions with 10% bovine serum in the medium, and split as appropriate before confluence was reached. 6TG was from Sigma (A4882), and a stock solution of 10 mM in 0.2M NaOH was prepared and kept in aliquots at -20°C. The cultures were washed and fresh medium with 6TG added at least once per week, in order to maintain the desired 6TG concentration. The CFDA (carboxyfluorescein diacetate) fluorescent dye exclusion test was used to determine cell viability, as described previously (13).

### 2.2. Bisulfite sequencing

DNA was extracted from the cells using the DNeasy kit (Qiagen, Crawley, UK). 0.5 µg of DNA was bisulfite-modified using the EZ DNA Methylation-Gold Kit, as recommended by the manufacturer (Zymo Research, Orange, CA). A promoter region was amplified by PCR as described below, and the PCR product was purified from a solubilized agarose gel slice using a silica column (Qiagen), cloned (TopoTA cloning kit, Invitrogen, Purchase, NY), and finally submitted to the local sequencing core facility for ABI sequencing (Applied Biosystems).

### 2.3. PCR

PCR primers were designed based on the HPRT and CDX1 5'-flanking regions given on the University of California at Santa Cruz website <http://genome.ucsc.edu>. For the sequencing PCR

reaction the primers bind to non-CpG sequences in order to yield products irrespective of the template's CpG methylation status (positions indicated in Fig. 1). The forward primer was 5'-ATTGAGTTGGGAGGGAAAGG, and the reverse primer was 5'-CCATTTCCACCTTCTCTTCCCA. Cycling conditions were 95°C for 12 min followed by 40 cycles of 95°C 1 min, 58°C 1 min 30 sec, 72°C 2 min 30 sec, and then finally 72°C for 10 min. The 25 µl reaction solution contained 0.2 µM each primer, 0.1 unit Hotstar Taq polymerase (Qiagen), 1.5 mM MgCl<sub>2</sub>, 200 µM each dNTP and 2 µl of the bisulfite-modified DNA. The PCR products were visualized after gel electrophoresis through 1.5% agarose containing ethidium bromide. The primer positions for the HPRT methylation-specific PCR are also shown in Fig. 1. These primers were designed to bind to DNA methylated at several CpG sites: the forward primer was 5'-AATTGGTAGGCGTCGGCGTAGGCGCGC, and the reverse primer was 5'-CCGACAAACCGAACTACTCACCACGAC. The cycling conditions were similar to those for the HPRT sequencing PCR, with the following differences: annealing at 63°C, elongation for 1 min 30 sec. For CDX1 sequencing PCR was performed as described earlier (14). For all PCR reactions, only the bisulfite-modified sense strand was amplified, starting with the reverse primer binding to this sense strand, and the complementary molecule thus produced serving as template for the forward primer.

#### ***2.4. Statistical Analysis***

The significance of all the 2x2 comparisons was assessed using Fisher's exact test.

### 3. Results

#### *3.1. Definition of 6TG Concentrations Used for Long-term Treatment of CRC Cell Lines*

The T84 and SW620 CRC cell lines were kept in medium containing up to 16  $\mu\text{M}$  6TG, and split as needed to keep the cultures proliferating at 20-80% confluence. In patients, plasma concentrations of up to 10  $\mu\text{M}$  6TG have been documented for the Lanvis pharmaceutical preparation (RxMed website <http://www.rxmed.com>). Expression of the HPRT gene in both lines was confirmed by RT-PCR (results not shown). Total cell number and the fraction of dead cells, as determined by the fluorescent dye exclusion test, were analysed after one week of culture (Table 1). No growth was seen at the highest concentrations of 6TG (8-16  $\mu\text{M}$ ), whereas sustained strong growth was seen at 1  $\mu\text{M}$  6TG, with both T84 and SW620 reaching 25-60% of the total cell number achieved by the untreated controls. T84 had a slightly higher growth rate than SW620, and there was no apparent effect of 6TG on cell death for either of the two cell lines. Paradoxically, the more marked growth retardation of SW620 in 6TG was coupled with less death. A similar sensitivity to 6TG was also seen with other CRC lines (COLO201, COLO678 and SW1116; results not shown). Based on these findings, 1  $\mu\text{M}$  6TG was considered to provide an optimal level of cytostatic drug stress on the CRC lines during long-term culture. T84 was still proliferating after one month in 1  $\mu\text{M}$  6TG (see T84 data at 17 days in Table 1). A minor reduction of the growth rate was also seen at 0.25  $\mu\text{M}$  6TG for SW620 (results not shown). For all long-term experiments, a 16  $\mu\text{M}$  6TG culture was set up, in addition to cultures at 0 and 1  $\mu\text{M}$ . Only a few cells were occasionally detectable after one month of culture. This suggests that cells harvested from cultures maintained for several weeks in low concentrations of 6TG do not significantly represent outgrowth of the rare HPRT-negative variants likely to be present at the start of the culture. Such variants are expected to occur at a frequency of



approximately  $10^{-6}$  in CRC lines (15), while for cloning of HPRT- mutants much higher 6TG concentrations, namely around 10-15  $\mu\text{M}$ , are usually used (16).

### ***3.2. Analysis of CpG Methylation in the HPRT Promoter Region***

The HPRT gene has been extensively studied (17-19). There is very good evidence indicating that its expression is regulated by a CpG-rich approximately 500 bp upstream region. Due to the X chromosome location of HPRT, and its house-keeping function in the nucleotide salvage pathway, human cells in general carry only a single active allele. However, which CpGs are most important for the control of HPRT gene expression has not been clearly established. In the present work, we have chosen to study the 357 bp region 5' to exon 1 because of its relatively high homology with the corresponding mouse sequence (61%, as compared to 34% in the further upstream 250 bp) (Fig. 1). This 357 bp segment contains a total of 43 CpG sites, and several transcription factor-binding motifs including, for example, four GGGCGG Sp1 sites. After DNA extraction and bisulfite-modification, the promoter region was PCR-amplified, cloned and sequenced. In accordance with published data (19), the promoter in male lymphocytes was virtually free from methylated CpGs, whereas all 6 clones from female lymphocytes showed dense methylation (Fig. 2A). This over-representation of the inactive female allele indicates that bisulfite-modified molecules containing relatively few long thymine-rich stretches (due to the conversion by bisulfite of cytosine to thymine, while methylated cytosines remain as cytosine) are preferentially amplified by the current PCR protocol. T84 cells, originating from a tumour of a male patient, showed a very low level of methylation (Fig. 2B, upper panel), similar to that in male lymphocytes. T84 cells exposed for 28 days to 6TG showed no signs of the heavy methylation found in female lymphocytes (Fig. 2B, lower panel). However, the total number of methylated CpGs among the total of 12 clones analysed from the 6TG-treated cultures (4.7%) was significantly higher than that in the 11 clones from untreated cells (1.7%). These results are

given in Table 2, together with an estimate of the possible artefactual contribution to the scoring of methylated CpGs arising from bisulfite-modification errors. In the analysed 376 bp HPRT promoter region there is a total of 88 cytosines at non-CpG positions, all of which are assumed to be unmethylated, and therefore should be converted to thymines by bisulfite. The number of cytosines remaining after bisulfite-modification at these 88 positions can be used as a measure of the bisulfite failure rate assuming this is the same for C at CpG and non-CpG sites. Thus, for example, the 1.5% of errors for 6TG untreated T84 suggests that the observed figure of 1.7% of C's at CpG sites reflects mainly bisulfite modification failures, whereas for the 6TG-treated T84 cells subtraction of the 2.1% errors from the 4.7% of observed cytosines at CpG sites indicates a true presence of 2.6% methylated CpG sites, the difference presumably being due to the effects of culturing in the presence of low concentrations of 6TG. There was no significant change in the numbers of methylated C sites at the non-CpG positions. Line SW620 showed a slightly lower increase in the number of methylated C sites at CpG positions, but this was not significant because fewer clones were analysed.

### ***3.3. Analysis of CpG Methylation in the CDX1 5'-Flanking Region***

CDX1 is involved in colonic epithelium differentiation and was chosen as a representative gene with no known specific role in 6TG toxicity. In a previous report on a panel of 37 CRC lines, we found evidence for a role for CDX1 as tumour suppressor in colorectal cancer (14), with 12 of the analysed lines showing either CDX1 silencing due to promoter methylation or a pattern of partial methylation. We now find lymphocytes to have heavy CDX1 promoter methylation, in accordance with our previous result for a lymphoblastoid cell line, suggesting, as expected, that CDX1 is not expressed in these cells (Fig. 3A). In our earlier study, T84 was positive for CDX1 mRNA and showed no signs of heavy promoter methylation as judged by methylation-specific PCR. In line with these published data, we now see a sparse CDX1 promoter methylation among

the 15 analysed T84 clones grown in the absence of 6TG. There is, in particular, very little methylation at the 4 CpG sites shown to be strongly correlated with gene expression (14) (Fig. 3B, upper panel). Interestingly, however, the region containing the 29 CpGs before the 4 CpG sites differs significantly in the proportion of CpG sites with methylated C versus that containing the 5 CpG sites after, and neither show any significant change with growth in 6TG (including control and 6TG data, 7.2% versus 18%,  $p=0.001$ ). T84 cells exposed to 6TG for 28 days showed a slightly higher overall proportion of methylated CpGs, similar to the results for HPRT, with an increase from a relatively high basal level of 8.2% to 11.1% in the 6TG-treated culture (Fig. 3B, lower panel, and Table 3). However, essentially all of the increase was in the 4 CpG sub-region previously found to correlate with gene expression (marked with a box in Fig. 3), where the change from 7% to 27% was highly significant with a  $p$  value of 0.006, which remains significant even allowing for some selection of the comparisons being made. The bisulfite modification errors were at a clearly lower level, similar to that seen in the HPRT experiments (2.5% and 3.0%, respectively).

### ***3.4. Methylation-Specific PCR in Search of Rare Heavy Promoter Methylation Events***

Although sequencing revealed no signs of cells with a densely methylated HPRT promoter suggestive of gene silencing, a low frequency of such variants could not be excluded. Recently two groups have, for example, presented evidence for a “seeding” two-step mechanism operating during tumour suppressor gene silencing (20-23). The first step is suggested to involve a low-density methylation occurring in a high fraction of the tumour population, similar to that we have observed for both HPRT and CDX1, while the second step involving high-density promoter methylation leads to gene inactivation seen only in a low fraction of the “seeded” cells, presumably as a result of selection for reduced gene expression. Stimulated by these reports, we performed methylation-specific PCR, using primers binding only to methylated CpG stretches.

This assay was sensitive enough to detect as little as one methylated copy among 100 unmethylated molecules (Fig. 4, lane 5). However, no PCR band was seen with the same 6TG-treated T84 cells that were used in figures 2 and 3, suggesting that the frequency of T84 cells becoming heavily methylated during 6TG-treatment was less than 1% (Fig. 4).

## 4. Discussion

We report here a significant, though low-level, increase in promoter CpG methylation in colorectal cancer cells treated with a cytostatic drug, 6TG, at a clinically relevant concentration. The data, which show variability in methylation within the promoter regions of two genes both in controls and following 6TG treatment, indicate the potential complexity of factors controlling DNA methylation at CpG sites. In addition to the fact that the observed sparse methylation was statistically significant for both of the studied genes, a case can be made in support of the relevance of our observations. The determination of cytosine methylation at CpG sites is based on the conversion of non-methylated cytosines to thymine, whereas a 5'-methylated cytosine is resistant, resulting in a cytosine rather than thymine on sequencing analysis of bisulfite-modified DNA. Since cytosines located at non-CpG positions are considered not to become methylated, it is possible to estimate the error rate of the bisulfite process by scoring the number of cytosines remaining as a cytosine at non-CpG sites. For the HPRT promoter in untreated T84 cells, the frequency of such methodological errors was no different from the frequency of methylation observed at CpG sites (1.5% and 1.7%, respectively). Assuming a uniform error rate at all cytosine positions, true frequencies of CpG methylation in T84 cells are therefore  $1.7 - 1.5 = 0.2\%$  for no 6TG and  $4.7 - 2.1 = 2.6\%$  for 6TG-treated cells. These figures suggest that during 6TG treatment CpG methylation went from a virtually methylation-free state (0.2%) to a sparsely methylated level of 2.6%. Superficially, the corresponding picture for the CDX1 gene was not as clear-cut, with a true basal CpG methylation frequency of  $8.2 - 2.5 = 5.7\%$ , going up to  $11.1 - 3.0 = 8.1\%$  in 6TG-treated T84 cells. However, this overall analysis hides the fact that essentially all the change in the frequency of methylation occurred in the region of 4 CpGs (from 7% to 27% in that region) which has been shown to be critical for the control of expression of the CDX1 gene (14). Thus, among the total excess of 16 methylated CpG's in 6TG-treated cells, 12 (75%) occurred in a region representing only 11% (4/38) of the total analysed CpG content.

If, as we believe our data show, there is a real, though low-level drug-associated increase in promoter methylation, does this represent a toxic and non-specific effect, or does it in any way relate to the inactivation of tumour suppressor genes in cancer? As already discussed, it has been suggested that 'seeds of methylation' may be an important precursor to dense methylation (23). Thus, CpG silencing of a glutathione-S-transferase (GSTP1) construct in prostate cancer cells has been shown to require treatment with HpaII methylase, mediating sparse methylation (20). Similar data come from gastric cancer cell lines where low-density methylation in most cells, as we have found in 6TG-treated cells, was associated with dense methylation in a small fraction of the cells for all the five analysed genes including E-cadherin (22). These reports support that our findings of sparse methylation may reflect a mechanism which provides the basis for an increased frequency of gene silencing in cancer cells during anti-cancer treatment, presumably following selection for reduced gene expression. Our findings are also in agreement with previous reports on the cumulative influence of epigenetic changes in multiple genes for emergence of drug resistance, on the assumption that drugs other than 6TG may act in a similar way to increase methylation at CpG sites (6).

Our evidence supports the view that the CpG methylation seen in our HPRT promoter clones does not represent the outgrowth of a rare subpopulation of cells already present before 6TG treatment. Firstly, there is no reason to assume that such a sparse promoter methylation would in any way affect gene expression or yield a selectable phenotype. Secondly, there was a continuous increase in cell number at the 6TG concentration (1  $\mu\text{M}$ ) used by us, which argues against significant enrichment of preformed variants. Furthermore, a 16  $\mu\text{M}$  6TG culture (a growth-inhibitory concentration used for the selection of HPRT-negative cell clones) was always run in parallel with the 1  $\mu\text{M}$  culture. A steady decrease in cell number was then observed, with outgrowth of clones occurring in only a small minority of all culture flasks.

In conclusion, our data show that treatment with low levels of the cytotoxic drug 6TG leads to low-level increases of methylation at CpG sites, which suggests that this may also be seen with other drug treatments. In that case, such drug treatments may be analogous to treatments which increase the mutation rate, in that increasing the level of non-specific CpG methylation may increase the probability of functionally relevant methylation changes occurring, which are then selected for. Future work is needed to document promoter methylation in association with other treatment modalities such as ionizing radiation and alkylating agents, as well as to test whether inhibition of CpG methylation may interfere with the development of drug resistance.

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### **Conflict of interest statement**

None declared

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**Table 1. Sensitivity of CRC cell lines to 6TG**

<b>CRC</b>	<b>6TG (<math>\mu</math>M)</b>	<b>Total cell number, relative to absence of 6TG (% dead cells)</b>	
		<b>7 days 6TG</b>	<b>17 days 6TG</b>
<b>T84</b>	<b>0</b>	<b>100 (7)</b>	<b>100 (3)</b>
	<b>1</b>	<b>60 (10)</b>	<b>67 (4)</b>
	<b>2</b>	<b>22 (11)</b>	<b>13 (3)</b>
	<b>4</b>	<b>4 (11)</b>	<b>8 (7)</b>
	<b>8</b>	<b>2 (9)</b>	<b>1 (5)</b>
	<b>16</b>	<b>1 (14)</b>	<b>&lt;1 (6)</b>
<b>SW620</b>	<b>0</b>	<b>100 (1)</b>	<b>n.d.</b>
	<b>1</b>	<b>25 (2)</b>	<b>n.d.</b>
	<b>2</b>	<b>14 (1)</b>	<b>n.d.</b>
	<b>4</b>	<b>2 (2)</b>	<b>n.d.</b>
	<b>8</b>	<b>1 (1)</b>	<b>n.d.</b>
	<b>16</b>	<b>&lt;1 (2)</b>	<b>n.d.</b>

**Table 2. HPRT promoter: bisulfite DNA sequencing results**

Cells	Number of sequenced clones	6TG (µM / days)	C at CpG sites (= methylated CpG)	C at non-CpG site (= bisulfite failure)
Male lymphocytes	7	0	4/301 (1.3%)	6/616 (1.0%)
Female lymphocytes	6	0	168/258 (65.1%)	21/528 (4.0%)
T84	11	0	8/473 (1.7%)	15/968 (1.5%)
T84	12	1 / 28d	24/516 (4.7%) <sup>a</sup>	22/1056 (2.1%) <sup>b</sup>
SW620	2	0	0/86 (<1.2%)	0/176 (<0.6%)
SW620	2	0.25 / 7d	0/86 (<1.2%)	0/176 (<0.6%)
SW620	5	1 / 7d	4/215 (1.9%) <sup>c</sup>	1/440 (0.2%)

Footnotes - all significance tested by Fisher's exact test, 2 sided (except where indicated), results as p values:

a control versus 6TG, p= 0.0108

b NS

c control and 0.25 6TG versus 1 6TG, p=0.1323 (one sided p=0.0941)

**Table 3. CDX1 promoter: bisulfite DNA sequencing results**

Cells	Number of sequenced clones	6TG ( $\mu$ M / days)	C at CpG sites (= methylated CpG)	C at non-CpG site (= bisulfite failure)
Female lymph	6	0	162/228 (77.1%)	14/702 (2.0%)
T84	15	0	47/570 (8.2%)	44/1755 (2.5%)
T84	15	1 / 28d	63/570 (11.1%) <sup>a</sup>	53/1755 (3.0%) <sup>b</sup>

Footnotes - all significance by Fisher's exact test, 2 sided except where indicated, p values given:

a p=0.1321 ( one sided 0.0661)

b NS

-329      seq F →

bisulfite attgagttg ggagggaaag gggttttgtt gggggagttb tggtttttt tgggagaaa ttttatggt tatttagtga

human actgagttg ggagggaaag gggcttcgct gggggagcct cggcttctc tgggagaaa tcccacggc tacctagtga

mouse ggctgcgta tggccagtac catttctc agaaagaaa ttatcaggcc cacctagtca

-249      methyl F →

bisulfite gtttgtaaat tgtaggtgt tggtgtaggt gtgtgggtg ggttgggggt ggggtttgtg gggtgtggtg ggtgggtag

human gcctgcaaac tgtaggcgc cggcgtaggc gcgcgggcgg ggccgggggc ggggcctgcg gggcgtggcg gggcggggcag

mouse gataagatt ccggaactgc ctttggtggc gcgcgagcgg agccggggac ggagcctgcg ggccgag--a gggcggggccg

-169

bisulfite aggggtgggt ttgtttttt ttagtttag gtggttgta tgagtttta ggtgaatntt ttggtttt tggtggtgt

human agggcggggc ctgcttctc tcagcttcag gcggctgcga cgagccctca ggcgaacctc tcggctttc cgccgggcgc

mouse agggcgggagc ctggccggcc tgagcctgag gcggcgaggg agagc-gttg ggcttacctc actgctttc ggagcggtag

-89

bisulfite tgttttttgt tggtttttg ttttttttt tgttttgta ttggttttt tttttgagt agttagttt tggtttggt

human cgcctcttg tgcgctcgc cctcctctc tgctccgca ccggcttct cctcctgagc agtcagccc cgccgggc

mouse cacctct--      -----      -----      -----ccg ccggcttct cctca----- -gaccgccg cgagccgacc

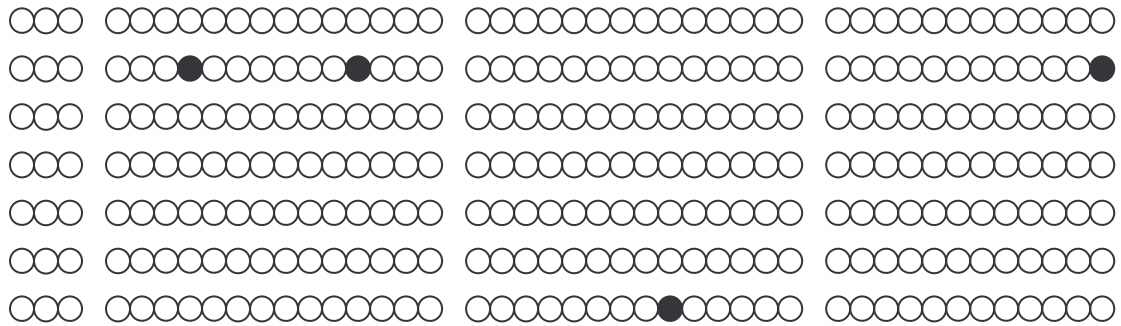
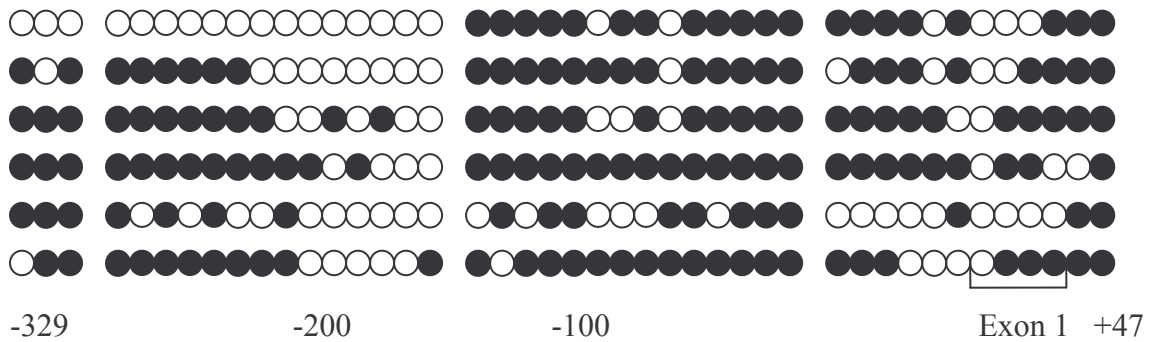
-9      methyl R ← +47      seq R ← +290

bisulfite ggttttgta tgtgatttg tagttttgt gttggtgta gtagttt gtttg / tgggaagagaaggtggaatgg

human ggctccgta tgcgaccgc cagccctggc gtcgtggtga gcagctcggc ctgccg / tgggaagagaaggtggaatgg

mouse ggtcccgtca tgccgaccgc cagtcccagc gtcgtggtga gccaaggggt ccagca

**Fig. 1 - Sequences of the 5'-flanking regions for human and mouse HPRT. The bisulfite-modified sequence is human DNA, assuming all CpG sites are unmethylated. CpG's are underlined. Mouse portions homologous to the human sequence are in bold. Positions relative to the human exon 1 start are shown above the bisulfite line. The arrows indicate the PCR primer positions.**

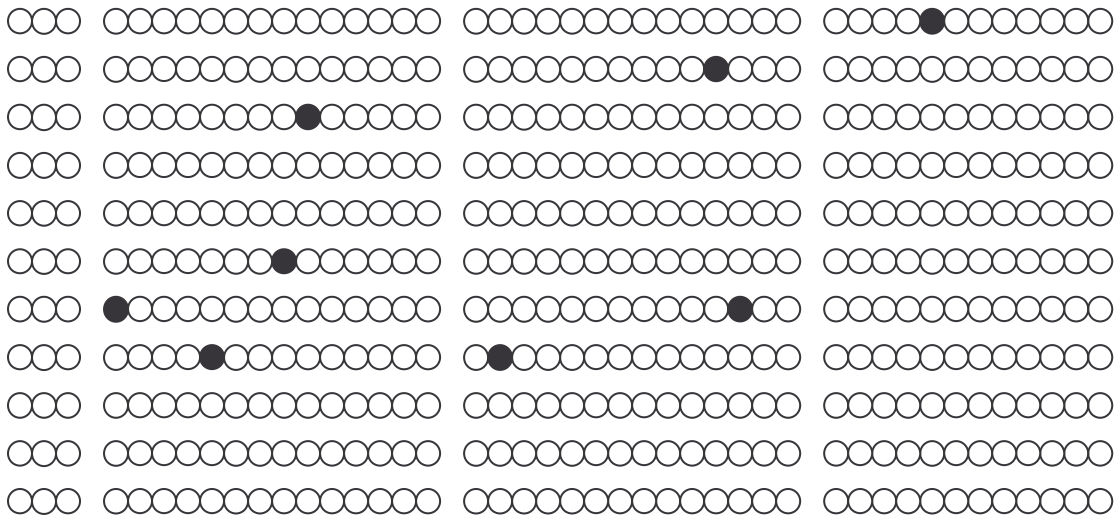
**(a)****Male lymphocytes****Female lymphocytes**

**Fig. 2 - HPRT promoter bisulfite DNA sequencing results for individual CpG sites. (a) 7 clones from male lymphocytes and 6 from female lymphocytes. (b) 11 clones from untreated T84 cells and 12 from 6TG-treated T84. Each of the 43 CpG sites is shown as a circle, and methylated CpGs are highlighted in black. The base positions relative to the exon 1 start site are shown. Each line represents one clone.**

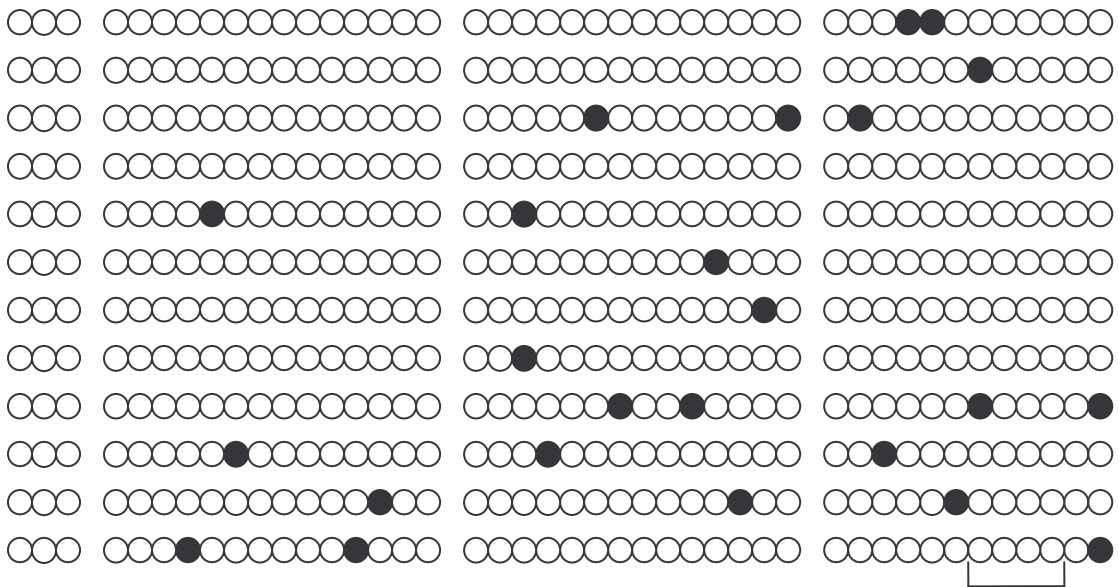


**(b)**

**T84, no 6TG**



**T84, 1uM 6TG for 4 weeks**



-329

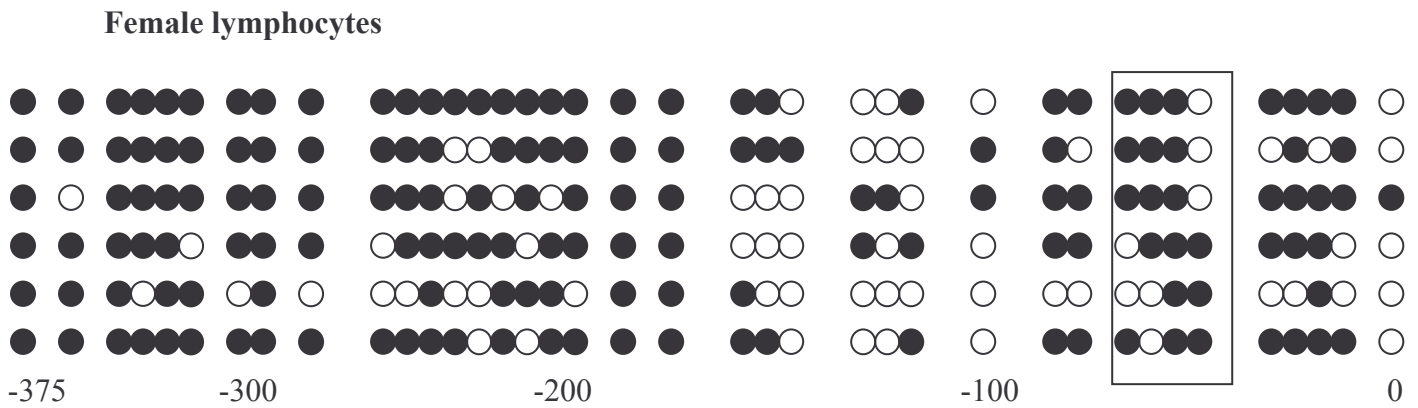
-200

-100

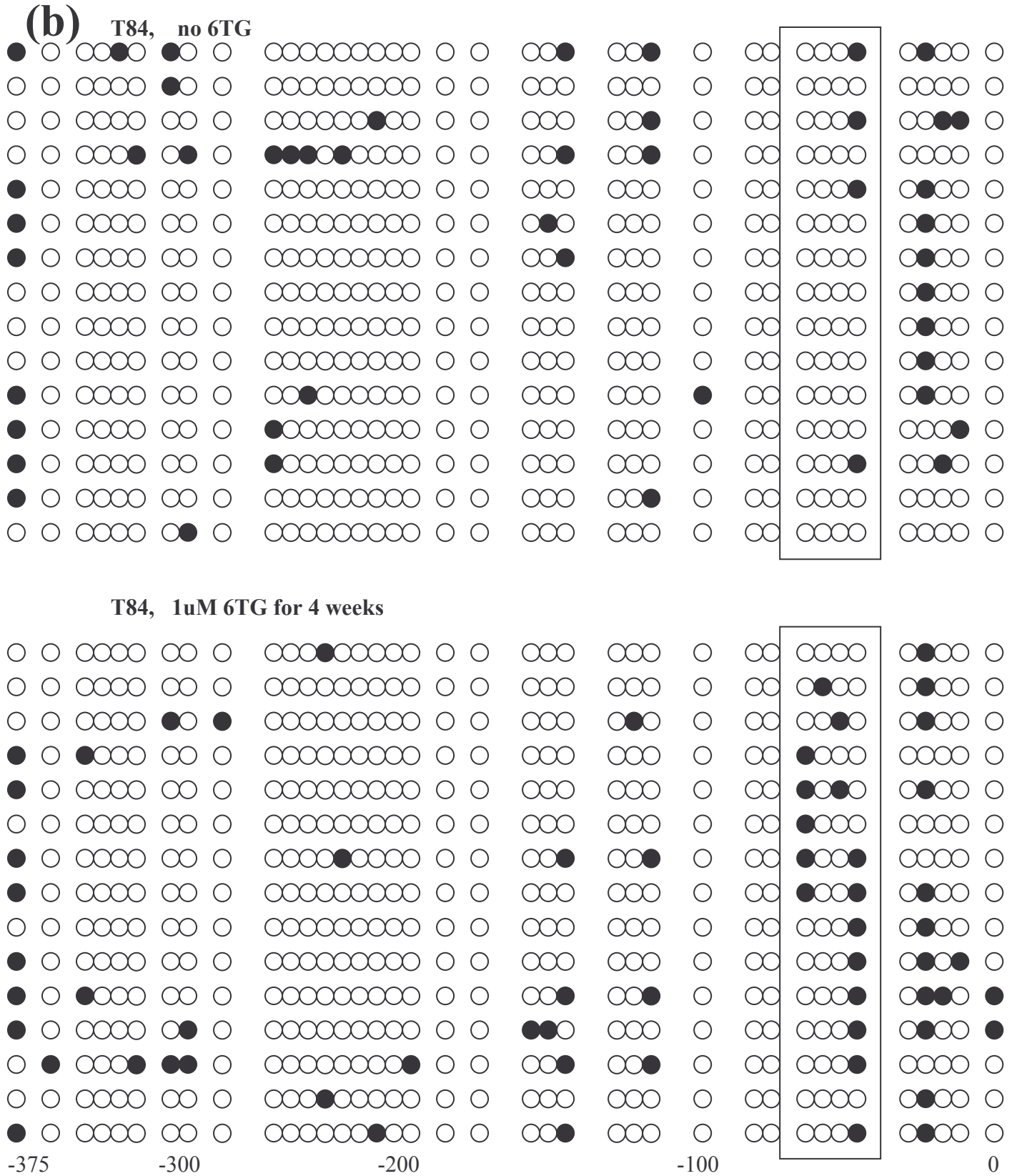
Exon 1 +47

**Fig. 2b** - Please see previous page for the Fig 2 legend.

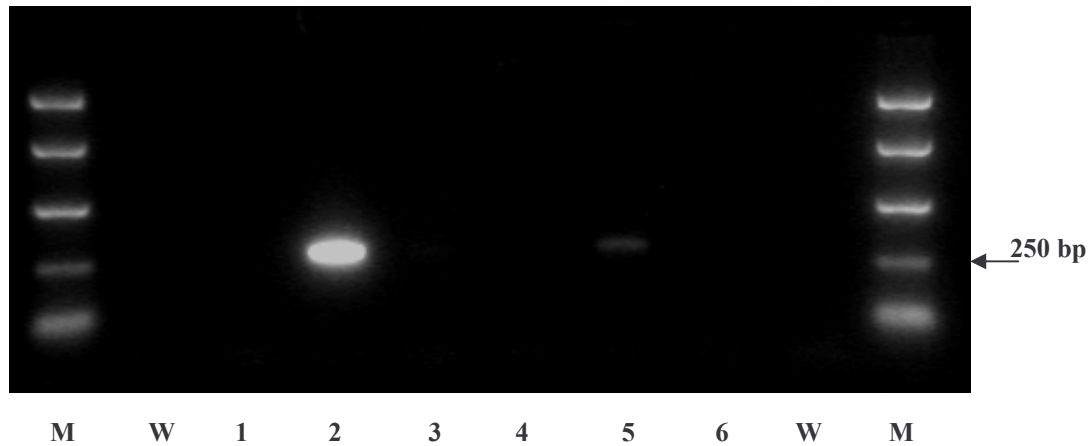
(a)



**Fig. 3 - CDX1 promoter bisulfite DNA sequencing results for individual CpG sites. (a) 6 clones from lymphocytes. (b) 15 clones from untreated and 6TG-treated T84 cells, respectively. Each of the 38 CpG sites is shown as a circle, and methylated CpGs are highlighted in black. The base positions relative to the transcription start site are shown. The box denotes the 4 CpG sites correlating strongly with gene expression (14). Each line represents one clone.**



**Fig. 3b.** Please see previous page for the Fig 3 legend.



**Figure 4. No evidence for clones with high-density HPRT promoter methylation in 6TG-treated T84. Methylation-specific PCR was performed with primers binding only to methylated CpG stretches (as shown in fig. 1). Bisulphite-modified DNA was from female lymphocytes (lane 2), T84 without 6TG-treatment (lane 3), T84 after 4 weeks with 1  $\mu$ M 6TG (lane 4), male lymphocytes spiked with a 100x dilution of the female lymphocyte DNA solution used in lane 2 (lane 5) (a faint band could be seen in the illuminated gel), male lymphocytes as in lane 5 with no spiking (lane 6). Lane 1: Female lymphocyte native DNA not modified with bisulphite. W=water control, M=size marker.**