# Chapter 6

## MHC class I pathway genes not encoded within the MHC

## 6.1 Introduction

To date there are at least 12 known genes whose products are involved in the MHC class I antigen presentation pathway. This was discussed in chapter 5. The aim of the pDMR screen (chapter 5) was to identify DMRs within the MHC region on the human chromosome 6 which can be associated with the MHC class $I^-$ phenotype. However as four of the genes involved in the pathway are not encoded within the MHC region, there may be additional genomic regions (outside the MHC) for which methylation patterns may be important. In order to investigate this further I performed expression and DNA methylation analyses for these four genes: beta-2 microglobulin (*B2M*), *ERp57, calnexin* (*CANX*) and *calreticulin* (*CALR*). I studied these genes using the same cell lines as for the MHC encoded genes (chapter 5). The experimental approach employed in this chapter involved use of real time qPCR and bisulphite sequencing.

## 6.2 Non-MHC encoded MHC class I pathway components

B2M is an invariant small polypeptide chain referred to as the 'light' chain and is encoded on chromosome 15 at location 42,790,967 – 42,797,651. For stable expression on the cell surface, MHC class I molecules are always associated with B2M. In the absence of B2M, MHC class I molecules are not stably expressed on the cell surface (Hughes et al., 1997).

ERp57 is a member of the protein disulphide isomerase (PDI) family of thiol oxidoreductases (Garbi et al., 2007). Recent studies have shown that, together with TAPBP, it is an essential structural component required for the stable assembly of the MHC peptide-loading. ERp57 and TAPBP are involved in the formation of disulfide bonds of MHC class I molecules (figure 5.1) (Garbi et al., 2006; Kienast et al., 2007). *TAPBP* is encoded on chromosome 15 at location 41,825,882 – 41,852,093.

CARL is a calcium binding lectin that recognises N-linked glycans bearing a terminal glucose residue, an intermediate in oligosaccharide maturation, present on incompletely folded ER glycoproteins. CALR associates with MHC class I dimers and interacts poorly with free MHC class I heavy chains. *CALR* deletion results in low cell surface MHC class I expression (Culina et al., 2004). *CALR* is encoded on chromosome 19 at location 12,910,392 – 12,916,274.

CANX is an endoplasmatic reticulum (ER) lectin similar to CALR (Williams and Watts, 1995). One difference is that the latter can bind to newly synthesised MHC class I heavy chains as well. *CANX* is encoded on chromosome 5 at location 179,058,536-179,091,243.

.

### 6.3 Expression analysis of *B2M, ERp57, CRT* and *CANX genes*

Expression analysis of these four genes was done in the same way as for the MHC-encoded MHC class I pathway genes (section 5.3.1). According to this analysis *B2M* is the only gene that is down-regulated (fold >1.5) in all cancer cell lines. *ERp57, CALR* and *CANX* showed almost normal expression levels in most of the cell lines, except for few cases (three cell lines for *CALR* and *CANX* and one cell line for *ERp57*) where a up-regulation (fold > 1.5) was observed in some cell lines (figure 6.1).

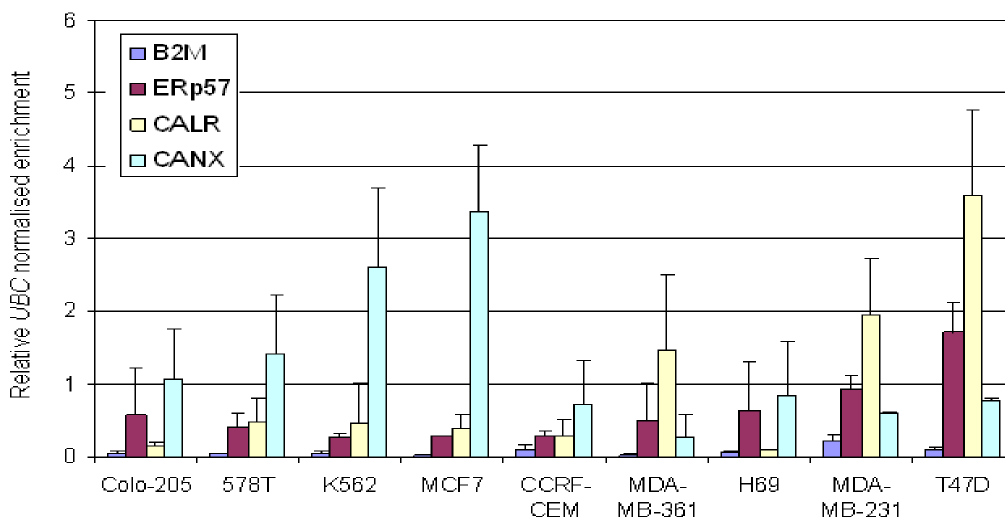

Figure 6.1. **Relative expression of non-MHC encoded MHC class I pathway genes.** mRNA levels of *B2M, ERp57, CALR* and *CANX* were determined by quantitative RT-PCR. After normalizing expression to UBC (section 2.2.2.4.3) the fold change in expression levels was calculated relative to the two control cell lines. Figure shows data corresponding to two biological replicates and three technical replicates of each (six measurement in total).

Expression analysis for these genes was also performed on mRNA extracted from MCF7 and 578T cells that were treated with 5-aza-CdR, as described in chapter 5 (section 5.4). Methylation inhibition affected only the expression of *B2M* in MCF7 cells. Specifically, expression of *B2M* increased in a 5-aza-CdR dose-depended manner up to 9-fold (figure 6.2). This effect is very similar to that corresponding to the *TAP2* gene (figure 5.4) in MCF7 cells.

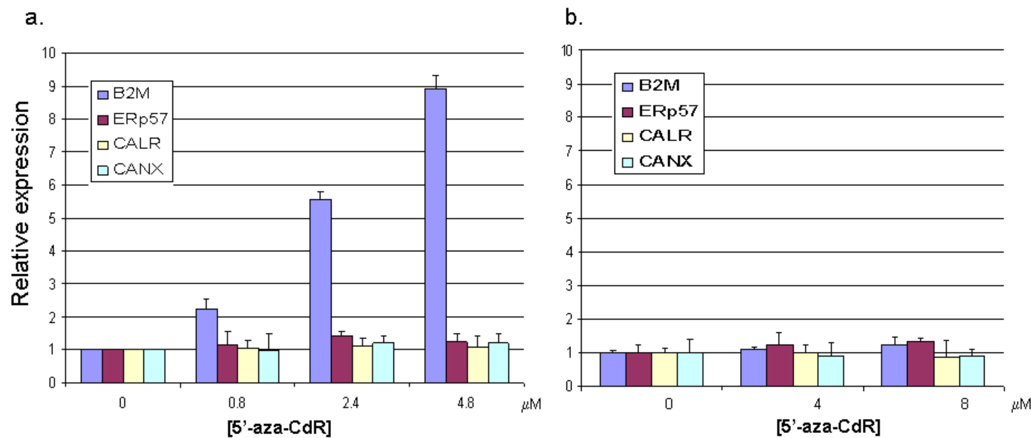


Figure 6.2: **Gene expression after DNA methylation inhibition in two cancer cell lines.** mRNA levels of *B2M, ERp57*, *CANX* and *CALR* were determined in MCF7 (a) and 578T (b) in both untreated and 5-aza-CdR treated cells. After normalizing expression to *UBC,* the fold change in expression was calculated relative to untreated cells. The mean of six measurements (two biological replicates and three technical replicates for each) is shown in part a. The mean of three technical replicates is shown in part b.

Hence, it is possible that DNA methylation is implicated only in *B2M* expression and only in MCF7 cells. As 5-aza-CdR treatment has no effect on *B2M* expression in 578T, it is likely that different cancer cell lines exploit different mechanisms to silence the same genes. Treating additional cell lines with the methylation inhibitor would be

informative. In an effort to elucidate this further I studied methylation across the encoding region of the *B2M* gene as described in the following section.

### 6.4 Methylation analysis of the *B2M* gene.

*B2M* is encoded within a 7kb region on chromosome 15 and has four exons (figure 6.3a). It has a CpG island covering about 200 bp of the 5'UTR, the first exon and about 500bp of intron 1. I aimed to generate bisulphite sequencing data for all CpG sites within the *B2M* gene. Methylation data for 20% of the CpG sites covering the whole gene were generated (figure 6.3b).

According to these data there are 3 CpGs in the 5'UTR and 8 CpGs at the end of intron 1 and beginning of exon 2, which are hypermethylated in all cancer cell lines compared to the shared controls. Their methylation status was tested after 5-aza-CdR treatment (see section 5.4) and in all cases methylation dropped to 20% (figure 6.3c). Although, the hypermethylation of these sites could be correlated with *B2M* expression, 5-aza-CdR treatment has no effect on B2M expression levels in 578T cells (figure 6.2). Hence, I cannot conclude that DNA methylation affects *B2M* expression.

However, it is worth noting the 9-fold increase of B2M mRNA levels following 5-aza-CdR treatment of MCF7 cells. Interestingly, this cell line is the only one that is hypermethylated close to the CpG island in intron 1 (figure 6.3a,b) and also has the lowest level of *B2M* expression compared to other cell lines (figure 6.1). A similar observation was made in chapter 5 for the *PSMB8* gene (section 5.6). It is possible that down-regulation of the *B2M* gene below a certain threshold leads to additional methylation, but treatment of additional cell lines with methylation inhibitors is necessary to verify this speculation.

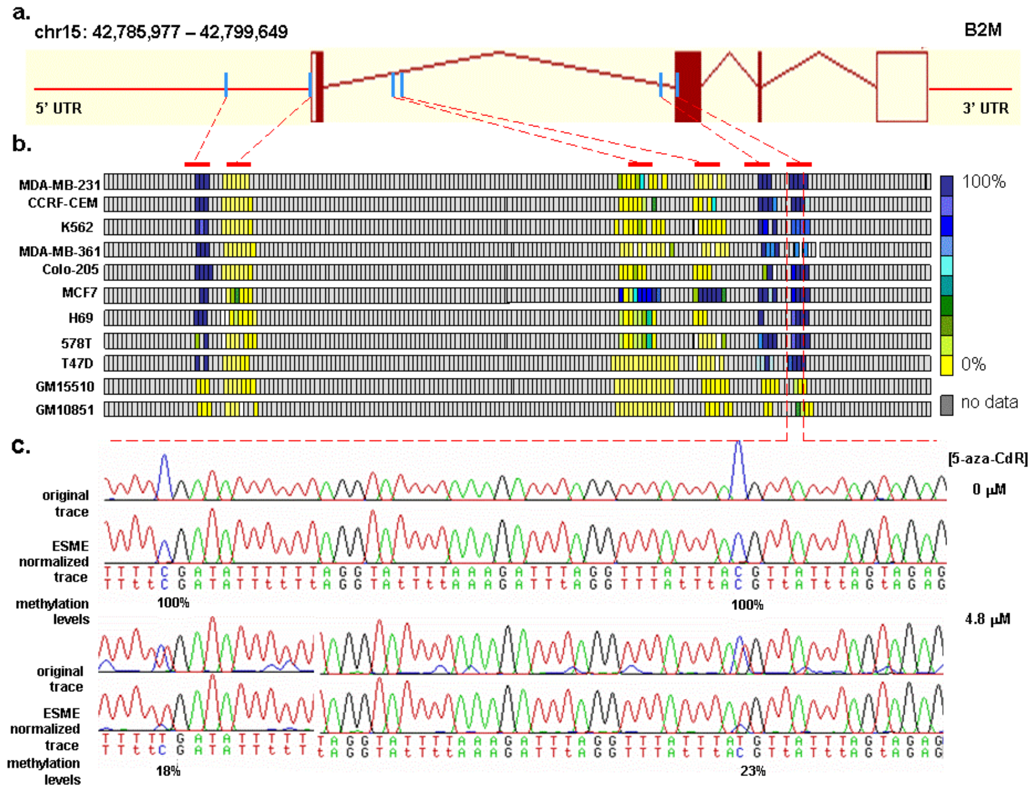


Figure 6.3. **Methylation analysis of the *B2M* gene**. Methylation analysis of six regions (average size 300-400bp) covering parts of the *B2M* gene was performed by bisulphite sequencing. (a). Diagram of the *B2M* gene. Regions (six in total) analysed by bisulphite sequencing are indicated by blue lines. (b). *B2M* methylation analysis data. Each square represents a CpG site. Total number of CpG sites within the *B2M* gene is shown. The colour code indicates methylation percentage as calculated by ESME analysis. (c). Methylation levels after 5-aza-CdR treatment. DNA extracted from MCF7 5-aza-CdR treated and untreated cells were subjected to bisulphite sequencing analysis. Data were analysed using ESME. Traces corresponding to 5-aza-CdR treated and untreated samples as well as traces before and after ESME normalization values are shown. Concentration of 5-aza-CdR used is indicated. Methylation values for each CpG are shown. This figure shows a representative part of the sequencing data generated corresponding for the amplicon in exon-2.

## 6.5 Discussion

In this chapter the four genes, *B2M*, *ERp57*, *CANX* and *CALR*, involved in the MHC pathway and encoded outside the MHC, were analysed. Expression analysis revealed that only *B2M* is down-regulated in the nine cancer cell lines tested. Treatment of two cell lines with DNA methylation inhibitors resulted in *B2M* up-regulation in only one (MCF7). Bisulphite sequencing analysis revealed methylation

differences between the cancer cell lines and the shared controls. However, because of no induction of expression in 578T cells after 5-aza-CdR treatment, methylation within the *B2M* gene could not be correlated with expression. The induction observed in MCF7 5-aza-CdR treated cells may be explained by an MCF7-specific silencing mechanism involving DNA methylation; different cancer cell lines may use different mechanisms for gene silencing.

*ERp57*, *CANX* and *CALR* did not show down-regulation in the cell lines studied here and did not respond to 5'-aza-CdR treatment. Hence their methylation status was not studied further. The slight up-regulation of these genes in some of the cell lines may contribute to the MHC class $I^-$ phenotype. Further experiments including inhibition of expression of these genes and studying the effect on MHC class $I^-$ phenotype will be informative.

## 6.6 Conclusion

*B2M*, *ERp57*, *CANX* and *CALR*, are genes encoded outside the MHC region, but their products are involved in the MHC class I pathway. Expression analysis revealed that only the *B2M* gene was down-regulated in the cell lines displaying the MHC class $I^-$ phenotype.