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Epigenetic silencing of *MHC2TA* transcription in cancer

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ABSTRACT

Lack of expression of major histocompatibility complex (MHC) molecules of both classes is frequently noted on tumour cells [1]. It is thought that in this way tumour cells escape immunosurveillance. The genes encoding both classes of MHC molecules are localized on the distal part of chromosome 6 (6p21.3). The class II transactivator (CIITA), encoded by the *MHC2TA* gene, is essential for transcriptional activation of all *MHC-II* genes, while it has a helper function in the transcriptional regulation of *MHC-I* genes (with the exception of human leukocyte antigen (*HLA*)-*G*) and of the gene encoding β2-microglobulin (β2*m*) [2]. Here we discuss our current knowledge on the expression characteristics of *MHC2TA* and argue for an important role of epigenetic factors and mechanisms in the transcriptional silencing of *MHC2TA* in cancer cells.

1. MHC-II function and regulation

MHC-II genes encode the polymorphic human leukocyte antigen (HLA)-DR, -DQ and -DP glycoprotein's, which are expressed as αβ heterodimers on the cell surface. MHC-II molecules play an essential role in the initiation of antigen-specific immune responses by virtue of their ability to present antigenic peptides to the T-cell receptor (TCR) of $CD4^+$ T lymphocytes [3,4]. Constitutive expression of MHC-II molecules therefore is normally restricted to specialized antigen-presenting cells (APCs) of the immune system, while on other cell types their expression can be induced by various inflammatory cytokines [5,6]. Inactivation of *MHC-II* genes therefore may be one of the mechanisms through which cells create an immune privilege and through which tumours may escape recognition by the host immune system.

Studies with cell lines established from patients with an MHC-II deficiency, also referred to as bare lymphocyte syndrome (BLS), have revealed that the absence of MHC-II molecule expression in these patients is not due to mutations in the genes encoding MHC-II molecules but due to mutations in transcription factors that regulate MHC-II expression [7]. A schematic overview of the elements and factors critical for activation of MHC-II promoters is presented in Fig. 1. Mutations have been described in individual components of the RFX complex (consisting of RFX5, RFXB/ANK and RFXAP) or CIITA, which account for the observed deficiency in MHC-II molecule expression [8]. Several studies have demonstrated that the RFX complex binds in a mutual cooperative fashion with the transcription factors CREB/ATF, and NFY to the SXY-module found in all *MHC-II* and accessory genes (i.e. *invariant chain*, *HLA-DM* and *HLA-DO*) [9]. This multiprotein complex bound to

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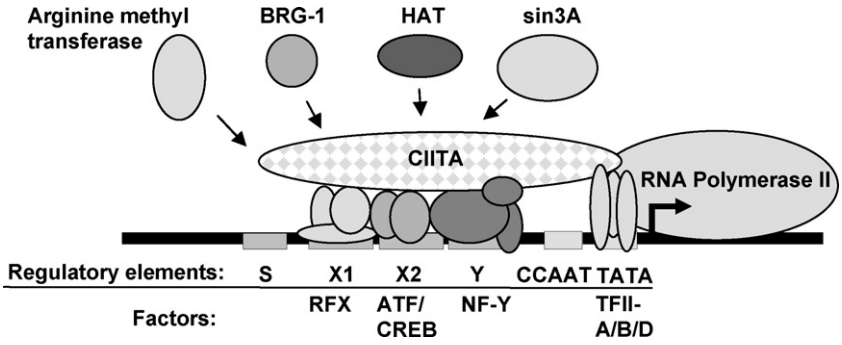

Fig. 1 – Elements and factors governing *MHC-II* gene transcription. Shown is the conserved SXY-module, which is bound by the multiprotein complex comprised of RFX, CREB/ATF and NF-Y. CIITA binds to this multiprotein complex bound to the SXY-module through interactions with RFX5, RFXB/ANK, CREB, NF-YB and NF-YC and acts as a platform for recruitment of various chromatin remodelling activities.

the SXY-module acts as an enhanceosome in the transcriptional activation of *MHC-II* genes. The SXY-module is also present in the promoters of *MHC-I* (with the exception of *HLA-G*) and *β2m* genes [10]. However, binding of this multiprotein complex to the SXY-module is not sufficient for MHC-II expression. Transcriptional activation of *MHC-II* genes requires the recruitment of CIITA to the MHC-enhanceosome [11–13]. On one hand, CIITA interacts with most of the components of the MHC-enhanceosome, and on the other hand also interacts with components of the basal transcription initiation complex. In this way CIITA connects the enhanceosome with the gene transcription initiation machinery [14–16]. Of the four transcription factors essential for *MHC-II* transcriptional activation, which were identified through the analysis of BLS-derived cell lines, only CIITA has the same restricted expression pattern as MHC-II and therefore is considered to be the master-regulator for *MHC-II* gene expression [17].

The transcriptional regulation of *MHC2TA* is controlled by a 14 kb multi-promoter region that harbours four-independent promoter units (Fig. 2A) [18]. Of these promoters, CIITA-PIV has been shown to be the promoter predominantly involved in IFNγ-inducible *MHC2TA* expression in human non-haematopoietic cells, such as fibroblasts and epithelial cells [18,19]. Promoter PI (CIITA-PI) is solely utilized for the expression in dendritic cells (DCs), whereas PIII (CIITA-PIII) is active in B-cells, DCs, monocytes and in activated human T-cells [18–22]. In addition to CIITA-PIV, IFNγ can also activate the CIITA-PIII

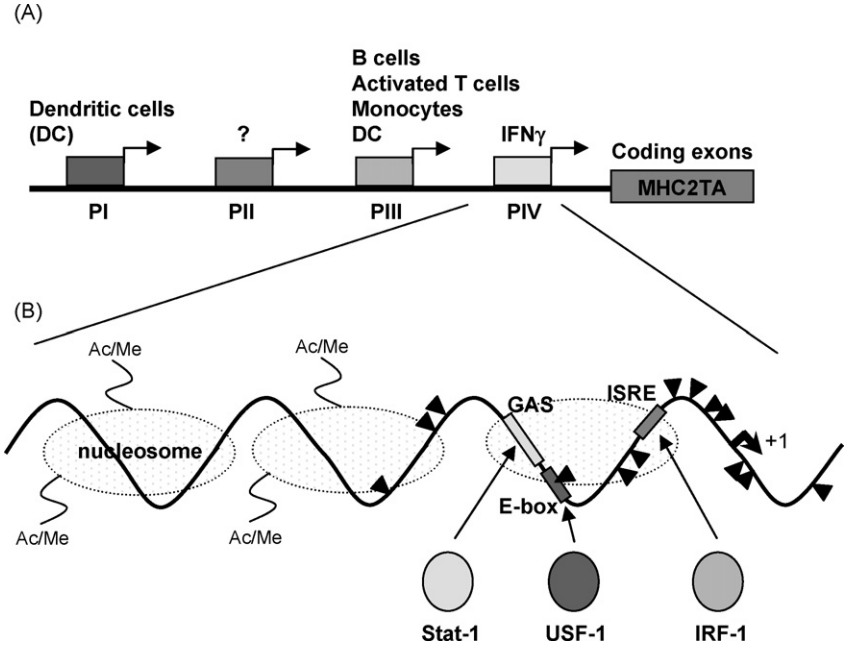

Fig. 2 – (A) Genomic organisation of the 14 kb *MHC2TA* multi-promoter region, encompassing CIITA-PI, -PII, -PIII and -PIV. (B) Factors and elements critical for IFNγ-mediated activation of CIITA-PIV. Stat-1 and USF-1 bind in a cooperate fashion to the GAS and adjacent E-box, while IRF-1 interacts with the ISRE. The positions of the various CpG dinucleotides analyzed in our studies are indicated by arrow heads. Ac/Me represent histone tail modifications evaluated by ChIP and include histone H3-triple methylated lysine 27 and acetylated histones H3 and H4.

promoter region. This activation has been shown to be mediated through a 4 kb PIII upstream regulatory region located 2 kb upstream of the CIITA-PIII core promoter region [23,24]. The function of CIITA-PII is still poorly understood.

2. Epigenetic control of gene transcription

Epigenetic changes are modifications in the architecture of chromatin without a change in the DNA sequence. In this way, global gene activation and local control of gene-specific transcription is exerted by components of the epigenetic machinery through the accessibility of regulatory DNA sequences to transcriptional activators and repressors. Post-translational modifications of histones have been proposed to establish a ''code'' which is read by enzymatic activities that determine the chromatin structure. In this way, the ''histone code'' establishes the cellular repertoire of expressed genes. Modifications of chromatin components include methylation of DNA at CpG dinucleotides and post-translational modifications of histone tails, which in most cases are reversible [25]. Furthermore, DNA and histone modifying enzymatic activities are intimately linked.

It is widely recognized that cancer is as much an epigenetic disease as it is a genetic and cytogenetic disease, and aberrant DNA methylation profiles and specific histone modifications have been associated with disease stages. Perturbations and deregulation of each of these epigenetic mechanisms are central to cancer development and progression, as they can lead to oncogene activation, chromosomal instability, and silencing of tumour suppressor genes [26]. Histone methylation plays an important role in chromatin dynamics and gene expression. The mechanisms that underlie gene repression by histone methylation are known to involve methylation of histone H3-lysine 9 (K9-H3) and -lysine 27 (K27-H3), which is catalyzed by the conserved SET-domain of histone methyltransferases (HMTases). The level of methylation at a specific lysine residue in histone H3 is achieved through the activity of various HMTases. The HMTase SUVAR39H1 principally catalyzes tri-methylation, while the HMTase G9a catalyzes mono- and di-methylation of K9-H3. The HMTase EZH2 catalyzes tri-methylation of K27-H3 (3Me-K27-H3) [27–29].

Tri-methylation of K9-H3 by SUVAR39H1, in general, results in gene repression because it signals the recruitment of heterochromatin protein (HP) 1 into chromatin [30]. Furthermore, it has been demonstrated that the HP1α isoform of HP1 interacts with the DNA methyltransferase (Dnmt) 1 and 3a, thereby integrating DNA modifying enzymatic activities [31]. Methylation modified CpG dinucleotides in DNA form recognition sequences of methyl–DNA binding proteins (e.g. MeCP2), which upon binding interact with histone deacetylases to suppress gene transcription. In this way post-translational histone tail and DNA modifications become integrated entities. Methylation of K9-H3 by G9a also leads to gene repression albeit in the absence of HP1 recruitment [30].

It has been well documented that polycomb group (PcG) proteins play a critical role in the maintenance of cellular identity and cell fate through heritable changes in gene expression. In particular, they are responsible for preservation of gene silencing in for example X-chromosomal inactivation and stem cell self renewal [32–34]. PcG proteins work by forming multimeric complexes with enzymatic activities. In this respect, two main PcG complexes have been characterized, which act as epigenetic chromatin modifiers. Polycomb repressive complex (PRC) 2 contains the HMTase EZH2 [35,36], and is thought to be involved in the initiation of gene silencing, whereas PRC1 is implicated in the stable maintenance of gene repression through mitoses. Recently it was found that EZH2 interacts with Dnmt's, and in this way EZH2 recruits Dnmt activities to target promoters for CpG methylation [37]. Once chromatin is marked by the activity of PRC2, PCR1 is recruited for maintenance of the transcriptional silent state which is achieved amongst others through additional histone modifications [38].

3. Epigenetic control of *MHC-II* and *MHC2TA* transcription

As mentioned above, CIITA is an integral part of the MHC-enhanceosome which controls activation of *MHC-II* gene promoters [2]. Others and we have shown that CIITA acts also as a platform to integrate chromatin-modifying activities into the enhanceosome (Fig. 1). CIITA interacts with histone acetyltransferases (HAT, e.g. CBP/p300, GCN5 and P/CAF), with BRG-1 (a component of the general chromatin remodeller SWI/SNF), the histone deacetylase Sin3a, and with an arginine methyl transferase [10,14,39]. As such CIITA bridges genetic and epigenetic mechanisms, which play an essential role in the transcriptional control of *MHC-II* expression to meet with local requirements for an adequate immune response.

With respect to transcriptional control of *MHC2TA*, several studies, including ours, have revealed that epigenetic mechanisms play a critical role in the transcriptional control of *MHC2TA* in several cancer types (e.g. teratocarcinoma, neuroblastoma, choriocarcinoma, squamous cell carcinoma, T-leukaemia, breast cancer, colorectal and gastric cancer) [40–51]. The lack of MHC-II expression in cell lines originating from various tumour types is due to lack of *MHC2TA* transcription upon IFNγ induction or upon activation (e.g. in T-leukaemia). The lack of IFNγ-induced *MHC2TA* transcription was found associated with CpG dinucleotide methylation in CIITA-PIV DNA [40–46,49–52]. However, besides CpG dinucleotide methylation, it has also been suggested that the lack of IFNγ-induced transcription of *MHC2TA* in several cancer types is associated with histone deacetylase activities [47,48,53,54].

Transcriptional activation of *MHC2TA* by IFNγ requires the assembly of IRF-1, Stat-1 and USF-1 on CIITA-PIV (Fig. 2B) [52,55]. The IFNγ-activated factor Stat-1 binds directly in a cooperative fashion with the ubiquitously expressed factor, USF-1, to the GAS/E box motif in CIITA-PIV. Indirectly, Stat-1 activates the transcription factor IRF-1, which subsequently participates in the activation of CIITA-PIV through binding to the ISRE in CIITA-PIV. Morris et al. have shown that in the choriocarcinoma cell lines JEG3 and JAR (trophoblast-derived) CpG dinucleotide methylation of CIITA-PIV DNA severely impairs recruitment of IRF-1, Stat-1 and USF-1 to CIITA-PIV following IFNγ exposure [52]. The lack of factor recruitment to CIITA-PIV therefore would explain the lack of *MHC2TA* transcriptional activation in these cells.

In the next sections we will describe in more detail the epigenetic regulation, both by DNA methylation and histone modifications, in two cancer types: T-cell leukaemia and uveal melanoma.

4. Lack of *MHC2TA* transcriptional activation in T-leukaemia

We have shown that, unlike normal peripheral T-cells, *in vitro* stimulation of T-leukaemia cell lines with well-known T-cell activation agents did not result in the induction of CIITA and congruent MHC-II molecule expression [46] (see Table 1). Because other T-cell activation markers like IFNγ, IL-4, CD69, and CD45RO were readily induced in the stimulated T-leukaemia cells, we eliminated the possibility that general T-cell activation pathways were corrupted [46]. Additionally, we showed in a transient promoter-reporter assay that CIITA-PIII, which is the principal T-cell employed *MHC2TA* promoter, was readily activated in CIITA-deficient T-leukaemia cells to levels similar to MHC-II expressing T lymphoma cells. These observations reveal that all essential transcription factors for CIITA-PIII activation were present in the leukaemia T-cells [46]. Further analyses showed absence in factor binding to CIITA-PIII and hyper-methylation of CIITA-PIII in the CIITA-deficient T-leukaemia cells. Subsequent demethylation of DNA with 5-AZA-2′-deoxycytidine resulted in re-expression of CIITA-PIII and HLA-DRA in these leukaemia T-cells. Moreover, we also found hyper-methylation of CIITA-PIII in HLA-DR-deficient primary leukaemia T-cells [46]. Therefore, the defect in CIITA expression in leukaemia T-cells correlated with DNA hypermethylation, which blocks factor assembly on CIITA-PIII resulting in impairment of its activation [44,46].

Since DNA methylation and histone modifications work in concert in accessibility of gene promoters, we have extended our research on the epigenetic mechanisms involved in the silencing of *MHC2TA* transcription in leukaemia T-cells also to histone modifications at the CIITA-PIII region. Using chromatin immunoprecipitation (ChIP) assays we found that the level of acetylated histone H3 and histone H4 in CIITA-PIII chromatin in T-leukaemia was strongly reduced when compared with CIITA-expressing T lymphoma cells (Table 2). The opposite was noted for methylation modifications in CIITA-PIII chromatin which are involved in transcriptional silencing (van Eggermond et al., in preparation, Table 2). These data suggest in addition to DNA methylation modifications, the involvement of histone methylation modifications in transcriptional silencing of *MHC2TA* in T-leukaemia.

Table 1 – Overview of used MHC-II negative and positive T-cell malignancies

	Type of tumour
MHC-II$^-$ cell lines	
Jurkat	Human acute T-cell leukaemia
Molt	Human acute T-cell lymphoblastic leukaemia
HSB-2	Human acute T-cell lymphoblastic leukaemia
MHC-II$^+$ cell lines	
HUT78	Human cutaneous T lymphocyte lymphoma
HH	Sezary syndrome aggressive cutaneous T-cell lymphoma
Karpas	Human CD30$^+$ Anaplastic large T-cell lymphoma

Table 2 – Overview of the expression characteristics and epigenetic DNA and histone modifications involved in MHC-II expression in T-cell malignancies

	MHC-II$^-$ cells	MHC-II$^+$ cells
HLA-DR surface expression	Absent	Present
CIITA promoter III/IV transcripts	Absent	Present
CIITA promoter III/IV transcription factors	Present	Present
CIITA promoter III/IV methylation	Present	Absent
Level of Histone H3/H4 acetylation	Low	High
Level of Histone H3 trimethylation	High	Low

5. Epigenetic mechanisms involved in deficient IFNγ-induced expression of MHC-II in uveal melanoma

Malignant uveal melanoma cells vary in their expression characteristics of MHC-II molecules. Whereas constitutive and IFNγ-inducible expression can be observed in cutaneous melanoma, uveal melanoma cells frequently fail to express MHC-II molecules upon IFNγ stimulation. This is because uveal melanoma cells lacking MHC-II expression fail to express CIITA following exposure to IFNγ as determined by various RNA quantification analyses including real-time RT-PCR using established uveal melanoma cell lines (Table 3; Holling et al., submitted for publication). Our studies have revealed that the lack of IFNγ-inducible expression of CIITA in uveal melanoma cells is not caused by a defective IFNγ-signalling pathway or lack of transcription factors critical for activation of CIITA-PIV, the principal IFNγ-responsive promoter of *MHC2TA*. This is similar to what is observed in CIITA-deficient leukaemia T-cells with respect to CIITA-PIII and suggest the involvement of epigenetic mechanisms in

Table 3 – Overview of the DNA methylation status of CIITA-PIII and CIITA-PIV in uveal melanoma

Cell line	MHC-II expression		CIITA-PIII	CIITA-PIV
	−IFNγ	+IFNγ		
OCM-3	+	+	Methylated	Unmethylated
OMM1	–	–	Partial	Unmethylated
OMM1.3	–	–	Unmethylated	Unmethylated
Mel270	–	–	Partial	Unmethylated
Mel285	–/+	+	Methylated	Methylated
92-1	–	–	Partial	Unmethylated
OCM-1	–	+	Methylated	Methylated

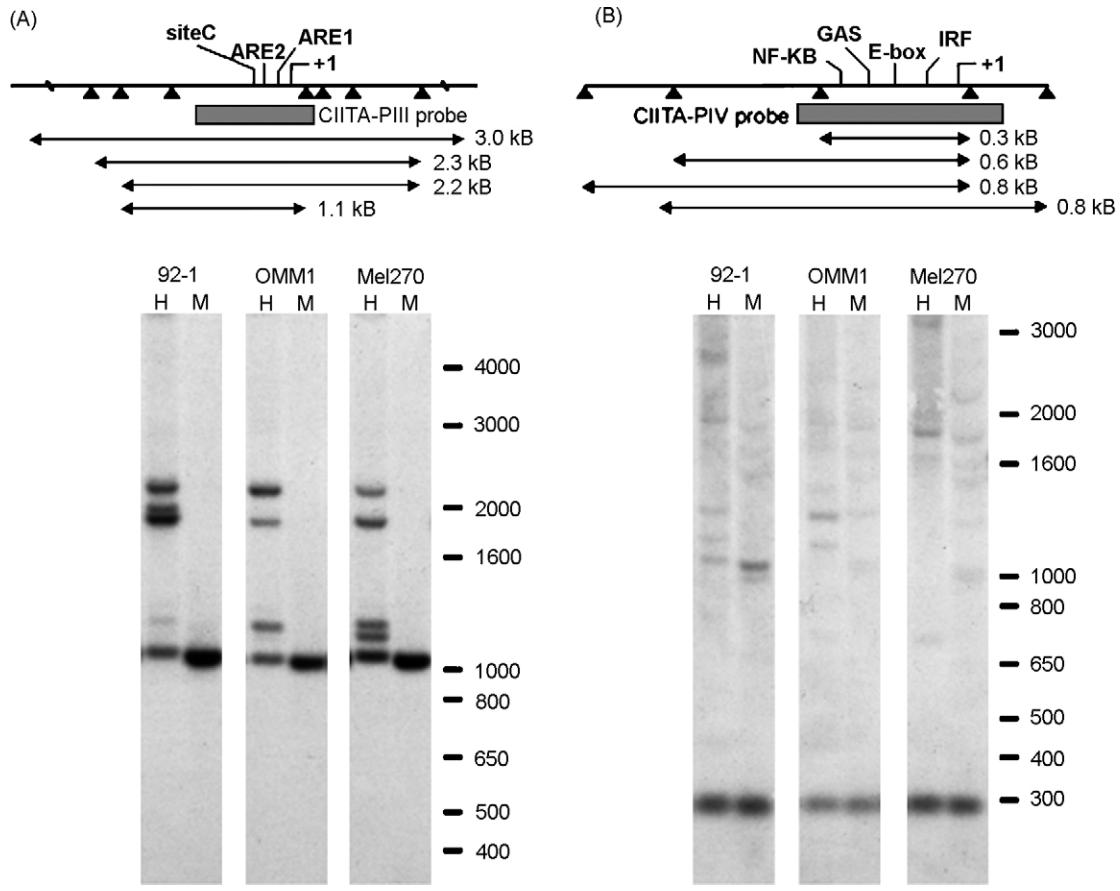

Fig. 3 – Methylation pattern of the genomic CIITA-PIII and -PIV region in uveal melanoma. (A) Top: Schematic overview of the CIITA-PIII promoter region. Known important regulatory transcription factor binding sites and the transcriptional start site are depicted. Restriction sites of the methylation-insensitive MspI (M) enzyme and the methylation sensitive HpaII (H) enzyme are indicated by arrow heads. Possible generated DNA fragment sizes are indicated as well as the location of the probe used. Bottom: CIITA-PIII methylation pattern as determined by Southern blot analysis on HindIII/HpaII (H), or HindIII/MspI (M) digested genomic DNA of the uveal melanoma cell lines 92-1, OMM1 and Me1270. HpaII does not cleave at 5'-CC^{Mc}GG-3' while its isoschizomer MspI cleaves both methylated and unmethylated 5'-CCGG-3'DNA. (B) Top: Overview of the CIITA-PIV promoter region. Bottom: CIITA-PIV methylation pattern as determined by Southern blot analysis. Shown are the digests with HindIII/HpaII (H), or HindIII/MspI (M) of genomic DNA from the uveal melanoma cell lines 92-1, OMM1 and Me1270.

transcriptional silencing of IFNγ-mediated transcriptional activation of *MHC2TA* through CIITA-PIV in uveal melanoma. To investigate this we have evaluated DNA methylation and histone modifications associated with transcriptional silent genes. The examination of the DNA methylation status of both CIITA-PIII and CIITA-PIV by using the isoschizomeric restriction enzymes *Hpa*II/*Msp*I revealed the existence of various levels of CpG dinucleotide methylation at 5′-CCGG-3′ sites (Fig. 3 and Table 3; Holling et al, submitted for publication).

However, the DNA methylation pattern we observed in CIITA-PIII and CIITA-PIV promoter region did seem not to have any correlation with the expression of MHC-II (either constitutive or IFNγ-inducible; Fig. 3 and Table 3). This was of a surprise because, as mentioned above, previous studies have shown that lack of IFNγ-inducible expression of MHC-II is associated with a methylated *MHC2TA* promoter region. This is particularly noted in various tumour types including trophoblast-derived and developmental tumours which have a methylated CIITA-PIV [49,50]. In the case of uveal melanoma, CIITA-PIV methylation was not associated with lack of MHC-II expression in several cell lines (Fig. 3 and Table 3). In addition to this, we also found that these cell lines displayed both a partial or complete unmethylated CIITA-PIII region. Moreover, in contrast to the general view that cell lines with inducible MHC-II expression have an unmethylated *MHC2TA* promoter region is our finding that DNA of both CIITA-PIII and CIITA-PIV in the MHC-II-inducible uveal melanoma cell line OCM-1 is methylated (Table 3; Holling et al., submitted for publication).

Since DNA methylation does not account for the silent state of the *MHC2TA* gene in the uveal melanoma cell lines lacking IFNγ-inducible expression of CIITA, we determined by ChIP-analysis the level of histone modifications which are associated with silent chromatin and transcriptionally inactive genes. These analyses have revealed that CIITA-PIV chromatin in OMM1.3 cells contained high levels of triple-methylated histone-H3-lysine 27 (Holling et al., submitted for publication).

These observations suggest that this histone methylation modification is the most important epigenetic regulator of IFNγ-inducible expression of CIITA in uveal melanoma.

In summary, the combined results of our studies have revealed an important contribution of histone methylation modifications in transcriptional silencing of *MHC2TA* promoters in T-leukaemia and in uveal melanoma. Further investigations are needed to determine if these histone modifications also play an important role in the regulation of *MHC2TA* in other tumour types. Moreover, DNA methylation at CpG dinucleotides does not seem to play a dominant role in transcriptional silencing of *MHC2TA* in uveal melanoma. Further studies are aimed now at linking these chromatin modifications with specific histone and DNA methyltransferases that account for the observed silencing of *MHC2TA* in cancer.

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