

# Aberrant patterns of DNA methylation in B cell lymphoma

its role in disease development and/or maintenance  
and possible epigenetic therapies

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## **Abstract**

During B-cell maturation genetic and epigenetic changes can occur that facilitate the development of B-cell lymphoma. Among the epigenetic changes there is DNA methylation, hypermethylation of the tumor suppressor genes (TSGs) by DNA methyltransferases (DNMTs) can be a factor in the development of B cell lymphoma. There are different types of B-cell lymphoma and this review focusses on Burkitt lymphoma (BL), Follicular lymphoma (FL) and Diffuse large B-cell lymphoma (DLBCL). In these B-cell lymphomas we analyse which TSGs are hypermethylated, what the roles of these TSGs are in tumor development/maintenance, and what the possible epigenetic therapies to de-methylate the hypermethylated TSGs are.

The promoter of BIM, DAPK, P16, DSP, and DLC-1 are hypermethylated in BL, FL and DLBCL. Inactivation of these genes causes poorer overall survival and disease free survival. However these genes are not hypermethylated in all the patients.

The application of epigenetic therapies to cause re-expression of the TSGs is promising. Especially nucleoside analogues, which trap the DNMTs are promising. Also gene upregulating effects have been found of these drugs on P16, DAPK and BIM.

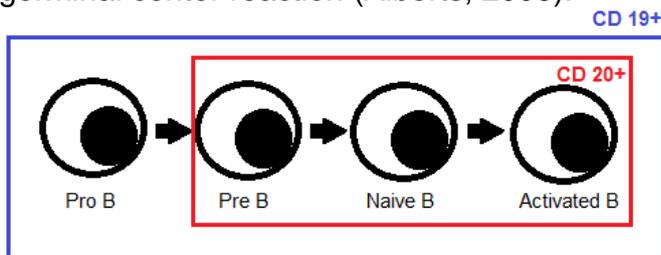
The other type of epigenetic therapy is the non-nucleoside analogues, that block the DNMT enzymes. It is not known yet if these drugs have an upregulating effect on the genes studied in this review.

These findings conclude that the TSGs BIM, DAPK, P16, DSP, and DLC-1 are hypermethylated in a part of the patients with B-cell lymphoma. With epigenetic therapies these genes can be reactivated and this has a positive effect on the overall survival of the patients with hypermethylation of these genes. However with the epigenetic therapies it is important to notice that not only the TSGs become hypomethylated, this could also happen to the oncogenes, so it is important for development of a good epigenetic therapy that it only reactivates the aberrant silenced genes.

## Introduction

Normal B-cells are white blood cells, also called lymphocytes, that take care of the antibody response in the body. B-cells develop in the bone marrow, hematopoietic stem cells become common lymphoid progenitor then pro-B- cell(CD19+), pre-B- cell(CD20+) and they end as naïve B-cells when they enter the bloodstream, shown in figure 1 (Alberts, 2008).

When there is an immune response, dendritic cells recognize and take up pathogens and fragments of infected cells at the site of infection and they phagocytise them. Afterwards they present small particles of the pathogen on their membrane, the antigens. Then they migrate with the antigens to a nearby lymph node and present the antigens to the naïve B-cells and activate them. The naïve B-cells create germinal centers in the secondary lymphoid organs where they mature during the germinal center reaction (Alberts, 2008).



**Figure 1.** CD19+ receptor is present in Pro B-cells and further. CD20+ receptor is present from Pre-B-cell and further.

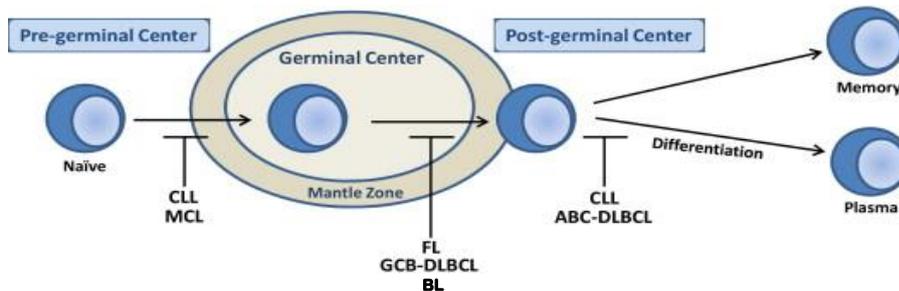
B-cells maturation has three different stages; first naïve B-cells exist at the pre-germinal center and have not been in contact with antigens. When naïve B-cells encounter antigens they move to the germinal center and undergo clonal expansion, somatic hyper mutation, selection, class switching. These steps make the antibody a better match with the encountered antigen. After this the B-cells differentiate into memory cells and plasma cells. Plasma cells are the B-cells that secrete antibodies. The antibodies bind to antigens, they neutralize loose antigens and mark cells with antigens on their membrane for destruction, or phagocytosis (Taylor et al., 2013; Alberts, 2008). Memory B-cells persist for long periods after antigen exposure and ensure a sustained immune protection and a rapid response against previously encountered foreign antigens by recall (Gatto & Brink, 2010).

During B-cell maturation, oncogenic events can occur. During somatic hyper mutation, translocations and other genetic changes can occur which facilitate tumor development. However, only one oncogenic event is not enough to cause cancer, as stated in the multiple hit theory. The first steps in tumorigenesis are often activation of oncogenes, and in later steps the tumor suppressor genes are often inactivated (Alberts, 2008).

There are two types of B-cell lymphoma, 89% of these are non-Hodgkin Lymphomas (NHLs), the others are Hodgkin Lymphomas (Taylor et al., 2013). When normal B-cells become malignant the normal regulation of cell differentiation is disturbed and an accumulation of cells at a particular stage of normal B-cell differentiation arises. As shown in figure 2, chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma (MCL) occur if cells do not differentiate after the naïve stage. If cells do not differentiate further in the germinal center follicular lymphoma (FL), germinal center B-cell-like diffuse large B-cell lymphoma (GCB-DLBCL) and Burkitt lymphoma (BL) will arise. When cells do not differentiate to memory or plasma cells CLL and

activated B-cell-like DLBCL will arise (Taylor et al., 2013). The mentioned B-cell lymphomas are all NHLs.

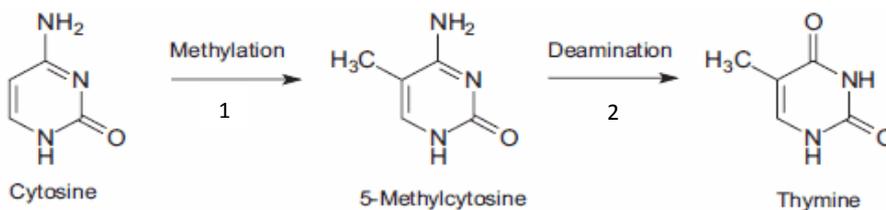
This review will take a closer look at BL, FL, and DLBCL. FL and DLBCLs are the most common NHLs, 40% are FL and 50% are DLBCL. DLBCLs can be divided into two subclasses; GCB-DLBCLs and ABC-DLBCLs. BL is a clinically aggressive lymphoma and is most common in childhood (85-90% of the cases), only 5% of the adult B-cell lymphomas are BL (Richter-Larrea et al., 2010; Taylor et al., 2013; De et al., 2013).



**Figure 2.** B cell maturation and development of B cell lymphomas (Taylor et al., 2013)

### DNA methylation

Epigenetic alterations play a role in development of lymphoid malignancies. Epigenetic alterations are heritable alterations in gene expression without changing the DNA sequence. In DNA methylation a methyl group is added at the 5'-position of a cytosine at the pyrimidine ring within a cytosine-guanine dinucleotide (CpG) by DNA methyl transferases (DNMTs), shown in figure 3 (Taylor et al., 2013).

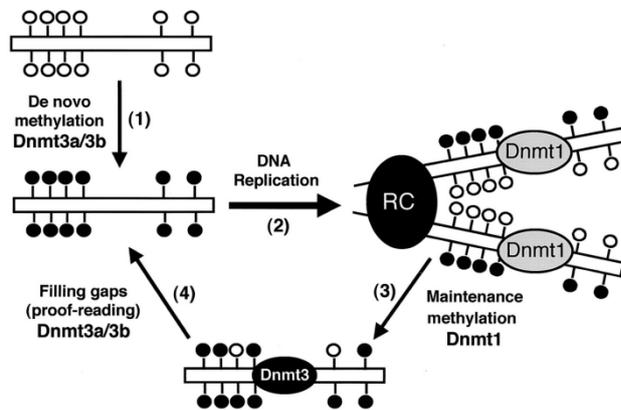


**Figure 3.** DNA Methylation and deamination. Step 1: The methyl group is transferred from the co-factor S-adenocyl-L-methionine (SAM) to the 5<sup>th</sup> position of the cytosine pyrimidine ring. Step 2: Deamination of methylated cytosine (Sigma-Aldrich, 2007)

When methyl groups are added to the promoter regions of the DNA the transcription factors and other factors necessary for gene transcription cannot bind to DNA so the methylated gene cannot be expressed. DNMT3A and 3B are responsible for 'de novo' methylation and target primary unmethylated CpGs. DNMT 1 is responsible for the maintenance of methylation during replication and is specific for hemimethylated DNA. However, it has been found that DNMT1 alone is incapable of perfect maintenance methylation; DNMT3A and 3B are also required for methylation maintenance, as shown in figure 4 (Jones & Liang, 2010; Walton et al., 2011; Jones, 2012; Taylor et al., 2013).

CpG islands in the genome co-localize with the promoters of all of the constitutively expressed genes and they also co-localize with about 40% of the tissue-specific genes (Walton et al., 2011). There are no CpG islands in front of promoters of genes that are not constitutively expressed because spontaneous deamination takes place there. The methylated cytosine is converted in a thymine in spontaneous deamination when the gene is almost always methylated during evolution, shown in figure 3 (Zemojtel et al., 2011).

Information about the phenotype of normal and malignant cells is determined by the methylation patterning of the DNA (Shaknovich & Melnick, 2011). DNA methylation patterns are tissue specific, changed methylation of the DNA leads to aberrant gene expression and can facilitate the development of B-cell lymphoma. However, as mentioned earlier there are more oncogenic events necessary to develop cancer (Jones & Liang, 2010; Taylor et al., 2013).



**Figure 4.** DNA methylation by DNMTs. After replication DNMT1 makes sure there is maintenance of methylation by methylating hemimethylated DNA strands. DNMT3 checks if methylation happened where it should and methylates the places that should be methylated and are not yet by proof-reading (Chen et al., 2003)

### *Tumor suppressor genes*

The tumor suppressor genes (TSGs) were discovered in the 1970s and early 1980s. TSGs are antigrowth genes and they can retain or suppress cell proliferation (Weinberg, 2014). TSGs can protect cells from undergoing malignant transformation by inducing apoptosis, inhibiting cellular migration and metastasis, and by impeding deregulated cell cycle progression (Hayslip & Montero, 2006).

The TSGs are involved in tumor development when both copies of the gene are lost or inactivated. Aberrant gene promoter methylation of TSG is associated with gene silencing and is equivalent to inactivated or mutated genes (Hayslip & Montero, 2006; Weinberg, 2014).

The identification of aberrant methylated genes may provide a better understanding of the pathogenesis of B-cell lymphoma and development of novel tumor markers and therapeutic targets (Ferrareso et al., 2014). Research has been done to restore the normal methylation patterns and the focus has mainly been on inhibition of DNA Methyltransferases (DNMTs) (Zhao et al., 2013). This review investigates which tumor suppressor genes are hypermethylated in B-cell lymphoma (DLBCL, Burkitt lymphoma and FL), what their roles are in tumor development/maintenance, and what are the possible epigenetic therapies to de-methylate the hypermethylated tumor suppressor genes?

## Chapter 1. Hypermethylation of tumor suppressor genes

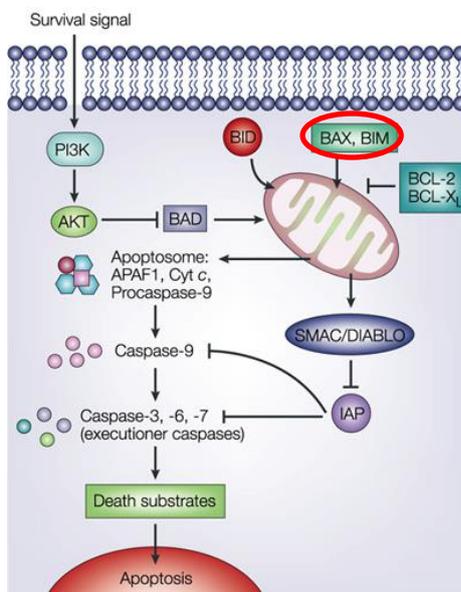
The reason that CpG islands of tumor suppressor genes become hypermethylated and other CpG islands do not might be because hypermethylation of TSGs can assist in giving a selective advantage of survival to the tumor cell. Hypermethylation can take place by an increase in DNA methyltransferase activity in tumor cells, compared to normal cells. The mRNA transcripts of DNMT1 and DNMT 3b are increased in haematological malignancies; this could cause an increase in methylation (Esteller, 2003).

Different types of TSGs become hypermethylated in different kinds of B-cell malignancies. BL, FL, and DLBCL are three types of NHLs that arise during the germinal center reaction, and for this reason they have many properties that are alike. This chapter will reveal some of the TSGs that are hypermethylated in BL, FL, and DLBCL, and the effect on disease development and maintenance will be mentioned. First a few well known genes that are hypermethylated in the B-cell lymphomas will be discussed (BIM, DAPK, P16) and afterward a few recently discovered hypermethylated tumor suppressor genes (DSP, DLC-1).

### 1.1 BH3-only family member protein (BIM)

The BIM gene is located on chromosome 2q13 and is an essential initiator of apoptotic cell death. Defects in the capacity of cells to undergo apoptosis provides tumor cells with an intrinsic survival advantage and can grant inherent resistance to chemotherapy for these cells. BIM plays a role in the intrinsic apoptotic pathway and is part of the BH3-only pro-apoptotic group of the BCL2 family (Richter-Larrea et al., 2010; Harada & Grant, 2012).

Figure 5 shows the role of BIM in the apoptotic pathway. BIM is one of the factors that activates the mitochondria to release cytochrome c, this activates the caspases that cause apoptosis and inhibits the apoptosis inhibitors (Igney & Krammer, 2002).



**Figure 5.** The intrinsic apoptotic pathway (Igney & Krammer, 2002)

BIM is frequently inactivated by epigenetic abnormalities in different B-cell lymphomas. Hypermethylation of the BIM promoter is present in germinal center-derived B-cell lymphomas. Promoter methylation is correlated with loss of expression of BIM at RNA and protein levels. The TSG BIM is in BL hypermethylated in about

46% (n=37) of the patients and in 83% (n=17) of the cell lines (Mestre-Escorihuela et al., 2007; Richter-Larrea et al., 2010).

#### *Disease development/maintenance*

BIM inactivation might be sufficient to evade apoptosis and this way cause tumor progression and maintenance (Richter-Larrea et al., 2010; Harada & Grant, 2012). When BIM is methylated (n=17), patients with germinal center derived B-cell lymphoma have a significantly lower complete remission and also shorter overall survival compared to patients without BIM promoter methylation (n=19) (Richter-Larrea et al., 2010).

Myc mutations might play a role in epigenetic silencing of BIM. Cases of BL where box I Myc mutations are present (spanning amino acid position 57-60), were more frequent to have BIM hypermethylation, namely in 50% of the cases (n=24), compared to 37,5% of the cases when mutations were not present (n=16). Especially in BL BIM is frequently silenced by hypermethylation, this could be because BL is a myc driven cancer (Richter-Larrea et al., 2010).

A mouse knockout study showed that BIM plays an important role in hematopoietic cell homeostasis when it is knocked out it causes an excess of lymphoid and myeloid cells (Harada & Grant, 2012). Another knockout study in mice showed that BIM is also required for death of germinal center-derived memory B cells and plasma cells. The proper termination of immune responses and apoptosis of activated B and T cells are mostly dependent on BIM (Fischer et al., 2007).

#### **1.2 Death-associated protein kinase (DAPK)**

The DAPK gene is located on chromosome 9q34.1 and is a calcium-calmodulin-dependent serine/threonine kinase. This kinase participates in different apoptosis pathways. DAPK contributes to interferon- $\gamma$  induced apoptosis, tumor necrosis factor- $\alpha$  induced apoptosis, and Fas-induced apoptosis (Hayslip & Montero, 2006). IFN- $\gamma$  and TNF- $\alpha$  both cause activation of DAPK, and activation of DAPK induces apoptosis. DAPK also induces apoptosis when DAPK is overexpressed (Lindström & Wiman, 2002). DAPK expression causes apoptosis by upregulation of the expression of p53, a protein that induces apoptosis (Michie et al., 2010).

The promoter of DAPK is hypermethylated in BL, FL and DLBCL resulting in loss of expression of the DAPK protein. The DAPK was hypermethylated in 100% of the BL patients (n=10), in 86% of the FL 86% of the patients (n=29), and in 59% of the DLBCL patients (n=46) (Lindström & Wiman, 2002; Shiramizy & Mick, 2003; Hayslip & Montero, 2006; Amara et al., 2008).

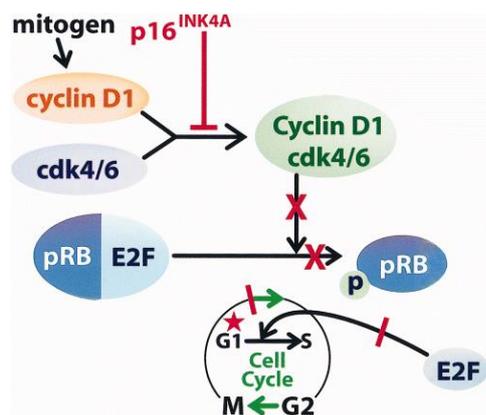
#### *Disease development/maintenance*

DAPK is a positive mediator of cell death. Hypermethylation of DAPK causes a significant poorer overall survival and disease free survival compared to patients with no DAPK promoter methylation (n=46). DAPK functions as a TSG in at least two different stages of tumorigenicity. The first is apoptotic checkpoint functioning early during cell transformation. The second is occurs later in the cancer development, during metastasis. This was studied in lung cancer, in which re-expression of DAPK in mice the ability of lung cancer cells to form metastasis was suppressed. However if this also works in B-cells has to be studied. Silencing of the DAPK causes suppression of cell death (Inbal et al., 1997; Kissil et al., 1997; Amara et al., 2008).

DAPK might also play a key role in response to chemotherapy because of its role in apoptosis. 81% of patients (n=16) who failed treatment response showed a hypermethylated DAPK promoter (Amara et al., 2008). By suppression of cell death and a possible role in development of resistance to chemotherapy DAPK hypermethylation plays a large role in development and maintenance of B-cell lymphoma.

### 1.3 Cyclin-dependent kinase inhibitor P16

The gene of P16 is located on chromosome 9q21 and is also called INK4a or CDKN2A. The role of P16 in normal cells is inhibition of cyclin-dependent kinases important for G1 cell cycle arrest (Amara et al., 2008). Normally cells do not express P16, however when there is DNA damage or there are too many mitogens in the cell P16 is expressed. P16 then binds to cyclin D-Cdk4/6 and inhibits this cyclin-Cdk complex and this causes that phosphorylation of pRb is prevented (Lindström & Wiman, 2002). If pRB is not hyperphosphorylated there is a cell cycle arrest because the E2F protein stays attached to pRB and cannot activate the next cyclin-Cdk complexes necessary to continue the cell cycle. However, if P16 is hypermethylated there is no inhibition of the phosphorylation of pRb and there will be no cell cycle arrest. In this way P16 plays a role in tumor progression (Weinberg, 2014). The role of P16 in cell cycle progression is shown in figure 6.



**Figure 6.** Mechanism of P16 activation causing cell cycle arrest (Natarajan et al., 2005)

Hypermethylation of P16 is common in NHLs and is associated with tumor progression (Esteller, 2003). This was studied by comparing low-grade and high-grade neoplasms. 35 of 41 high-grade neoplasms had P16 hypermethylation and the low-grade neoplasms showed normal P16 expression (Villuendas et al., 1998). In most cases there is a lack in mRNA and protein expression when the P16 gene promoter is hypermethylated (Lindström & Wiman, 2002). P16 hypermethylation was present in 42% of the BL patients (n=19) and in 89% of the BL cell lines (n=19), 31% of the FL patients (n=17), and 52% of the DLBCL patients (n=46) (Klanby et al., 1998; Hayslip & Montero, 2006; Amara et al., 2008).

#### *Disease development/maintenance*

Whenever the P16 gene promoter is hypermethylated in B-cell lymphoma, cells have a selective advantage, because cell cycle arrest can be prevented when there is DNA damage. This causes more genomic instability and more chances of multiple genetic hits that help develop a tumor cell (Amara et al., 2008).

The prognostic impacts of P16 promoter hypermethylation are not completely clear yet, however there are indications that there is a significant correlation between P16

hypermethylation and shortened overall survival(n=32) (Zainuddin et al., 2011; Krajnovic et al., 2013). Hypermethylation is often found in patients(n=46) with aggressive tumors and advanced clinical stages, and it is associated with a worse overall survival (Amara et al., 2008).

#### **1.4 Desmoplakin (DSP)**

The DSP gene is located on chromosome 6p24. The DSP protein is involved in the Wnt signalling pathway (Bethge et al., 2013). When DSP is expressed cell proliferation, anchorage-independent growth, migration and invasion are inhibited, and cells are also more sensitive to apoptosis induction by anticancer drugs. However, these data have been found in lung cancer, so if DSP has the same role B-cell lymphoma still has to be investigated. Though the inactivation of the DSP gene in lung cancer also happens by promoter methylation so there is a good possibility that there are more similarities (Yang et al., 2012).

Hypermethylation in the NHLs was compared with normal CD19+-B-cells. The promoter of the DSP gene was hypermethylated in 17% of the BL patients (n=7), in 25% of the FL patients(n=14), and in 40% of the DLBCL(n=10) (Bethge et al., 2013).

##### *Disease development/maintenance*

Over expression of DSP by transfection with plasmids of the DSP gene, caused reduced expression of the Wnt/B-catenin target genes Axin2 and matrix metalloproteinase MMP14. The Wnt pathway causes cell proliferation and cell migration and might play a role in metastasis. MMP14 causes cells to go through the extra cellular matrix and this way this might play a role in metastasis. Axin2 plays a role in the regulation of the Wnt/B-catenin pathway (Yang et al., 2012; Weinberg, 2014).

So when DSP is epigenetically silenced tumor cells will probably proliferate more. The tumor cells will also be capable of anchorage-independent growth, migration and invasion which all promote metastasis formation. However B-cells are not anchored, so the effect of DSP hypermethylation in B-cell lymphoma needs more research.

#### **1.5 Deleted in liver cancer-1 (DLC-1)**

The DLC-1 gene is located on chromosome 8p21.3-22 and is a Rho family GTPase. The Rho family GTPases play roles in the regulation of different kinds of cellular processes, like cell cycle progression, cell morphology, cell migration, and actin cytoskeleton organization (Feng et al., 2011).

The promoter of DLC-1 is hypermethylated in NHLs, so also in BL, FL, and DLBCL. The promoter of the DLC-1 gene was hypermethylated in 100% of the BL patients(n=6), in 83% of the FL patients(n=30), and 85% of the DLBCL patients(n=14) (Shi et al., 2007; Ying et al., 2007; Pike et al., 2008; Feng et al., 2011).

##### *Disease development/maintenance*

The DLC-1 gene was re-expressed in cell lines to see the effect of DLC-1 on disease development/maintenance, this was accomplished using a plasmid with the whole gene that was transfected into NHL cell lines. The Raji and Daudi cell lines were used and they were transfected a the plasmid with the DLC-1 gene or with an empty plasmid(negative control). When the DLC-1 gene is re-expressed in NHLs the proliferation and migration of tumor cells is inhibited in vitro. DLC-1 also reduced the expression of Bcl-2, and this induces apoptosis. The effect of DLC-1 on cell migration

is probably caused by inactivation of Rho-GTPases proteins which regulate many cellular functions in response to extracellular factors(Feng et al., 2011). Consequently, when DLC-1 is silenced by gene promoter hypermethylation in B cell lymphoma cell proliferation and migration is increased and apoptosis is decreased.

Table 1 shows a short summary of the results. The table shows which genes were hypermethylated and what the normal function of the gene is.

**Table 1.** *Hypermethylated TSG genes and normal function*

<b>TSG hypermethylation</b>	<b>Function of gene</b>	<b>Burkitt Lymphoma</b>	<b>Follicular lymphoma</b>	<b>Diffuse Large BC lymphoma</b>
<b>BIM</b>	Pro-apoptotic	46%(n=37)	*	*
<b>DAPK</b>	Apoptosis	100%(n=10)	86%(n=29)	59%(n=46)
<b>P16</b>	Impedes mitosis	42%(n=19)	31%(n=17)	52%(n=46)
<b>DSP</b>	Wnt-signaling	17%(n=7)	25%(n=14)	40%(n=10)
<b>DLC-1</b>	Rho family GTPase	100%(n=6)	83%(n=30)	85%(n=14)

\* Hypermethylation of the TSG is present, however the amount of patients that have this methylation is unknown.

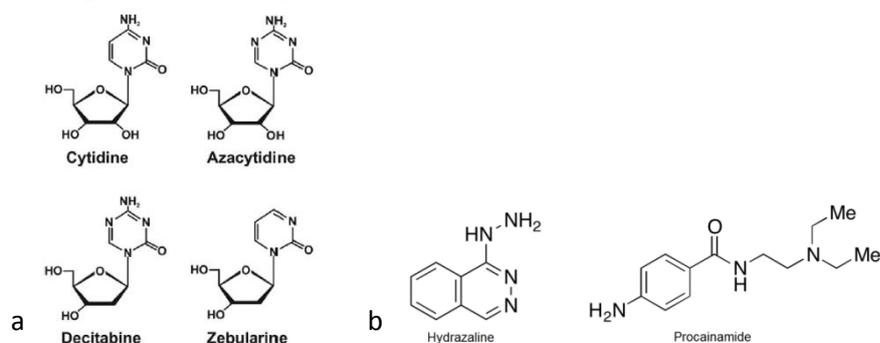
## Chapter 2. Effects of epigenetic therapies on B-cell lymphoma

Inactivation of TSGs plays an important role in the development of cancer and in the development of resistance to chemotherapy in cancer. During carcinogenesis the pro-apoptotic and pro-differentiation pathways that are disabled are often the same pathways that are necessary for the chemo response (Gnyszka et al., 2013). Epigenetic changes are responsible for carcinogenesis in similar way as genetic mutations, both cause targeted genes to be silenced, which can lead to no functional gene products (Gnyszka et al., 2013). Epigenetic modifications like DNA hypermethylation are reversible. To reverse DNA hypermethylation DNMT inhibitors are being studied as epigenetic modulators, which is also what this chapter is about (Zhao et al., 2013). DNA hypermethylation is also an attractive and selective tumor specific therapeutic cancer treatment because hypermethylation occurs infrequently in normal cells (Gnyszka et al., 2013). First some nucleoside analogue DNMTs and afterwards some non-nucleoside analogue DNMTs will be discussed.

### 2.1 DNA methyltransferase inhibitors **Nucleoside analogues**

Nucleoside analogues as DNMT inhibitors (DNMTIs) inactivate DNMTs by forming a covalent bond between the DNMT and the DNA (Clozel et al., 2013). At this moment there are two by the US Food and Drug Administration (FDA) approved DNMTIs; azacytidine and decitabine (5-aza-2'-deoxycytidine). However, these DNMTIs are currently not used for BL, FL, and DLBCL. But the DNMTIs are used for other cancers like acute myeloid leukaemia and chronic myelomonocytic leukaemia (Gnyszka et al., 2013).

Azacytidine and decitabine are analogues of cytidine modified in the 5<sup>th</sup> position of the pyrimidine ring, shown in figure 7a. Azacytidine is a ribonucleoside and is mostly incorporated in the RNA and to a lesser degree in DNA. Decitabine is a deoxyribose analogue and is only incorporated into the DNA strands (Gnyszka et al., 2013).

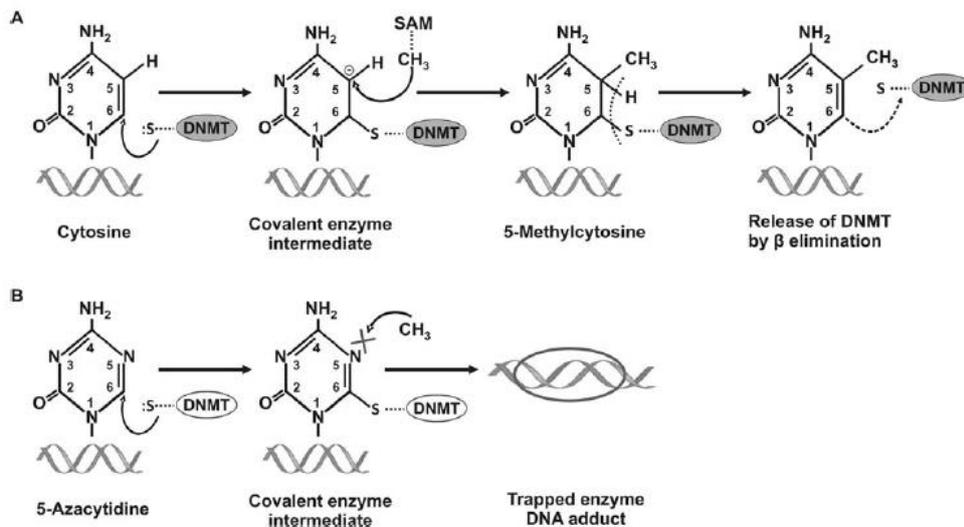


**Figure 7.** 7a) Molecular structures of Cytidine, Azacytidine, Decitabine, and Zebularine. Changes in Azacytidine en Decitabine compared to Cytidine are at the 5<sup>th</sup> position of the pyrimidine ring; there is Nitrogen instead of Carbon (Gnyszka, Jastrzebski, & Flis, 2013).

7b) Molecular structures of non-nucleoside DNMTIs Hydrazaline and Procainamide. (Toronto Research Chemicals Inc. Products for innovative research, 2013)

The DNMTIs disrupt the interaction between DNA and DNMTs because there is nitrogen instead of carbon in the 5 position of the modified pyrimidine build into the DNA. The DNMTIs stay covalently bound to the DNMT and the DNA and with this the function of the DNMT is blocked. The functionality of the DNA is also compromised and this triggers DNA damage signalling, this causes degradation of the trapped DNMTs. By this mechanism further methylation of cytosine residues is inhibited and this causes a passive loss of cytosine methylation after replication in the daughter cells (Gnyszka et al., 2013). As a result there is a gradual depletion of methylated

CpGs when the cells divide (Clozel et al., 2013). The mechanism of normal methylation and the effect of DNMTs on methylation are shown in figure 8b.



**Figure 8.** Methylation of DNA by DNMTs.

8a) Normal methylation. DNMTs bind to cytosine in the DNA. Afterwards DNMT is released.

8b) No methylation is possible because there is nitrogen on the 5<sup>th</sup> position of the pyrimidine ring of cytosine. DNMT will stay covalently bound and a DNA adduct will arise, signalling DNA damage.

(Gnyszka et al., 2013).

DNMT inhibitors probably have a particular large effect in tumors with high proliferation rates (Clozel et al., 2013). The DNMTIs are administered intravenously. For the DNMT inhibitors to be activated, they need to be incorporated in the genome of rapidly dividing cells during the S-phase of the cell cycle (Yang et al., 2010). The S phase is necessary because the cell cycle then enables selective and effective incorporation of the DNMTIs into the DNA. This reduces the hypo methylation in normal cycling cells, because normal cells divide at a slower rate and incorporate less of these drugs into their DNA, so there is less of an effect in normal cells on DNA methylation (Cortez & Jones, 2009; Gnyszka et al., 2013). However not all cells proliferate slowly so this could also have an effect on fast proliferating normal cells, like epithelial cells of the intestine.

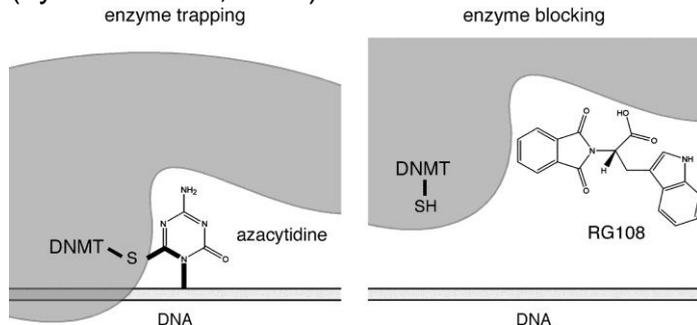
DNMTIs could be used prior to chemotherapy to cause de-methylation and re-expression of genes that play a role in chemotherapy sensitivity. In patients with DLBCL administration of DNMT inhibitors caused chemo sensitization (n=5) (Clozel et al., 2013).

The effect of DNMTIs is not yet known for all the TSGs discussed in the previous chapter, however for some it is known. The epigenetic silencing of BIM can be pharmacologically reversed with DNMTIs. When DNMTIs are used as treatment the expression of BIM can increase 1,5-4 times and this decreases the cell viability. These data have been obtained in a study of 36 patients samples and 20 cell lines (Richter-Larrea et al., 2010). 5'-azadeoxycytidine also causes DNA demethylation and was capable to restore DAPK expression in B-cell lines. These data have been found in a study of 10 different B-cell cell lines at different stages of B-cell lymphoma (Kissel et al., 1997). Also P16 can be re-activated by decitabine and azacytidine, these results have been found in a study with 3 different cell lines (Gnyszka et al., 2013).

No long term negative effects have been found of DNMTIs in patients yet. However some problems of the DNMTIs are that they have a poor bioavailability, they are instable in physiological media, and they are highly toxic. At this moment the DNMTI Zebularine is investigated to be used, Zebularine is a more stable and less toxic DNMTI. The molecular structure of Zebularine is shown in figure 5 (Gnyszka et al., 2013).

## 2.2 DNA methyltransferase inhibitors **Non-Nucleoside analogues**

Non-Nucleoside DNMTIs do not have to be incorporated into the DNA. The DNMTs are not trapped, as with nucleoside analogues, but are blocked, shown in figure 9 (Lyko & Brown, 2005).



**Figure 9.** The different mechanisms of DNMT inhibition. Enzyme trapping by nucleoside analogues and enzyme blocking by non-nucleoside analogues (Lyko & Brown, 2005).

The mechanism of these DNMTIs in blocking DNA methylation can happen by different compounds. One compound is hydralazine, a vasodilator that works as a blocker by the interaction between the nitrogen of Hydralazine with the lysine-162 and the arginine-240 of the DNMT. Another compound is procainamide, an antiarrhythmic agent, the blocking mechanism is the same as for Hydralazine, however Procainamide preferably binds to DNMT1 (Yang et al., 2010). For the molecular structures of Hydralazine and Procainamide see figure 7b.

Another option for DNMTI by non-nucleoside analogues is by using short chain oligodeoxynucleotides and microRNAs. An example of a short chain oligodeoxynucleotide is MG98, it binds to the 3'UTR of the DNMT1 mRNA and with prevents translation of mRNA. However this is not yet validated and used in clinical settings (Yang et al., 2012).

The use of microRNA miR29a can also inhibit DNMTs. miR29a targets DNMT2A/B and DNMT1 also by binding to the 3'UTR of the DNMTs and prevents translation into protein. These mechanisms both reduce the DNA methylation (Yang et al., 2010).

RG108 is also a non-nucleoside DNMTI that can inactivate the DNMT1 enzyme because it fits into the catalytic pocket of the DNMT1 enzyme (Yoo & Jones, 2006). The effects of non-nucleoside analogues on the TSGs discussed in the previous chapter are unknown at this moment.

The disadvantage of non-nucleoside analogue DNMTIs is that they have an effect of all cells in the body. The nucleoside analogues get incorporated especially in fast proliferating cells so especially in tumor cells, however the non-nucleoside analogue DNMTIs can block the DNMT enzymes in all cells. So, also in the slow proliferating cells the DNMTIs could be present and active and this can cause hypermethylated oncogenes to become hypermethylated and active.

## **Discussion**

In this review we have taken a closer look at hypermethylated TSGs in three different types of NHLs, namely BL, FL, and DLBCL. This review focusses on the NHLs because 89% of the B-cell lymphomas are NHLs. The hypermethylated TSGs that have been found are BIM, DAPK, P16, DSP, and DLC-1. There is not always a clear role of the hypermethylated TSGs in the NHLs, but the most prominent roles that these genes play in disease development and maintenance is increasing of proliferation, reducing apoptotic mechanisms of the cells and evading of cell cycle checkpoint regulations.

Also the epigenetic therapies to reverse hypermethylation in these gene were reviewed. There are different epigenetic therapies that are already available or in trial that reverse the methylation of the TSGs. The effects and working mechanism of nucleoside analogues and non-nucleoside analogues were analysed. Nucleoside analogues trap the DNMTs that cause methylation, and the non-nucleoside analogues block the DNMT enzymes. Both mechanisms result in a decreased amount of methylation in dividing cells.

In table 1 the percentage of patients with hypermethylation of the TSGs is shown. Especially DAPK and DLC-1 seem to have promising results, because a high amount of patients have these genes hypermethylated (59-100%). BIM and P16 hypermethylation is present in approximately 50% of the patients. Re-expression of these genes can have an increasing effect on apoptosis, and can decrease cell proliferation and cell migration.

DSP is also hypermethylated in NHL patients with a range of 17-40% of the patients. However the that role DSP hypermethylation plays in the development of NHLs is debatable because DSP proteins play a role in the adhesions of cells, and B-cells do not adhere to each other. So, if DSP hypermethylation plays any role in NHLs has to be further investigated (Genecards, 2014).

The impact of the studies about gene hypermethylation is low. The studies have been done with a low amount of patients which makes it impossible to draw hard conclusions from these data. In one study DLC-1 promoter hypermethylation of 100% was found, however the study contained only 6 patients. These results are all promising results which might be important for new therapy methods in the future, so more research should be done.

However it is important to take into account that not all patients have hypermethylation of the TSGs, the TSGs can also be inactivated in different ways, like deletions or inversions. For therapeutic approaches it is important that patients that do not have hypermethylated TSGs also do not get DNMTIs because DNMTIs can have harmful effects. They can cause demethylation of genes like oncogenes and cause more tumor progression this way.

The therapies for the reduction of DNA hypermethylation are promising therapies for patients that have hypermethylated TSGs. Because of the higher proliferation rate of tumor cells the nucleoside analogue DNMTIs will be incorporated more in the DNA of the tumor cells during proliferation than in the normal cells (Cortez & Jones, 2009). However there are also fast proliferating normal cells, but because the bioavailability of DNMTIs is not really good the DNMTs probably be more incorporated into the tumor cells than into fast proliferating normal cells. A reason for this is that the arteries that go through the tumors are of less quality than normal arteries so particles like DNMTIs can pass the arteries that go through the tumor more easily (Weinberg, 2014).

However, there is still reduction in methylation in normal cells, which could have negative side effects. If in normal cells oncogenes are not expressed because of hypermethylation and DNMTs are added that reduce the amount of hypermethylation, the oncogenes can become active and new tumor cell development can be facilitated. However at this moment no long term negative effects are found when patients use DNMTs (Gnyszka et al., 2013).

Especially for azacytidine and decitabine the results are promising in NHLs, and they already have been approved by the FDA for a few other types of cancers. These nucleoside analogues also have shown to have a demethylating effect of BIM, DAPK and P16. However of these genes, only for BIM effects of re-expression has been found in patients, DAPK and P16 re-expression is only tested in cell lines. So more research has to be done because at this moment the results have a low impact, but the results are promising.

Non-nucleoside analogues block the DNMT enzymes. Among the non-nucleoside analogues there are Hydrazaline, Procainamide, MG98, miR29a, and RG108. The non-nucleoside DNMTs might be less toxic than the nucleoside DNMTs because they are not incorporated into the DNA (Yang et al., 2010). However there are at this point no results that the non-nucleoside analogue DNMTI treatment could result in re-expression of the silenced TSGs mentioned in this review (BIM, DAPK, P16, DSP, and DLC-1). So if they want to use the non-nucleoside analogues for NHLs in the future much more research has to be done.

Many of the chemotherapies work by activating the apoptotic pathways, and these pathways are often inactivated during carcinogenesis. So as said in chapter 2, activating the TSGs with DNMTs, and with this activating the inactivated apoptotic pathways has a positive effect on treatment response (Clozel et al., 2013; Gnyszka et al., 2013). The chemotherapy often induces BIM to induce apoptosis (Harada & Grant, 2012), so when the gene promoter of BIM is hypermethylated the chemotherapy has no way to induce apoptosis and chemo resistance can arise. Therefore demethylation of the TSGs can also have a positive effect on chemo response by activating the apoptotic pathways.

The use of DNMTs prior to chemotherapy to cause de-methylation and re-expression of the TSGs has been found to cause chemo sensitization in DLBCL. However, this has a low impact because the study had only 5 participants, but the results are promising (Clozel et al., 2013).

The research for novel compounds that target DNMTs and decrease methylation should be continued. It is important to find new less toxic and more selective DNMTs that work only in tumors. At this moment research is done about reactivation of only the aberrant silenced genes, this could give promising results and give therapies with less side effects (Gnyszka et al., 2013).

Overall, the results are promising, some more than others. However the amount of participants in the studies was not high enough to make hard conclusions. Also more research has to be done for the epigenetic therapies that should decrease the amounts of hypermethylation.

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