

# Epigenetic Regulation and Therapy in Lymphoid Malignancies

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## Introduction

Lymphoid malignancies usually represent malignant counterparts of lymphocytes at discrete stages of normal lymphocyte differentiation; this disease is characterized by uncontrolled proliferation and survival of clonal neoplastic lymphoid cells. Major breakthroughs in the past have contributed to our understanding of the genetic failures and the changed biology in lymphoma cells that underlie the initiation and progression of the disease. It is now recognized that not only genetic but also epigenetic alterations are similarly important in this process. Enormous evidence has accumulated in the past decades that establishes the importance of epigenetic modifications in cancer and has resulted in shifting the focus from entirely genetic-based studies to integrated studies involving both genetic and epigenetic alterations. Since these alterations do not change the DNA sequences and are pharmacologically reversible, they have been regarded as optimal targets for what is now known as epigenetic therapy. In this review, we will discuss our current understanding of normal epigenetic processes, outline our knowledge of epigenetic alterations, discuss advances in the understanding of post-translational histone modifications (DNA acetylation and methylation) and microRNA in normal and in lymphoma cells, and explore novel therapies. We will also discuss how this information is being used to improve current therapy of this disease.

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## Targeting Histone Deacetylation in the Treatment of Lymphoma

Histone acetylation plays a critical role in the regulation of gene expression. In general, hyperacetylated histones are associated with transcriptionally active genes, whereas hypoacetylated histones are associated with suppressed transcription. Histone acetyltransferase and histone deacetylase (HDAC) controls these changes and the dynamic balance between chromatin structure and gene expression of proteins involved in regulation of a variety of functions (Sharma et al. 2010). Therefore, HDAC is considered closely associated with a variety of tumors (Hagelkruys et al. 2011). As a promising new generation of targeted anti-cancer drugs, HDAC inhibitors (HDACi) have a higher response rate and have been highly focused in recent years. The anti-tumor activity of HDACi has been confirmed from a number of experiments, and I/ II clinical trials have shown that HDACi can have an effect on refractory cutaneous T cell lymphomas (CTCL), Hodgkin lymphoma (HL), myeloid tumors, and solid tumors (Mehnert and Kelly 2007). Presently, vorinostat and romidepsin have been approved by the US FDA for the treatment of relapsed CTCL.

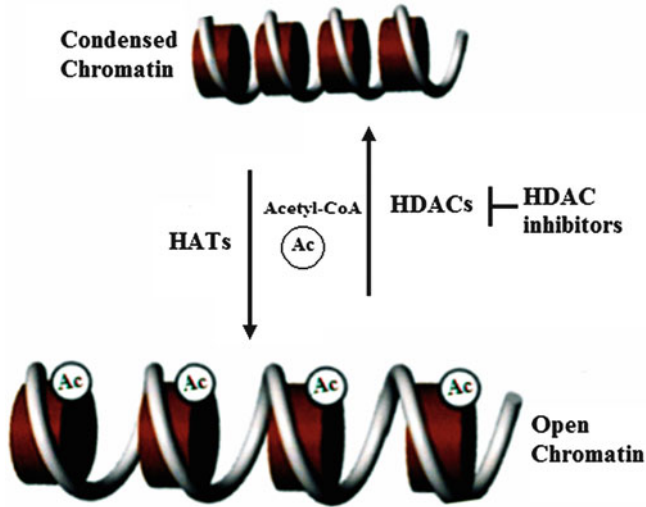
### Concept of HDAC

Studies have shown that histone acetylation and deacetylation modifications play important roles in the regulation of gene expression and tumor development. There are two types of enzymes that determine the degree of histone acetylation: histone acetyltransferase and HDAC.

HDACs are enzymes involved in the remodeling of chromatin and have a key role in the epigenetic regulation of gene expression. They catalyze the removal of acetyl groups from lysine residues in histone amino termini, leading to chromatin condensation and transcriptional repression. In addition, the activity of non-histone proteins can be regulated through HDAC-mediated hypo-acetylation (Fig. 1) (Hagelkruys et al. 2011).

### Classification of HDAC

To date, eighteen HDACs have been identified in humans. They have been grouped into two major categories: zinc-dependent HDACs and NAD-dependent HDACs. These have been further classified into four major classes based on their homology to yeast HDACs, their subcellular localization, and their enzymatic activities. The class I HDACs (1, 2, 3, and 8) are homologous to the yeast RPD3 protein, can generally be detected in the nucleus, and show ubiquitous expression in various human cell lines and tissues. Class II HDACs (4, 5, 6, 7, 9, and 10) share homologies with



**Fig. 1** Histone acetyltransferase (HAT) and histone deacetylase (HDAC) determine the degree of histone acetylation. The extent of acetylation and deacetylation on different positions of core histones is determined by the antagonistic activity of histone acetylases (HAT) and histone deacetylases (HDAC) and alters the nucleosomal conformation of both transformed and non-transformed cells

the yeast Hda1 protein and can shuttle between the nucleus and the cytoplasm. The class IIb HDACs, HDAC6 and 10, are found in the cytoplasm and contain two deacetylase domains. HDAC6 has unique substrate specificity with a tubulin deacetylase domain specific for the cytoskeletal protein tubulin. The class III HDACs (SIRT1, 2, 3, 4, 5, 6, and 7) are homologues of the yeast protein Sir2 and require NAD<sup>+</sup> for their activity to regulate gene expression in response to changes in the cellular redox status. SIRT1 has been shown to interact with p53, then deacetylate it, resulting in repression of p53-mediated transcriptional activity. SIRT1 can negatively regulate multiple pathways, including both tumor suppressors (p53, FOXO) and oncogenic proteins (survivin,  $\beta$ -catenin, NF- $\kappa$ B). HDAC11 is the sole member of the class IV HDACs. There is similarity to the catalytic core regions of both class 1 and 2 enzymes; however, HDAC11 does not have a strong enough identity to be placed in either class (Fig. 2) (Copeland et al. 2010).

## Role and Classification of HDAC Inhibitors (HDACi)

HDACi alters gene transcription by inhibiting histone deacetylase activity and remodeling chromatin architecture. The rapid development in understanding the biology of lymphoid cells has led to the development of HDACi as a promising class of mechanism-based agents for the treatment of lymphoma. All currently

| HDAC I | HDAC II | HDAC III | HDAC IV |
|--------|---------|----------|---------|
| HDAC 1 | HDAC 4  | SIRT 1   | HDAC 11 |
| HDAC 2 | HDAC 5  | SIRT 2   |         |
| HDAC 3 | HDAC 6  | SIRT 3   |         |
| HDAC 8 | HDAC 7  | SIRT 4   |         |
|        | HDAC 9  | SIRT 5   |         |
|        | HDAC 10 | SIRT 6   |         |
|        |         | SIRT 7   |         |

**Fig. 2** Histone deacetylase (HDAC) classes based on function and DNA sequence similarity

**Table 1** Classification of histone deacetylase inhibitors

| HDAC inhibitors         | Compounds   | Pharmacologic profile   |
|-------------------------|---|---|
| Short chain fatty acids | Valproic acid, phenylbutyrate   | Short plasma half life, rapid metabolism, non-specific mode of action |
| Hydroxamic acids        | TrichostatinA, vorinostat (SAHA), PXD-101 (belinostat), LBH589 (panobinostat), ITF2357, pyroxamide, oxamflatin, scriptaid | Potent HDAC I/II/IV inhibitors, also called pan-HDAC inhibitors       |
| Benzamides              | MGCD0103, MS-275, CI-994, SK-7041   | MGCD0103 is a class I specific HDAC inhibitor                         |
| Cyclic peptides         | Romidepsin, etinostat (MS-275)  | Very potent HDAC inhibitor with IC50s in low nanomolar range          |
| Situin inhibitors       | Niacinamide, sirtinol   | Class II specific HDAC inhibitors                                     |

available HDACs mainly inhibit class 1 and 2 HDACs. Many structurally diverse compounds can bind to HDACs and inhibit their enzymatic activity. These compounds include hydroxamates, cyclic peptides, aliphatic acids, benzamides, and electrophilic ketones.

HDACi can be divided into the following five types based on their chemical structure (Table 1):

1. Hydroxamic acids, including trichostatin A, vorinostat (SAHA), panobinostat (LBH-589), and others. They are the earliest discovered and most extensively studied HDAC inhibitors so far. Hydroxamic acids have strong activity and simple structure, with major effects on the Class I and Class II HDACs.

2. Short-chain fatty acids, such as butyric acid, benzene acid, and valproic acid. Compounds of this type possess a relatively simple structure, less selectivity, low inhibitory activity and bioavailability, and fast metabolism *in vivo*.
3. Cyclic tetrapeptide classes, including trapoxin and depsipeptide (FK228). This class can be further divided into two subclasses: Aoe-containing inhibitors and that without Aoe moiety. Moreover, Aoe-containing cyclic peptide could irreversibly bind and inhibit HDACs, while the other subclass without Aoe moiety refers to reversible inhibitors.
4. Benzamide comprises MS-275, MGCD103 and CI-994 and so on. The activity of these inhibitors is lower than that of the corresponding hydroxamic acids and cyclic peptide compounds, but this type has a high selectivity with class I HDAC.
5. Electrophilic ketones include a variety of trifluoromethyl ketones. When trifluoromethyl ketone carbonyl is reduced to hydroxyl groups, then it loses its activity.

## Mechanisms of Action of HDACis

HDAC inhibitors exert a myriad of biological effects, including induction of differentiation/apoptosis, cell-cycle/mitotic arrest and induction of autophagic cell death. HDACis can modify gene expression and altering the acetylation status of transcription factors and other proteins involved in transcription, and can lead to cell death by activating apoptosis by both the intrinsic and the extrinsic pathways, also, HDACis can cause DNA damage through production of ROS, and blocking the activity of the chaperone protein Hsp90.

Abnormal regulation of cell cycle is an important mechanism of tumorigenesis. Studies have shown that a variety of HDACi can induce cyclin-dependent kinase inhibitor p21 and activate the expression of apoptosis precursor protein. p21 is an important differentiation-related genes, and plays an important role for growth arrest by blocking cyclin-dependent kinase activity. Richon et al found that cell cycle kinase inhibitor p21 protein levels were significantly increased after giving HDACi for 6 ~ 24 hours, while the levels of cyclin A and D1 were decreased. Researches also indicate that HDACi can also induce p16, cyclin E, thioredoxin binding protein 2 (TBP2) and other cell cycle regulators.

It is noteworthy that though HDACi can cause high degree of histone acetylation, these genes in the activation of transcription are less than 2%, so we conclude that the HDACi on gene expression is selective.

HDACi can also regulate non-histone acetylation. The substrate proteins of HSO involved in tumor cell proliferation and apoptosis and related to anti-tumor properties of the regulation. Yang et al show that by inducing acetylation of HSP90, Panobinostat induce tumor cell growth arrest and apoptosis. HDACi-induced HSP90 acetylation achieved by inhibiting the activity of HDAC6 .

HDACi mainly through the death receptor pathway or the mitochondrial pathway activate caspase and induces tumor cell apoptosis. Bokelmann reports suggest that valproic acid and vorinostat can activate pro-apoptotic factors Bid and Bim, while

inhibiting anti-apoptotic Bcl-2 factor. Zhao and others found that excessive expression of E2F1 which is regulated by the pro-apoptotic factor Bim leading to cell apoptosis. Interestingly, Rosato et al have shown that HDACi can still induce cell death even deal all the cells with inhibitors of apoptosis. So we believe HDACi can also cause non-apoptotic cell-death. At present, most reports consider this is related to autophagy, but the specific mechanism remains unclear. Some believe that the mechanisms of HDACi-induced cell death also including the expression of Bcl-2 family with BH-3 region, P53 pathway, and ROS regulation (Mehnert and Kelly 2007; Cotto et al. 2010; Copeland et al. 2010).

## Clinical Application of HDACis in Lymphoma

The rapid development in understanding the biology of lymphoid cells has led to HDACi developed as a promising class of mechanism-based agents for the treatment of lymphoma (Zain and O'Connor 2010).

All currently available HDACis mainly inhibit class I and II HDACs. Many structurally diverse compounds can bind to HDACs and inhibit their enzymatic activity, including hydroxamates, cyclic peptides, aliphatic acids, benzamides, and electrophilic ketones. Presently, vorinostat and romidepsin have been approved in the United States for the treatment of relapsed and refractory CTCL (Shakovich and Melnick 2011).

### *Hodgkin Lymphoma*

With current therapies available, such as chemotherapy, radiotherapy, and hematopoietic stem cell transplantation, the cure rate of Hodgkin lymphoma (HL) is higher than 85%. However, for patients without complete remission, the prognosis is relatively poor and the median survival time is about 3 years. The latest clinical trials have shown that HDACi has a promising effect on the relapsed or refractory HL (Buglio and Younes 2010).

A phase II clinical trial of oral panobinostat (LBH589), conducted in 28 patients with relapsed or refractory HL, reported clinical efficacy in 2 cases (complete response; CR) and 24 cases of progressive response (PR), with maximum tolerated dose (MTD) defined as 40 mg/day. Subsequently, in another following phase II clinical trial in which panobinostat was dosed at MTD, of all 61 relapsed or refractory HL patients enrolled, one patient achieved CR, ten patients achieved PR, and 31 patients showed stable disease (SD). The common toxicity was thrombocytopenia, which was reversible by decreasing the dose. Panobinostat demonstrated an encouraging clinical activity in relapsed or refractory HL with fewer side effects (Suredd et al. 2010).

Mocetinostat (MGCD0103) is an amino phenylbenzamide inhibitor specific for HDAC classes I and IV. Researches have demonstrated that mocetinostat has a

potent anti-proliferative activity in HL cell lines. Furthermore, mocetinostat can induce tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) expression and secretion. In a phase II clinical trial in relapsed or refractory HL, mocetinostat was administered at a dose of 110 mg three times per week. Among the enrolled 20 patients, 7 had CR. However, as a result of an intolerable side effect, an additional ten patients were enrolled at a dose of 85 mg three times per week. The disease control rates in the 85 mg and 100 mg cohorts were 35% and 25%. Of four patients who died, all in the 100 mg cohort, two cases may have resulted from the pharmaceutical administration. However, mocetinostat, at 85 mg three times per week, was shown to be an effective single agent for relapsed or refractory HL with a manageable safety profile (Buglio et al. 2010).

### ***B Cell Non-Hodgkin Lymphoma***

B-cell non-Hodgkin lymphoma (NHL) is the most common type of lymphoma and has been readily classified into aggressive subtypes and indolent diseases. Diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) are common types of NHL. Because of frequent relapse in B-cell lymphomas, novel effective treatments are clearly needed. At present, there are no HDACis approved specifically for the treatment of B-cell lymphomas, but a strong rational and clinical trials of some HDACis have shown potent activity for B-cell NHL (Crump et al. 2008a).

Vorinostat (SAHA), a pan-HDACi of class I and II, is reported to be the first clinically effective HDACi in B-cell lymphomas, because it helped a transformed B-cell lymphoma patient achieve a CR. In an oral vorinostat phase I clinical trial in ten patients with B-cell malignancy, four patients achieved CR/PR (3 with follicular and 1 with mantle cell lymphoma), with MTD of oral vorinostat defined as 400 mg daily. The common related adverse events were anorexia, hyperlipidemia, albuminuria, fatigue, nausea, diarrhea, hypocalcemia, and an abnormal hematology (thrombocytopenia, anemia, leukopenia). However, adverse hematology reactions were reversible, with patients recovering from these events through a period of rest. In a phase II trial of vorinostat in the DLBCL, 1 of 18 patients enrolled achieved CR, with the time to remission more than 468 days. Thus, vorinostat, as a single agent, does not show the expecting activity in the treatment of B-cell lymphoma (Watanabe et al. 2010; Crump et al. 2008b).

Belinostat is another hydroxamic acid derivative, without preferential activity versus a special class of HDACs. In a phase I clinical trial of belinostat for treatment of advanced B cell tumor, with belinostat administered by shirt infusion at different doses (600, 900, and 1,000 mg/m<sup>2</sup>/day) in 16 patients with relapse or refractory B cell lymphoma, five patients, including two patients with DLBCL, two with chronic lymphocytic leukemia (CLL), and one with multiple myeloma achieved disease stabilization. The adverse events reported were nausea, vomiting, fatigue, and diarrhea. Importantly, there were two patients, both with multiple myeloma, who suffered renal failure, the cause of which was not clear and needed further research.

**Table 2** Clinical trials of HDACi in the CLTL

| Agent      | Phase | Sponsor or author                       | Number of cases | ORR          | mDOR   | mTTP   | mTTR  |
|------------|-------|---|-----------------|--------------|--------|--------|-------|
| Vorinostat | I/II  | Duvc M                                  | 33              | 8%           | 15.1w  | 30.2w  | 11.9w |
| Vorinostat | II    | Olsen                                   | 74              | 29.7%        | >185d  | 4.9 m  | 55d   |
| Romidepsin | II    | NCI                                     | 71              | 34%<br>(4CR) | 13.7 m | 15.1 m | 2 m   |
| Romidepsin | Iib   | Gloucester<br>Pharmaceutical<br>company | 96              | 34%<br>(6CR) | 15 m   | 8 m    | 2 m   |

Data in another phase I clinical trial of oral belinostat in recurrent refractory lymphoma also showed an acceptable safety profile and more importantly signs of clinical efficiency in terms of stable disease. Particularly, clinical responses were reported in all patients with mantle cell lymphoma (Gimsing et al. 2008; Zain et al. 2009).

### *T Cell Non-Hodgkin's Lymphoma*

T cells and NK cell tumors account for about 12% of NHL, with 15–20% being aggressive lymphomas. Presently, the 5-year survival rate for these patients was only 10–30%. Relapsed or refractory lymphoma is still a significant clinical problem to be solved (Zain and O'Connor 2010).

In a phase I/II clinical trial of oral vorinostat, in which a total of 33 patients with relapsed or refractory CTCL were enrolled, the overall response rate (ORR) was 24.4%, the median time to respond (mTTR) was 11.9 weeks, and the median duration of response (mDOR) was 15.1 weeks. Consequently, the 400 mg/day dose was considered optimum in terms of response-toxicity profile for evaluation in a phase II multicenter trial. The related dose-limited toxicities included gastrointestinal disorders, anorexia, dehydration, fatigue, and bone marrow depression.

In a subsequent phase II study conducted in 74 relapsed or refractory CTCL patients, the ORR was 29.7% (including 1 CR), the mTTR, by far shorter, was just 8 weeks, and the mDOR was more than 185 days. The most common toxicities were diarrhea, fatigue, nausea, and anorexia. Compared with other drugs, vorinostat has the advantage of short time to respond, a good tolerance, and convenient oral administration. The agent has already been approved by the US FDA for treatment of relapsed or refractory CTCL in 2006 (Duvic et al. 2007; Olsen et al. 2007).

In a phase II study conducted by the National Cancer Institute, 71 patients with relapsed or refractory CTCL were given intravenous romidepsin at 14 mg/m<sup>2</sup>. The results showed that the ORR was 34%, a CR was observed in four patients, mTTR was 2 months, and mDOR was 13.7 months. The related side effects included fatigue, anorexia, and hematologic abnormalities.

The activity of romidepsin in CTCL was defined further in another phase Iib trial conducted by Gloucester Pharmaceuticals. The results showed that romidepsin was



associated with an ORR of 34% in the overall population, with four patients achieving a CR. The mDOR was 13 months, resulting in romidepsin being regarded as an important drug in treatment of relapsed or refractory CTCL. However, this drug should be carefully used in patients with heart disease because the cause of cardiac side effects is not yet exactly known. The agent was approved for the treatment of relapsed or refractory CTCL by the US FDA in 2009 (Table 2) (Piekarz et al. 2009; Bates et al. 2008).

## ***Conclusion***

As a new class of targeted antitumor drugs in epigenetics, HDACi regulates gene expression mainly through changing the histone acetylation and modulating chromatin structure. Compared with other chemotherapy agents, HDACi is found to have higher selectivity to tumor cells than normal ones. They can effectively target tumor cells to growth arrest, differentiation, and apoptosis, while have little effect on normal cells. HDACi has become the focus of clinical research; at present, many HDACis have shown promising activity in the clinical research malignant lymphoma treatment. Among them, vorinostat and romidepsin were approved for the treatment of relapsed or refractory CTCL. Other studies, such as romidepsin used in peripheral T-cell lymphoma (PTCL) and MGCD0103 in HL, have demonstrated remarkable and prospecting clinical effect.

However, there are still many problems to be solved, such as the specific anti-cancer mechanism and the relationship between concentration and duration of HDACi with the clinical effect. In addition, because of the short half-life and fast metabolism rate of HDACi, endeavors to find the proper delivery methods and dosage and methods to improve pharmacodynamic stability are also needed.

## **Histone Methylation in Lymphoma**

As a significant epigenetic regulation mechanism, histone methylation plays an important role in many biological processes. In cells, there are various histone methyltransferases and histone demethylases working cooperatively to regulate the histone methylation state. Upon histone modification, effector proteins recognize modification sites specifically and affect gene transcriptional process. Histone lysine methylation generally happens in lysine (K) residues and arginine residues. Histone H3 K4, K9, K27, K36, K79, and H4 K20 can be methylated. Post-translational modification of chromatin and regulation of gene transcription through amino-terminal residues of histone is an important part of epigenetics. Enhancer of zests homolog 2 (EZH2) is the catalytic component of a polycomb group protein that mediates repression of gene transcription, silencing of the target genes, and leading to tumorigenesis through its histone 3 lysine 27 methyltransferase activity. Because EZH2 gene is highly expressed in human malignancies and is capable of promoting

cell proliferation, and facilitating malignant tumor cell diffusion, the progression of lymphoma has been shown to be closely associated with EZH2 dysregulation.

## Histone Methylation

Histone lysine methylation is catalyzed by different specific histone lysine methyltransferases (histone lysine (K) methyltransferases). Histone lysine methylation carries histone H3 and H4; for both, there are a total of six protein lysine residue methylation sites. The lysine side chain could be methylated, dimethylated, and trimethylated.

Histone arginine methylation plays a key role in the regulation of gene transcription, with such modification mediated by protein arginine methyltransferases (PRMTs). The methylation modification of PRMT1 and PRMT4 are related to activation of gene transcription, whereas methylation modifications of PRMT5 and PRMT6 are associated with inhibition of gene transcription. Histone arginine methylation is a dynamic reversible process. The arginine residues are related to a variety of covalent modifications, and these modifications are present in multiple sites, with transcription of genes having different effects and which could further regulate other physiological functions within cell, such as cell development, dynamic chromatin regulation, and DNA replication and repair (Wang et al. 2008).

## Histone Demethylation

Histone demethylases mainly contains PADI4, LSD1, and JmjC domain-containing proteins.

PADI4 (peptidyl arginine deiminase, type IV) is a member of a gene family that encodes enzymes responsible for the conversion of arginine residues to citrulline residues. This gene may play a role in granulocyte and macrophage development, leading to inflammation and immune response. Multiple arginine sites mainly on histone 3 and histone 4 can be catalysed by PADI4 (Liu et al. 2011).

LSD1 (lysine-specific demethylase 1), a nuclear homolog of amine oxidases, functions as a histone demethylase and transcriptional corepressor, represses transcription via histone demethylation, and specifically demethylates histone H3 lysine 4. LSD1 is a kind of protein with the functions of demethylation and transcriptional repressor. Experiments confirmed that the inhibition of LSD1 by interfering RNA (RNAi) can lead to increased H3-K4 methylation, so that the target gene is expressed and LSD1 achieves transcriptional repression by demethylation. In 2004, LSD1 was identified as the first histone demethylase. It contains a C-terminal amine oxidase domain that is responsible for the demethylase activity through a FAD-dependent mechanism. An N-terminal SWIRM domain that is important for the stability and chromatin targeting of LSD1 was also found in other chromatin regulators. The mechanism of LSD1-mediated demethylation requires a protonated nitrogen for the reaction to

proceed, the substrate specificity of it is limited to mono- or dimethylated lysine residues. LSD1 regulates gene expression mainly through three aspects: (1) through the integration of the SANT2 structure domain of CoREST and the target gene, causing demethylation of H3K4 and then gene transcription repression; (2) through binding of LSD1 and androgen/estrogen receptor can cause the demethylation of H3K9, leading to activation of hormone receptor-dependent transcription; and (3) through demethylation of H3K4, DNMT3L, which is the positive regulator of DNA methylation transferase (DNMTs) and could combine with unmethylated K4, promoting expression of DNMTs and resulting in DNA re-methylation (de novo methylation) and the inhibition of gene transcription (Escoubet-Lozach et al. 2009).

Unlike LSD1, the JmjC domain-containing proteins that have been tested do not require a protonated nitrogen and are able to reverse all three states of lysine methylation. In vivo, the overexpression of it can reduce dimethylation of H3-K36 level. We could consider JmjC domain protein as a demethylase. When compared with LSD1, first, they have different reaction mechanisms; second, the LSD1 protein family only has a small part of the proteins associated with demethylation, whereas the JmjC domain protein is a large family with the potential ability to regulate and inhibit different histone methylation. Finally, the LSD1 demethylation reaction requires protonated nitrogen, thus limiting its three methylated histone methylation capacity (Pfau et al. 2008).

## Role of EZH2 in Lymphoma

A recently identified human gene (EZH2) is the human homologous genes of the zeste gene enhancer of *Drosophila*. It is an important member of the polycomb gene family. EZH2 is highly expressed in a variety of tumors and can promote cell proliferation and facilitate malignant tumor cell differentiation (Chase and Cross 2011). EZH2's role in the tumor has become the focus of current research.

EZH2 is a subunit of PRC2, and its SET domain catalyzes trimethylation of H3K27, a histone modification associated with transcriptional silencing. H3K27me3 helps recruit PRC1 to chromatin; it is thought that PRC1 is the effector of PcG-mediated silencing and long-term epigenetic memory.

In the PcG family, EZH2 plays a central role. PcG and TrxG (trithorax group) are widely conserved genes and play an important role in cellular memory system to prevent cell identity changes. The system is established from the early embryonic development, throughout the development process, and until adulthood. Through the formation of different components of the PcG and TrxG protein complex, they regulate the chromatin "active" or "inactive" status, maintaining corresponding gene transcription or inhibition. As an important PcG protein, EZH2 participates in the formation of chromatin structure, gene expression regulation, and growth control. EZH2 affects the formation of PcG and TrxG protein complexes, development and differentiation of hematopoietic cells, X chromosome inactivation, and even the process of tumorigenesis (Wang et al. 2004).

## ***The Role of EZH2 in Cancer***

The EZH2 gene is expressed in all fetal and adult tissues but is significantly reduced in the adult heart, brain, and kidney. There are many reports that the EZH2 gene is highly expressed in human malignancies. High expression of the EZH2 gene was first found in hematological malignancies, including bone marrow cancer, Hodgkin disease, non-Hodgkin lymphoma, and mantle cell lymphoma. Subsequently, prostate cancer, breast cancer, bladder cancer, liver cancer, colorectal cancer, and gastric cancer were found to have high expression of the EZH2 gene, while EZH2 in the corresponding normal tissues is not expressed or shows only a small amount of expression (Chase and Cross 2011).

Deregulation of PcG gene expression in experimental model systems has clearly been linked to oncogenesis. Because of the crucial effects of EZH2 in individual development and cell growth, especially in normal lymphoid organs, B-cell differentiation, and proliferation of lymphoid tissue, EZH2 was first studied in hematopoietic malignancies. BMI-1 and EZH2 are involved in two different types of PcG complexes that regulate specific binding of target gene expression in normal B lymphocytes. Germinal center (GC) B lymphocyte development at different stages is related to different types of PcG gene expression. Because the PcG complex could affect pluripotent hematopoietic stem cells (HSCs) and directed differentiation potential through chromatin modifications, the imbalanced expression of EZH2 may lead to lymphomas. Research has also indicated that EZH2 may be involved in the malignant disease process. BMI-1 gene in mice shows the imbalance in the gene leads to B-cell and T-cell lymphoma, and its absence causes serious developmental defects in the hematopoietic system (Chase and Cross 2011; Wang et al. 2004; Velichutina et al. 2010).

## ***EZH2 and Lymphoma***

Lymphoma represents a group of malignancies that originate in the lymph nodes or other lymphoid tissues. There is increasing evidence that the up-regulated EZH2 in normal GC B cells as well as the deregulation of PcG expression are related to the formation of lymphomas (Velichutina et al. 2010).

The overexpression of EZH2 has been reported in mantle cell lymphoma and adult T-cell leukemia/lymphoma cells. In Burkitt lymphoma, the overexpression of c-Myc contributes to stimulate EZH2 expression. Hodgkin/Reed-Sternberg (H/RS) cells co-express BMI-1 and EZH2 (Dukers et al. 2004). Within the B-cell lineage, it was shown that EZH2 is highly expressed in lymphoid progenitors, and EZH2 deficiency induces defects in early lymphopoiesis (Sneeringer et al. 2010). Moreover, EZH2 mutations also exert influence on lymphomagenesis. A missense mutation in the EZH2 SET domain was discovered in a sizeable fraction of DLBCLs, and a truncation mutation of EZH2 was found in only 2 of 221 other tumors, suggesting that mutation of EZH2 is a lesion specific to lymphoma.

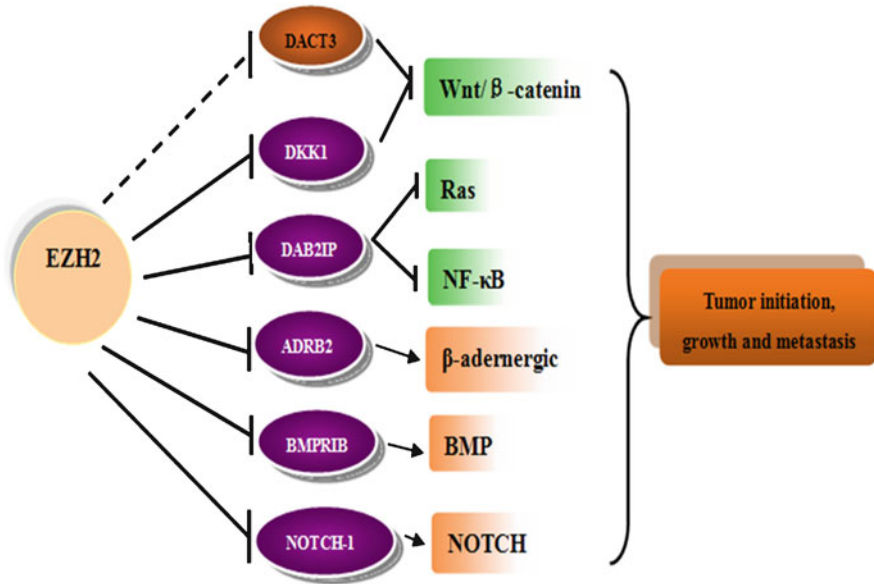
There is increasing evidence that the deregulation of PcG expression is related to the formation of lymphomas. Also, the irregular expression profile of BMI-1 and EZH2 in B-NHL suggests that the distinct balance between the BMI-1 and EZH2-containing PcG complex is disturbed in these lymphomas. Furthermore, the extent of irregular PcG expression is correlated with the type of lymphoma and therefore the clinical behavior (Park et al. 2011).

The expression of PcG complexes during GC reaction is linked to the differentiation status of follicular B cells and a mutually exclusive pattern of BMI-1/RING1 and EZH2/EED. PcG proteins are in reactive centroblasts and centrocytes. These observations suggest that expression of PcG complexes is highly regulated during GC reaction and that PcG proteins may contribute to antigen-specific B-cell maturation. Research has also shown that adult T-cell leukemia/lymphoma cells (Sasaki et al. 2011) have deregulated polycomb repressive complex 2 with over-expressed EZH2 and that there is the possibility of a new therapeutic strategy targeting histone methylation in this disease.

On the basis of the discovery that EZH2 is a mutant oncogene in germinal B cell lymphomas and the fact that the extent of abnormal PcG expression correlated with the type of lymphoma and therefore the clinical behavior, a new therapeutic strategy against EZH2 may exist for mechanism-based epigenetic treatment of patients with these lymphomas. Important steps toward this goal include further delineation of the lymphoma types in which this mutation occurs and development of assays amenable to detection of these mutations in routine clinical specimens.

### ***The Mechanism of Action of EZH2***

Beside PcG, with the function of controlling expression of Hox genes, a growing number of studies have revealed the function of PcG in stem cell function, differentiation, development, and cell cycle control. PcG proteins have been identified as important proteins in tumorigenesis due to their potential to repress tumor suppressor genes. Through the repression of the INK4/ARF locus, which encodes both p16INK4a, which prevents inactivation of the tumor suppressor RB, and p19ARF, which stabilizes the tumor suppressor p53, PcG plays a central role in cell proliferation. Genome-wide mapping by chromatin immunoprecipitation experiments of PcG members in human and murine stem cells has shown that several genes involved in regulation of cell development and more importantly, the localization and expression patterns of PcG genes change during the stages of differentiation. The PcG proteins maintain cellular identity by preserving chromatin states against chromatin-disrupting processes during cell cycle transitions. For example, during the process of DNA replication, H3K27-methylated histones recruit PRC2 and PRC1 complexes to nucleosomes of the nascent DNA strand to continue gene silencing, then PRC1 and PRC2 provoke changes in chromatin structure. At present, PRC1 is believed to recognize the PRC2-methyl mark and the PRC2 complex is considered to be required for the repressive capacity of PRC1 complex. Furthermore, PcG



**Fig. 3** EZH2-mediated epigenetic regulation of signaling pathways. EZH2 activates oncogenic signaling cascades and inhibits pro-differentiation pathways through epigenetic silencing of the negative regulators and positive effectors, respectively, thereby establishing an oncogene-tumor suppressor signaling paradigm in driving tumor initiation, growth and progression. As DACT3 is repressed via H3K27me3, EZH2 is implicated to be its epigenetic regulator

genes play a major role in regulating hematopoietic function, especially for HSCs. For instance, BMI-1-deficient mice have severely impaired HSC self-renewal, and bone marrow progenitors lacking BMI-1 have a restricted proliferative potential; overexpression of EZH2 confers long-term repopulating potential on HSCs, preventing its exhaustion after replicative stress; and expression of one null EED allele or 2 hypomorphic EED alleles results in greater lymphoproliferation and a greater risk of developing hematologic tumors. Additionally, a gene expression signature of PRC2 target genes predicts poor outcome in patients with breast and prostate cancer, implying that there might exist a window to improve therapeutic efficiency for neoplasms (Tsang and Cheng 2011; Bracken et al. 2003; Yap et al. 2011).

Genome-wide searches of PRC2 target genes in mammalian cells including fibroblasts, ES, and cancer cells also revealed cohorts of signaling components that control cell differentiation. Emerging data indicate that EZH2 has a master regulatory function in controlling key signaling pathways in cancers by transcriptional repression of signaling molecules. EZH2 activates oncogenic signaling cascades and inhibits pro-differentiation pathways through epigenetic silencing of the negative regulators and positive effectors, respectively, thereby establishing an oncogene-tumor suppressor signaling paradigm in driving tumor initiation, growth and progression.

## ***Conclusion***

In summary, the roles for EZH2 in normal GC B-cells are to favor cellular proliferation by directly repressing several tumor suppressor genes; to create a repression state similar to that found in stem cells that might foster self-renewal and prevent premature differentiation; and to maintain and stabilize a transcriptional repression program (as shown in Fig. 3).

Preclinical data and results suggest that therapeutic targeting of EZH2 might have significant antilymphoma effects and support the rationale for development of inhibitors of the EZH2 SET domain (Hang et al. 2011).

## **Role of miRNAs in Lymphoma**

MicroRNAs (miRNAs) are an abundant class of short regulatory (~22 nt) noncoding RNAs widely expressed in all metazoan eukaryotes and highly conserved throughout different organisms. Biogenesis of miRNAs is a complex process starting in the nucleus and ending in the cytoplasm (Li et al. 2011). miRNAs are encoded by genes that are presumably transcribed into single or clustered primary transcripts, which are processed to produce the mature miRNAs. These, in turn, are incorporated into a ribonucleoprotein complex called RNA-induced silencing complex (RISC) and guide the RISC to the target mRNA. RISC plus miRNA mediate target gene downregulation by mRNA cleavage or translational repression, mainly through binding to 3'UTR of target mRNA. miRNAs are involved in critical biologic processes, including development, cell differentiation, apoptosis, and proliferation. Recently, a growing body of evidence has implicated specific miRNAs in the pathogenesis of lymphoma.

## **MiRNAs in B Cell Lymphomas**

### ***Chronic Lymphoid Leukemia (CLL)***

It was in CLL that a role of miRNAs in cancer was first reported by Calin et al. in 2002. The miR-15a/16-1 cluster at chromosome 13q14 within the most commonly deleted region in CLL was found to be down-regulated in 68% of cases with the deletion. Subsequently, these miRNAs have been shown to target Bcl-2 and remove the suppressor influence on anti-apoptotic Bcl-2, which is overexpressed consistently in CLL. Unique miRNA signatures have been generated dependent on VH gene mutation and ZAP70 status. The miR-29 family and miR-223 have been found consistently to be down-regulated in association with unmutated VH genes and are decreased dramatically in patients with poor prognosis independently of the prognostic marker classification used.

Possibly of as much importance in CLL associated with the very poor prognostic genotype, 17p13/TP53 deletion is consistently associated with down-regulation of miR-34a (Merkel et al. 2010). Members of the miR-34 family have been discovered to be direct p53 targets and mediate some of the p53-dependent effects. While miR-34b/c are not expressed in CLL cells, low miR-34a levels are a sensitive indicator of the activity of the p53 axis in CLL (Moussay et al. 2010) and are associated with p53 inactivation and chemotherapy-refractory disease irrespective of 17p deletion/TP53 mutation status. Among patients harboring a 17p deletion, low expression of the miR-223, miR-29c, miR-29b, and miR-181 family were associated strongly with disease progression, confirming the adverse effects of low levels of miR-29 and miR-223 in CLL progression. In a separate study, the 17p-deleted miRNA signature comprised high levels of miR-15a, miR-21, and miR-155 and low levels of miR-34a and miR-181b. In addition, miR-21 expression stratified survival in patients with 17p-deleted CLL and among cohorts of patients with CLL with a variety of chromosomal aberrations. Among both the good- and poor-prognosis subgroups, miR-181b levels identified patients who required early therapy and who thus need closer follow-up. Furthermore, studies have identified miRNAs that may predict clinical resistance of CLL to fludarabine and a similar expression profile emerges with miR-29a, miR-181a and miR-221 among those being expressed differentially (Moussay et al. 2010).

### ***Diffuse Large B-Cell Lymphoma (DLBCL)***

miR-155 was increased in several types of B cell lymphomas. SHIP1 has been identified as a target of miR-155 and is down-regulated by miR-155 in DLBCL as a consequence of autocrine stimulation by TNF- $\alpha$ . Another target of miR-155 is SMAD, which is suppressed by high levels of miR-155 and protects DLBCL cells from the growth-inhibitory effects of transforming growth factor- $\beta$  (Rai et al. 2010). It is reported that miR-96, miR-182, miR-589, and miR-25 were shown to be significantly up-regulated in 7q+DLBCL, which were associated with lower death rate and better overall survival in patients treated with R-CHOP (Chigrinova et al. 2011). The expression of miR-18a correlated with overall survival, whereas the expression of miR-181a and miR-222 correlated with progression-free survival, suggesting that the expression of specific miRNAs may be useful for DLBCL survival prediction (Alencar et al. 2011). Myc-mediated repression of miRNA-34a promotes high-grade transformation of B-cell lymphoma by dysregulation of FoxP1 gastric marginal zone B-cell lymphoma of MALT type (MALT lymphoma). In addition, the miR-34a showed the strongest anti-proliferative properties through the targeted gene FoxP1, which promotes DLBCL proliferation, elucidating a novel Myc- and FoxP1-dependent pathway of malignant transformation and suggesting miR-34a replacement therapy as a promising strategy in lymphoma treatment (Craig et al. 2011a).



### ***Mantle Cell Lymphoma (MCL)***

miR-16-1 regulates the expression of cyclin D1 (CCND1) in MCL, which is associated with a poor prognosis, whereby miR-16-1 binding sites within the CCND1 mRNA 3'UTR are deleted and miRNA regulation of CCND1 is altered. miR-17-5p/miR-20a from the miR-17\_92 cluster were expressed at high levels concomitantly with MYC and associated with poor disease prognosis. Subsequently, Zhao et al. (2010) found that 18 miRNAs were down-regulated and 21 were up-regulated in MCL compared with normal B lymphocytes, with the most frequent alterations being decreased miR-29, miR-142 and miR-150 and increased miR-124a and miR-155. Patients with significantly down-regulated miR-29 had a shorter survival compared with those who expressed relatively high levels of miR-29. Down-regulation of the miR-29 family was associated with CDK6 overexpression, and CDK6 was found to be a direct target of miR-29. Zhang et al. demonstrated that Myc acts as a repressor of miRNA-15a/16 by recruiting HDAC3, by the way that both c-Myc and HDAC3 co-localized to the two promoters of the miR-15a/16-1 cluster gene, DLEU2, suggesting the role of HDAC3 in Myc-induced miR-15a/16-1 changes and reveal novel mechanisms for c-Myc-driven miRNA suppression and malignant transformation in aggressive B-cell malignancies (Zhang et al. 2011). Follicular dendritic cells protect B-cell lymphoma cells against apoptosis, in part through activation of a miR-181a-dependent mechanism involving down-regulation of Bim expression, indicating that cell-cell contact controls tumor cell survival and apoptosis via miRNA in mantle cell and other non-Hodgkin lymphomas (Lwin et al. 2010).

### ***Mucosa-Associated Lymphoid Tissue (MALT)***

miR-203 is decreased in MALT lymphoma, which results from extensive promoter hypermethylation of the miR-203 and coincides with dysregulation of the miR-203 target ABL1. Reexpression of miR-203 can prevent tumor cell proliferation, and ABL inhibitors prevent tumor cell growth, supporting that the transformation from gastritis to MALT lymphoma is epigenetically regulated by miR-203 promoter methylation and identifying ABL1 as a novel target for the treatment of this malignancy (Craig et al. 2011b).

### ***Follicular Lymphoma (FL)***

The miR-155, miR-221 and miR-21 are overexpressed similarly in FL as they are in DLBCL, with no significant differences between expression levels in these different lymphomas. A signature of four miRNAs (miR-330, -17-5p, -106A, -210) has been

proposed as a diagnostic classifier to discriminate FL, DLBCL, and non-neoplastic lymph node, but this has not been confirmed by other series.

### ***Burkitt's Lymphoma (BL)***

It had been reported that the down-regulation of hsa-miR-9\* seems to specifically identify a particular subset of BL cases lacking MYC translocation and determined that the hsa-miR-9\* is able to modulate E2F1 and c-Myc expression, suggesting it as a promising novel candidate for tumor cell marker (Onnis et al. 2010). The expression of hsa-miR-127 was shown to be strongly upregulated in EBV-positive BL samples compared with the EBV-negative cases. In addition, it was shown that hsa-miR-127 is involved in a B-cell differentiation process through posttranscriptional regulation of BLIMP1 and XBP1. These findings suggest that the overexpression of miR-127 may represent a key event in the lymphomagenesis of EBV-positive BL, likely by blocking the B-cell differentiation process (Leucci et al. 2010). miR-26a, repressed by MYC, was also found to be deregulated in primary human Burkitt lymphoma samples, and expression of miR-26a influenced cell cycle progression by targeting the oncogene EZH2, suggesting that MYC modulates genes important to lymphomagenesis via deregulation of miRNAs. Overexpression of let-7a decreased Myc mRNA and protein. Down-regulation of Myc protein and mRNA using siRNA MYC also elevated let-7a miRNA and decreased Myc gene expression. These findings with let-7a add to the complexity of MYC regulation and suggest that dysregulation of these miRNAs participates in the genesis and maintenance of the lymphoma phenotype in Burkitt lymphoma cells.

### ***Others***

MiR-21, miR-19, and miR-92a levels in cerebrospinal fluid (CSF) collected from patients with primary central nervous system lymphoma (PCNSL) indicated a significant diagnostic value, and, more importantly, combined miRNA analyses resulted in an increased diagnostic accuracy with 95.7% sensitivity and 96.7% specificity. This shows that the CSF miRNAs are potentially useful tools as novel noninvasive biomarkers for diagnosis of PCNSL (Baraniskin et al. 2011). It is shown that the plasma miR-92a values in NHL were extremely low when compared with healthy subjects, and the very low plasma level of miR-92a increased in the complete response (CR) phase but did not reach the normal range, with plasma levels lower again in the relapse phase, indicating that the plasma miR-92a value could be a novel biomarker not only for diagnosis but also for monitoring lymphoma patients after chemotherapy (Ohyashiki et al. 2011).

## MicroRNAs in T-Cell lymphomas

### *NK/T Cell Lymphoma*

It was observed that circulating miR-221 was decreased in patients with NK/T cell lymphoma and a reverse correlation with performance status and the overall survival after treatment, indicating that plasma miR221 may be a diagnostic and prognostic marker for NK/T cell lymphoma (Guo et al. 2010). miR-21 and miR-155 expression levels were significantly greater in NK-cell lymphoma. Reducing expression of miR-21 or miR-155 led to up-regulation of phosphatase and tensin homologue (PTEN), programmed cell death 4 (PDCD4), or Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1). Moreover, transduction with either miR-21 or miR-155 led to down-regulation of PTEN and PDCD4 or SHIP1 with up-regulation of phosphorylated AKTser473. These results provide important new insight into the pathogenesis of NK-cell lymphoma/leukemia and suggest targeting miR-21 and/or miR-155 may represent a useful approach to treating NK-cell lymphoma/leukemia. It was shown that low miR-146a expression is an independent poor prognostic factors for NK/T cell lymphoma. miR-146a overexpression can inhibit nuclear factor-kB (NF-kB) activity, suppress cell proliferation, induce apoptosis, and enhance chemosensitivity. TNF receptor-associated factor 6, a target of miR-146a and a known NF-kB activator, was down-regulated by miR-146a. Promoter methylation of miR-146a gene was observed, as well as in NK/T-cell lymphoma tissues with low miR-146a expression, and miR-146a expression was induced by the conversion of methylation status with a demethylating agent. These results suggest that miR-146a might function as a potent tumor suppressor in NK/T-cell lymphomas and be useful for patient assessment and therapeutic targeting (Paik et al. 2011).

### *Mycosis Fungoides (MF)/Sézary Syndrome*

The most significant differentially expressed microRNA were miR-155 and miR-92a (both up-regulated), while miR-93 showed the highest up-regulation in tumor stage mycosis fungoides (MF) by the miRNA microarray, unfortunately, when these miRNAs were validated by miRNA-qPCR on additional test groups. None of the miRNAs was up-regulated in tumor stage MF of those previously shown to be up-regulated in SS, and only 2 of the 19 miRNAs down-regulated in tumor stage MF were also down-regulated in SS (van Kester et al. 2011). miR-21 expression is increased in Sézary cells when compared with CD4+ T cells from healthy donors. Silencing of miR-21 in Sézary cells results in increased apoptosis, suggesting a functional role for miR-21 in the leukomogenic process (van der Fits et al. 2011).

It showed that expression of miR-223 can distinguish SzS samples from healthy controls and patients with mycosis fungoide. The miR-342 plays a role in the pathogenesis of Sézary syndrome by inhibiting apoptosis, thus describing a novel mechanism of regulation for this miRNA via binding of miR-199a\* to its host gene. Moreover, it also provides the first in vivo evidence for down-regulation of the miR-17-92 cluster in malignancy and demonstrates that ectopic miR-17-5p expression increases apoptosis and decreases cell proliferation in Sézary syndrome cells (Ballabio et al. 2010). A total of 45 miRNAs have been shown to be differentially expressed between Sézary syndrome and controls. Using predictive analysis, 19 miRNAs, including miR-21, miR-214, miR-486, miR-18a, miR-342, miR-31, and let-7 members, were also found. Moreover, a signature of 14 miRNAs, including again miR-21, was identified, which may be able to discriminate patients with unfavorable and favorable outcomes (Narducci et al. 2011).

### ***Anaplastic Large Cell Lymphoma (ALCL)***

It was reported that the ALK(+) ALCL cell lines and biopsy specimens express a low level of miR-29a and that this down-modulation requires an active NPM-ALK kinase and that the low expression of miR-29a, probably through methylation repression, plays an important regulatory role in MCL-1 overexpression that could promote tumor cell survival by inhibiting apoptosis. Enforced miR-29a expression was found to modulate apoptosis through inhibition of MCL-1 expression in ALCL cell lines and in a xenografted model, with a concomitant tumor growth reduction. Thus, synthetic miR-29a represents a potential new tool to affect tumorigenesis in these lymphomas (Desjobert et al. 2011).

### ***Cutaneous T Cell Lymphomas (CTCL)***

Data have shown that the most induced (miR-326, miR-663b, and miR-711) and repressed (miR-203 and miR-205) miRNAs distinguish CTCL from benign skin diseases with >90% accuracy. A qRT-PCR-based classifier consisting of miR-155, miR-203, and miR-205 distinguishes CTCL from benign disorders with high specificity and sensitivity and with a classification accuracy of 95%, indicating that miRNAs have a high diagnostic potential in CTCL (Ralfkiaer et al. 2011).

## **Conclusion and Perspective**

In summary, although it is clear that the functional importance of miRNAs in lymphoma is gaining momentum rapidly, we still have much to learn from these tiny molecules and further research on their target genes, specific transcription factors

and their interactions is needed to integrate miRNAs into the regulatory network in the genesis of lymphoma. Recent researches have described remarkably effective inhibition of miRNAs *in vivo*, thus providing an entry point into the promising new discipline of microRNA therapeutics. Undoubtedly, the coming years will bring possibilities of designing miRNAs mediated targeting therapies and make miRNAs specifically attractive as new tools for the diagnosis, prognosis, and therapy of lymphoma. It is to be hoped that some of these will be efficient and will benefit future lymphoma patients.

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