DNA methylation of microRNA genes in multiple myeloma

Kwan Yeung WONG[1], Xiaojun HUANG[2], Chor Sang CHIM[1*]

[1]Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Hong Kong, China; [2]Department of Hematology, Peking University Institute of Hematology, Beijing, China.

*Corresponding Author: Dr CS Chim :

Tel: +852 22553879; Fax: +852 28162187; Email: jcschim@hku.hk

Keywords: multiple myeloma; methylation; tumor suppressor; miRNA; *miR-34*; *miR-124*; *miR-194*; *miR-203*

Abstract

DNA methylation is one of the heritable epigenetic modifications, leading to repressed gene expressions and consequent phenotypic alterations without changing the DNA sequence. MicroRNA (miRNA) is a novel class of short non-coding RNA molecules regulating a wide range of cellular functions through translational repression of their target genes. Recently, epigenetic dysregulation of tumor suppressor miRNA genes by promoter DNA methylation has been implicated in human cancers, including multiple myeloma (MM). This article presents a brief overview of the pathogenesis of MM, the role of DNA methylation in cancer biology, methods of DNA methylation analysis, miRNA biology, dysregulation of miRNAs in MM, and summaries the current data on the role of DNA methylation of tumor suppressive miRNAs in MM.

Multiple myeloma

Multiple myeloma (MM) is a cancer arising from neoplastic proliferation of plasma cells. In the US, it is the second most common form of hematological malignancies, which accounts for approximately 15% of all hematological malignancies or approximately 1% of all malignant diseases [1]. Interestingly, the incidence of MM appears to be higher in Western than Asian countries [2].

Clinical stages of MM

The disease starts with immortalization of a post-germinal center B cell, which will then home to the bone marrow and clinically present as asymptomatic monoclonal gammopathy of undetermined significance (MGUS) (Figure 1). MGUS progresses to symptomatic MM at a rate of 1% per year, and hence is considered as the precursor of MM [3]. Symptomatic MM is characterized by the presence of end-organ damages which include hypercalcemia, renal failure, anemia, and bone lesions (CRAB) [4]. About 15% of all newly diagnosed MM patients are preceded by an additional intermediate stage, known as smoldering MM (SMM), which will evolve into symptomatic MM at a higher rate of 10% per year [5]. At the terminal stage, MM cells become independent of the bone marrow stroma, and hence extramedullary MM, such as plasma cell leukemia, may occur [6].

Molecular genetics of MM

Based on gene expression profiling, universal upregulation of D-type cyclins (cyclin D1, D2, or D3) is a hallmark of all MM [7]. However, MM remains a highly heterogeneous disease with variable losses and gains of chromosomes, and can be categorized into

non-hyperdiploid and hyperdiploid MM [8-10] (Figure 1). It has also been found that this ploidy dichotomy can also be detected even in MGUS, the precursor of symptomatic MM, and hence an early event of myelomagenesis [10,11].

Non-hyperdiploid MM, constituting approximately half of all MGUS and MM, can be further subdivided into 3 categories, known as hypodiploid (chromosome number up to 44/45; and/or DNA index less than 0.95), pseudodiploid (chromosome number between 44/45 to 46/47; and/or DNA index between 0.95 to 1.05) and near-tetraploid (chromosome number greater than 75; and/or DNA index greater than 1.75). Majority of the non-hyperdiploid MM is associated with a primary translocation which involves juxtaposition of a strong immunoglobulin heavy chain gene enhancer locus to a partner oncogene important for myelomagenesis [12,13]. In MM patients, approximately 15% of patients harbor t(11;14)(q13;q32), which is associated with upregulation of oncogenic cyclin D1 (*CCND1*) [13]. Another 15% of patients carry t(4;14)(p16.3;q32), which involves dysregulation of fibroblast growth factor receptor 3 (*FGFR3*) and wolf-hirschhorn syndrome candidate 1 (also known as MM SET domain, *MMSET*) [14,15]. Less frequent but distinguishable primary translocations, known as t(14;16)(q32;q23), t(6;14)(p21;q32) and t(14;20)(q32;q11), are found in approximately 5%, 3% and 2% of patients, leading to dysregulation of v-maf musculpaponeurotic fibrosarcoma oncogene homolog (avian) (*MAF*) (also known as *c-MAF*), cyclin D3 (*CCND3*) and v-maf musculpaponeurotic fibrosarcoma oncogene homolog B (avian) respectively [16-19].

In contrast to the non-hyperdiploid MM, hyperdiploid MM constitutes another half of all MGUS and MM, due to trisomies of odd-numbered chromosomes including 3, 5, 7, 9, 11,

15, 19 or 21, resulting in a chromosome number between 46/47 to 75, as measured by conventional karyotyping; and/or a DNA index between 1.05 to 1.75. In contrast to other odd-numbered chromosomes frequently involved in hyperdiplody, chromosome 13 is often deleted instead [20,21]. Moreover, trisomy of chromosome 11, to which *CCND1* is localized, has been shown to result in direct upregulation of *CCND1* [22,23]. While the underlying mechanism leading to the aforementioned dichotomy of MM remains to be elucidated, it is noteworthy that, clinically, hyperdiploid MM patients are associated with better prognosis and treatment outcomes than the non-hyperdiploid MM patients [24-26].

During disease progression, secondary translocations and other genetic aberrations, including deletion of the short arm of chromosome 17 [del(17p)] and mutations of *RAS* genes, etc, are involved [27]. Unlike primary translocations, which involve juxtaposition of the strong immunoglobulin heavy chain gene enhancer locus to a partner oncogene, the mechanisms of secondary translocations are less well-defined but appears unrelated to the error-prone B cell-specific DNA modification events. For instance, at the time of disease progression, about 15% of MM patients carry secondary immunoglobulin heavy chain translocation involving v-myc myelocytomatosis viral oncogene homolog (avian) (*c-MYC*) (8q24), which confers proliferative advantage to MM cells [27,28].

Based on fluorescence *in situ* hybridization (FISH) analysis, del(13) is detected in 20% to 50% of MGUS, and approximately 50% of MM. Notably, 90% of del(13) is characterized by monosomy 13, and interstitial deletion of 13q14 occurs in the remaining 10% of cases [20,21]. Despite that del(13) was once believed to impart poor prognosis, recently, the

prognostic impact of del(13) has been shown to be mediated by its strong association with unfavorable risk factor of t(4;14) [29,30].

By interphase FISH analysis, del(17p), which is the locus for tumor suppressor protein TP53, is generally found in less than 10% of MM patients at diagnosis. However, presence of del(17p) at diagnosis is a powerful negative prognostic factor for MM [31,32]. A recent study of MM patients uniformly receiving bortezomib-based induction therapy prior to autologous stem cell transplantation further confirmed that del(17p) is associated with an inferior event-free survival (median time: 14 vs. 36 months) and overall survival (4-year OS: 50% vs. 79%) as compared with those without del(17p). Therefore, the adverse impact of del(17p) appears not abolished by the use of targeted therapy [33].

RAS mutations, predominantly *K*- and *N-RAS* at codon 12, 13 and 61, but not *H-RAS*, is present in more than half of MM at diagnosis but not in MGUS, suggesting that the *RAS* mutations is at least a marker of the transition from MGUS to MM [34-37].

Last but not least, the bone marrow microenvironment is very important in the pathogenesis of MM for homing of MM plasma cell to the bone marrow and secretion of growth-stimulating cytokines to the MM plasma cells. The homing of MM plasma cell is a chemotaxis mechanism mediated by the bone marrow stromal cells secreted chemokine (C-X-C motif) ligand 12 or stromal cell-derived factor 1, which binds to its specific receptor, chemokine (C-X-C motif) receptor 4, expressed on the MM plasma cell. Moreover, upon cell-to-cell interactions between the bone marrow stromal cells and the MM plasma cells, a

multitude of cytokines are secreted, and hence favor the proliferation and survival of MM plasma cells by autocrine and paracrine signaling [e.g. interleukin 6 (IL6), insulin-like growth factor 1, tumor necrosis factor alpha and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB)], angiogenesis [e.g. vascular endothelial growth factor and basic fibroblast growth factor] and osteolysis [e.g. tumor necrosis factor (ligand) superfamily, member 11, tumor necrosis factor receptor superfamily, member 11a and tumor necrosis factor receptor superfamily, member 11b] [27,38,39].

DNA methylation

DNA methylation and cancer

DNA methylation refers to catalytic addition of a methyl group (-CH) to carbon 5 position of a cytosine ring in a CpG dinucleotide [40-44]. CpG dinucleotide cluster in any genomic region of over 200 bp in length with a high GC content of more than 50% and observed/expected CpG ratio larger than 0.60 is known as a CpG island [45,46]. In human, CpG island is associated with at least 50% of gene promoters. Methylation of a promoter-associated CpG island will lead to recruitment of histone methyltransferase, methyl-CpG-binding domain (MBD) protein and histone deacetylase, resulting in formation of a compact heterchromatin configuration, which precludes the binding of transcription factor complex, and hence silencing of the associated gene [46,47].

In normal cells, a majority of genes with promoter-associated CpG islands are usually unmethylated, associated with a euchromatin configuration, and hence are generally transcriptionally ready or active (Figure 2). However, a fraction of genes with

promoter-associated CpG islands are methylated, and hence silenced in normal cells including genetic imprinting and X-chromosome inactivation [48,49].

On the other hand, in cancer cells, genes with promoter-associated CpG islands are aberrantly methylated in a tumor-specific manner, leading to gene silencing. In particular, hypermethylation of promoter-associated CpG islands of tumor suppressor genes, resulting in decreased or loss of gene expressions, and hence loss of tumor suppressor functions, has been implicated in carcinogenesis [40,41,43,44,50-52] (Figure 2). Furthermore, in cancers, hypermethylation of the tumor suppressor genes may serve as a second hit, in addition to deletion or mutation of the other allele, thereby fulfilling the Knudson's two-hit hypothesis [53].

In MM, by genome-wide or gene-specific approaches, aberrant DNA methylation has been found to mediate the loss of a number of protein-coding tumor suppressor genes regulating cell cycle progression, cell signaling or apoptosis, including cyclin-dependent kinase inhibitor 2A (*CDKN2A*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*), death-associated protein kinase, secreted frizzled-related protein 2 and suppressor of cytokine signaling 1 (*SOCS1*), etc [54,55].

Methods of DNA methylation analysis

Over the years, techniques of DNA methylation analysis have evolved from qualitative to quantitative in fashion, and from locus-specific to genome-wide in scale [56-58] (Table 1). Bisulfite conversion, which chemically deaminates or modifies unmethylated cytosine to

uracil, and hence translating an epigenetic variation (methylated or unmethylated) into a genetic difference (C or U), is an important procedure fundamental to most of the later methods [59,60].

(I) Candidate gene-specific methods

Before the integration of bisulfite conversion into DNA methylation research, earlier techniques mostly depend on enzymatic digestion and high-performance liquid chromatography (HPLC). For the methylation-sensitive restriction enzyme-based analysis, for example, DNA methylation pattern of CpG dinucleotides embedded in a CCGG sequence can be detected by the use of isoschizomer pair of *Hpa*II and *Msp*I, by which methylated CCGG can be digested by *Msp*I but not by *Hpa*II, together with gel electrophoresis and Southern blotting [61]. However, the use of restriction enzyme digestion is limited by the requirement of large amount of DNA and the availability of restriction enzyme cut site at the locus-of-interest. Moreover, it is less informative about the methylation pattern over a stretch of CpG dinucleotides and prone to generate false-positive results due to incomplete digestion. In addition to enzymatic digestion, HPLC was also employed in DNA methylation analysis in the early days [62]. However, the use of HPLC is also limited by the requirement of large amount of DNA and the need of skillful and tedious operation.

Later, Frommer et al. first incorporated bisulfite conversion into DNA methylation research, leading to the advent of bisulfite genomic sequencing (BGS), and subsequently a qualitative method, known as methylation-specific PCR (MSP). Moreover, bisulfite conversion is integrated to a number of quantitative methods, namely, combined bisulfite

restriction analysis (COBRA), methylation-sensitive single nucleotide primer extension (Ms-SNuPE), MethyLight, pyrosequencing and MassARRAY.

BGS generates the highest resolution map of the DNA methylation status of every single cytosine residue within a locus-of-interest, by the use of simple techniques including bisulfite conversion, PCR, cloning and sequencing [63]. Bisulfite-converted DNA is first amplified by primers, which do not span any CpG site and hence allow unbiased amplification of both methylated and unmethylated alleles. The amplicons are then cloned and sequenced. However, it is labor-intensive and hence is limited to small number of locus-of-interest and samples.

MSP is currently the most popular technique used in studying DNA methylation of locus-specific CpG sites because of its specificity and simplicity [64]. DNA methylation status of any given CpG site is revealed by PCR amplification of bisulfite-converted DNA with two sets of PCR primers, one specific to the methylated sequence and the other to the unmethylated sequence. With validated specific primers and PCR conditions, the methylation status in a large number of samples can easily be obtained. However, it is not a quantitative method.

COBRA is highly similar to one of the two classic methods for the use of restriction enzyme, however, with the incorporation of bisulfite conversion, it becomes a high-throughput and quantitative technique [65]. Upon bisulfite conversion, unmethylated *Bst*UI recognition sites CGCG will be converted to TGTG, whereas methylated *Bst*UI sites

remain unchanged. Followed by *Bst*UI digestion and gel electrophoresis, methylation of a locus-of-interest can be quantified by [100% X intensity of (digested fragments/ both digested and undigested fragments)].

Ms-SNuPE is a quantitative DNA methylation analysis method derived from primer extension technique [66]. In brief, primer extension is performed on bisulfite-treated locus-of-interest with ^{32}P-labeled dCTP or dTTP, which enable differentiation and quantification of the methylated or unmethylated template. However, the use of radioactive isotopes hinders the popularity of this technique.

MethyLight, also known as quantitative MSP, enables simultaneous detection and quantification of bisulfite-treated methylated and unmethylated templates by two specific TaqMan probes labeled with different fluorophores [67]. By the use of real-time PCR, MethyLight is regarded as a high-throughput, sensitive and quantitative method in DNA methylation research.

Pyrosequencing, which originally designed to study single-nucleotide polymorphism by indirect detection of pyrophosphate (PPi) released during DNA synthesis, has also been applied to detect the C and T difference generated by bisulfite conversion [68,69]. During a primer extension process on a bisulfite-treated template, PPi is released in an equimolar fashion according to the number of incorporated nucleotides, resulting in a proportional conversion of PPi to ATP by sulfurylase, and hence a quantifiable firefly luciferase signal

driven by the ATP. However, such a high-throughput, accurate and quantitative method is limited by the length of individual read, which is only about 60 to 100 bp.

MassARRAY, a technique involving a combination of bisulfite conversion, *in vitro* transcription, RNA digestion and MALDI-TOF mass spectrometry, is another high-throughput quantitative DNA methylation analysis method [70]. In brief, bisulfite-treated locus-of-interest is amplified with an *in vitro* transcription tag, which allows later *in vitro* transcription. The transcripts will then be digested into fragments without affecting any of the original CpG sites. Based on mass difference arise from methylated (resulting G) and unmethylated (resulting A) on the fragments, quantification of the methylated or unmethylated fragment is enabled. However, this technology requires sophisticated operation.

(II) Genome-wide methods

Recently, coupled with bisulfite conversion, methylation-sensitive restriction enzyme digestion or methylation-sensitive antibody purification, genome-wide analysis of DNA methylation is made possible with different kinds of DNA microarrays and high-throughput sequencing methods [57,71-73].

Bisulfite-converted DNA, for instance, can be subjected to Infinium Methylation Assay (Illumina), which allows quantitative analysis of > 485,000 specific CpG dinucleotides per sample. By methylation-specific single-base primer extension, specific fluorescence-labeled nucleotides will be incorporated, and hence a ratio of different fluorescent signals indicating

the methylation status [74,75]. Other array platforms include Affymetrix, Agilent and NimbleGen. Alternatively, bisulfite-converted DNA libraries can be generated from sonication or restriction enzymes prior to high-throughput sequencing, resulting in whole-genome bisulfite sequencing (WGBS) or reduced representation bisulfite sequencing (RRBS), which is able to generate DNA methylome with single-base resolution [76,77].

Moreover, methylation-sensitive restriction enzyme may be used to enrich methylated or unmethylated DNA for different kinds of tiling arrays and high-throughput sequencings. For instance, *Bst*UI or *Hpa*II digestion (cleaves unmethylated DNA) will lead to enrichment of methylated sequences, whereas *Msp*I or McrBC digestion (cleaves methylated DNA) will result in enrichment of unmethylated sequences, which are followed by array or sequencing profiling [78,79].

Alternatively, with the development of methylation-sensitive antibodies, such as MECP2 (methyl-CpG-binding protein 2), MBD1 and MBD2, which binds to methylated CpG sites, immunoprecipitation of methylated sequences prior to DNA microarrays or high-throughput sequencing is enabled and collectively known as MeDIP (methylated DNA immunoprecipitation)-chip, when it is analyzed by DNA microarray, or MeDIP-seq, when it is analyzed by high-throughput sequencing method [80,81].

MicroRNA (miRNA)

History and biogenesis

miRNA is a class of short non-coding RNA molecules of 20 to 30 nucleotides (nts) in length [82]. miRNAs inhibit the translation of their own target genes via binding of the miRNA seed region (i.e. the 2nd to 7th nts from 5' to 3' of the mature miRNA) to the three prime untranslated region (3'UTR) of the target gene, and hence involve in the regulation of various cellular activities, including development, differentiation, proliferation, and apoptosis [83-86] (Figure 3).

The first miRNA was identified in *Caenorhabditis elegans*, in which *lin-4* miRNA was found controlling the gradual decrease of LIN-14 protein expression from developmental stage L1 to later larval stages, through binding to the 3'UTR of *lin-14* messenger RNA (mRNA), leading to translational inhibition of the *lin-14* mRNA [87,88]. Later, with more small non-coding RNAs discovered in *C. elegans*, this class of RNAs was collectively named as miRNAs by Thomas Tuschl, David Bartel, and Victor Ambros [89-91].

Most miRNA genes are associated with RNA polymerase II promoter and are generally first transcribed into primary (pri-) miRNA (>100 nts) by RNA polymerase II in the nucleus [92]. A pri-miRNA transcript is first stabilized by 5'capping and 3'polyadenylation, and then further processed into precursor (pre-) miRNA by RNase III Drosha and its co-factor Pasha [93,94]. A pre-miRNA (60 to 80 nts) forms a hairpin or stem-loop structure, followed by export into the cytoplasm through Ran-GTP-dependent exportin 5 [95,96]. In the cytoplasm, the pre-miRNA is further processed by RNase III Dicer into mature miRNA duplex (22 to 25 nts), which will then be loaded into a RNA-induced silencing complex (RISC) [94]. The functional mature miRNA strand of the duplex is retained in the RISC for recognizing the mRNA target through sequence complementarity between the miRNA seed region and the

3'UTR of the target gene. Subsequently, the target gene is inhibited by either translational inhibition or mRNA degradation mediated by the miRNA-associated RISC [97,98].

Precise miRNA expression is essential for normal cellular functions, including apoptosis, proliferation, and differentiation [83-86]. Conversely, dysregulation of miRNA expression is implicated in various diseases, including cancers.

miRNA, DNA methylation and Knudson's hypothesis

Mechanistically, miRNAs play a role in the regulation of DNA methylation in cancers. *miR-29* family miRNAs are downregulated in cancers including acute myeloid leukemia (AML) and non-small cell lung cancer [99,100]. Moreover, it has been shown that the *miR-29* family miRNAs are tumor suppressive miRNAs targeting DNA methyltransferase (*DNMT*) 3A and 3B, which are responsible for initiation of *de novo* DNA methylation [101,102]. Indeed, restoration of the *miR-29* family miRNAs in lung cancer cells led to inhibition of cellular proliferation, induction of apoptosis and reduced tumorigenicity in mice. Furthermore, by targeting *DNMT3A* and *3B*, restoration of the *miR-29* family miRNAs resulted in re-expression of hypermethylated tumor suppressor genes, including *CDKN2B* and estrogen receptor 1 in AML cells, and fragile histidine triad and WW domain containing oxidoreductase in lung cancer cells by gene hypomethylation. Therefore, *miR-29* family miRNAs are involved in locus-specific hypermethylation of tumor suppressor genes via inhibition of *DNMTs*.

In breast and cervical cancers, *CDKN2A* and *SOCS1* are important tumor suppressor genes inactivated by DNA hypermethylation. [103,104]. On the other hand, *miR-24* and

miR-155 are oncogenic miRNAs over-expressed in breast and cervical cancers [105,106]. Furthermore, *miR-24* and *miR-155* have been shown to target the 3'UTR of, and hence repress *CDKN2A* and *SOCS1* respectively. [106,107]. Therefore, in addition to gene hypermethylation, tumor suppressor genes can be translationally repressed by oncogenic miRNAs, suggesting that the Knudson's hypothesis can potentially be fulfilled by a complex co-operation of gene alterations with one allele inactivated by gene deletion, mutation or hypermethylation, and the other allele by miRNA targeting.

Thus, these data suggested that miRNAs play multifaceted role in carcinogenesis. Later part of this article focuses on the role of DNA methylation of tumor suppressor miRNAs in MM.

Dysregulation of miRNAs in MM

Dysregulation of miRNAs has been implicated in MM [108,109]. In brief, by miRNA expression profiling using hybridization or Taqman low-density array, miRNAs were found aberrantly expressed in MM cells as compared with their healthy counterparts [110-116]. Moreover, miRNA signatures were found associated with distinct genetic subtypes, such as t(4;14), t(11;14) and t(14;16), and different clinical stages of MM [110,112,114-117]. Furthermore, of these dysregulated miRNAs, some were found to be involved in the regulation of cell cycle, proliferation and apoptosis. For examples, *miR-21* and *miR-17-92* were identified as oncogenic miRNAs, leading to enhanced survival and reduced apoptosis of MM [110,118]. In particular, upregulation of *miR-21*, a downstream target of activator of transcription 3 (STAT3), was found to potentiate the proliferative IL6-mediated signal transducer and STAT3 signaling in MM. In contrast, *miR-15a* and *miR-16-1* were found to be tumor suppressor miRNAs, resulting in increase of apoptosis and suppression of NFκB

pathway in MM cells [111,119]. Moreover, these studies showed that some known tumor suppressor miRNAs, such as *let-7*, *miR-29* and *miR-193*, are downregulated in MM.

Downregulation of miRNA expression in cancers may be mediated by various mechanisms, ranging from epigenetic inactivation, gene mutation or copy number loss to defective miRNA biogenesis or post-transcriptional processing [120]. Of these, DNA methylation is associated with repression of miRNAs possessing promoter-associated CpG islands [121]. Furthermore, the expressions and functions of these tumor suppressor miRNAs can be reversed and restored by DNA hypomethylation treatment [122]. Therefore a better understanding of epigenetic inactivation of tumor suppressor miRNA genes is essential for the biology and treatment in human cancers including MM. Recently, the following studies described the role of DNA methylation of tumor suppressor miRNA genes, including *miR-34a*, *miR-34b/c*, *miR-194-2-192*, *miR-203* and *miR-124-1*, in MM.

Aberrant methylation of miRNA in MM

miR-34a

The tumor suppressor protein TP53 plays a central role in the tumor suppression network, in response to carcinogenic cellular stress and DNA damage, through the induction of apoptosis, cell cycle arrest and senescence [123]. Deletion of the short arm of chromosome 17, to which *TP53* gene is localized, confers an adverse impact on event-free and overall survival of MM patients [29,124]. However, homozygous deletion or mutation of the *TP53* gene is rarely found in MM patients [32,125]. Therefore, it was hypothesized that

the dysregulation of the TP53-mediated tumor suppression may be due to inactivation of other tumor suppressive components along the TP53 pathway in MM.

Recently, the *miR-34* gene family members (*miR-34s*), including *miR-34a*, *miR-34b* and *miR-34c*, have been shown to be direct transcriptional targets and tumor suppressive effectors downstream to the TP53 [126-131]. Deletion or mutation of the *TP53* gene abrogates the *miR-34s* expression, leading to attenuated TP53-mediated tumor suppression activities, and hence loss of translational repression of the *miR-34s* target genes, such as B-cell CLL/lymphoma 2, *CCND1*, cyclin E2, cyclin-dependent kinase (CDK) 4, *CDK6*, *E2F*, v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian) and sirtuin 1, etc [132-139].

With the presence of promoter-associated CpG island at each of the *miR-34a* (1p36) and *miR-34b/c* (11q23) promoters, frequent DNA hypermethylation of the *miR-34s* gene, leading to silencing of the *miR-34s*, and hence upregulation of the *miR-34s* target genes has been found in a wide range of solid cancers, including bladder, breast, colon, lung, melanoma, neuroblastoma, prostate, and ovarian cancer, etc [126,127,129,130,140-143].

In hematological cancers, Chim et al. studied the methylation status of the *miR-34a* in a broad spectrum of primary samples, consist of AML, chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), MM, non-Hodgkin's lymphoma (NHL) and Philadelphia chromosome negative (Ph-ve) myeloproliferative diseases (MDS) [144,145]. Both of these studies showed that the promoter-associated CpG island of

the *miR-34a* was unmethylated in normal controls but aberrantly methylated in 50% of the hematological cancer cell lines, including human myeloma cell lines (HMCLs). Treatment with 5-aza-2'-deoxycytidine led to demethylation of the *miR-34a* promoter and consequent re-expression of the *pri-miR-34a* transcript in cells homozygously methylated for the *miR-34a*. Among primary samples at diagnosis, the *miR-34a* promoter was preferentially methylated in 18.8% NHL (p=0.018), 5.5% MM, 4.0% CLL and 2.2% MDS and none of ALL, AML and CML. Furthermore, in MM, with paired primary samples of at diagnosis and at relapse/progression, it was also shown that methylation of the *miR-34a* promoter remained infrequent even at the time of disease relapse/progression. Therefore, in contrast to the frequent methylation of *miR-34a* in epithelial cancers [142,146], methylation of the *miR-34a* promoter appears unimportant in MM pathogenesis and progression.

miR-34b/c

In contrast to *miR-34a*, methylation of the *miR-34b/c* was implicated in the progression of MM [147]. In a recent study, the promoter-associated CpG island of the *miR-34b/c* was shown to be unmethylated in normal controls but aberrantly methylated in 75% of the HMCLs, in which the expression of mature *miR-34b* inversely correlated with the methylation status of the *miR-34b/c* promoter. Moreover, hypomethylation treatment with 5-aza-2'-deoxycytidine led to demethylation of the *miR-34b/c* promoter and concomitant re-expression of the mature *miR-34b* in myeloma cells homozygously methylated for the *miR-34b/c*, thereby confirming miRNA silencing was associated with promoter hypermethylation. Furthermore, restoration of the *miR-34b* led to inhibition of cellular proliferation and concomitant increase of apoptosis in MM cells, thereby confirming the tumor suppressor role of the *miR-34b* in MM. In primary samples, hypermethylation of the

miR-34b/c promoter occurred in only 5.3% diagnostic MM but 52.2% relapsed MM samples ($p<0.001$). Moreover, in 12 MM patients with paired samples at both diagnosis and relapse, apart from one showing methylation of the *miR-34b/c* promoter at both diagnosis and relapse, hypermethylation of the *miR-34b/c* promoter was acquired at the time of relapse in six (54.5%) patients. Therefore, *miR-34b/c* promoter methylation is acquired at relapse, and hence a biomarker of disease progression in MM.

Therefore, the frequent methylation of *miR-34b/c* in MM at relapse is consistent with the notion that inactivation of other components of the TP53 tumor suppression network may be involved in MM.

miR-194-2-192

In addition to the *miR-34s*, which mediates TP53-associated tumor suppression, Pichiorri et al. found that the expression of *miR-194-2-192* (11p13.1) miRNA cluster was also TP53-dependent, and could be silenced by DNA hypermethylation in HMCLs [148]. First, they showed that the expression of the *miR-194-2-192* cluster was higher in HMCLs with intact TP53 expression as compared with those with deleted or mutated *TP53*. Moreover, in cells with intact *TP53*, treatment with Nutlin-3a, a small-molecule inhibitor of Mdm2, TP53 E3 ubiquitin protein ligase homolog (mouse), upregulated expression of TP53, and consequently *miR-194-2-192* cluster in both HMCLs and primary MM plasma cells. However, the authors found that, upon treatment with Nutlin-3a, despite successful activation of the wild-type TP53, the *miR-194-2-192* cluster was not uniformly re-expressed in all HMCLs and primary MM plasma cells. In search of the mechanism for this discordance, the authors showed that the promoter-associated CpG island of the *miR-194-2-192* cluster was

hypermethylated, and hence, upon hypomethylation treatment, the *miR-194-2-192* cluster could be re-expressed in HMCLs possessing intact TP53 expression. Moreover, the tumor suppressive property of the *miR-194-2-192* cluster was demonstrated by, upon overexpression of the *miR-194-2-192* cluster, the inhibition of cellular proliferation (by growth assays) and blockage of MM cell migration (by trans-well assay). The possible tumor suppressor role of the *miR-194* was further illustrated in another study, in which high expression of the *miR-194* was associated with superior overall survival in MM patients [116].

miR-203

Epigenetic inactivation of the tumor suppressor miRNA *miR-203*, localized to 14q32, was reported in CML, hepatocellular carcinoma and a wide range of hematological malignancies [145,149-151]. While juxtaposition of the 14q32 immunoglobulin heavy chain enhancer to an oncogene partner occur in approximately 50% of MGUS and SMM, > 75% of MM, and > 80% of PCL, leading to upregulation of oncogenes, such as *CCND1*, *CCND3*, *FGFR3*, *MMSET*, and *MAF* [12,13], double-stranded DNA breaks inherent with the translocation may result in DNA loss [152], and hence potential loss of tumor suppressor gene or miRNA. Wong et al. hypothesized that hypermethylation of the promoter-associated CpG island, and hence silencing of *miR-203* might contribute to the development of MM, by fulfilling the Knudson's two-hit hypothesis [53]. Therefore, the authors studied the methylation status of *miR-203* in HMCLs, together with primary samples from patients with MGUS, MM at diagnosis and MM at relapse/progression, by MSP [153]. This study showed that the promoter-associated CpG island of *miR-203* was unmethylated in normal controls but homozygously methylated in 25% of the HMCLs, in which hypomethylation treatment led to demethylation of the

miR-203 promoter and concomitant re-expression of the mature *miR-203*. Furthermore, based on the same search result yielded by both bioinformatics algorithms miRanda and TargetScan, the authors further validated cyclic AMP responsive element binding protein 1 (*CREB1*) mRNA as a novel direct target of the *miR-203*, by luciferase assay. Upon overexpression of *miR-203*, the luciferase activity was reduced in cells transfected with constructs carrying wild-type *CREB1* 3'UTR but not in cells transfected with constructs carrying mutant *CREB1* 3'UTR, thereby confirming *CREB1* as a target of *miR-203*. Moreover, restoration of *miR-203* led to downregulation of CREB1 protein and inhibition of cellular proliferation of MM cells. In primary samples, hypermethylation of the *miR-203* promoter occurred at similar frequency in MGUS, MM at diagnosis and 21% MM at relapse/progression (MGUS: 25%, MM at diagnosis: 24%, MM at relapse/progression: 21%; p=0.973). Therefore, *miR-203* methylation may be an early event in the development of myelomagenesis.

miR-124-1

One of the first and most well-defined epigenetically silenced tumor suppressor miRNAs is *miR-124-1*. Hypermethylation of the *miR-124-1* has been reported in majority of patients with ALL, brain, cervical, colon and liver cancers, leading to direct inhibition of CDK6 translation by binding on the 3' UTR of the *CDK6* mRNA, thereby tumor suppressive [151,154-157]. Later, Wong et al. reported a study of the *miR-124-1* methylation in different types of hematological malignancies, including MM [158]. The promoter-associated CpG island of the *miR-124-1* was shown to be unmethylated in normal controls but frequently methylated in 75% of the HMCLs. Upon 5-aza-2'-deoxycytidine treatment of MM cells with homozygous methylation of *miR-124-1*, re-expression of the mature *miR-124* was associated

with demethylation of the *miR-124-1* promoter and a euchromatic trimethyl H3K4 histone code, leading to repression of CDK6 expression. In primary samples, surprisingly, hypermethylation of the *miR-124-1* promoter was detected only in 2% primary MM samples at diagnosis or relapse/progression. Together, the authors reasoned that frequent methylation of the *miR-124-1* promoter in human myeloma cell lines was a result of *in vitro* passaging, and hence unimportant in the pathogenesis of MM.

Conclusion and future perspectives

Current data on DNA methylation of miRNAs in MM focuses on the loss of tumor suppressor miRNAs due to promoter DNA hypermethylation. Epigenetic inactivation of these tumor suppressor miRNAs is involved in the pathogenesis (*miR-194-2-192* and *miR-203*) and progression (*miR-34b/c*) of MM. Hence, DNA methylation of miRNAs can potentially be biomarker for diagnosis or relapse in MM. Importantly, while deletion or inactivating *TP53* mutation is infrequent in MM, abrogation of the TP53 tumor suppression machinery can be achieved by inactivation of the TP53 transcriptional targets, such as the *miR-34* family members and *miR-194*, by DNA methylation.

Moreover, methylation of these tumor suppressor miRNAs can be reversed by hypomethylation treatment, leading to restoration of corresponding expression and tumor suppressor function of these miRNAs. Epigenetic therapy has recently emerged as a state-of-the-art strategy in cancer treatment [159,160]. For instance, pharmacological grade DNA methyltransferase inhibitors have been approved for the treatment of myelodysplastic syndrome [161]. Therefore, these findings on methylation of tumor suppressor miRNAs may provide a foundation for the use of epigenetic drugs in the treatment of MM. In addition,

these data also suggest the potential use of tumor suppressor miRNA mimics as a cancer therapy in tumors lacking certain critical tumor suppressor miRNAs [162].

Last but not least, these data highlight the importance of methylation of tumor suppressor miRNAs in MM with respect to the disease pathogenesis, diagnosis and therapy. Therefore, future genome-wide analysis of DNA methylation of miRNAs in MM will allow identification of novel miRNAs important in myelomagenesis.

Funding

This work was supported by the Hong Kong Research Grants Council General Research Fund (Ref: 763409M) awarded to C.S.C. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest Statement: None declared.

References:

1. Siegel, R.*, et al.* (2011) Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin.*, **61**, 212-236.
2. Landgren, O.*, et al.* (2009) Patterns of monoclonal gammopathy of undetermined significance and multiple myeloma in various ethnic/racial groups: support for genetic factors in pathogenesis. *Leukemia*, **23**, 1691-1697.
3. Kyle, R.A.*, et al.* (2002) A Long-Term Study of Prognosis in Monoclonal Gammopathy of Undetermined Significance. *N. Engl. J. Med.*, **346**, 564-569.
4. International Myeloma Working Group (2003) Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. *Br. J. Haematol.*, **121**, 749-57.
5. Kyle, R.A.*, et al.* (2007) Clinical Course and Prognosis of Smoldering (Asymptomatic) Multiple Myeloma. *N. Engl. J. Med.*, **356**, 2582-2590.
6. Sher, T.*, et al.* (2010) Plasma cell leukaemia and other aggressive plasma cell malignancies. *Br. J. Haematol.*, **150**, 418-427.
7. Bergsagel, P.L.*, et al.* (2005) Molecular Pathogenesis and a Consequent Classification of Multiple Myeloma. *J. Clin. Oncol.*, **23**, 6333-6338.
8. Chng, W.J.*, et al.* (2007) Molecular Dissection of Hyperdiploid Multiple Myeloma by Gene Expression Profiling. *Cancer Res.*, **67**, 2982-2989.
9. Chng, W.J.*, et al.* (2006) Ploidy status rarely changes in myeloma patients at disease progression. *Leuk. Res.*, **30**, 266-271.
10. Smadja, N.V.*, et al.* (1998) Chromosomal analysis in multiple myeloma: cytogenetic evidence of two different diseases. *Leukemia*, **12**, 960.
11. Chng, W.J.*, et al.* (2005) A validated FISH trisomy index demonstrates the hyperdiploid and nonhyperdiploid dichotomy in MGUS. *Blood*, **106**, 2156-2161.
12. Bergsagel, P.L.*, et al.* (2001) Chromosome translocations in multiple myeloma. *Oncogene*, **20**, 5611-22.
13. Avet-Loiseau, H.*, et al.* (2002) Oncogenesis of multiple myeloma: 14q32 and 13q chromosomal abnormalities are not randomly distributed, but correlate with natural history, immunological features, and clinical presentation. *Blood*, **99**, 2185-2191.
14. Richelda, R.*, et al.* (1997) A Novel Chromosomal Translocation t(4; 14)(p16.3; q32) in Multiple Myeloma Involves the Fibroblast Growth-Factor Receptor 3 Gene. *Blood*, **90**, 4062-4070.
15. Chesi, M.*, et al.* (1998) The t(4;14) Translocation in Myeloma Dysregulates Both FGFR3and a Novel Gene, MMSET, Resulting in IgH/MMSET Hybrid Transcripts. *Blood*, **92**, 3025-3034.

16. Shaughnessy, J.*, et al.* (2001) Cyclin D3 at 6p21 is dysregulated by recurrent chromosomal translocations to immunoglobulin loci in multiple myeloma. *Blood*, **98**, 217-223.
17. Chesi, M.*, et al.* (1998) Frequent dysregulation of the c-maf proto-oncogene at 16q23 by translocation to an Ig locus in multiple myeloma. *Blood*, **91**, 4457-4463.
18. Hanamura, I.*, et al.* (2001) Ectopic Expression of MAFB Gene in Human Myeloma Cells Carrying (14;20)(q32;q11) Chromosomal Translocations. *Jpn. J. Cancer Res.*, **92**, 638-644.
19. Fonseca, R.*, et al.* (2004) Genetics and Cytogenetics of Multiple Myeloma. *Cancer Res.*, **64**, 1546-1558.
20. Avet-Loiseau, H.*, et al.* (2000) Chromosome 13 abnormalities in multiple myeloma are mostly monosomy 13. *Br. J. Haematol.*, **111**, 1116-1117.
21. Fonseca, R.*, et al.* (2001) Deletions of chromosome 13 in multiple myeloma identified by interphase FISH usually denote large deletions of the q arm or monosomy. *Leukemia*, **15**, 981.
22. Bergsagel, P.L.*, et al.* (2005) Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. *Blood*, **106**, 296-303.
23. Soverini, S.*, et al.* (2003) Cyclin D1 overexpression is a favorable prognostic variable for newly diagnosed multiple myeloma patients treated with high-dose chemotherapy and single or double autologous transplantation. *Blood*, **102**, 1588-1594.
24. Smadja, N.V.*, et al.* (2001) Hypodiploidy is a major prognostic factor in multiple myeloma. *Blood*, **98**, 2229-2238.
25. Fonseca, R.*, et al.* (2003) The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma. *Blood*, **102**, 2562-2567.
26. Debes-Marun, C.S.*, et al.* (2003) Chromosome abnormalities clustering and its implications for pathogenesis and prognosis in myeloma. *Leukemia*, **17**, 427-436.
27. Kuehl, W.M.*, et al.* (2002) Multiple myeloma: evolving genetic events and host interactions. *Nat. Rev. Cancer*, **2**, 175-187.
28. Avet-Loiseau, H.*, et al.* (2001) Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumors. *Blood*, **98**, 3082-3086.
29. Avet-Loiseau, H.*, et al.* (2007) Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myélome. *Blood*, **109**, 3489-3495.
30. Fonseca, R.*, et al.* (2009) International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. *Leukemia*, **23**, 2210-2221.
31. Fonseca, R.*, et al.* (2003) Clinical and biologic implications of recurrent genomic aberrations in myeloma. *Blood*, **101**, 4569-4575.

32. Chng, W.J*., et al.* (2007) Clinical significance of TP53 mutation in myeloma. *Leukemia*, **21**, 582-584.
33. Avet-Loiseau, H*., et al.* (2010) Bortezomib Plus Dexamethasone Induction Improves Outcome of Patients With t(4;14) Myeloma but Not Outcome of Patients With del(17p). *J. Clin. Oncol.*, **28**, 4630-4634.
34. Bezieau, S*., et al.* (2001) High incidence of N and K Ras activating mutations in multiple myeloma and primary plasma cell leukemia at diagnosis. *Hum. Mutat.*, **18**, 212-224.
35. Liu, P*., et al.* (1996) Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. *Blood*, **88**, 2699-2706.
36. Rasmussen, T*., et al.* (2005) Possible roles for activating RAS mutations in the MGUS to MM transition and in the intramedullary to extramedullary transition in some plasma cell tumors. *Blood*, **105**, 317-323.
37. Chng, W.J*., et al.* (2008) Clinical and biological significance of RAS mutations in multiple myeloma. *Leukemia*, **22**, 2280-2284.
38. Hideshima, T*., et al.* (2007) Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat. Rev. Cancer*, **7**, 585-598.
39. Podar, K*., et al.* (2008) Bone marrow microenvironment and the identification of new targets for myeloma therapy. *Leukemia*, **23**, 10-24.
40. Esteller, M. (2008) Epigenetics in Cancer. *N. Engl. J. Med.*, **358**, 1148-1159.
41. Laird, P.W. (2005) Cancer epigenetics. *Hum. Mol. Genet.*, **14**, R65-R76.
42. Robertson, K.D. (2005) DNA methylation and human disease. *Nat. Rev. Genet.*, **6**, 597-610.
43. Herman, J.G*., et al.* (2003) Gene Silencing in Cancer in Association with Promoter Hypermethylation. *N. Engl. J. Med.*, **349**, 2042-2054.
44. Jones, P.A*., et al.* (2002) The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.*, **3**, 415-428.
45. Fazzari, M.J*., et al.* (2004) Epigenomics: beyond CpG islands. *Nat. Rev. Genet.*, **5**, 446-455.
46. Ioshikhes, I.P*., et al.* (2000) Large-scale human promoter mapping using CpG islands. *Nat. Genet.*, **26**, 61-63.
47. Kim, T.H*., et al.* (2005) A high-resolution map of active promoters in the human genome. *Nature*, **436**, 876-880.
48. Bird, A. (1999) MOLECULAR BIOLOGY:DNA Methylation de Novo. *Science*, **286**, 2287-2288.
49. Singal, R*., et al.* (1999) DNA Methylation. *Blood*, **93**, 4059-4070.

50. Robertson, K.D*., et al.* (2000) DNA methylation: past, present and future directions. *Carcinogenesis*, **21**, 461-467.
51. Chim, C.S*., et al.* (2002) Hypermethylation of gene promoters in hematological neoplasia. *Hematol. Oncol.*, **20**, 167-176.
52. Costello, J.F*., et al.* (2001) Methylation matters. *J. Med. Genet.*, **38**, 285-303.
53. Knudson, A.G. (2001) Two genetic hits (more or less) to cancer. *Nat. Rev. Cancer*, **1**, 157-162.
54. Chim, C.S*., et al.* (2008) Gene hypermethylation in multiple myeloma: lessons from a cancer pathway approach. *Clin Lymphoma Myeloma*, **8**, 331-9.
55. Walker, B.A*., et al.* (2011) Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. *Blood*, **117**, 553-562.
56. Dahl, C*., et al.* (2003) DNA methylation analysis techniques. *Biogerontology*, **4**, 233-50.
57. Laird, P.W. (2010) Principles and challenges of genome-wide DNA methylation analysis. *Nat. Rev. Genet.*, **11**, 191-203.
58. Shen, L*., et al.* (2007) Methods of DNA methylation analysis. *Curr. Opin. Clin. Nutr. Metab. Care*, **10**, 576-581.
59. Hayatsu, H*., et al.* (2008) Bisulfite modification for analysis of DNA methylation. *Curr. Protoc. Nucleic Acid Chem.*, **6**, 1-6.10.
60. Hayatsu, H*., et al.* (1970) Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. *Biochemistry*, **9**, 2858-2865.
61. Felsenfeld, G*., et al.* (1982) Methylation and gene control. *Nature*, **296**, 602-603.
62. Eick, D*., et al.* (1983) Quantitative determination of 5-methylcytosine in DNA by reverse-phase high-performance liquid chromatography. *Anal. Biochem.*, **135**, 165-171.
63. Frommer, M*., et al.* (1992) A Genomic Sequencing Protocol that Yields a Positive Display of 5-Methylcytosine Residues in Individual DNA Strands. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 1827-1831.
64. Herman, J.G*., et al.* (1996) Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 9821-9826.
65. Xiong, Z*., et al.* (1997) COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.*, **25**, 2532-2534.
66. Gonzalgo, M.L*., et al.* (1997) Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res.*, **25**, 2529-2531.
67. Eads, C.A*., et al.* (2000) MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.*, **28**, e32-00.

68. Colella, S*., et al.* (2003) Sensitive and quantitative universal Pyrosequencing™ methylation analysis of CpG sites. *Biotechniques*, **35**, 146-151.
69. Ronaghi, M*., et al.* (1996) Real-Time DNA Sequencing Using Detection of Pyrophosphate Release. *Anal. Biochem.*, **242**, 84-89.
70. Ehrich, M*., et al.* (2005) Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A. of the United States of America*, **102**, 15785-15790.
71. Zilberman, D*., et al.* (2007) Genome-wide analysis of DNA methylation patterns. *Development*, **134**, 3959-3965.
72. Kondo, Y*., et al.* (2010) DNA methylation profiling in cancer. *Expert Rev. Mol. Med.*, **12**, e23.
73. Zuo, T*., et al.* (2009) Methods in DNA methylation profiling. *Epigenomics*, **1**, 331-345.
74. Walter, K*., et al.* (2012) DNA Methylation Profiling Defines Clinically Relevant Biological Subsets of Non-small Cell Lung Cancer. *Clin. Cancer Res.*.
75. Tsai, H.-C*., et al.* (2012) Transient Low Doses of DNA-Demethylating Agents Exert Durable Antitumor Effects on Hematological and Epithelial Tumor Cells. *Cancer Cell*, **21**, 430-446.
76. Lister, R*., et al.* (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, **462**, 315-322.
77. Kim, J.H*., et al.* (2011) Deep sequencing reveals distinct patterns of DNA methylation in prostate cancer. *Genome Res.*, **21**, 1028-1041.
78. Ordway, J.M*., et al.* (2006) Comprehensive DNA methylation profiling in a human cancer genome identifies novel epigenetic targets. *Carcinogenesis*, **27**, 2409-2423.
79. Oda, M*., et al.* (2009) High-resolution genome-wide cytosine methylation profiling with simultaneous copy number analysis and optimization for limited cell numbers. *Nucleic Acids Res.*, **37**, 3829-3839.
80. Cheung, H.H*., et al.* (2010) Genome-wide DNA methylation profiling reveals novel epigenetically regulated genes and non-coding RNAs in human testicular cancer. *Br. J. Cancer*, **102**, 419-427.
81. Yoshinao, R*., et al.* (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. *BMC Genomics*, **11**.
82. Bartel, D.P. (2004) MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*, **116**, 281-297.
83. Schickel, R*., et al.* (2008) MicroRNAs: key players in the immune system, differentiation, tumorigenesis and cell death. *Oncogene*, **27**, 5959-5974.
84. Le, M.T.N*., et al.* (2009) MicroRNA-125b Promotes Neuronal Differentiation in Human Cells by Repressing Multiple Targets. *Mol. Cell. Biol.*, **29**, 5290-5305.

85. Felli, N.*, et al.* (2009) MicroRNA 223-dependent expression of LMO2 regulates normal erythropoiesis. *Haematologica*, **94**, 479-486.

86. Garzon, R.*, et al.* (2008) MicroRNAs in normal and malignant hematopoiesis. *Curr. Opin. Hematol.*, **15**, 352.

87. Lee, R.C.*, et al.* (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, **75**, 843-854.

88. Wightman, B.*, et al.* (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*, **75**, 855-862.

89. Lagos-Quintana, M.*, et al.* (2001) Identification of Novel Genes Coding for Small Expressed RNAs. *Science*, **294**, 853-858.

90. Lau, N.C.*, et al.* (2001) An Abundant Class of Tiny RNAs with Probable Regulatory Roles in Caenorhabditis elegans. *Science*, **294**, 858-862.

91. Lee, R.C.*, et al.* (2001) An Extensive Class of Small RNAs in Caenorhabditis elegans. *Science*, **294**, 862-864.

92. Lee, Y.*, et al.* (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.*, **23**, 4051-4060.

93. Cai, X.*, et al.* (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA*, **10**, 1957-1966.

94. Lee, Y.*, et al.* (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature*, **425**, 415-419.

95. Lee, Y.*, et al.* (2002) MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J.*, **21**, 4663-70.

96. Yi, R.*, et al.* (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.*, **17**, 3011-3016.

97. Zeng, Y.*, et al.* (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 9779-9784.

98. Zeng, Y.*, et al.* (2002) Both Natural and Designed Micro RNAs Can Inhibit the Expression of Cognate mRNAs When Expressed in Human Cells. *Mol. Cell*, **9**, 1327-1333.

99. Garzon, R.*, et al.* (2008) MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood*, **111**, 3183-3189.

100. Yanaihara, N.*, et al.* (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell*, **9**, 189-198.

101. Fabbri, M.*, et al.* (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 15805-15810.

102. Garzon, R.*, et al.* (2009) MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood*, **113**, 6411-6418.
103. Szalmas, A.*, et al.* (2009) Epigenetic alterations in cervical carcinogenesis. *Semin. Cancer Biol.*, **19**, 144-152.
104. Hinshelwood, R.*, et al.* (2008) Breast cancer epigenetics: normal human mammary epithelial cells as a model system. *J. Mol. Med.*, **86**, 1315-1328.
105. Wang, X.*, et al.* (2008) Aberrant Expression of Oncogenic and Tumor-Suppressive MicroRNAs in Cervical Cancer Is Required for Cancer Cell Growth. *PLoS One*, **3**, e2557.
106. Jiang, S.*, et al.* (2010) MicroRNA-155 Functions as an OncomiR in Breast Cancer by Targeting the Suppressor of Cytokine Signaling 1 Gene. *Cancer Res.*, **70**, 3119-3127.
107. LaI, A.*, et al.* (2008) p16^{INK4a} Translation Suppressed by miR-24. *PLoS One*, **3**, e1864.
108. Pichiorri, F.*, et al.* (2011) MicroRNAs: New Players in Multiple Myeloma. *Front. Genet.*, **2**.
109. Benetatos, L.*, et al.* (2012) Deregulated microRNAs in multiple myeloma. *Cancer*, **118**, 878-887.
110. Pichiorri, F.*, et al.* (2008) MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 12885-12890.
111. Roccaro, A.M.*, et al.* (2009) MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma. *Blood*, **113**, 6669-6680.
112. Lionetti, M.*, et al.* (2009) Identification of microRNA expression patterns and definition of a microRNA/mRNA regulatory network in distinct molecular groups of multiple myeloma. *Blood*, **114**, e20-e26.
113. Unno, K.*, et al.* (2009) Identification of a novel microRNA cluster miR-193b-365 in multiple myeloma. *Leuk. Lymphoma*, **50**, 1865-1871.
114. Gutierrez, N.*, et al.* (2010) Deregulation of microRNA expression in the different genetic subtypes of multiple myeloma and correlation with gene expression profiling. *Leukemia*, **24**, 629-637.
115. Zhou, Y.*, et al.* (2010) High-risk myeloma is associated with global elevation of miRNAs and overexpression of EIF2C2/AGO2. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 7904-7909.
116. Corthals, S.*, et al.* (2011) MicroRNA signatures characterize multiple myeloma patients. *Leukemia*, **25**, 1784-1789.
117. Lionetti, M.*, et al.* (2009) Integrative high-resolution microarray analysis of human myeloma cell lines reveals deregulated miRNA expression associated with allelic imbalances and gene expression profiles. *Genes Chromosomes Cancer*, **48**, 521-531.

118. Loffler, D.*, et al.* (2007) Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood*, **110**, 1330-1333.
119. Corthals, S.L.*, et al.* (2010) Micro-RNA-15a and micro-RNA-16 expression and chromosome 13 deletions in multiple myeloma. *Leuk. Res.*, **34**, 677-681.
120. Deng, S.*, et al.* (2008) Mechanisms of microRNA deregulation in human cancer. *Cell Cycle*, **7**, 2643-6.
121. Weber, B.*, et al.* (2007) Methylation of human microRNA genes in normal and neoplastic cells. *Cell Cycle*, **6**, 1001-5.
122. Lujambio, A.*, et al.* (2007) CpG island hypermethylation of tumor suppressor microRNAs in human cancer. *Cell Cycle*, **6**, 1455-9.
123. Vogelstein, B.*, et al.* (2000) Surfing the p53 network. *Nature*, **408**, 307-310.
124. Avet-Loiseau, H.*, et al.* (1999) P53 deletion is not a frequent event in multiple myeloma. *Br. J. Haematol.*, **106**, 717-719.
125. Imamura, J.*, et al.* (1994) p53 in hematologic malignancies. *Blood*, **84**, 2412-2421.
126. Tarasov, V.*, et al.* (2007) Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle*, **6**, 1586-93.
127. Chang, T.C.*, et al.* (2007) Transactivation of miR-34a by p53 Broadly Influences Gene Expression and Promotes Apoptosis. *Mol. Cell*, **26**, 745-752.
128. He, L.*, et al.* (2007) A microRNA component of the p53 tumour suppressor network. *Nature*, **447**, 1130-1134.
129. Raver-Shapira, N.*, et al.* (2007) Transcriptional Activation of miR-34a Contributes to p53-Mediated Apoptosis. *Mol. Cell*, **26**, 731-743.
130. Bommer, G.T.*, et al.* (2007) p53-Mediated Activation of miRNA34 Candidate Tumor-Suppressor Genes. *Curr. Biol.*, **17**, 1298-1307.
131. Corney, D.C.*, et al.* (2007) MicroRNA-34b and MicroRNA-34c Are Targets of p53 and Cooperate in Control of Cell Proliferation and Adhesion-Independent Growth. *Cancer Res.*, **67**, 8433-8438.
132. Luan, S.*, et al.* (2010) MicroRNA-34a: A Novel Tumor Suppressor in p53-mutant Glioma Cell Line U251. *Arch. Med. Res.*, **41**, 67-74.
133. Zenz, T.*, et al.* (2009) Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, TP53 mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. *Blood*, **114**, 2589-2597.
134. Mraz, M.*, et al.* (2009) MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes. *Leuk. Lymphoma*, **50**, 506-509.

135. Mraz, M.*, et al.* (2009) miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. *Leukemia*, **23**, 1159-1163.

136. Zenz, T.*, et al.* (2009) miR-34a as part of the resistance network in chronic lymphocytic leukemia. *Blood*, **113**, 3801-3808.

137. Dijkstra, M.K.*, et al.* (2008) 17p13//TP53 deletion in B-CLL patients is associated with microRNA-34a downregulation. *Leukemia*, **23**, 625-627.

138. Ji, Q.*, et al.* (2008) Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres. *BMC Cancer*, **8**, 266.

139. Hwang, C.I.*, et al.* (2011) Wild-type p53 controls cell motility and invasion by dual regulation of MET expression. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, 14240-14245.

140. Corney, D.C.*, et al.* (2010) Frequent Downregulation of miR-34 Family in Human Ovarian Cancers. *Clin. Cancer Res.*, **16**, 1119-1128.

141. Gallardo, E.*, et al.* (2009) miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer. *Carcinogenesis*, **30**, 1903-1909.

142. Lodygin, D.*, et al.* (2008) Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. *Cell Cycle*, **7**, 2591-600.

143. Tivnan, A.*, et al.* (2011) MicroRNA-34a is a potent tumor suppressor molecule in vivo in neuroblastoma. *BMC Cancer*, **11**, 33.

144. Chim, C.S.*, et al.* (2010) Epigenetic inactivation of the miR-34a in hematological malignancies. *Carcinogenesis*, **31**, 745-750.

145. Chim, C.S.*, et al.* (2011) Methylation of miR-34a, miR-34b/c, miR-124-1 and miR-203 in Ph-negative myeloproliferative neoplasms. *J. Transl. Med.*, **9**, 197.

146. Wong, K.Y.*, et al.* (2011) DNA methylation of tumor suppressor miRNA genes: a lesson from the miR-34 family. *Epigenomics*, **3**, 83-92.

147. Wong, K.Y.*, et al.* (2011) Epigenetic inactivation of the MIR34B/C in multiple myeloma. *Blood*, **118**, 5901-5904.

148. Pichiorri, F.*, et al.* (2010) Downregulation of p53-inducible microRNAs 192, 194, and 215 Impairs the p53/MDM2 Autoregulatory Loop in Multiple Myeloma Development. *Cancer Cell*, **18**, 367-381.

149. Bueno, M.J.*, et al.* (2008) Genetic and Epigenetic Silencing of MicroRNA-203 Enhances ABL1 and BCR-ABL1 Oncogene Expression. *Cancer Cell*, **13**, 496-506.

150. Chim, C.S.*, et al.* (2011) Epigenetic inactivation of the hsa-miR-203 in haematological malignancies. *J. Cell. Mol. Med.*, **15**, 2760-2767.

151. Furuta, M.*, et al.* (2010) miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. *Carcinogenesis*, **31**, 766-76.

152. Van Gent, D.C.*, et al.* (2001) Chromosomal stability and the DNA double-stranded break connection. *Nat. Rev. Genet.*, **2**, 196-206.

153. Wong, K.Y.*, et al.* (2011) Epigenetic silencing of MIR203 in multiple myeloma. *Br. J. Haematol.*, **154**, 569-578.

154. Agirre, X.*, et al.* (2009) Epigenetic Silencing of the Tumor Suppressor MicroRNA Hsa-miR-124a Regulates CDK6 Expression and Confers a Poor Prognosis in Acute Lymphoblastic Leukemia. *Cancer Res*, **69**, 4443-4453.

155. Silber, J.*, et al.* (2008) miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med.*, **6**, 14.

156. Wilting, S.*, et al.* (2010) Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol. Cancer*, **9**, 167.

157. Lujambio, A.*, et al.* (2007) Genetic Unmasking of an Epigenetically Silenced microRNA in Human Cancer Cells. *Cancer Res.*, **67**, 1424-1429.

158. Wong, K.Y.*, et al.* (2011) Epigenetic Inactivation of the miR-124-1 in Haematological Malignancies. *PLoS One*, **6**, e19027.

159. Yoo, C.B.*, et al.* (2006) Epigenetic therapy of cancer: past, present and future. *Nat Rev Drug Discov*, **5**, 37-50.

160. Kaiser, J. (2010) Epigenetic drugs take on cancer. *Science*, **330**, 576-578.

161. Kuendgen, A.*, et al.* (2008) Current status of epigenetic treatment in myelodysplastic syndromes. *Ann. Hematol.*, **87**, 601-611.

162. Bader, A.G.*, et al.* (2010) The Promise of MicroRNA Replacement Therapy. *Cancer Res.*, **70**, 7027-7030.

163. Chng, W.J.*, et al.* (2006) Prognostic factors for hyperdiploid-myeloma: effects of chromosome 13 deletions and IgH translocations. *Leukemia*, **20**, 807-813.

164. Wong, K.Y. (2011) Promoter DNA Methylation of Tumour Suppressor MicroRNA Genes in Multiple Myeloma. *Department of Medicine*. The University of Hong Kong, Hong Kong, vol. Doctor of Philosophy, pp. 188.

165. Toyota, M.*, et al.* (2008) Epigenetic Silencing of MicroRNA-34b/c and B-Cell Translocation Gene 4 Is Associated with CpG Island Methylation in Colorectal Cancer. *Cancer Res*, **68**, 4123-4132.

166. Pigazzi, M.*, et al.* (2009) miR-34b Targets Cyclic AMP-Responsive Element Binding Protein in Acute Myeloid Leukemia. *Cancer Res*, **69**, 2471-2478.

Legends:

Figure 1: Clinical stages and molecular genetics of multiple myeloma. *CCND*: cyclin D; *CDKN2C*: cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4); *FGFR3*: fibroblast growth factor receptor 3; HMCL: human myeloma cell line; *MAF*: v-maf musculoaponeurotic fibrosarcoma oncogene homology (avian); *MAFB*: v-maf musculoaponeurotic fibrosarcoma oncogene homology B (avian); MGUS: monoclonal gammopathy of undetermined significance; *MMSET*: Wolf-Hirschhorn syndrome candidate 1; NF-κB: nuclear factor of kappa light polypeptide gene enhancer in B-cells; PCL: plasma cell leukemia; SMM: smoldering multiple myeloma. Derived from Smadja et al., 2001; Kuehl et al., 2002; Debes-Marun et al., 2003; Fonseca et al., 2003; Chng et al., 2006 [24-27,163].

Figure 2: Role of DNA methylation in normal and cancer cells. In normal cell, CpG dinucleotides (lollipops) of promoter-associated CpG island are generally unmethylated (blue lollipops) and is associated with a euchromatin histone configuration, which allows access of transcription factors (TFs), histone acetyltransferases (HATs), H3K4 histone methyltransferase (HMT), and RNA polymerase complex, for gene transcription. In cancer cell, promoter-associated CpG island are aberrantly hypermethylated (black lollipops) by DNA methyltransferase (DNMT), leading to recruitment of histone deacetylase (HDAC) and HMT, results in a heterochromatic histone configuration, and hence gene silencing. (Co-Act, co-activator; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; MBD, methyl-CpG-binding domain protein; Pol II, RNA polymerase II; TAF, TBP-associated factor; TBP, TATA-binding protein; TF, transcription factor). Modified from Wong et al., 2011 [146].

Figure 3: Mechanisms of miRNA biogenesis and function. Most miRNA genes are transcribed by RNA polymerase II (Pol II) into primary (pri-) miRNA, which is processed into precursor (pre-) miRNA stem-loop by Drosha and Pasha. Pre-miRNA is transported into cytoplasm via exportin 5, and is processed into mature miRNA duplex by Dicer. The functional strand, which recognizes target gene, is loaded into RNA-induced silencing complex (RISC). Partial complementary binding of the miRNA to the 3' untranslated region of target gene through the miRNA seed region will result in translation inhibition of target gene. Perfect complementary binding of the miRNA will induce mRNA degradation of target gene. Extracted from PhD thesis of K.Y.W. [164] (ORF, open reading frame; Pol II, RNA polymerase II; RISC, RNA-induced silencing complex; UTR, untranslated region)

Table 1: Common DNA methylation analysis methods

	Locus-specific	Genome-wide
Qualitative	MSP	
Quantitative	BGS	Infinium
	COBRA	WGBS
	Ms-SNuPE	RRBS
	MethyLight	Enzyme-chip
	Pyrosequencing	Enzyme-seq
	MassARRAY	MeDIP-chip
		MeDIP-seq

Keys: BGS, bisulfite genomic sequencing; chip, DNA microarray; COBRA, combined bisulfite restriction analysis; MeDIP, methylated DNA immunoprecipitation; MSP, methylation-specific polymerase chain reaction; Ms-SNuPE, methylation-sensitive single nucleotide primer extension; seq, high-throughput sequencing; RRBS, reduced representation bisulfite sequencing; WGBS, whole-genome bisulfite sequencing.

Table 2: List of tumor suppressor miRNAs hypermethylated in multiple myeloma

miRNA	Genomic location	Key known target	Hypermethylated in other cancer type	Reference
miR-34a	1p36	BCL2 CDK4/6 E2F3	Prostate Ovary	[140,142,144]
miR-34b/c	11q23	CCNE2 CREB MET	Colon Ovary	[131,147,165,166]
miR-124-1	8p23	CDK6	Colon ALL	[154,157,158]
miR-194-2-192	11q13	MDM2	Nil	[148]
miR-203	14q32	BCR-ABL CREB	CML	[149,153]

Keys: BCL2, B-cell CLL/lymphoma 2; BCR-ABL, breakpoint cluster region c-abl oncogene 1, non-receptor tyrosine kinase; CCNE2, cyclin E2; CDK4/6, ; CREB, cAMP responsive element binding protein; E2F3, E2F transcription factor 3; MET, met proto-oncogene; MDM2, Mdm2, TP53 E3 ubiquitin protein ligase homolog (mouse).

Figure 1

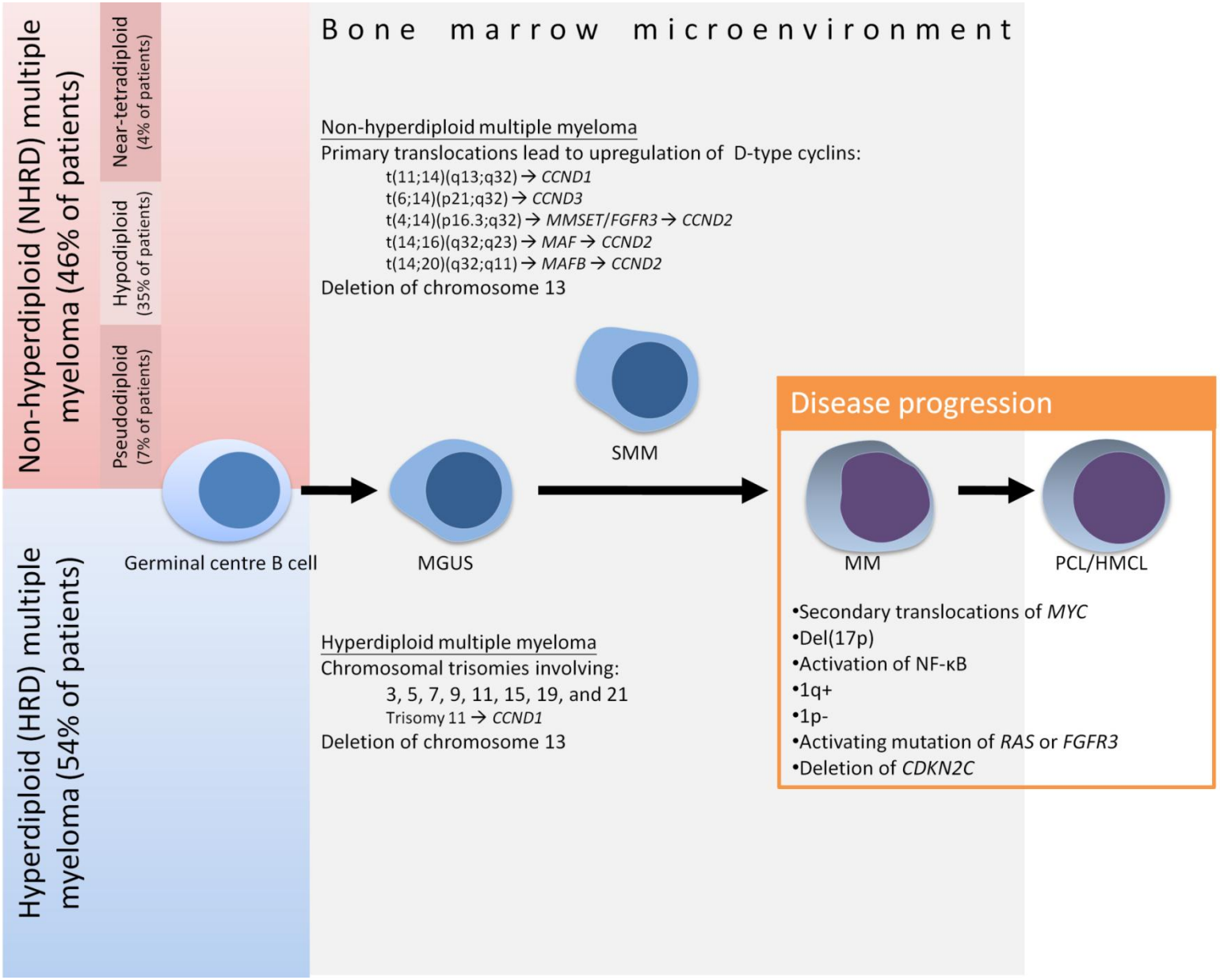

Non-hyperdiploid (NHRD) multiple myeloma (46% of patients)
Near-tetradiploid (4% of patients)
Hypodiploid (35% of patients)
Pseudodiploid (7% of patients)
Hyperdiploid (HRD) multiple myeloma (54% of patients)
Bone marrow microenvironment
Non-hyperdiploid multiple myeloma
Primary translocations lead to upregulation of D-type cyclins:
t(11;14)(q13;q32) → CCND1
t(6;14)(p21;q32) → CCND3
t(4;14)(p16.3;q32) → MMSET/FGFR3 → CCND2
t(14;16)(q32;q23) → MAF → CCND2
t(14;20)(q32;q11) → MAFB → CCND2
Deletion of chromosome 13
SMM
Disease progression
Germinal centre B cell
MGUS
MM
PCL/HMCL
•Secondary translocations of MYC
•Del(17p)
•Activation of NF-κB
•1q+
•1p-
•Activating mutation of RAS or FGFR3
•Deletion of CDKN2C
Hyperdiploid multiple myeloma
Chromosomal trisomies involving:
3, 5, 7, 9, 11, 15, 19, and 21
Trisomy 11 → CCND1
Deletion of chromosome 13

Figure 2

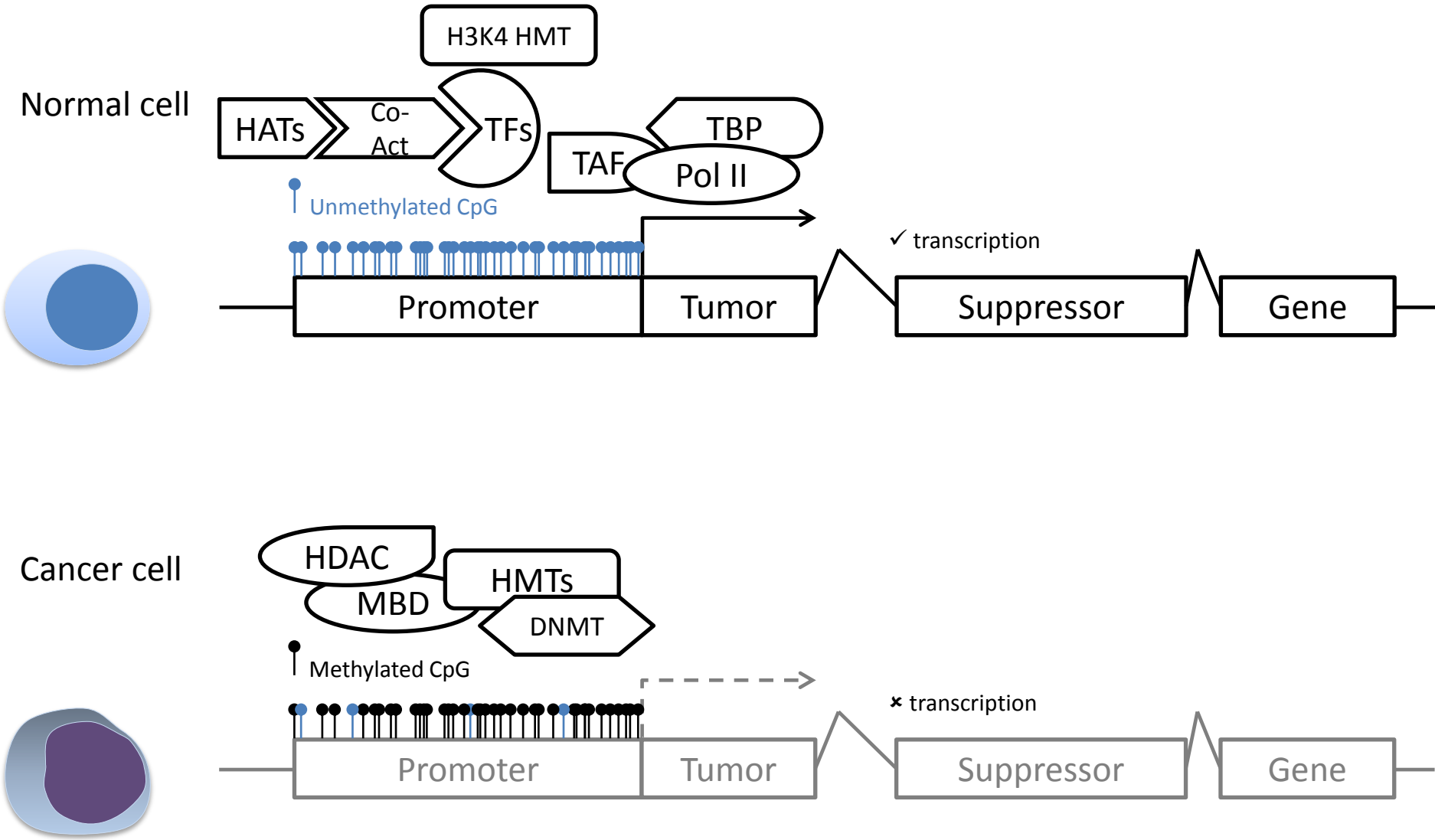
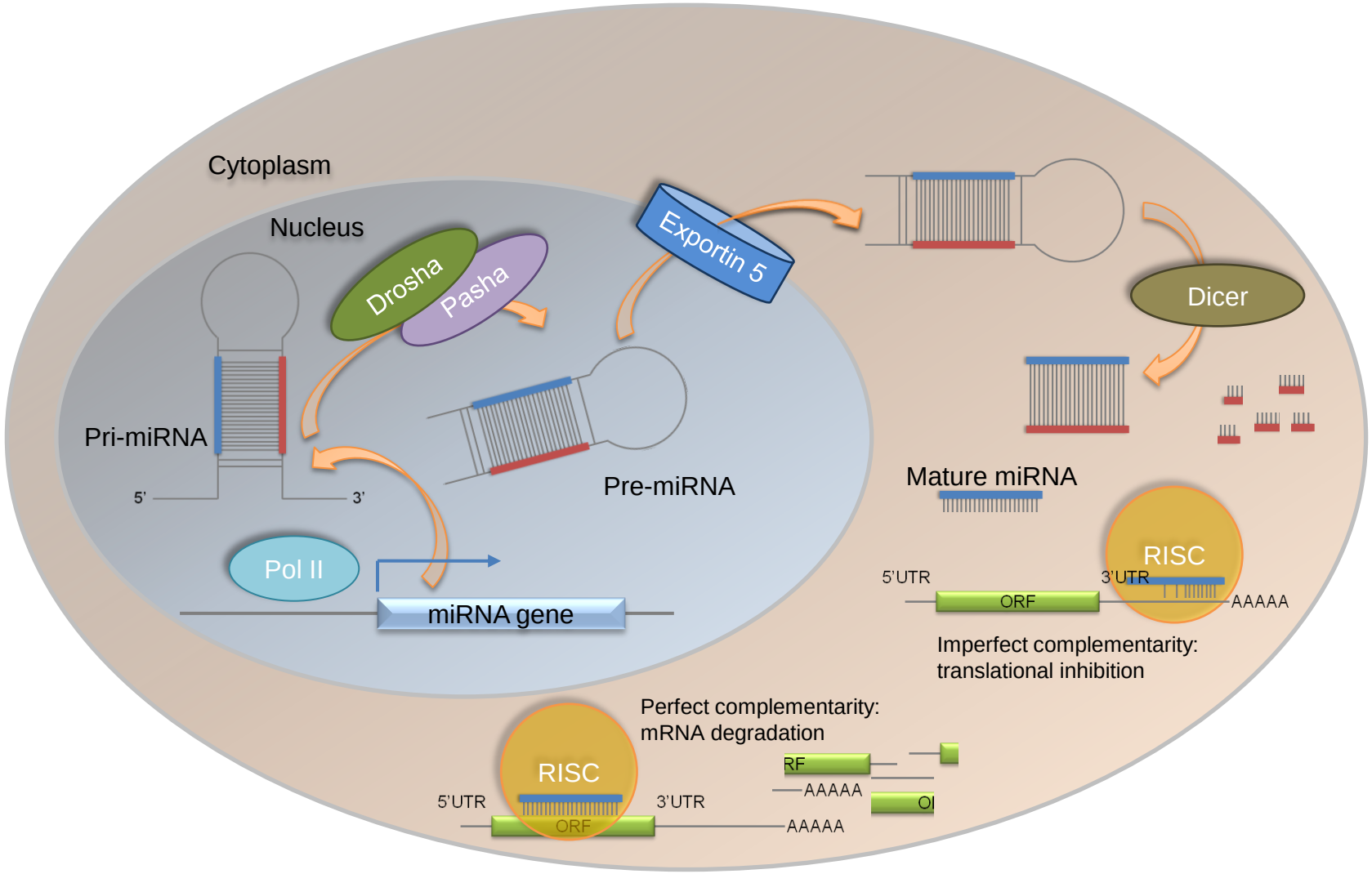

Normal cell
H3K4 HMT
HATs
Co-
Act
TFs
TBP
TAF
Pol II
Unmethylated CpG
✓ transcription
Promoter
Tumor
Suppressor
Gene
Cancer cell
HDAC
HMTs
MBD
DNMT
Methylated CpG
× transcription
Promoter
Tumor
Suppressor
Gene

Figure 3


Cytoplasm
Nucleus
Drosha
Pasha
Exportin 5
Dicer
Pri-miRNA
5'
3'
Pre-miRNA
Mature miRNA
Pol II
miRNA gene
RISC
5'UTR
3'UTR
ORF
AAAAA
Imperfect complementarity:
translational inhibition
Perfect complementarity:
mRNA degradation
RISC
5'UTR
3'UTR
ORF
AAAAA
RF
AAAAA
OI