DNA Methylation in Multiple Myeloma

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Introduction

- What is DNA methylation?
  - Epigenetics
  - Other linked mechanisms
  - What does it do?
  - Why is it important generally?
  - Why is it important in cancer biology?

- How is it measured?
  - What has been done in myeloma?

-Our own work with DNA methylation arrays
  - investigation of plasma cell types
  - integration with previous gene expression and cytogenetic datasets
Epigenetics is the study of heritable changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence, hence the name *epi*- (Greek: επί- over, above) - genetics

Changes in gene expression not caused by mutation, deletion, amplification or translocation

1. DNA methylation
2. Histone modifications
1. DNA Methylation

DNA is composed of four bases – A, G, C, T

The ‘fifth base’ is known as methyl-C
  - methyl-C is only found in a CpG dinucleotide

CpG dinucleotides are under-represented in the human genome
  - expect 4.4% in a 42% GC rich genome but only find ~1%
  - not uniformly distributed throughout the genome
  - found in clusters called ‘CpG islands’
  - CpG islands are found at genic regions (7% mostly unmethylated),
    or in repetitive sequences (41% mostly methylated)

Controlled by DNA methyltransferases (DNMTs)
  - DNMT1 - maintains DNA methylation
  - DNMT3a/b - de novo DNA methylation
CpG Islands Are Found Surrounding The Start of Genes

CpG islands have a loose definition:
- 50% C/G
- CpG Obs/Exp > 0.6

Albumin

CDKN2A (p16)
2. Histone Modifications

DNA is wrapped around histone proteins – complex termed chromatin

Histone tails can be modified through:
- methylation
- acetylation
- phosphorylation
- ubiquitylation

All these changes have a substantial influence on the chromatin structure and gene function and this influence is dependent on the type and location of the modification

For example:
H3K9-me and H3K27-me are associated with inactive transcription

H3K9-ac is associated with active transcription

Controlled by many proteins:
HMTs – histone methyltransferases e.g. MMSET
HAT – histone acetyltransferases
Histone Demethylases e.g. UTX

HDAC – histone deacetylases
DNA methylation is intrinsically linked to chromatin remodelling
- Euchromatin (active) has an open structure, lack of DNA methylation and specific histone modifications (e.g. acetylated H3K9)
  - Active transcription
- Heterochromatin (inactive) has a closed structure and is associated with DNA methylation and specific histone modifications (e.g. methylated H3K9 and H4K20)
  - Inactive transcription

The state is controlled by many proteins that interact with both the histones and CpG dinucleotides in the DNA

HMTs can recruit DNMTs resulting in increased methylation of histones and DNA
Methylation and Biology

• DNA methylation is important in normal cellular function

In development:
  - methylation changes allow cells to develop into different cell types, or revert to stem cells

Imprinting:
  - whereby alleles inherited from paternal or maternal source have different methylation patterns
    - Angelman Syndrome, Prader-Wili Syndrome
  - result of loss of expression of the relevant gene
Methylation in Cancer

Evidence in many cancer types of abnormal methylation patterns compared to normal tissues

1- Wave of loss of DNA methylation (hypo-methylation) across the genome
   - results in genome instability, may lead to chromosomal alterations such as amplifications, deletions or translocations
   - mostly found in repetitive elements

2- Gene-specific hyper-methylation
   - increased methylation at tumour suppressor genes
   - associated with loss in expression
   - mostly found in genic regions

Initiating factors not known

Changes in methylation can alter TSGs or oncogenes
   - increase in methylation of TSG
   - decrease in methylation of oncogene
   - methylation of other regulatory elements – transcription factors, miRNAs etc.
Approaches to Analysing the Methylome

The diagram illustrates various approaches to analysing the methylome based on the number of CpGs analysed per sample genome. The approaches are categorized by the number of samples analysed:

- **High number of samples (10^5-10^6)**: MethyLight, EpiTYPER, MSP, COBRA, Pyrosequencing, Southern blot, GoldenGate, Infinium, Enzyme–chip, MeDIP–chip, BSPP, BC–seq, Enzyme–seq, RRBS, MeDIP–seq, WGSBS.
- **Lower number of samples (10^3-10^4)**: Sanger BS.

The diagram shows a range of methods, with some highlighted boxes indicating specific techniques or clusters of similar approaches.
Methylation in Myeloma

- Methylation in myeloma has generally been studied on a gene-by-gene basis
- Identified hypermethylation of specific genes e.g. *CDH1* (E-cadherin), *SOCS1*, *CDKN2A*, *CDKN2B*, *TGFBR2* etc.
- Hypermethylation of these genes are associated with poor outcome
- Use of demethylating agents in treating patients suggests that the methylome is important in myeloma

- Whole methylome analysis can be used to identify new targets and mechanisms of disease progression
Differential repetitive DNA methylation in multiple myeloma molecular subgroups

Valentina Bollati, Sonia Fabris¹, Valeria Pegoraro, Domenica Ronchetti¹, Laura Mosca¹, Giorgio Lambertenghi Deliliers¹, Valeria Motta, Pier Alberto Bertazzi, Andrea Baccarelli and Antonino Neri¹,*

genetic instability are poorly understood. Epigenetics relate to stable and heritable patterns of gene expression and genomic functions that do not involve changes in DNA sequence (6). In mammals, DNA methylation, the most investigated epigenetic hallmark, is a reversible mechanism that modifies genome function and chromosomal stability.

Tumor and Stem Cell Biology

DNA Methylation Analysis Determines the High Frequency of Genic Hypomethylation and Low Frequency of Hypermethylation Events in Plasma Cell Tumors

Bodour Salha¹, Angela Baker¹, Gregory Ahmann², Daniel Auclair³, Rafael Fonseca², and John Carpten¹

Abstract

Multiple myeloma (MM) is a plasma cell malignancy of the bone marrow, which evolves from a premalignant stage called monoclonal gammopathy of undetermined significance (MGUS). In some patients, an intermediate stage referred to as smoldering multiple myeloma (SMM) is clinically recognized, with the full-bone malignancy termed MM. We conducted a study to assess differential CpG methylation at 1,500 genic loci during MM progression and profiled CD138⁺ plasma cells from MGUS, SMM, and MM specimens; human myeloma cell lines; and normal plasma cell (NPC) samples. We showed that the number of differentially methylated loci (DML) increased with tumor grade, and the vast majority were due to hypomethylation. Hierarchical clustering analysis revealed samples that coclustered tightly with NPC. These cases, referred to as "normal-like," contained significantly fewer DML when compared with their non-normal-like counterparts and displayed overall methylation levels resembling NPC. This study represents one of the first methylome interrogation studies in MM and points toward global hypomethylation at genic CpG loci as an important and early mechanism driving myelomagenesis. Determining the set of critical genes and pathways based on the methylome myeloma is expected to lead to an improved understanding of biological mechanisms involved in myelomagenesis. Cancer Res; 70(17) 6934–44. ©2010 AACR.
Myeloma Biology

- Myeloma occurs during the normal maturation of B cells into Plasma cells
- Generate a non-malignant state known as MGUS
- Further events transform the cell into a malignant clone causing disease symptoms
- Late stage disease can result in bone marrow independence

Does DNA methylation play a part in this process?
- Myeloma genomes characterised by primary events: IGH Translocations, hyperdiploidy
- Copy number abnormalities: gain 1q, deletion of 1p, 6q, 8p, 13q, 16q, 17p
Myeloma IX Samples

Bone marrow-derived CD138+

Lysis

AllPrep spin columns
Simultaneous extraction of RNA and DNA

RNA
Gene Expression U133 Plus 2.0
258 samples

DNA
Genomic Analysis 500K Mapping Set
115 samples

CELLS
FISH and cytogenetics >1700

DNA
Genomic Analysis

PBL

CLINICAL DATA
OS, PFS, etc.
Illumina Infinium Global Methylation Array

- Human methylation 27 bead array
- 27,578 informative CpG sites
- 500 ng DNA

$$\frac{M}{M+U} = \text{proportion methylated}$$

1.0 fully methylated
0.0 fully unmethylated
Validation of methylation by BSP sequencing

E-cadherin

KMS11

RPMI-8226

$\text{r}^2 = 0.9954$
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**Germinal centre** → **MGUS** → **Myeloma** → **Plasma Cell Leukemia**
Overall Methylation Differs Between Cell Types

- Little variation in methylation between MGUS samples (A)
- Large variation between MGUS and MM samples (B) and between MM samples (C)
- Large variation between MM and PCL samples (D) and between PCL samples (E)
- Malignant phenotypes are more heterogeneous than non-malignant phenotypes (F)
Methylation status defines myeloma progression

Supervised clustering based on sample cell type

A

Heatmap Key

Value

B cell  NPC  MGUS  MM  PCL  HMCL

Hypo-methylated

Hyper-methylated

B

B cell  PC  MGUS  MM  PCL  HMCL

Hypo  637  14  **4620**  425  41
Hyper  222  9  784  **1148**  426
Methylation changes in disease progression

MGUS $\rightarrow$ MM Hyper-methylation
784 probes (2.8%)
675 unique genes (5.5%)

MGUS $\rightarrow$ MM Hypo-methylation
4620 probes (16.7%)
2876 unique genes (23.3%)
De-methylation of non-CpG island probes

- Genome Hypo-methylation is restricted to non-CpG island probes (▲) and not CpG island probes (■)

- Indicative of global hypomethylation and genome instability
MGUS → MM: Gene Specific hypermethylation

Hypermethylation of tumor suppressor genes

Negative regulation of cell cycle
(58 genes; CASP2, CDH1 (E-cadherin), CDKN2A (p16), CDKN2B (p15), CDKN1C (p57), DCC)

Cell adhesion molecules (152 genes, \( P=1.3\times10^{-10} \))

Development (61 genes, \( P=7.9\times10^{-18} \))
Are there cytogenetic specific methylation groups?

Unsupervised clustering of methylation data by individual samples

Annotated by copy number changes, hyperdiploidy, translocation and disease time point
Methylation status is defined by primary events

- Non-malignant cells cluster separately
- Copy number abnormalities do not cluster
- Methylation clustering is defined by primary events – translocations and hyperdiploidy
- \( t(4;14) \) samples are most different, and may be due to MMSET over-expression
- PCL samples do not cluster separately, but remain within their cytogenetic group

Cell type: B cell, NPC, MGUS, MM, PCL, HMCL

Translocation:
- \( t(4;14) \), \( t(11;14) \), \( t(6;14) \), \( t(14;16) \), \( t(14;20) \)

Hyperdiploid

IGH split
t(4;14)-specific CpG Hypermethylation

A. PAX1
B. CDKN2A
C. APC
D. SOCS2
Validation of Array Results By BSP

\[ APC \]

\[ \beta\text{-value} \]

\[ t(4;14) \]

\[ 0.57 \]

\[ \text{No split} \]

\[ 0.07 \]

\[ SOCS2 \]

\[ \beta\text{-value} \]

\[ t(4;14) \]

\[ 0.54 \]

\[ \text{No split} \]

\[ 0.04 \]
# t(4;14) myeloma

Pathway analysis of hypermethylated genes in t(4;14) enrichment for:
- cellular development (P=2.6x10^{-29})
- cell signalling (P=6.1x10^{-27})
- cellular adhesion (P=7.3x10^{-25})

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### t(4;14) myeloma – correlation of methylation and expression

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Methylation and Expression Changes at the transition from Myeloma to Plasma Cell Leukaemia
Comparing methylation and expression changes

**Methylation**
161 MM samples vs 31 PCL samples

- P<0.05
- 1573 probes (1394 genes)

**Expression (U133 Plus 2.0)**
205 MM samples vs 10 PCL samples

- P<0.05, fold change >2
- Benjamini-Hochberg correction
- 3780 probes (2971 genes)

In PCL samples
- Increased: 1148
- Decreased: 425

In MM samples
- Increased: 3494
- Decreased: 286
Pathway analysis of changes in PCL (Ingenuity)

Methylation

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Expression

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<tr>
<td>3</td>
<td>View RNA Post-Transcriptional Modification, Molecular Transport, RNA Trafficking</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>View Gene Expression, Post-Translational Modification, Genetic Disorder</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>View Post-Translational Modification, Genetic Disorder, Metabolic Disease</td>
<td>32</td>
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</tbody>
</table>

Cellular movement is relevant to PC egress from the bone marrow into the peripheral circulation
Cell Adhesion Molecules are down-regulated in PCL samples

- PECAM1: -4 fold
- CXCL12: -17 fold
- VCAM1: -44 fold

Expression and Methylation

Signal Intensity

MGUS, MM, PCL

B cell, NPC, MGUS, MM, PCL, HMCL
Integration of methylation and expression changes in MM and PCL

- **Methylation**
  - 1277
  - 117 (35)

- **Expression**
  - 2854

26 genes ↑ expression and ↓ methylation
9 genes ↓ expression and ↑ methylation
Methylation of genes affects expression

Expression fold change $>1.5$, $P<0.05$, difference $>100$
Methylation $P<0.05$, difference $>0.2$

**Decreased expression with increased methylation in PCLs**
ACP1, ARIH2, CAV1, GLTRSC2, KIAA0652, QPRT, SLAMF1, SLC35B3, SPAG4, TPD52

**Increased expression with decreased methylation in PCLs**
AP2A2, BANF1, BMP8B, C3orf63, C9orf82, CASP8, CCND1, CHAC1, CX3CR1, DTNBP1, GNPNAT1, HUS1, IL10, LYAR, PSD4, RUNX2, SETD4, SLC7A11, SNRPB2, SNTB1, SPN, THUMPD1, TMEM41A
**SLAMF1 (CD150)** expression decreases as methylation increases.

- **Expression**
  - Fold change: -7.9
  - P = 0.03

- **Methylation**
  - Difference: 0.25
  - P < 0.001
As DNA methylation does not directly control cell adhesion molecules, are there other mechanisms at work?

Changes in expression of chromatin remodeling

**Upregulation in PCLs of:**
- SETDB1, SETD4, SMYD2, SETD5, SMARCA4,
- SMARCAD1, KTI12, PBRM1, CHMP1A, SMARCE1,
- SMARCC1, CHRAC1, HP1BP3

**Activation of SWI/SNF complex mediating chromatin remodeling**

**Control expression of cellular adhesion molecules**
As PCL samples cluster within the cytogenetic group to which they belong, we performed translocation-specific methylation analysis between MM and PCL:

- **t(11;14)**: 36 MM vs 9 PCL – 986 probes (882 increased methylation)
- **t(14;16)**: 7 MM vs 10 PCL – 162 probes (70)
- **t(4;14)**: 22 MM vs 5 PCL – 1559 probes (1553)

The Venn diagram shows the overlap of differentially methylated genes:

- **t(11;14)**: 803 (81%)
- **t(14;16)**: 96 (60%)
- **t(4;14)**: 1409 (90%)

The listed genes are:

- IMPG2
- TXNDC2
- RPS13
- NDUFS1
- HIST2H2AA
- TPD52
- DCC
- BANF1
- C1orf163
- CGB5
- DLL1
t(11;14)-specific methylation changes

**Methylation**
- t(11;14) 36 MM vs 9 PCL

**Expression**
- t(11;14) 34 MM vs 7 PCL

---

**Decreased expression with increased methylation in PCLs**
- ARIH2, CAV1, CLC, CPEB4, CTGF, FSTL1, GNAS, GPR125, LPHN2, MBNL2, MMP8, MPO, P2RX1, PDE4B, PEBP1, PELI1, PLD4, SLAMF1, SNX9, TF, TPD52
- Fold change -1.96 to -41.74

**Increased expression with decreased methylation in PCLs**
- BLZF1, CASP8, MVP, PARP12, PDE4DIP, RUNX3
- Fold change 1.75 to 4.34
Conclusions

• DNA methylation can be used to define myeloma cytogenetic subgroups and disease state
• MGUS>MM transition is characterised by genome-wide hypomethylation and gene-specific hyper-methylation
• t(4;14) samples have a more pronounced DNA hypermethylation signature which may be linked to MMSET over-expression
• copy number abnormalities do not drive clustering of methylation data, but primary events (translocations and hyperdiploidy) do
• PCL samples do not cluster separately, but instead remain within their original cytogenetic subgroups indicating fewer methylation changes at this transition
• Changes between MM and PCL are characterised by an increase in DNA methylation and a dramatic change in gene expression
• Decreased expression of CAMs in PCL samples is not associated with increased DNA methylation at their genomic location
Disease Stages and Genetic Events

**Primary Events**

- **Germinal centre B cell**
- **MGUS**
- **Smouldering Myeloma**
- **Intramedullary Myeloma**
- **Plasma Cell Leukaemia**

**Switch translocations**
- t(4;14) FGFR3/MMSET
- t(11;14) cyclin D1
- t(6;14) cyclin D3
- t(14;16) c-maf
- t(14;20) mafB

**Hyperdiploidy**
- 3, 5, 7, 9, 11, 15, 19, 21

**Deletion 13**
- Monosomy 13
- Interstitial deletion

**Epigenetic phenomena**
- Global hypomethylation
- Hypermethylation of tumour suppressor genes
- t(4;14)-specific hypermethylation

**Genetic Instability**
- Recurrent amplifications and deletions
- 17p-

**Activating Mutations**
- N-Ras
- K-Ras
- FGFR3

**Secondary Translocations**
- t(8;14) c-myc

**Secondary Events**
Acknowledgements

ICR
Prof Gareth Morgan
Faith Davies
David Gonzalez
Christopher Wardell
Kevin Boyd
David Johnson
Paola Leone
Matthew Jenner
Emma Smith

Salisbury
Fiona Ross
Laura Chiecchio

Milan
Antonino Neri

Aalborg
Mette Nyegaard

Cancer Research UK
National Institute for Health Research
Leukaemia Research
Myeloma UK
UK Myeloma Forum