DNA methylation: a potential clinical biomarker for the detection of human cancers

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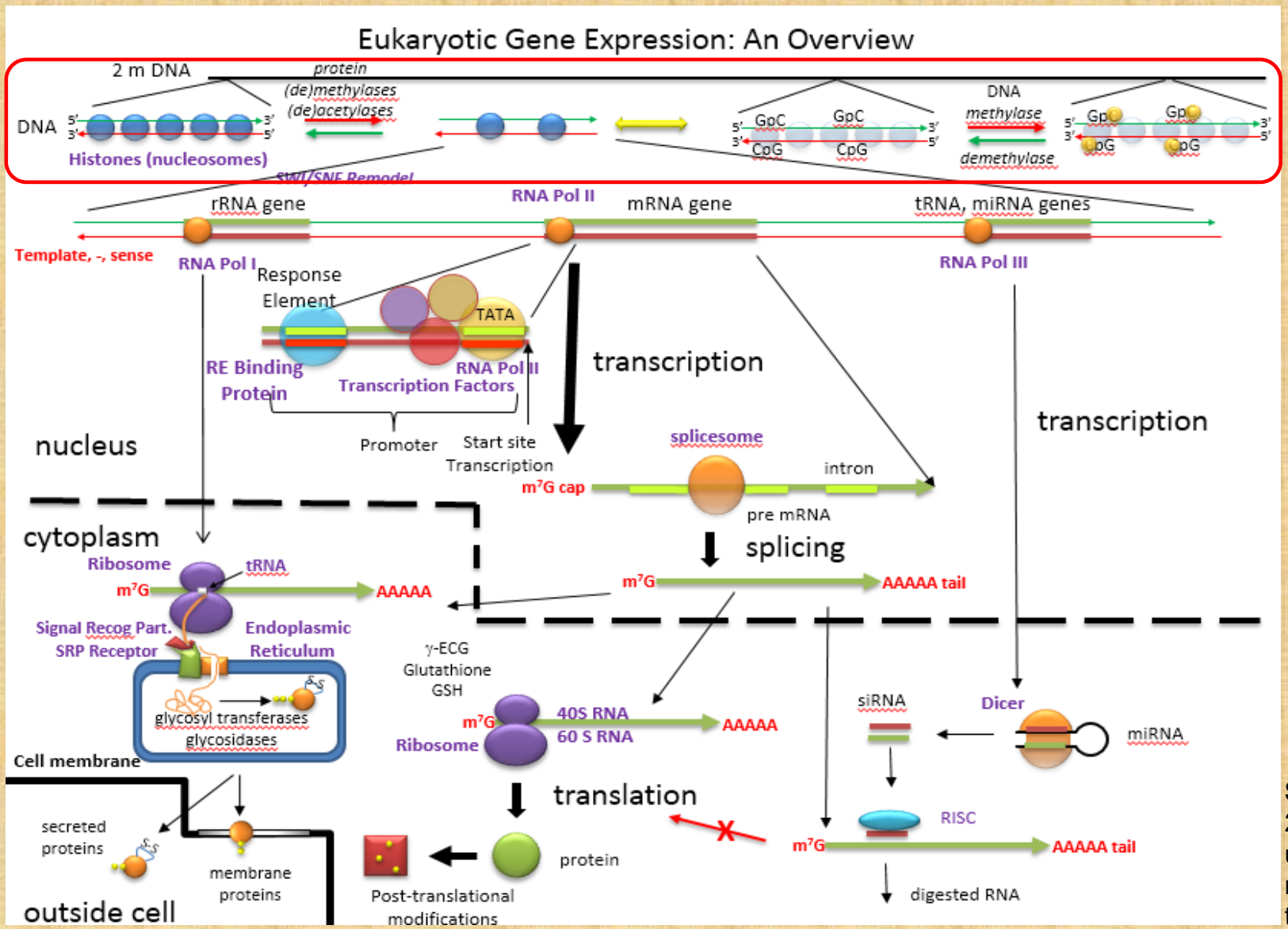


Source: cited from Jakubowski, 2016(http://employees.csbsju.edu/hjakubowski/classes/ch331/bind/olbindtransciption.html) on the 25th November 2016

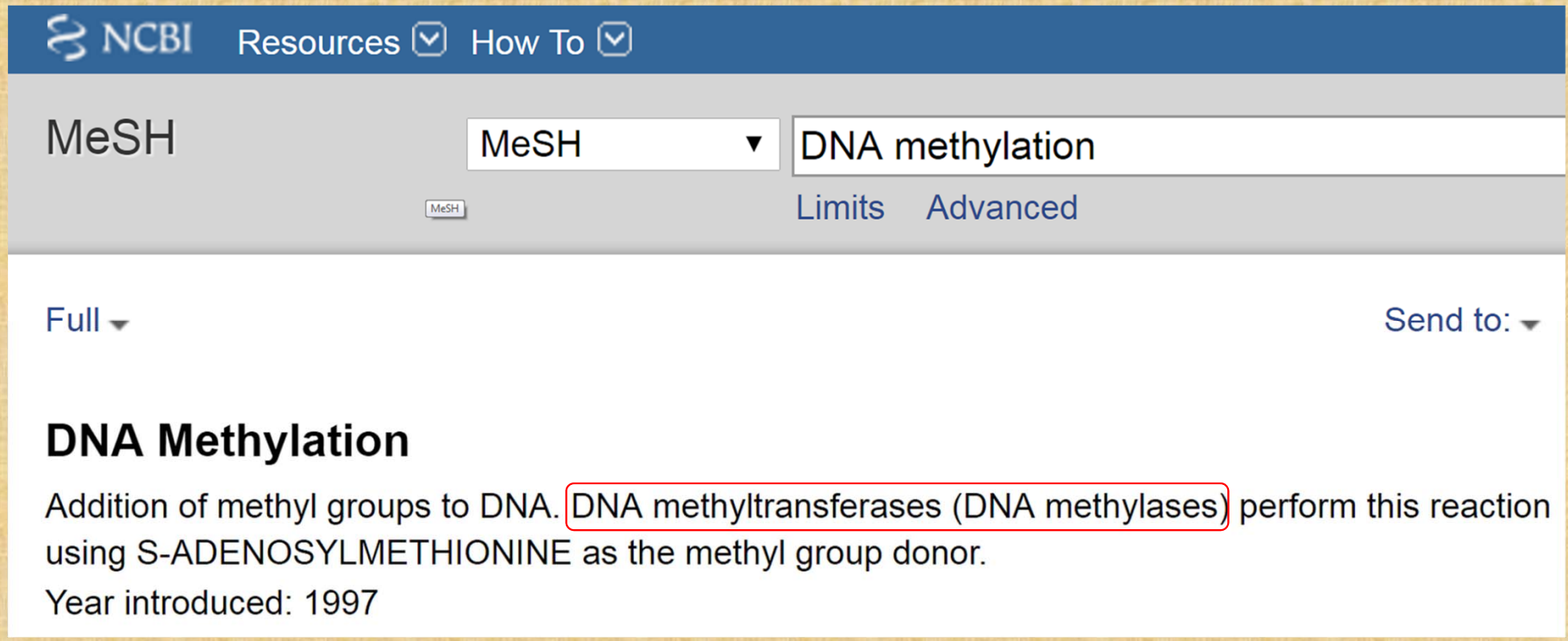
Objective

- The importance of understanding DNA methylation
- Concepts of DNA methylation
- DNA methylation as a clinical biomarker
- Techniques for DNA methylation analysis
  - Examples

A bit history about the discovery of DNA methylation

- DNA methylation was known not later than the time that DNA was classified as the genetic material (Avery et al., 1944; McCarty & Avery, 1946)
- Modified version of cytosine was discovered in 1948 by Rollin Hotchkiss
  - It contains a similar chemical property as thymine
  - Proposed to be responsible for gene regulation and cell differentiation (Holliday and Pugh, 1975; Compere and Palmiter, 1981)
- DNA methylation acted as an epigenetic factor was established around the 80s

In a molecular view…



DNA methylation…

- **Branch of epigenetics**
  - investigation of heritable change independent of the context of DNA sequence
- **Regulatory role in gene expression**
  - usually working together with microRNA (miRNA) or/and histone modifications
- **DNA methylation pattern in adult→ Hypermethylation/Hypomethylation**
  - either by DNA methyltransferase alone or
  - by the cooperation work of DNA methyltransferases and demethylases, which was discovered just a decade ago

Family of DNA methyltransferases (DNMTs)

- Recruitment of DNMTs during S phase of the cell cycle
- **DNMT1** - mainly found in mammals
    - Promote restoration of methylation state after DNA replication
- **DNMTs 3A/3B** - responsible for de novo methylation
- Members of DNMTs work collaboratively with one another at a target region of methylation
- Also, they interact with transcription initiation factors to alter particular gene expression

Family of DNA methyltransferases (DNMTs)

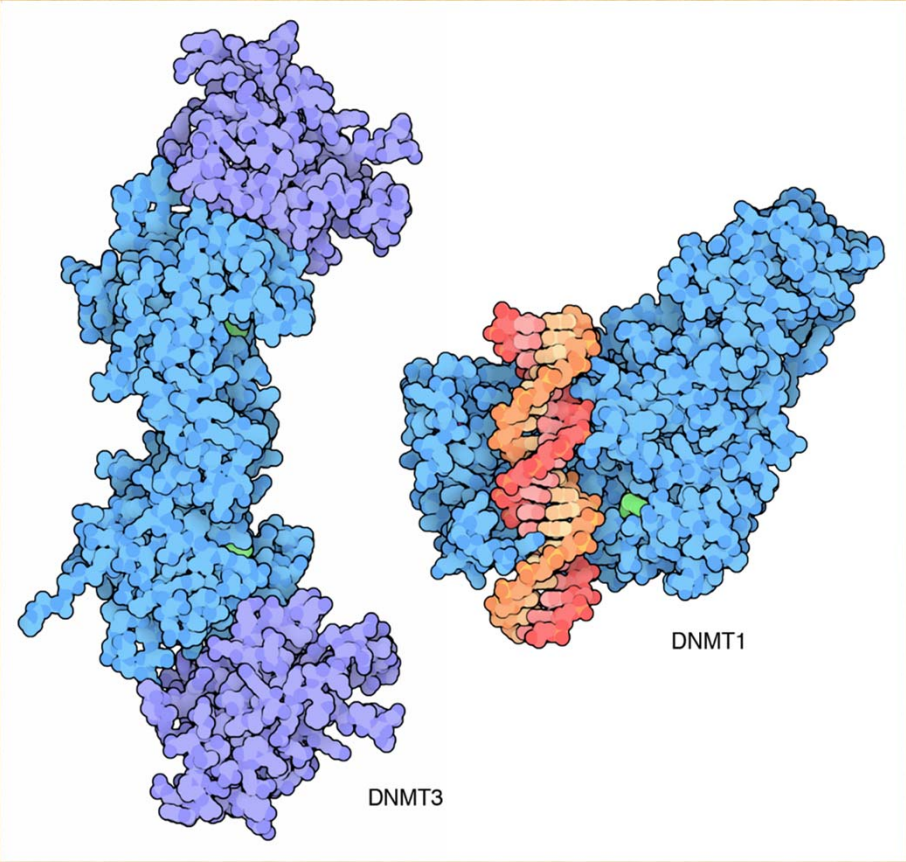


Figure 2a: Brief structure of DNMT3 and DNMT1 (cited from website PDB-101 DNA methyltransferases: http://pdb101.rcsb.org/motm/139)

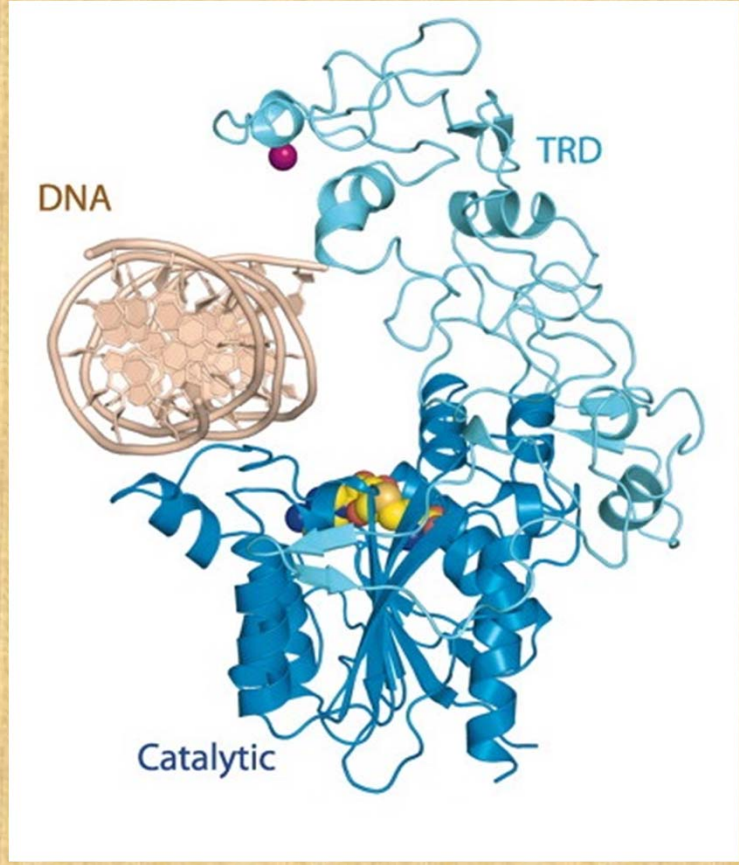


Figure 2b: The crystal structure of the mDNMT1(650–1602)–DNA 19-nucleotide oligomer complex (modified from Jikui Song et al. *Science* 2011;**331**:1036-1040)

DNA methyltransferases (DNMTs) activities

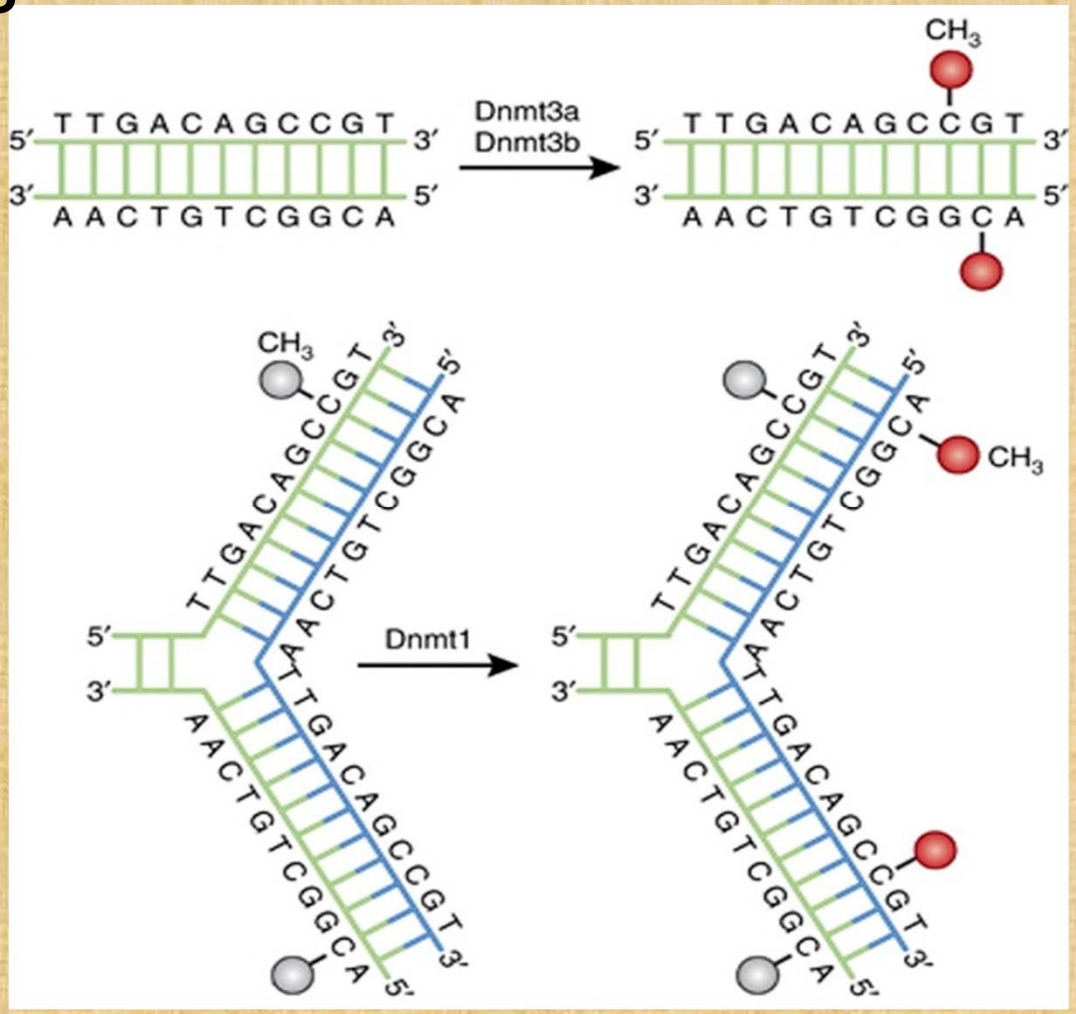


Figure 3a: Dnmts-mediated pathways of DNA methylation(modified from Lisa D Moore, et al. *Neuropsychopharmacology*. 2013 January;**38**(1):23-38.)

Tissue-specific DNA methylation

Embryonic development

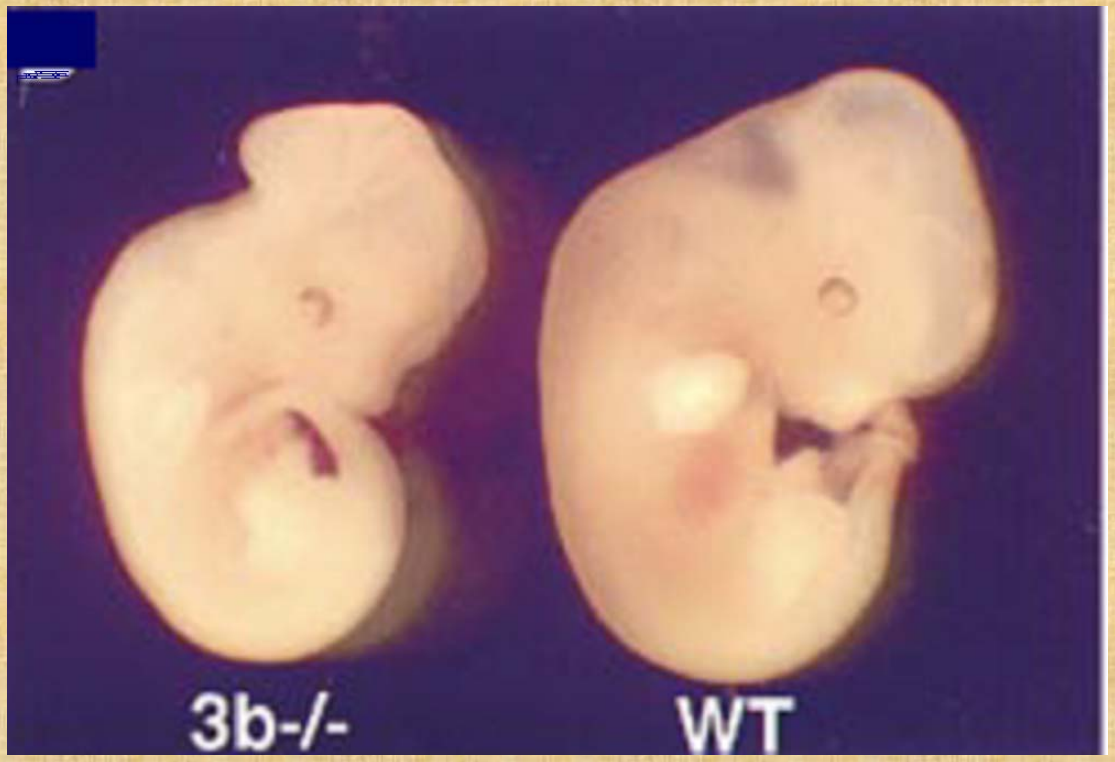


Figure: Dnmt3b double mutant embryo and its normal counterpart (wild type) (edited from Okano et al., 1999)

- Onset of developmental defects in various severity levels

Tissue-specific DNA methylation

Differential expression among tissues

- Dissection of embryo at specific positions with corresponding DNMTs expression sites
- E.g. Dnmt3b is discovered to be highly expressed in the anterior head region and eyes

Note:

- A, anterior; P, posterior; al, allantois; am, amnion; ch, chorion; ee, embryonic ectoderm; m, mesoderm; and ne, neuroectoderm.

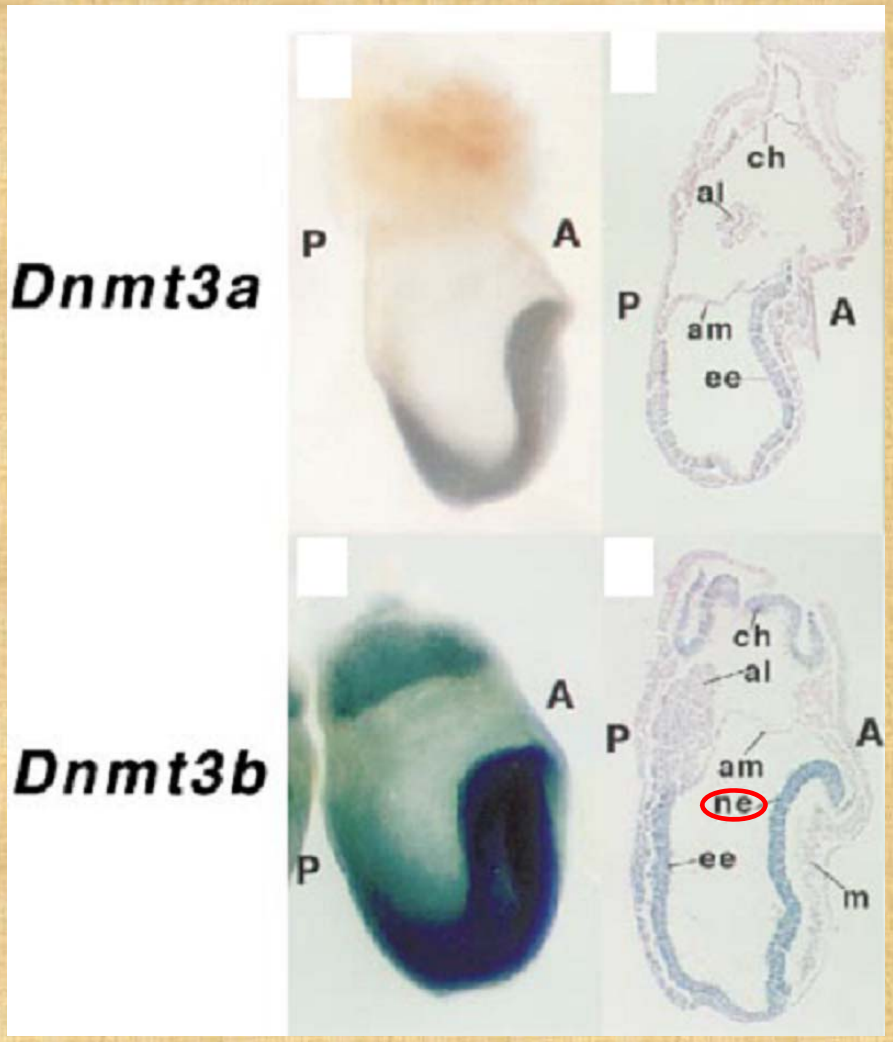


Figure: The patterns in expression of Dnmt3a and Dnmt3b during early period of embryonic development (edited from Okano et al., 1999)

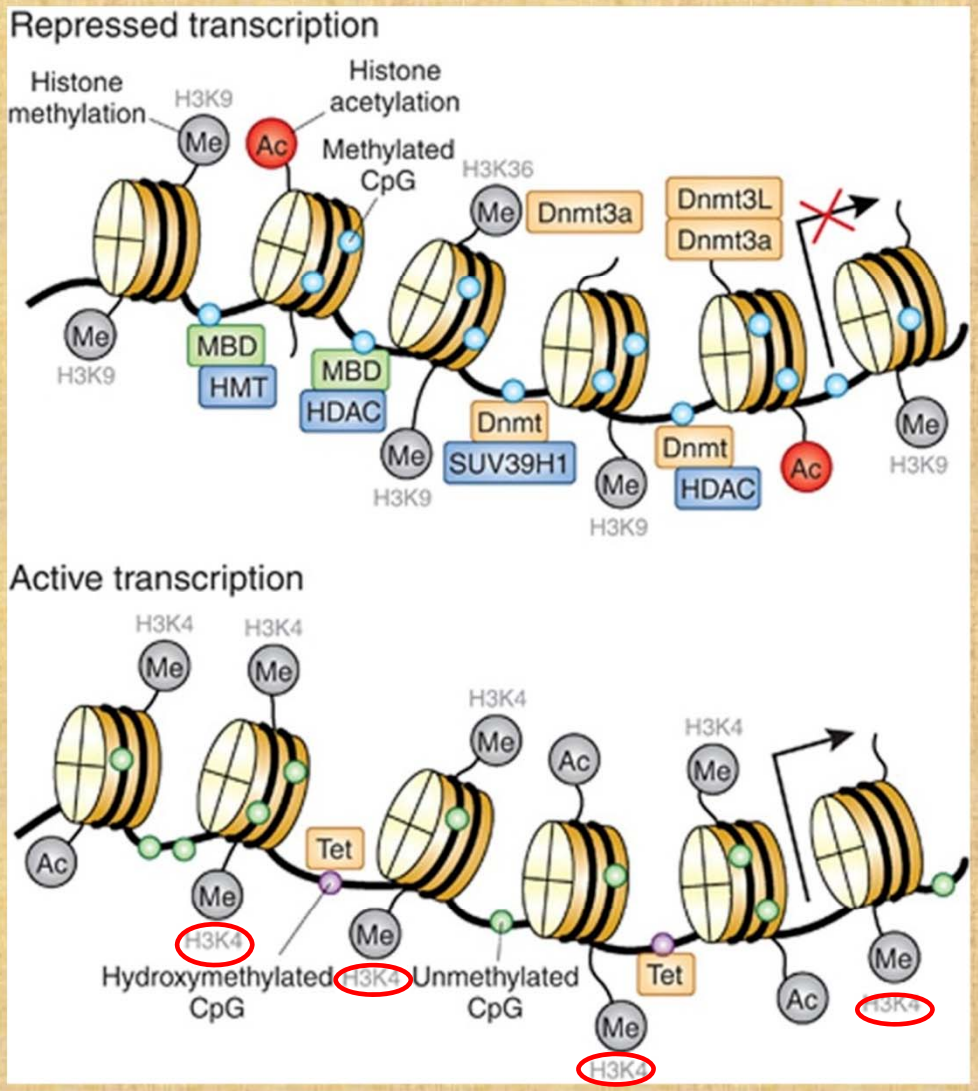


Figure 1: Components involved in active/repressed state of region in genome(cited from Lisa D Moore, et al. Neuropsychopharmacology. 2013 January;38(1):23-38.)

- Multiple epigenetic mechanisms work together to switch on or off the gene expression.
- DNA methylation is recognized by a number of components:
  - methyl-binding proteins such as MBDs (yellow)
  - DNA methyltransferases (Dnmts)
  - Histone deacetylases (HDACs)
  - Histone methyltransferases (HMTs)
- HDACs and HMTs target and modify the histone tails

H3K4 methylation

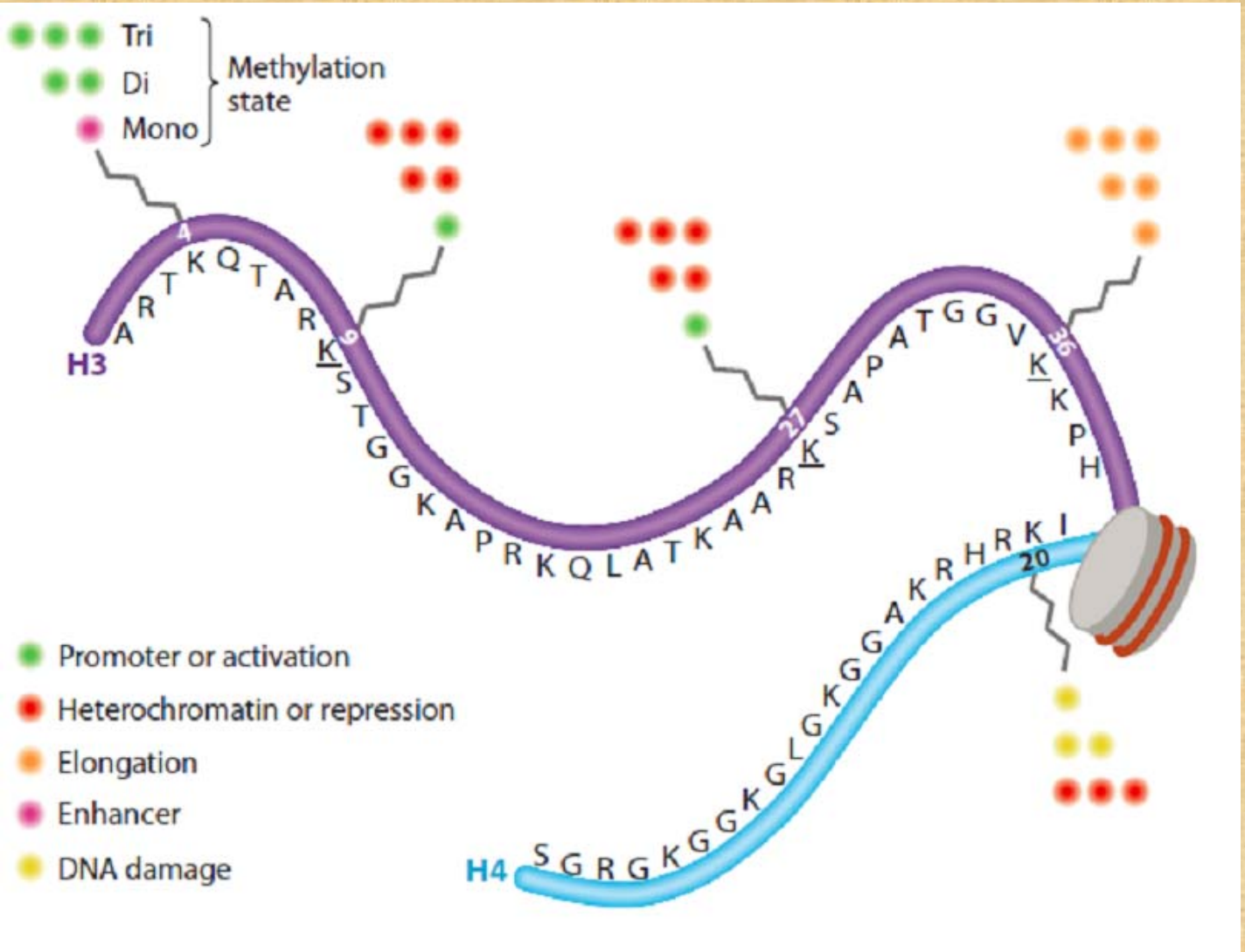


Figure: Overview of the major components involved in H3K4 methylation.(cited from Mosammaparast & Shi, 2010)

H3K4 methylation

- Abundant in non-methylated CpG islands
- Cfp1 (protein)– component of the modification complex that maintains the hypomethylated state of CpG islands
- The modification hinders the binding of DNMTs

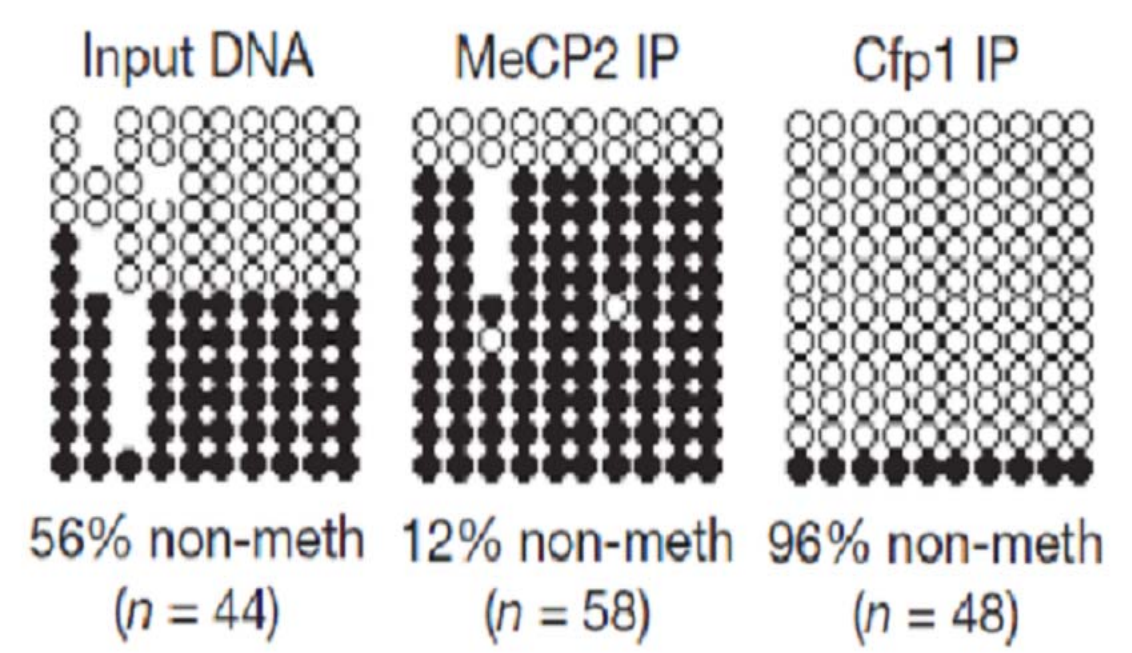


Figure: Bisulphite analysis of the input sample DNA, chromatin immunoprecipitated with Cfp1 antibodies and MeCP2 (Control), respectively. Note: Solid and open circles represent methylated and non-methylated
CpGs, respectively. Uncharacterized CpGs are represented as gaps. (cited from Thomson et al., 2010)

H3K4 methylation

- ‘ChIP-Seq’ – genome-wide, high-throughput DNA sequencing approach
- It is used to detect the distribution of particular protein of interest (e.g. Cfp1)

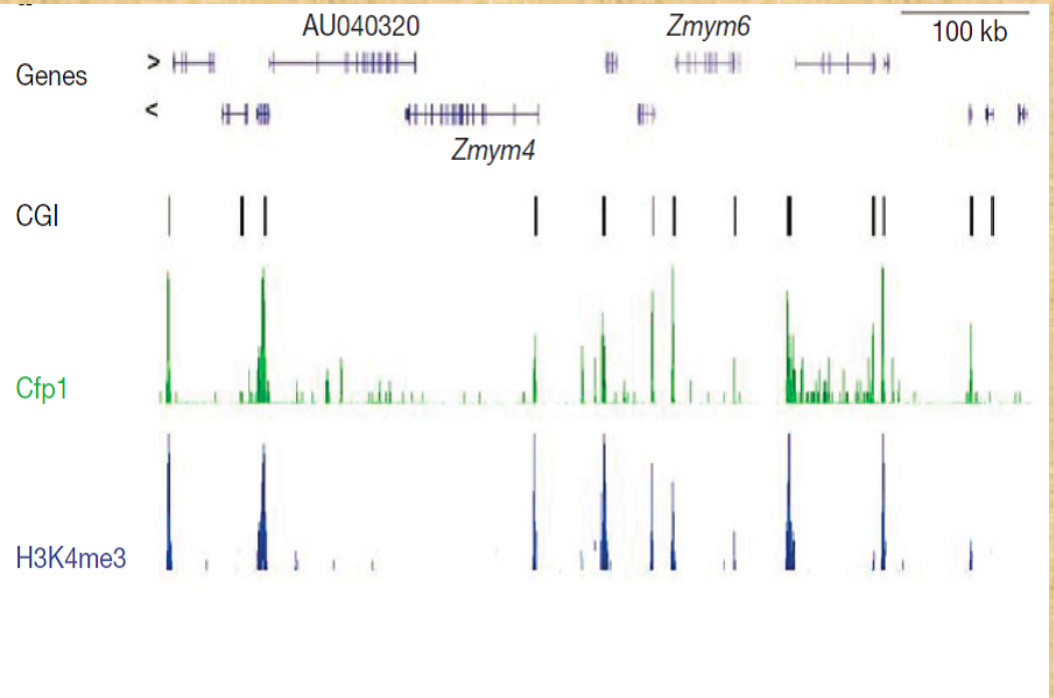


Figure: ChIP-seq analysis result with Cfp1 CpG islands distribution and corresponding methylation state (cited from Thomson et al., 2010)

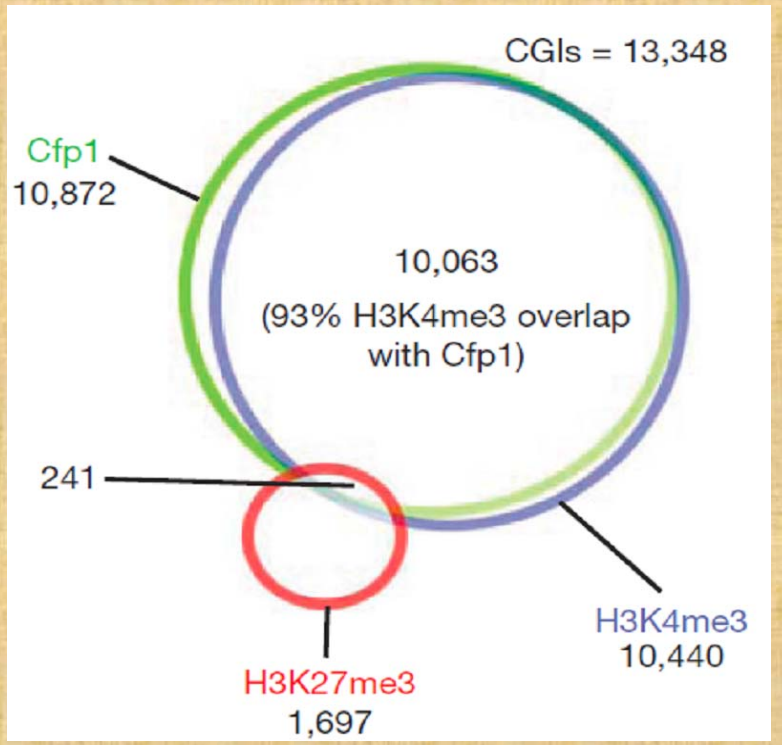


Figure: Representation of a close relationship between the expression of Cfp1 and H3K4me3 (cited from Thomson et al., 2010)

Hallmark of DNA methylation

- Silencing/Turning on specific genomic region
- **CpG islands**
  - Clusters of CpG dinucleotides
  - Most of these regions are unmethylated
  - In general, ‘Dnmts-free’ CpG sites are typical signals of active transcription
  - Could provide useful insights for the cohort study regarding particular association between DNA methylation and carcinogenesis

Active gene promoter is frequently bound by transcription factors

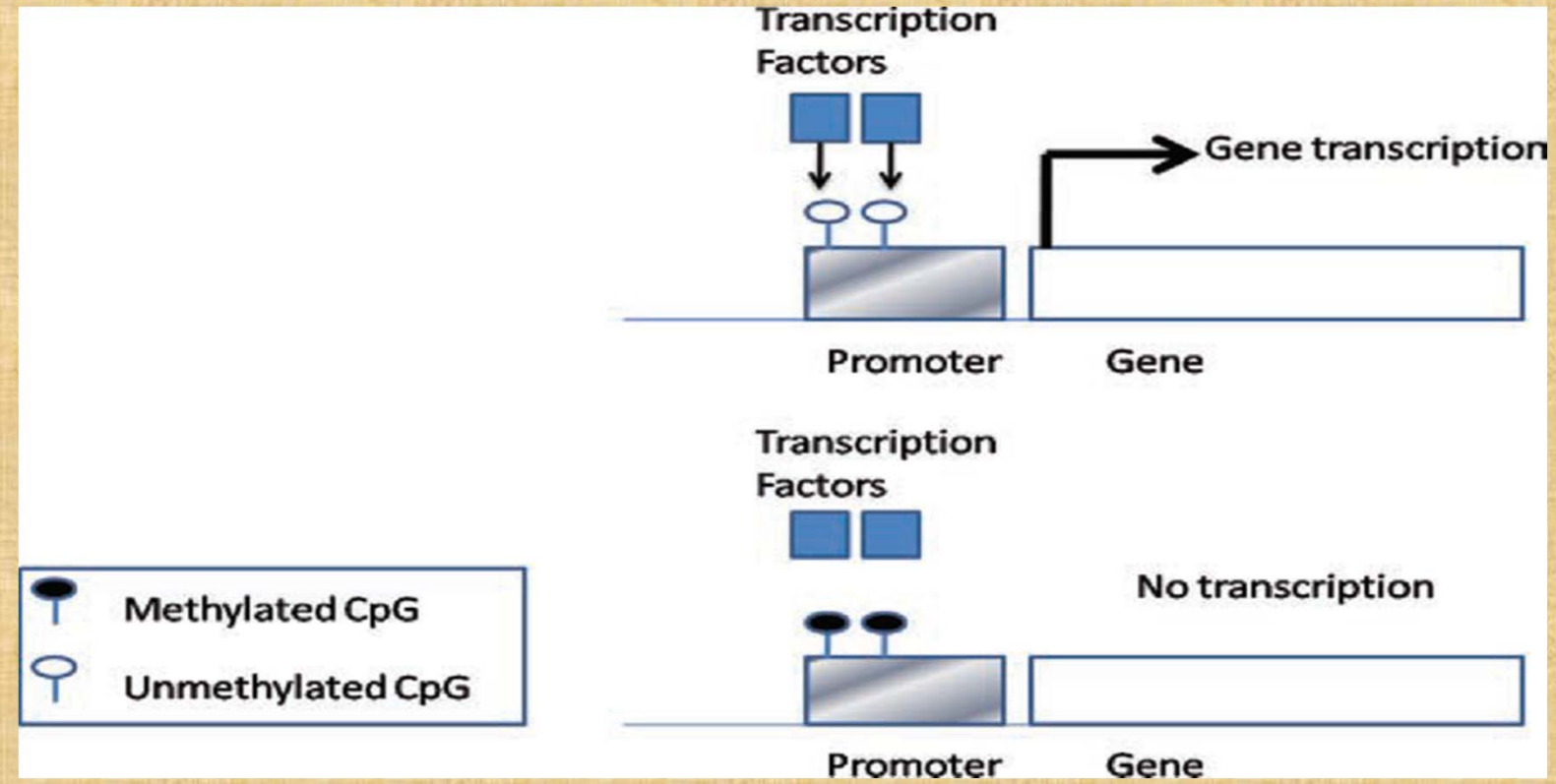


Figure: Gene regulation works with DNA methylation (modified from *SAC review* Lim & Maher, 2010)

Mechanisms for DNA methylation

- Three distinct roles in regulating DNA methylation are played by three corresponding classes of enzymes
  - 'Writers' – catalysis of methyl groups addition onto specific part of DNA (e.g. DNMTs)
  - 'Erasers' – promote DNA demethylation
  - 'Readers' – modify DNA methylation and subsequent bring changes to gene expression
- The connection between DNA methylation and other epigenetic mechanisms should be put into consideration

Histone demethylases

- **Participate in the regulation of methylation state**
- Discovery of the family of histone demethylases allow:
  - understanding the relationship between the levels of methylation on a particular residue and the corresponding effects (i.e. genome activation or repression)
  - Further search of potential therapeutic targets that can be used for drugs designing (e.g. chemical inhibitors)

Histone demethylation

- It is proposed that the prerequisite of the demethylation is the involvement of a histone exchange mechanism
- Discovery via analysing some biochemical reactions as well as bioinformatics data
- **Examples** of mechanisms:
  - Active histone exchange (Ahmad & Henikoff, 2002) and proteolytic removal of histone amino-termini (Allis et al., 1980)

Conventional ways to detect onset of cancer in human

- Biopsy of suspected tissue (e.g. cervical tissue) and analyze it under microscopy
- Subjects may usually feel discomfortable when undergoing Pap test for screening potential target (e.g. cervical cancer)
- Significant results from Pap test are largely depended upon careful observation as well as repeated testing
- Optimization of detection kits have been demandingly required
- Molecular test, like DNA methylation analysis, for detecting the presence of human cancer has been rapidly developed and refined

What is ‘biomarker’?

- **Definition** of the word ‘biomarker’
  - ‘A physical substance (e.g. molecule) or a metabolic process (e.g. cancer-induced response) indicates the existence of cancer’ (Laird, 2003)
- A typical assay of biomarker is governed by
  - Specificity
  - Sensitivity
- Application in the field of risk assessment/management for cancer detection and diagnosis

Methylation-specific PCR(MSP)-based techniques

- Remarkable ability in the discovery of potential gene markers
  - Association with disease progression, including human cancers
  - Prediction of certain clinical outcomes
  - Progression to the study in epidemiology
- The emerging field of genome-scale molecular diagnostics with single-base resolution

Nonbisulphite-based approaches

- Utilization of **pairs** of restriction endonucleases include:
  - methylation-sensitive member (e.g. *Hpa*II)
  - methylation-insensitive member (e.g. *Msp*I)
- Status of methylation can be recovered with downstream southern blotting or PCR
- Advantages of using these assays:
  - technically simple
  - economically favourable

Bisulphite-based approaches

- Gained popularity since the literature Frommer et al. published
- Based on the principle of deamination of ‘methylation-labeled free’ cytosine to uracil
- Followed by PCR amplification using primers which are non-complementary to each other

Detection of DNA methylation within genome

Table 2: Major approaches for analysis of DNA methylation (cited from Laird PW. Principles and challenges of genome-wide DNA methylation analysis. *Nat Rev Genetics* 2010;**11**:191-203.)

Pretreatment	Analytical step			
	Locus-specific analysis	Gel-based analysis	Array-based analysis	NGS-based analysis
Enzyme digestion	• *Hpa*II-PCR	• Southern blot • RLGS • MS-AP-PCR • AIMS	• DMH • MCAM • HELP • MethylScope • CHARM • MMASS	• Methyl–seq • MCA–seq • HELP–seq • MSCC
Affinity enrichment	• MeDIP-PCR		• MeDIP • mDIP • mCIP • MIRA	• MeDIP–seq • MIRA–seq
Sodium bisulphite	• MethyLight • EpiTYPER • Pyrosequencing	• Sanger BS • MSP • MS-SNuPE • COBRA	• BiMP • GoldenGate • Infinium	• RRBS • BC–seq • BSPP • WGSBS

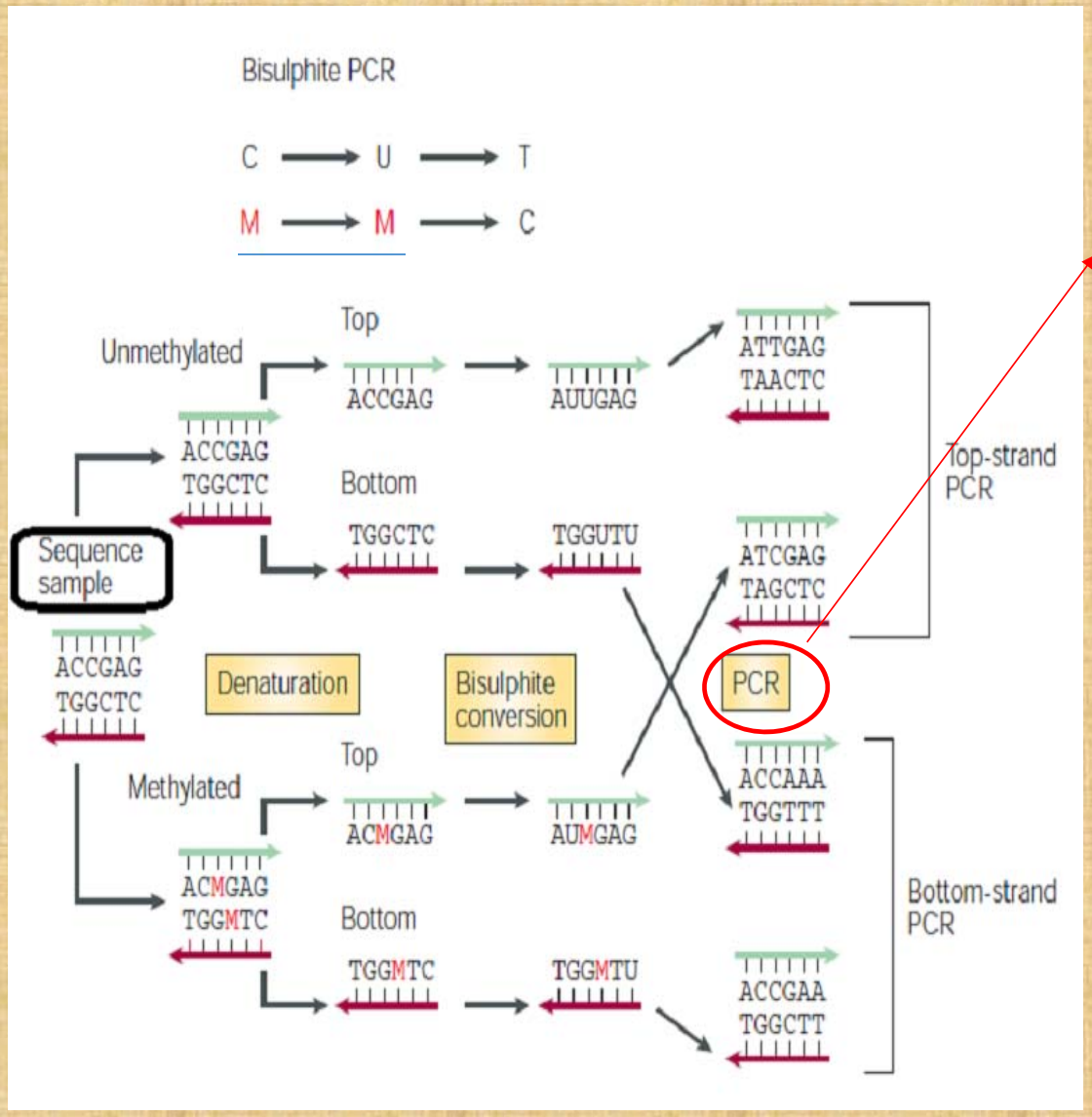


Figure: Principle and steps involve in the bisulphite conversion (edited from Laird, 2003)

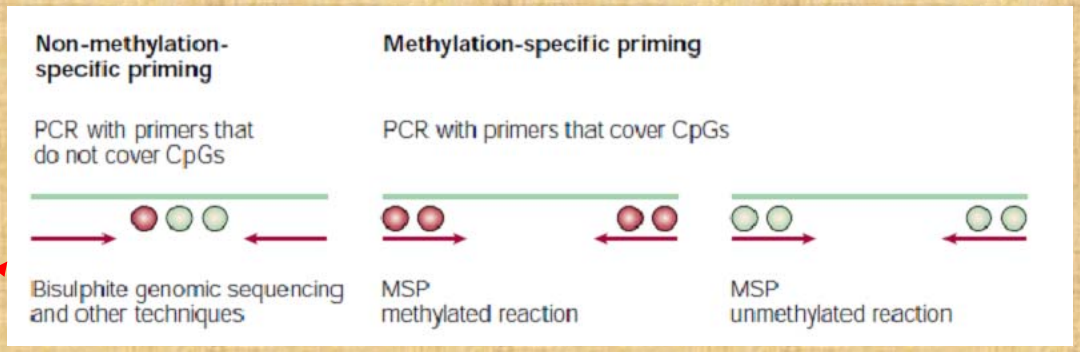


Figure: Choices of downstream PCR priming(edited from Laird, 2003)

- Methylation status can be revealed through the administration of primers possessing with specific chemical characteristics in PCR
- Approximate proportion of methylated cytosine residues in the sample can be shown via calculating the ratio of $\frac{C}{C+T}$

Some general principles for DNA methylation analysis

- Changes in DNA methylation can be defined in terms of:
  - Methylation content
  - Methylation level
  - Methylation pattern
  - Level profile
  - Pattern profile
- They have been used to provide distinct epigenetic information for DNA methylation analysis
  - E.g. - distribution of cytosine

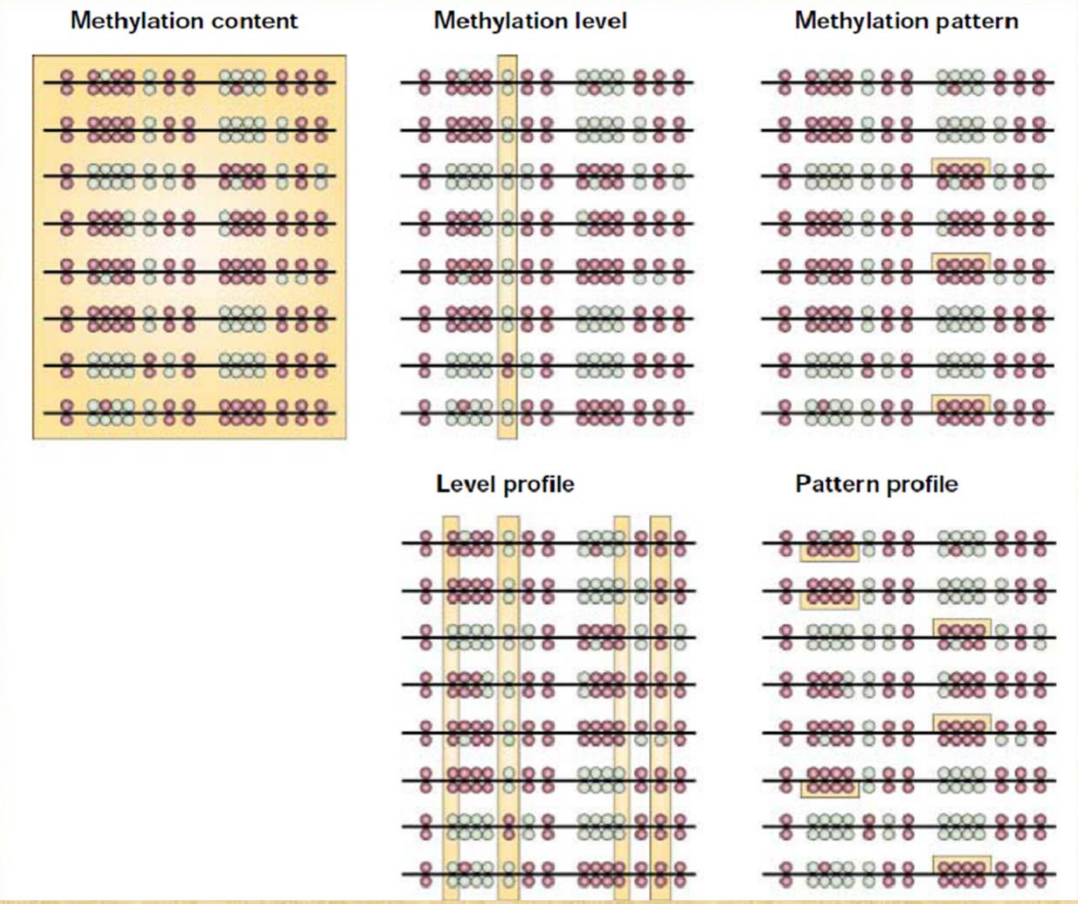


Figure 5: Common types of characterization for DNA methylation analysis (edited from Laird PW. The power and the promise of DNA methylation markers. *Nat Rev Cancer* 2003;**3**:253-66.)

Hypermethylation of CpG islands exists in human cancer

Oncogene (2002) 21, 5427–5440

www.nature.com/onc

CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future

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We have come a long way since the first reports of the existence of aberrant DNA methylation in human cancer. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes is now firmly established as an important mechanism for gene inactivation. CpG island hypermethylation has been described in almost every tumor type. Many cellular pathways are inactivated by this type of epigenetic lesion: DNA repair (hMLH1, MGMT), cell cycle ($p16^{INK4a}$, $p15^{INK4b}$, $p14^{ARF}$), apoptosis (DAPK), cell adherence (CDH1, CDH13), detoxification (GSTP1),

toma (Rb) gene in 1989 (Greger *et al*., 1989), only a few years after the first oncogene mutation was discovered in the H-*ras* in a human primary tumor. However, while genetic lesions in cancer took off from that point and almost monopolized the cancer research field, epigenetic researchers, even to this day, are still trying to catch-up. Not until 1994 was the idea that CpG island promoter hypermethylation could be a mechanism to inactivate genes in cancer fully restored as a result of the discovery that the Von Hippel-Lindau (VHL) gene also undergoes methylation-associated

Fundamental mechanisms responsible for tumour suppressor gene dysregulation

- According to Farrell et.al., 1999…

Mechanism	Gene tumour
Loss of heterozygosity (concomitant mutation in the retained allele)	*RB1* Retinoblastomas
Homozygous deletion (loss of both alleles)	*p16/CDKN2A* Head and neck cancers
Methylation of CpG islands (reduced or absent expression)	*p16/CDKN2A* Multiple tumour types

Inactivation of tumour suppressor gene p53 by CpG hypermethylation

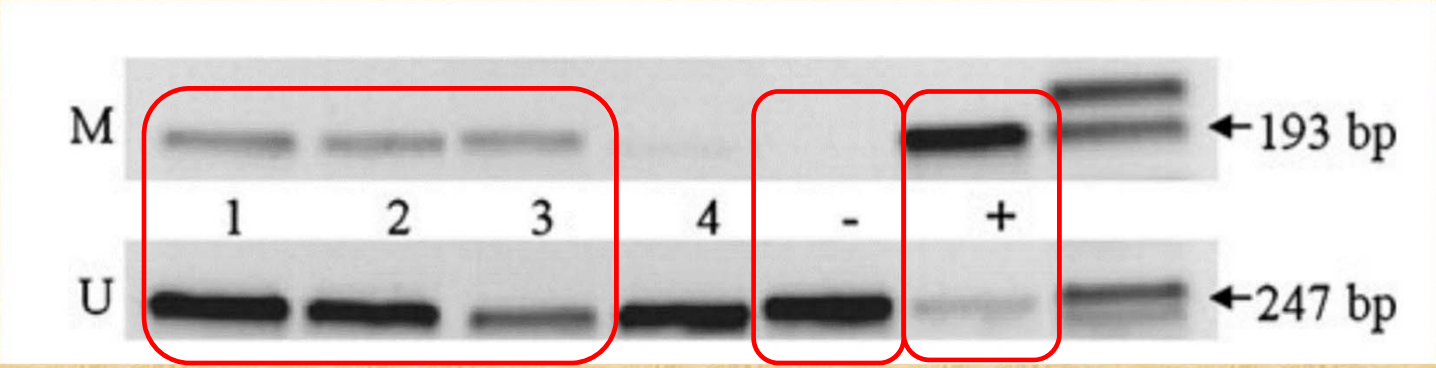


Figure 3: PCR results of the p53 promoter region in tumor samples (Chmelarova et al., 2012)

Note:

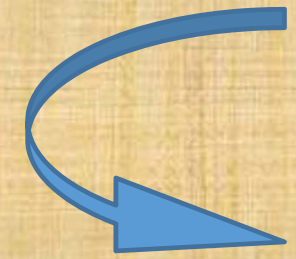
- (+) symbol universally methylated positive control DNA,
- (-) symbol universally unmethylated negative control DNA.
- Sample no. 1, 2, 3 have partial methylated promoter region of p53 gene and sample no. 4 has unmethylated promoter region of p53 gene

Interpretations:

- Dominant phenotypic effect on the p53 promoter region
- Degree of promoter methylation inversely correlates with the expression of p53 gene

Clinical application of potential methylation biomarkers

- Laird, 2003 mentioned that:
  - Methylation patterns - most remarkable in the sensitive disease detection
  - Profiling methods play a significant role in approaches of stratification
- Methylation markers outweigh genetic markers in terms of tumour prevalence
  - e.g. detection of mutations/deletions within proto-oncogenes
- Aim at early detection of human cancers
  - Could be achieved by remote imaging of particular methylation biomarkers that can target the stage of disease progression
  - Minimize the risk of 'missing out' the development of certain types of cancers (e.g. pancreatic cancer and lung cancers etc.)



Hypermethylated genes in cancer and their associated tissue types

Gene name	Gene function	Cancer type
APC	WNT signalling	Prostate, colon, lung, bladder
AR	Androgen receptor signalling	Prostate
BMAL1	AHR signalling	Leukaemia, lymphoma
BRCA1	DNA damage response	Breast, ovarian
CDH1	Cell–cell adhesion	Breast, prostate
CDH11	Cell–cell adhesion	Colon, breast, oesophagus, gastric, liver
CDH13	Cell–cell adhesion	Lung, head and neck
CDKN2A	Cell cycle control	Lymphoma, colon, stomach, prostate
CDKN2B	Cell cycle control	Leukaemia
DAPK1	Programmed cell death control	Lung, head and neck, bladder
EMP3	Signal transduction	Glioma
ESR1	Oestrogen receptor signalling	Breast
GSTP1	Detoxification	Prostate, liver, lung
IGFBP3	Signal transduction	Colon, lung, ovarian, prostate
LGALS3	Extracellular matrix protein	Prostate
MASPIN	Peptidase inhibitor	Pancreas
MGMT	DNA repair	Colon, glioma, lymphoma, prostate, lung
miR-148a	Metastasis suppression	Metastasis
miR-34b and *miR-34c*	Metastasis suppression	Metastasis
miR-9	Metastasis suppression	Metastasis
miR-200s	Epithelial–mesenchymal transition	Colon, bladder, squamous cell carcinoma
MLH1	DNA repair	Colon, endometrium, stomach

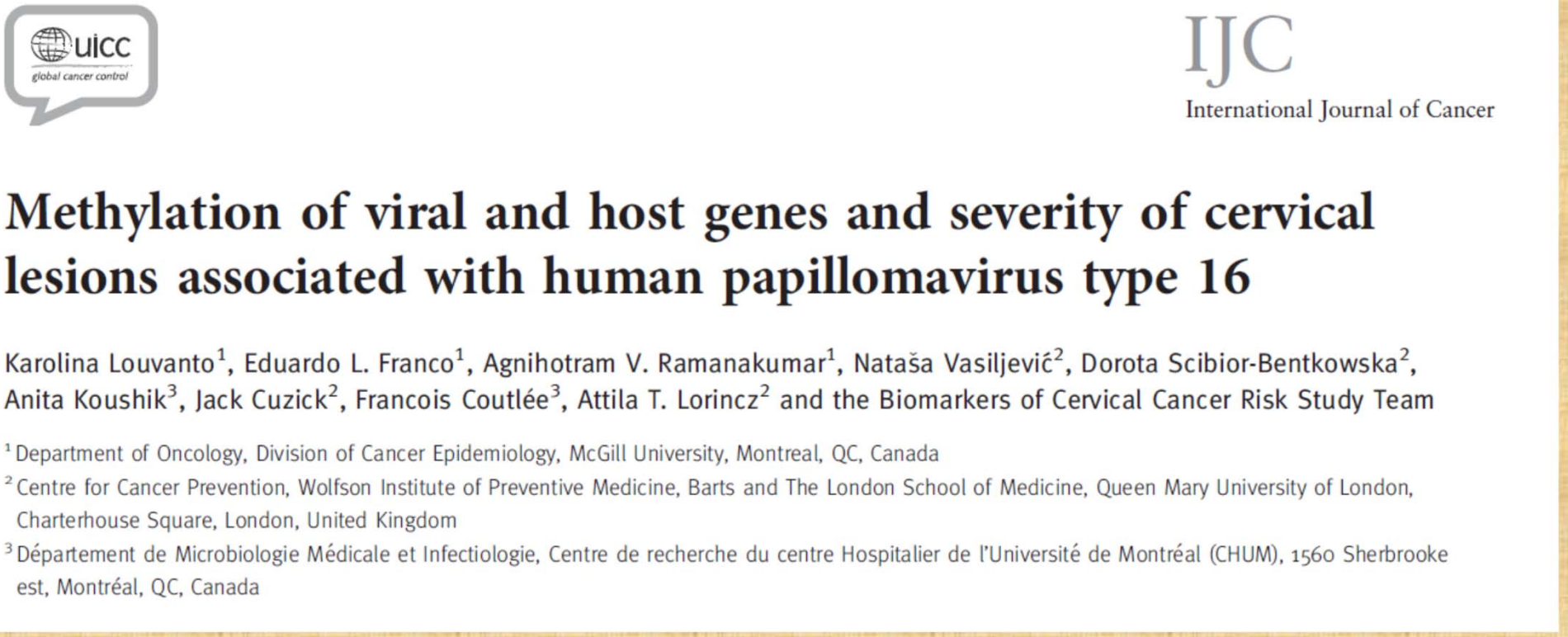
Clinical application of potential methylation biomarkers

***GSTP1* - tumour suppressor gene (**Hopkins et al., 2007; Henrique & Jerónimo, 2004)

- *GSTP1* methylation correlate with over 90% prostate cancer cases (Lee, et al. 1994 & Jeronimo, et al. 2001)
  - GSTP1: glutathione-S-transferase P1 enzyme
  - Prostate cells that cannot express GSTP1 would be more prone to DNA damage than their normal counterparts
- Easy extraction of samples – simply from body fluids (urine, serum and plasma) of the patients

Clinical application of potential methylation biomarkers

Case study:



UICC
global cancer control

IJC
International Journal of Cancer

Methylation of viral and host genes and severity of cervical lesions associated with human papillomavirus type 16

Karolina Louvanto[1], Eduardo L. Franco[1], Agnihotram V. Ramanakumar[1], Nataša Vasiljević[2], Dorota Scibior-Bentkowska[2], Anita Koushik[3], Jack Cuzick[2], Francois Coutlée[3], Attila T. Lorincz[2] and the Biomarkers of Cervical Cancer Risk Study Team

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Clinical application of potential methylation biomarkers

Material and methods:

- Bisulphite conversion of extracted DNA with specific DNA methylation kit
- Three gene sites were chosen
  - *EPB41L3* (human gene) - regulation of cytoskeleton
  - *LMX1A* (human gene) - transcription factor involving in body development
  - HPV16 (viral gene) - L1 region
- Lesion categories
  - NILM < CIN1 < CIN2 < CIN3 < SCC, ADC
- Comparison of methylation status among these categories and detection of associations

Clinical application of potential methylation biomarkers

Sample information of the methylation analyses:

- 244 women (210 HPV positive + 34 HPV negative)
- HPV negative=normal screening Pap smear + no cervical abnormalities history

➢ Case-control study

➢ DNA were extracted from exfoliated cervical cells

Clinical application of potential methylation biomarkers

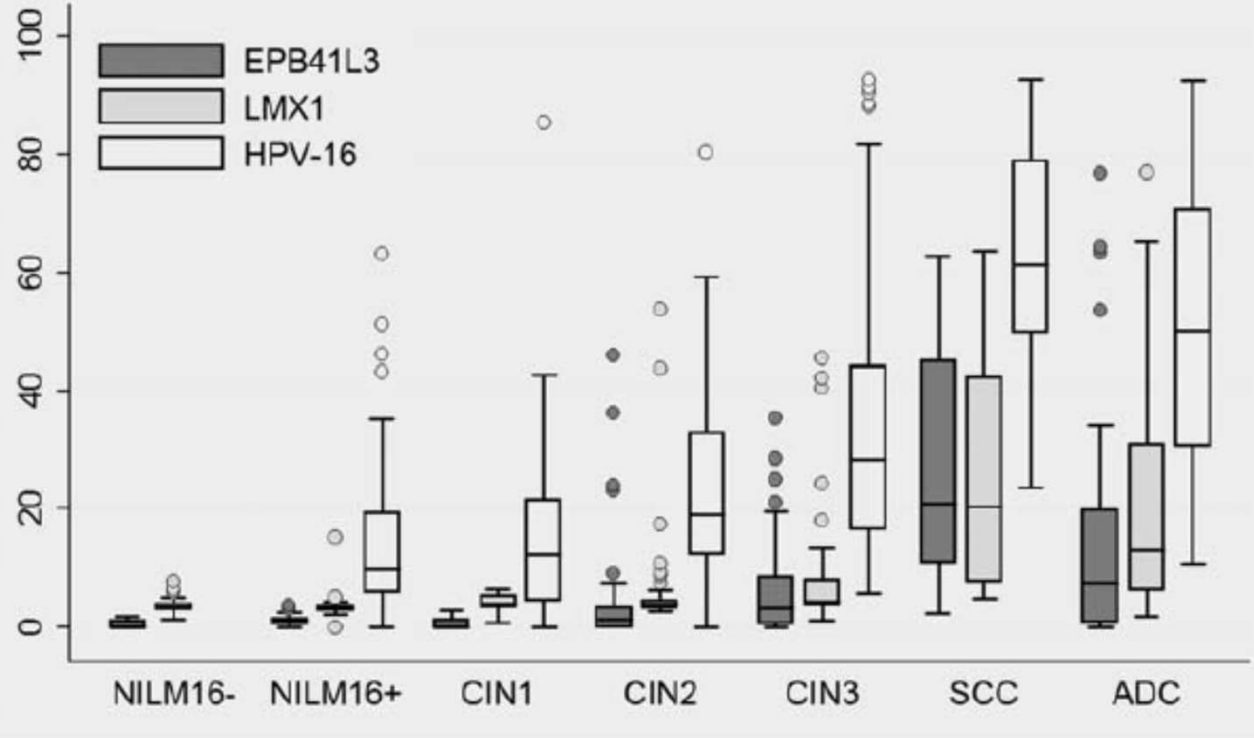

Figure 1. Box plots for average methylation levels of the host genes EBP41L3 (CpG 438, 427, 425) and LMX1 (CpG 260, 262, 266, 274); and the viral HPV16 L1 gene (CpG 6367, 6389) accord-

Source: Louvanto et al. 2015

Results:

- Higher average methylation were found at the HPV16 sites than the host gene sites
- No apparent correlation between viral and host gene methylation status

Clinical application of potential methylation biomarkers

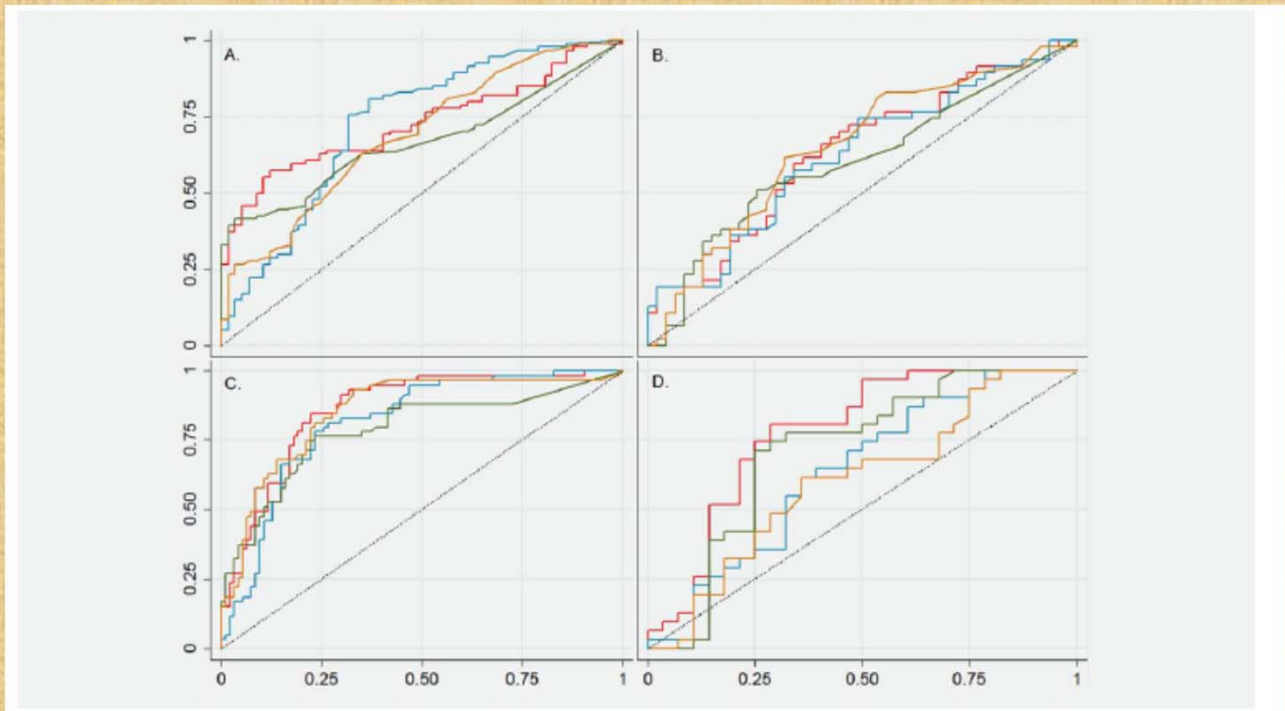

Figure 2. Receiver-operating characteristic (ROC) curves for the performance of the average DNA methylation at different viral (HPV16 in blue) and host genome sites (EPB41L3 in green and LMX1 in orange), as well as their corresponding linear predictors (red) in discriminating the following pairs of lesion categories: (a) CIN2/CIN3 vs. NILM16+/CIN1; (b) CIN3 vs. CIN2; (c) SCC/ADC vs. CIN2/CIN3 and (d) SCC vs. ADC. Abbreviations: NILM16+: no intraepithelial lesion or malignancy and HPV16-positive, CIN: cervical intraepithelial neoplasia (the number denotes the grade), SCC: squamous cell carcinoma, ADC: adenocarcinoma. Specimens in all groups being compared are HPV16 positive.

Source: Louvanto et al. 2015

Note:

A. CIN2/CIN3 vs. NILM 16+/CIN1
B. CIN3 vs. CIN2
C. SCC/ADC vs. CIN2/CIN3
D. SCC vs. ADC

Results:

- Best diagnostic performance was the comparison between the group (SCC/ADC vs. CIN2/CIN3)
- No methylation markers (including their combination usage) contribute promising discrimination in methylation status between CIN2 & 3 groups

Clinical application of potential methylation biomarkers

Discussion & Conclusion

- There is a trend of improving performance for contrasts of different lesion categories (e.g. CIN2/CIN3 vs. NILM 16+/CIN1; SCC/ADC vs. CIN2/CIN3)
- High sensitivity of detection level but meanwhile containing a relatively high false positive rate

→ Low specificity

- Current results should be put into test in real screening and triage processes in the coming future

Challenges and future prospects

- Standardization of current protocols in terms of methodology
- Demethylating drugs design and subsequent application in cancer therapies
- Continuous work of high-throughput methylation screening of patient samples in:
    - Detections of different stages of cancers with promising clinical specificity and sensitivity
    - Identification of various types of tumour
- Personalized medicines

Lastly…

- DNA methylation is mature enough to be used as a routine clinical biomarker for the detection of human cancers ONLY IF
  - more cohort data can be integrated and analysed
  - clinical data can be efficiently exchanged
- The need of establishing a cooperation platform that facilitate communication about some up-to-date research findings

Reference list:

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