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METHYLATION ANALYSIS ON WHOLE GENOME LEVEL. WHAT DID WE LEARN FROM BEN STUDIES?

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Abstract

Recent years have brought the interest of genetic research to the human epigenome. Its unique characteristics – being simultaneously stable and dynamic, present vast opportunities for cell function regulation, maintaining and executing different cell type programmes. Greatest efforts were invested in studying DNA methylation in comparison to histone modifications and miRNA studies.

The present review aims to shed some light on the key mechanisms governing the epigenetic characteristics of the human genome and how epigenetic patterns can be analysed on whole genome scale.

Here are presented the main advantages and disadvantages of different approaches to methylation analysis. This review presents a link between the methodology and some of its application in BEN research as an example of polygenic disorders.

Key words: BEN, epigenetics, methylation, whole-genome analysis.

Epigenetics and disease – where's the link?

Epigenetic characteristics represent heritable properties in genome function, which do not involve the primary DNA sequence. The dynamics of epigenetic characteristics ensure the stability of the epigenetic profile through cell generations and allow the cell to perform specific functions (differentiation) and to adapt according to different stimuli. The major tools that maintain the epigenetic apparatus include histone modifications, miRNA interference, and DNA methylation (Fig. 1). Epigenetic mechanisms allow the cells to respond reversibly and in a precise way to environmental cues, but also to preserve cell type specific gene programmes. Changes of the epigenetic pattern can lead to development of diseases- such as cancer, by altering the gene expression pattern of the cells. Epigenetic changes over time display familial clustering. Since we know that a great number of diseases "run in the family" and regular genetics have failed to build a comprehensive picture of genetic predisposition, the epigenetic pattern could be involved in transmitting "predisposition" over generations.

Histone modifications (acetylation, methylation, ubiquitination) build an intricate network that can change chromatin composition and thus alter the availability of the gene sequences to a number of transcription factors, enhancers or silencers, ultimately leading to change in the gene expression level.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules, about 21–25 nucleotides in length. Being partially complementary to one or more messenger RNA (mRNA) molecules their major function is to down regulate gene expression in a variety of manners, including translational repression, mRNA cleavage, and deadenylation. The DNA sequences coding for a great part of the miRNAs are located in the introns of their targeted genes, share their regulatory elements and have a similar expression profile. The human genome is presumed to encode over 1000 miRNAs, which may target about 60% of mammalian genes.

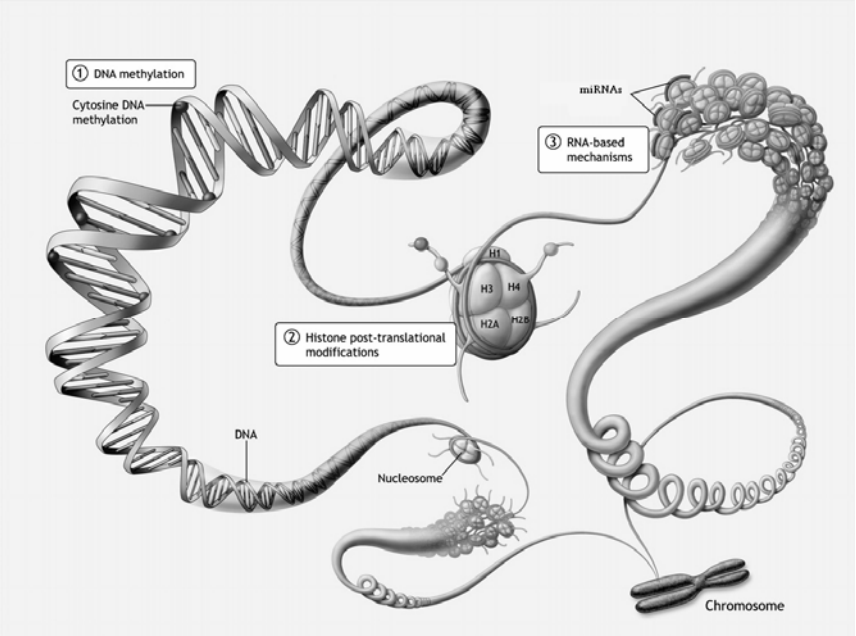

Figure 1 – Major mechanisms of epigenetic modifications – (1) DNA methylation, (2) histone modifications, (3) miRNAs adapted from Epigenetic Regulation of Vascular Endothelial Gene Expression by Charles C. Matouk, Philip A. Marsden

DNA methylation is in the centre of epigenetic research. In mammals DNA methylation is a chemical modification – covalently binding a methyl group to the 5th-carbon position predominantly of cytosines located in CG dinucleotide rich sequences, commonly referred to as CpGs. In contrast to the greater part of the CpG sites throughout the human genome the CpG islands located in gene promoter regions and other gene related DNA sequences are not constantly methylated. CpG islands are defined by size at least 200 bp; GC content more than 50%, and with an observed-to-expected CpG ratio greater than 60%. More than 50% of human genes contain a CpG island in their promoter region. DNA methylation in promoter regions is perceived as a gene silencing mark that leads to lower gene expression levels. Methylation is in close coordination with the other epigenetic mechanisms such as histone modifications, but the exact hierarchical relations are still poorly understood. CpG island methylation is involved physiologically in genomic imprinting, X inactivation and cell differentiation. As well as a fundament for physiological regulation, DNA methylation processes can be involved in disease. An aberrant methylation profile is observed in different cancers. In cancer the promoter-associated CpG islands of certain genes are hypermethylated; even more, these deregulated genes are different in different cancer types. Regions of differential methylation between cell types are not exclusive just to promoter regions – there are CpG islands reported in exons, introns, enhancer sites, and intergenic regions – raising the idea that they could regulate miRNAs, reverse strand transcripts and alternative splicing. General hypomethylation is observed in cancer and it mainly affects non-CpG island DNA sequences that are normally methylated. An aberrant methylation profile is reported even in premalignant stages – suggesting the possible contribution of epigenetic alterations in the very early carcinogenesis.

Approaches to genome-wide methylation analysis – what's on the table?

Great amount of effort were invested in creating high-throughput whole genome platforms for studying DNA methylation. Whole genome approach allows analyzing the full methylation pattern, giving a more comprehensive picture of methylation profiles.

Array based methodology

With the rapid development of the array-based technologies and their rising application

in DNA research and diagnostics it was no surprise that such platforms were introduced in methylation analysis. There are two major approaches to genome-wide DNA methylation analyses – bisulfite conversion and enrichment for the methylated fraction of a DNA sample.

Bisulfite conversion introduces chemical modifications in the primary DNA sequence by converting the unmethylated cytosines to uracil. Thus are created different sequence templates in methylated and unmethylated regions. The bisulfite conversion array based platforms rely on designing probes able to recognize methylated from unmethylated sequences. Limitations of these platforms are based on the probe design, the density of the array and the essence of bisulfite conversion. Because of the conversion there are in the worst situations 3 bases in the DNA sequences and this loss of complexity impedes the analysis. The cornerstone of the development of these techniques was the introduction of SNP detection using bead arrays. For each locus two allele-specific and two locus-specific oligonucleotides are required. The 3' end of the allele-specific oligonucleotides are synthesized to be complementary to either the bisulfite modified DNA (methylated allele) or the unmethylated allele. Moreover, a specific PCR primer site is attached to the 5' end of each oligonucleotide that is fluorescently labelled with different dyes. The 5' end of the locus specific oligos has a locus-specific sequence, its 3' end is a universal primer site and the middle part represents a molecular tag that designates the oligo to a certain genomic position. There is a difference between the two locus-specific probes – for one oligonucleotide probe its CpG island is considered to be methylated and for the other vice versa. So at the places where there is an oligospecific hybridization, a one-step primer extension reaction is performed to testify that matching has occurred at the allele-specific differently methylated sequence. Further, the locus-specific oligos are ligated to their corresponding partner and the products are amplified using PCR before their hybridization to a bead array. Methylation level is assessed by measuring fluorescence signal intensity from the primers of the allele specific oligos. There are platforms based on this approach that can simultaneously determine the methylation status of more than 27,000 CpG islands corresponding to ca. 14,000 genes. There needs to be no less than 17% difference in the absolute methylation level between samples so that they can be defined as differently methylated loci. The major pitfall in this analyzing strategy is the assumption that all CpGs in a given region are of the same methylation status. There is evidence that at certain points single CpGs can differ in their methylation status from all other CpGs in the region. If this happens to be the case, and a single CpG has a different methylation status, no allele specific product will be synthesized, and therefore the methylation status cannot be determined. Still, this methodology provides absolute methylation levels and is less time-consuming in comparison to others.

Methylation enrichment techniques rely on the extraction of the methylated fraction of whole genome DNA samples by methylation-specific binding proteins. The first step in this methodology is to achieve DNA fragments of 200–1500 bp. Most popular choices for DNA fragmentation are restriction enzyme digestion and ultrasound. Less frequently heat can also be applied. Specific proteins recognize and bind the methylated cytosines. This complex can be extracted by standard precipitation techniques on magnetic beads or other suitable carriers. Unbound (non-methylated) DNA is removed by several washing steps and then the methylated fraction is eluated from the DNA-protein complex. Essential for the approach is the protein used to extract the methylated DNA. MeDIP (Methylated DNA immunoprecipitation) protocol relies on an antibody that specifically recognizes methylated cytosines. MCIp (methyl CpG immunoprecipitation) is based on fusion protein of the Fc part of IgG1 and MBD2 (methyl-binding protein 2). MIRA protocol includes immunoprecipitation by a hybrid protein formed by MBD2 and glutathione transferase. One other protein reported to be used as a methylation DNA extraction facilitator is MeCP2. MeCP2 targets are concentrated in pericentromeric heterochromatin and there is evidence that it can also bind unmethylated DNA sequences. These preferential targeting can introduce a bias in the experiments. In contrast to MBD2 and MeCP2 antibodies do not have such sequence preferences, but MeDIP-based approaches give information about mean methylation in the region, so they

are not applicable to situations where the goal is the methylation status of a certain CpG island to be analyzed. MeDIP techniques show high dependence on the CpGs density. In general, precipitation-based techniques have less specificity and sensitivity compared to other approaches.

Methylation specific sequencing

Bisulfite modified DNA sequencing platforms based on second generation sequencing for whole genome analysis have been introduced to the research field. By such an approach the methylation status of every CpG locus could be determined. Second generation sequencing allow only the sequence for short segments of DNA (up to 600 bp) to be determined. So the whole of the genome methylation sequencing strategies seriously rely on software products to align and generate the whole genome sequence. This represents a formidable drawback, since the analysis is based on mathematical and statistical algorithms. Results from partial genome sequencing based on this platform have already been published. With the progress of genomic technologies these methylation analysis platforms may have their place in methylation studies. With the advent of NGS (next generation sequencing), protocols for whole genome methylation analysis are being developed. All require bisulfite conversion of the sample. The basic principle of all developed platforms is the same – DNA template immobilized to a solid surface in order to be parallel sequenced – thus generating thousands to millions of reads in a single run. All platforms present whole genome methylation data at a single base resolution.

Novel NGS-based methylation analysis methods are under development. They do not require prior bisulfite treatment – they can distinguish the methylated cytosines from the four standard DNA bases. Major advantages of these platforms include less bias during template preparation, possible longer read length, lower cost, higher speed and better accuracy.

What did we gain from methylation studies in BEN?

Balkan endemic nephropathy (BEN) represents a chronic tubulointerstitial nephritis indigenous to regions of the Balkan peninsula. BEN has late and subtle onset, affecting both genders with a slight female predominance in some regions. The disease shows familial clustering. BEN has a long preclinical period, a slow progression, and eventually leads to terminal renal failure. About 30–40% of those affected develop uroepithelial tumours of the upper urinary tract with varying degree of malignancy. Papillar carcinomas are most common among BEN-associated uroepithelial cancers. Malignancies are one of the most common causes of death in BEN and can develop before end-stage renal disease. Standard laboratory tests usually reveal no salt retention, low molecular weight proteinuria and normo-hypochromic normocytic anemia. With disease progression, decreased kidney concentration ability is observed. Ultrasound examination shows symmetrically reduced kidney size in advanced stages of the disease. Primary pathomorphological characteristics include diffuse cortical interstitial fibrosis and tubular atrophy.

More than 50 years after BEN cases were first reported there is still no consensus as to what the etiology of BEN is. Numerous studies and research projects have been able to shed some light on the factors leading to BEN development, nevertheless there still is not a comprehensive picture of BEN etiology. BEN appears to be a complex, multifactorial disease, a result of an intricate interplay between genetic background and environmental factors. Evidence supporting the involvement of environmental factors is inconclusive and none of the agents was proven to be able to explain every aspect of BEN on its own.

Numerous chemical elements, organic and non-organic compounds, viruses and microorganisms have been studied in the context of BEN. Heavy metals such as Mg, Mo, Cd, Pb As, Se, Ca, Cu show statistically significant differences in soil concentration between endemic and non-endemic regions, although no direct connection of a toxic effect of any heavy metal to the disease development can be unequivocally proven.

Aristolochic acid found in the seeds of Aristolochia clematis, a plant with a worldwide distribution including BEN endemic regions, was proposed as a possible causative agent.

The contribution of aristolochic acid to BEN etilology was based on its well-known nephrotoxic and carcinogenic properties. Still, the exact significance of this compound to BEN etiology has not been fully estimated. There are several reports on the link between aristolochic acid and malignancies in BEN patients.

The mycotoxin ochratoxin A was also researched. It is supposed to have a synergic effect with other agents in BEN development. Moreover, there is a geographical overlap between regions with a high ochratoxin A concentration in wheat and endemic nephropathy areas.

Viral etiopathogenesis of BEN was also explored – papers report on various viruses (Picorna virus, Polyoma virus, Herpes simplex 1 and 2, Adenovirus, Hepatitis B, Cytomegalovirus, Epstein-Barr virus) found in kidney samples of BEN patients, although a causative relation between these infections and BEN development could not be established indisputably.

Familial clustering of BEN suggests a genetic predisposition to the disease. A multifactorial model with a polygenic genetic predisposition was proposed by Toncheva et al. The hypothesis is that environmental factors require a certain genetic background in order to induce the disease development. The genetic predisposition to BEN was studied in many directions. Cytogenetic research showed higher folic-acid-induced chromosomal fragility and more frequent spontaneous chromosomal aberrations in BEN. Previous studies have implicated genes located in cytoband 3q25–3q26, genes coding xenobiotic metabolising enzymes, tumour-suppressor genes and proto-oncogenes. There is evidence that the share of rapid debrisoquine metabolisers is higher in BEN patients than in healthy controls, thus polymorphic variants in CYP2D6 causing sensitivity to various chemical agents are suspected in BEN pathogenesis. Partial LCAT deficiency was also studied in the context of BEN, since LCAT-deficient individuals show evidence of renal tubular injury. Cytogenetic research showed *in vitro* higher folic-acid-induced chromosomal fragility and more frequent spontaneous chromosomal aberrations. Some of the regions expressing fragility contain oncogenes – 1p36–C-SRC, 3p25–RAF1, 3q27–FIM3, 6q23–MYB, 1p13–NRAS, 6p11–KRAS1P.

BEN, with its complex interplay between environment and genetic background, can be explored as a model of polygenic disorder. It presents an excellent case for epigenetic studies and research on the way environment and genetics cooperate to cause disease.

We designed the study as a case-control study based on comparing methylation profiles among BEN patients and healthy controls. We opted for pool analysis, since we wanted to find repeated changes in the methylation pattern that are present in the greater part of the patients. We used DNA extracted from peripheral blood samples by standard phenol-chloroform extraction protocol. In epigenetic studies it is always best to analyse samples from the main affected organ – in our case the kidney. Unfortunately it is not always possible for such samples to be obtained, so it is permissible to use surrogate tissue such as blood. We formed our pools based on ethnic background and gender, since methylation status can differ between sexes and for us it was of interest to explore the different endemic regions separately.

We used an Agilent platform for whole genome methylation analysis which is based on immunoprecipitation (MeDiP) via an antibody against 5-methyl cystosine. Immunoprecipitation, DNA purification, labelling and hybridization were according to Agilent protocol. Slides were processed on Agilent scanner G2505. Data was extracted by Agilent Feature extraction software (v.11.0.1.1) and raw data analysis was performed by Agilent Genomic Workbench Lite (v6.5.0.18). The probe methylation status was assessed by BATMAN assay (Bayesian Tool for Methylation Analysis), a built-in function of the Agilent Genomic Workbench Lite. Software was designed for further analysis and interarray comparison. We defined genomic regions that are differentially methylated between patients and controls and compared them in the different ethnic groups and genders. Thus we defined genomic regions that have the same changes in the methylation pattern. Three genes showed different methylation in all groups. Interestingly, genes from several functional groups proved to be differentially methylated. Moreover different members of these pathways are involved in the different ethnic groups. Still, the significance of a large

percentage of DMRs remains unclear. So it presents a formidable challenge to screen through the experimental data and unequivocally define the importance of differential methylation to disease development. Our data suggests a possible involvement of immune dysregulation in BEN etiopathology.

In conclusion: epigenetics presents vast opportunities to explore disease aetiology. Whole genome methylation analysis platforms allow simultaneous analysis of hundreds and thousands of relevant loci, they present a comprehensive picture of the methylation pattern. The great amount of data that these experiments generate requires multi-step analysis; very often in-house software adjusted to the experimental needs has to be designed. The future evolution of the technology will allow methylation analyses to be conducted with greater precision and with less effort. In the future epigenetics promises to yield the answer to the riddles that many diseases present.

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Резиме

МЕТИЛАЦИОНИ АНАЛИЗИ НА НИВО НА ЦЕЛ ГЕНОМ. ШТО НАУЧИВМЕ ОД СТУДИИТЕ НА БЕН?

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Последниве години се појави интересирање за генетско истражување на хуманиот епигеном. Неговите единствени карактеристики – да биде истовремено стабилен и динамичен – даваат огромни можности за регулирање на функцијата на клетката, одржувајќи и извршувајќи различни видови програми на клетките. Големи напори беа вложени во проучувањето на ДНК метилација во споредба со модификациите на хистонот и студиите на miRNA.

Целите на прегледот е да се фрли малку светлина врз клучните механизми кои владеат со епигенетските карактеристики на човечкиот геном и како епигенетските модели може да се анализираат на скалата на целиот геном.

Овде се претставени главните предности и недостатоци на различни пристапи кон анализата на метилацијата. Овој преглед е врска помеѓу методологијата и некои од нејзините примени во истражувањето на БЕН, како пример на полигенска болест.

Клучни зборови: БЕН, епигенетика, метилација, анализа на цел геном.