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Current Genomics, 2015, *16*, 405-410

Epigenetics of Osteoporosis: Critical Analysis of Epigenetic Epidemiology Studies

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Abstract: Osteoporosis is a systemic disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and propensity to fracture. Environmental factors during early life, including those in utero, may influence bone mass during later life and, consequently, the risk of osteoporosis. Epigenetic mechanisms play central roles in the differentiation of bone cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively. A few studies have shown some differentially methylated genes in patients with osteoporosis. They include genes belonging to the Wnt pathway, which is an important regulator of osteoblast differentiation, and other genes involved in the development of the skeleton. Likewise, some miRNAs may be differentially expressed in these patients. However, those preliminary results need to be replicated in other cohorts. Unlike the genome, the epigenome is cell-specific and changes with aging and environmental factors. Therefore, the design and interpretation of epigenetic epidemiology studies pose a number of practical difficulties. A framework for the critical appraisal of these studies is proposed.

Keywords: Critical appraisal, DNA methylation, Epigenetics, microRNA, miRNA, Osteoporosis.

INTRODUCTION

Osteoporosis is characterized by reduced bone mass and architectural deterioration of bone tissue, which lead to a decreased bone strength and propensity to fractures. It is a prevalent disorder, affecting more than one third of post-menopausal women and 10-16% of men over 60 years of age [1, 2]. Bone mass accumulation starts at the intrauterine life and continuous during the growth period, so that bone mass reaches a peak by the third decade of life in humans. After remaining stable for some years, bone mass starts a progressive decline that accelerates in women during the 5-10 years following the menopause. Thus, osteoporosis may result from an inadequate accumulation of bone during the growth period, and/or from an accelerated loss of bone after the peak bone mass is attained [3, 4].

OSTEOPOROSIS DUE TO AN INADEQUATE PEAK BONE MASS

Osteoporosis is infrequent in young individuals. In fact, even subjects with an inadequate peak bone mass, due to an insufficient accumulation of bone mass during the growth period, rarely suffer osteoporosis-related fragility fractures (ie, fractures occurring in the absence of high-energy trauma). However, these individuals are at high risk for fractures in later life, because the universal age-related decrease of bone mass, superimposed on an already low bone mass, easily lowers it below the fracture threshold [5].

Peak bone mass appears to have a strong genetic component. Thus, direct correlations between the bone mass of parents of various ethnicities and their children have been reported [6, 7]. However, despite large cooperative efforts, the genes explaining the hereditary influence on bone mass have not been completely elucidated. Nevertheless, some genes, including members of the Wnt family, have been consistently identified as associated with bone mass in young individuals [8].

A number of acquired factors have profound influences in the growing skeleton. They include nutrient factors, exercise, comorbid disorders, etc. They may act either in utero or after birth. In fact, there is evidence for a relationship between in utero growth and bone mass during later years [9, 10]. For example, a meta-analysis of observational studies found a clear association between birth weight and bone mass later in life. Birth weight is associated more clearly with bone mineral content (BMC) than with bone mineral density (BMD) [11]. This suggests that the association is preferentially driven by a correlation of birth weight with the future bone size, rather than with bone density. Among the prenatal environmental factors influencing the skeletal mass of the offspring, the maternal vitamin D levels have received much attention [12].

These studies show that intrauterine events influence the skeletal phenotype. However, despite some suggestive data in experimental animals [13], it has not been elucidated to which extent true epigenetic mechanisms explain the influ-

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ence of intrauterine environmental factors on the human skeleton. Nevertheless, a few studies have suggested that the DNA methylation pattern at birth may influence skeletal homeostasis. Thus, some studies reported associations of the methylation of eNOS (an enzyme responsible for the synthesis of nitric oxide) and RXR (retinoid –X receptor) in cord blood with childhood bone mass [14, 15].

OSTEOPOROSIS DUE TO ACCELERATED BONE LOSS

The loss of bone mass is a phenomenon associated with normal aging. It depends on both genetic and environmental factors. Estrogens play a critical role in bone homeostasis, both in women and men. In line with this concept, several studies have shown an association of polymorphisms of the CYP19A1 gene and BMD [16-21]. CYP19A1 encodes aromatase, an enzyme that converts androgenic precursors into estrogens. Aromatase activity in fat, bone and other tissues is the main source of estrogens in men and postmenopausal women [22]. Large collaborative studies have revealed that allelic variants of other genes are also associated with BMD and fractures [23].

As reviewed in other papers in this issue and in previous reviews in the journal [24], epigenetic mechanisms play important roles in the differentiation and activity of bone cells. However, there is only scarce information about how epigenetic marks actually influence the risk of osteoporosis and other skeletal disorders.

We have explored the differences in DNA methylation between osteoporosis and osteoarthritis, two disorders with changes in bone mass in opposite direction. In line with the hypothesis that these disorders have a developmental component, among the genes differentially methylated we found several members of pathways involved in the development of the skeleton [25]. Also, in line with this concept, the methylation status of the promoters of sclerostin and other genes of the Wnt pathway have been suggested to influence the risk of osteoporosis [26, 27]. However, these results need to be replicated in other cohorts before drawing firm conclusions.

Non-coding RNAs are also involved in regulating the differentiation of osteoblasts and osteoclasts, the main players of bone remodeling [24, 28]. In fact, in studies analyzing the abundance of miRNAs in human tissue samples, several miRNAs were found differentially expressed in osteoporosis [29-32]. However, as shown in (Table **1**), the results were not replicated across studies. Therefore, additional investigations are needed to identify which miRNAs are actually involved in the pathogenesis of osteoporosis.

INTERPRETATION OF EPIGENETIC EPIDEMIOLOGY STUDIES

Current technologies allow performing candidate and epigenome-wide studies efficiently. Thus, not unexpectedly, the number of published studies on epigenetic epidemiology is increasing exponentially. However, a number of issues need to be taken into consideration to critically appraise those studies [33, 34]. Following the experiences of other disciplines and some consensus recommendations [35-38], a framework for the interpretation of epigenetic literature is proposed here (Table **2**).

Internal Validity

In order to confirm the internal validity of the study, potential sources of bias should be considered.

Study Subjects

First, the phenotype of the individuals needs to be clearly stated. If the study included diseased subjects, we should examine if the phenotype of the patients is representative of the whole disease spectrum or just of a particular stage or variant of the disorder. It is important to note that, differently from the genome, the epigenome may change with age and with environmental influences. Thus, the age of the individuals and other potential confounders, including previous therapies, need to be considered. On the other hand, if the study compared patients and controls, it is important to check if the control group was adequate for the patient group studied. Features such as age, sex, ethnicity and nutrition and other environmental influences should be comparable between both groups.

Sample Issues

The fact that the epigenome is cell-specific represents a difficulty inherent to epigenomic studies. In other words,

Table 1. Studies exploring miRNAs in patients with osteoporosis.

Author	Ref.	miRNA	Target Genes	Results
Li, 2009	[32]	miR-2861	RUNX2 HDAC5	Mutation causes osteoporosis in young subjects
Wang, 2013	[31]	miR-214	ATF-4	Promotes osteoporosis
Seeliger, 2014	[30]	miR-21 miR-23^{a}-24 miR-100 miR-125b	PDCD4 RUNX2 BMPR2	Differentially expressed in osteoporosis
Garmilla, 2015	[29]	miR-518 miR-187	WISP1 CTNBP1 IL6, TNF	Differentially expressed in osteoporosis

Table 2. Checklist for the critical appraisal of studies of epigenetic epidemiology.

1. Is the study valid?

a. Study subjects issues

- Accurate phenotype definition?
- Age and sex?
- Appropriate disease spectrum?
- Did they receive previous therapy?
- If the study included control individuals, were they appropriate?

b. Tissue (or other sample) issues

- Is the tissue relevant to disease pathophysiology?. If not, were the initial results confirmed in a relevant tissue?
- Was sample cell heterogeneity considered?
- If several tissues are compared, was the control tissue relevant and appropriate?

c. Technology issues and data analysis

- Is the technology properly described?
- How extensive genome coverage was attained?
- Is the technology precise and accurate?
  - Replication with the same procedure (precision)
  - Verification with alternative methods (accuracy)
- Was the analysis pipeline well described and appropriate?
  - Technical controls, batch correction, etc.
  - Statistical analysis. Type I and II errors

2. What are the results?

a. Single locus (CpG, individual miRNAs, etc)

b. Single gene and other genomic regions (promoters, TFBS, CGIs, etc.)

c. Network analysis: Pathways, GSEA, IPA, miRNA families, etc.

d. Functional assays: in silico, *in vitro*, *in vivo*

3. Are the results important and generalizable?

a. Is there a known (or new) biological rationale?

b. Were the results replicated in an independent group of individuals?

c. Was the possibility of reverse causation considered?

d. Are the results of pathogenetic, diagnostic, prognostic or therapeutic importance?

different from genetic sequences, epigenetic marks vary across cells. Hence, studies trying to identify the disease's epigenetic signatures must be performed in relevant tissues. However, getting the appropriate tissue samples may pose ethical and practical problems. Thus, in some cases it may be worthwhile to use accessible tissues or fluids (blood, saliva urine) as proxies in exploratory analyses. Additionally, this approach may provide information useful for utilizing epigenetic marks as biomarkers. Nevertheless, from a pathophysiological perspective, the results must be confirmed in samples of a relevant tissue, such as bone when studying skeletal disorders.

If samples from different individuals are compared, it is important to pay attention to differences in cell composition and the potential influence of this factor should be considered [39]. There are algorithms appropriate to perform cell composition adjustments in studies of DNA methylation of blood samples [40]. However, similar adjustments are more difficult when solid samples are analyzed. Laser-assisted microdisection followed by methods optimized for the analysis of CpG methylation of small number of cells may be an alternative [41]. Finally, in paired studies of diseased and control tissues of the same individual, the control tissue should be carefully selected depending on the study objectives. It is important to consider that not only the disease, but also the host responses may induce changes in tissue composition and epigenetic marks. For example, the epigenetic signature of tumor tissue is frequently compared with that of adjacent tumor-free tissue. However, the latter may have an altered cell composition due to the host immune response and, consequently, it may not be a good representation of the true normal tissue.

Technology and Data Analysis

As in all research papers, the methodology and the details of the technology used should be precisely explained. In particular, in epigenome-wide studies it is important to

ticular, in epigenome-wide studies it is important to consider the coverage attained. There are about 25 million CpGs across the genome. Exploring their methylation level may require costly whole bisulfitome sequencing. Alternative procedures frequently used include reduced representation bisulfite sequencing (RRBS), which explores about 5 million CpGs [42], and the popular Infinium 450K arrays. These represent a convenient and relatively inexpensive method to explore the DNA methylome. However, it is important to realize that these arrays interrogate about 485,000 CpG sites [43]; this is, they just explore about 2% of the potentially methylated CpGs. Thus, many regions remain unexplored. This fact must be considered especially in the case of studies with negative results.

As with other analytical tools, the reproducibility of the technique used can be assessed by including replicate aliquots of some samples that are analyzed several times with the same procedure (technical replicates). On the other hand, the accuracy of the procedure should be confirmed by using a different technique. For instance, data obtained with methylation arrays are commonly verified by using some techniques based on the sequencing of bisulfite-converted DNA, such as pyrosequencing or base specific cleavage and matrix-assisted later desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), the basis of the Epityper^{r} system.

Epigenome-wide analyses are susceptible to various technical biases (batch effects, color biases, etc.). Therefore, quality control issues need to be considered carefully. Some analytical pipelines and software packages to facilitate such analyses have been recently described [44-48].

As in other studies, sample size is an important issue. The statistical power of the study and consequently the probability of type II error (ie, obtaining a negative result when there are true differences between the studied groups) should be considered. Sample size and the variability of the measurement (ie, CpG methylation level, miRNA abundance, etc.) are major factors influencing the statistical power. Epigenome-wide studies explore potential between-group differences at many loci (up to several millions). Therefore, the inflation of type I error (ie, assuming as statistically significant a difference that was found just by chance) is an issue. In order to minimize it, the usual 0.05 threshold for statistically significance is no longer appropriate. It must be lowered down to a new threshold adjusted by the number of comparisons being made. Several methods exists to perform such addjustment, including the Bonferroni procedure, and the less stringent false discovery rate calculation [49].

What Are the Results

Single Locus, Genomic Regions and Networks

As a first step, data analysis is usually focused on the individual unit of analysis, such as the methylation level of single CpGs or the abundance of individual miRNAs. A deeper insight may be obtained by grouping procedures. For DNA methylation, after the analysis of individual CpGs, other common procedures include analyzing CpGs grouped in genomic regions of interest, such as gene bodies, promoter regions, CpG islands, transcription factor binding sites, etc.

Following this exploration, some type of network analysis should usually be performed. Simple pathways analysis and gene set enrichment analysis try to find out if the genes showing a special characteristic (for example, differential methylation between the groups studied) are overrepresented in a given "pathway" in comparison with the overall genome. Other studies try to find relationships between the genes or genomic regions identified as significant in the individual analysis. A number of commercial software packages, as well as other tools freely available on internet (DAVID, GESEA, WebGestalt, EnrichNet, NetworkAnalyst, etc.), help performing pathways and network analyses.

Functional Studies

Functional experiments enhance the conclusions of the study. For example, in a study showing that a gene promoter is more methylated in patients with a disease than in controls, the validity and relevance of this finding would increase if a reduced expression of the gene or repressive histone tail marks are shown in the patient group. In some cases, an in silico validation is appropriate, as available databases can be explored looking for data validating the hypothesis. However, depending on the objectives of the study, *in vitro* experiments may be needed to obtain functional data. They may include, for example, EMSAs and transfections of reporter vectors with methylated and unmethylated inserts, gain-of-function and loss-of-function experiments by transfecting miRNA mimics and antagonists, etc. Finally, *in vivo* experiments may provide the strongest support for the biological relevance of the findings.

Importance and Generalization of the Results

Biological Rationale

New, unexpected and even unexplained findings must not be merely discarded, for they may disclose previously unknown and potentially important information. Nevertheless, a biological rationale adds value to epidemiological findings. Such rationale may result form functional studies and other data in the literature.

Replication

The replication in independent cohorts of individuals is important. On the one hand, it supports the technical validity of the initial results; on the other hand, it confirms that the conclusions can be applied to populations other than that represented in the discovery cohort. Nevertheless, it is important to note that the absence of replication does not necessarily mean that the result in the discovery cohort was spurious. A number of factors, including the genetic background and environmental circumstances, have strong effects on the epigenome (Fig. **1**). Therefore, in some cases epigenetic differences may be observed only when the individuals are exposed to certain environmental factors or have a particular ethnic origin.

Direct and Reverse Causation

The genome is stable from conception. Therefore, the question of reverse causation is not important in genetic studies. However, it is certainly a cause of concern for the interpretation of epigenetic studies. In a study showing dif-

ferent epigenetic marks between a group of patients and a group of controls, we should ask the question whether the epigenetic differences are causing the disease or it is the other way around. In human studies this may be a very difficult to solve question. However, in some situations the comparison of epigenetic signatures in early and late stages of the disease may provide some useful clues.

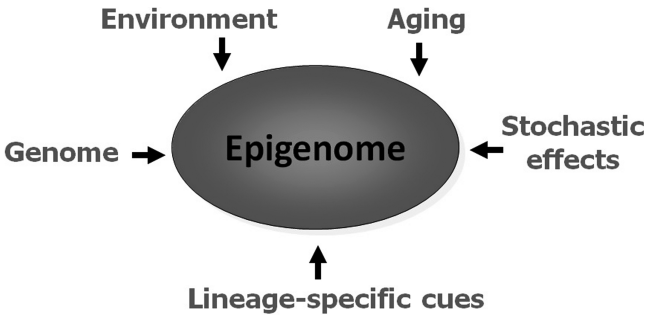

Fig. (1). Factors determining the epigenome.

Scientific and Clinical Relevance

Epigenetic studies are revealing new data that are very important from the scientific point of view, as they provide a better insight into the molecular mechanisms regulating cell differentiation and function. Their importance from a biomedical point of view is even higher if the studies open new windows to elucidate the pathogenesis of the disease, to use new biomarkers for establishing the diagnosis or the prognosis of the disease, and especially, to find therapeutic targets that may lead to more effective and safe treatments.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Supported by a grant from Instituto de Salud Carlos III-European Union FEDER funds (FIS PI 12/0615).

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Received: May 07, 2015 **Revised: June 15, 2015** **Accepted: June 15, 2015**