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FROM EPIGENETICS TO ANTI-DOPING APPLICATION: A NEW TOOL OF DETECTION

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ABSTRACT
Eukaryotic genomes transcribe up to 90% of the genomic DNA but only 1–2% of these transcripts encode for proteins, whereas the vast majority are transcribed as non-coding RNAs (ncRNAs). They are divided into short ncRNA, particularly microRNA (miRNA) and small interference RNA (siRNA), and long ncRNAs. Noteworthy, they are unexpectedly stable since they are protected from degradation through different mechanisms: package in exosomes/microvesicles structures, in apoptotic bodies, in HDL lipoprotein, or by RNA binding proteins. For several years already, biomarkers have been used to detect biological disease; in the last years, a requirement appeared to find some of them to unearth the signs of doping. The potential of ncRNAs as a biological candidate is strongly debated and it seems to have become the right tool in the anti-doping hands. In the recent years, the next-generation sequencing (NGS) technology was used by the World Anti-Doping Agency to draft the athlete biological passport (ABP), measuring the circulating miRNAs and applying these new biomarkers in anti-doping. NGS technology does not require any prior knowledge of ncRNAs, but the limit to employ this biomarker to detect performance-enhancing drug use must consider the intrinsic and extrinsic factors that might affect measurements.
Key words: pbiomarkers, doping, HDL, ncRNA, exosome

Introduction

Epigenetics is the study of those heritable changes in gene expression which do not cause changes in the primary sequence of DNA [1]. The main feature of epigenetic changes is that they can be copied from one generation to another, causing, in the long run, even changes in the phenotype.

Epigenetic changes determine the uniqueness of a particular phenotype through the expression of certain genes and repression of others. It is expressed by means of chromatin modification or by covalent modifications of histones that include acetylation and methylation of lysine residues, acetylation and phosphorylation of tyrosine residues, histidine phosphorylation, etc. The methylation processes may occur even after the replication to work of methyltransferases, causing the silencing or activation of particular genes via, respectively, hypermethylation and hypomethylation [2].

Many regulatory proteins bring epigenetic tags; these cause transcriptional changes of the genome. For example, the family of DNMT enzymes, DNA-methyltranferases (DNA MTase), determine DNA methylation of a cytosine at the 5' position. There are also lysine methyltransferases (HMTase) that add methyl groups to lysine residues in histones.

Other epigenetic regulators of particular importance were found to be the non-coding RNA (ncRNA) acting both at the transcriptional and translational level and play a crucial role in the formation of heterochromatin or silencing of certain genes.

They are divided into:

– short ncRNA, of which the best known are microRNA (miRNA) and small interference RNA (siRNA);

– long ncRNAs (lncRNA).

Several studies show that 90% of the human genome is transcribed, mostly as ncRNAs [3, 4]. The ncRNA are ranked housekeeping and regulatory on the basis of

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their function: the former are always expressed, while the latter are regulators of gene expression. The housekeeping ncRNA are snRNA (small nuclear), mRNA, tRNA, snoRNA (small nucleolar RNA). The regulatory ncRNA, with regard to their size, are classified into longer and short. When the molecule is composed of less than 200 nucleotides, it is called small ncRNA; these include miRNAs, Piwi-associated RNA (piRNAs) and siRNAs. If it is composed of more than 200 nucleotides, it is called long ncRNAs [5–7].

The lncRNA are classified into groups and subgroups, for example sense-RNAs when one or more exons overlap to another one transcribed on the same strand; intronic-RNA when derived from an intron; intergenic-RNA when localized between two genes [8]. Other groups are 3'-UTR associated RNAs that derive from 3'-untranslated regions of protein-coding transcript and TERRA's group, including telomeres, telomeric repeat-containing RNA [9].

These molecules are involved in numerous biological processes and in organizing chromatin to proteins [10, 11]. LncRNAs regulate the modifications of chromatin, transcription and post-transcriptional processes; moreover, they are involved in epigenetic reprogramming of specific sites and in maintaining molecular stoichiometry [12]. The lncRNA are able to remodel chromatin and histones and are also involved in alternative splicing [13].

The lncRNAs are expressed even in the central nervous system where they play an important role in the control of gene expression during development and are involved in the differentiation of neurons and in their destiny [14, 15].

The brain-derived neurotrophic factor (BDNF) is essential for synaptic plasticity, for memory, and for neuronal growth. An anti-BDNF (BDNF-AS) lcnRNA has been discovered that could constitute duplex dsRNA with BDNF mRNA in the brain [16]. Failure in the functionality of BDNF-AS determines the onset of neurite for an increased concentration of BDNF mRNA and protein.

Different studies have shown the involvement of lcnRNA in neurological disorders such as Alzheimer's disease [17], Parkinson's disease, neuroimmunological disorders [18], Huntington's disease, neuro-oncological disease. For this reason, these molecules could be used as targets for the treatment of the above pathologies, and several studies are still being performed in both animals and humans.

The ncRNA

In a classical view, RNA plays the fundamental role of an intermediate stream of genetic information from DNA to the final protein product, while in the light of the latest advances in molecular biology, aimed at understanding the cell system, profound revisions of this way of thinking are necessary. Historically, RNA has been characterized and formally divided into different classes on the basis of different properties and functions: the messenger RNA (mRNA) undergoes the process of translation by the ribosome for protein synthesis; the ribosomal RNA (rRNA) is essential for the assembly of ribosomal proteins, therefore playing a structural function, and for the link with mRNA; the transfer RNA (tRNA) is involved in the process of protein synthesis as transporting into the A site of the ribosome amino acids to be incorporated into the nascent polypeptide chain. Actually, in the recent years, a number of additional biological functions of RNA have been identified, not only in structural quality and as molecules with catalytic activity, but also – and especially – as regulators of gene expression in various cellular processes, like differentiation, development, or oncogenesis.

Less than 1.5% of the mammalian genome encodes proteins, about 5% is preserved, and recent large-scale studies have shown that most of the genome is transcribed in a complex repertoire of overlapping transcripts, often from both strands of DNA.

A significant part of these transcripts are processed in small and long RNA regulators [19], acting through a specific recognition sequence of other RNA or DNA to promote the development of several programs by modulating the structure of RNA, splicing and stability, transcription and translation, as well as chromatin structure [20].

The lncRNA

There are tens of thousands of lncRNAs, polyadenylated and non-transcribed from the mammalian genome [21, 22], many of which appear to be regulated during development, subjected, alternatively spliced, inducible under physiological conditions [23, 24].

A set of these lncRNAs is defined as macroRNA, transcribed by long non-coding regions expressed in mice; many of them are enriched in the brain, they have transcription antisense, and seem localized mainly in the nucleus [25].

It is known that thousands of lncRNAs are encoded in the human genome, expressed mostly in tissue-specific patterns, and pairing with DNA, other RNAs and proteins with a various mechanisms of action [26].

Many classes of ncRNAs are involved in the development of dendrites, transport and localization of mRNA, and local protein synthesis [27]. The central component of these processes is a protein that binds RNA, fragile X mental retardation protein (FMRP), the absence of which causes mental retardation, epilepsy, autism, and disorders related to anxiety. FMRP is part of an elaborate ribonucleoprotein complex which also includes FXR1P/2P, nucleolin, YB1/p50, Pur**α**, Staufen, IMP1 and kinesin 5, and ncRNA BC1 and BC200, preserved in the mouse and in humans.

In *Drosophila*, lncRNAs are involved in many stages of organogenesis and cell differentiation; showing a rapid

evolution, they are expressed during embryogenesis and are highly specific for the central and peripheral nervous system [23].

In mammals, lncRNAs also come from natural antisense transcripts [28], many of which are abundant in the nervous system, with expression profiles defined in the development and in different areas, and modulate the expression of genes involved in the morphogenesis of the brain, the proliferation of stem cells, stress response, cell polarity, cytoskeletal functions, neuronal survival, maturation, and synaptic plasticity [29].

Finally, lncRNAs are able to modulate mRNA levels by competing for miRNA binding [30].

The miRNA

These are fragments of about 21–25 nucleotides of single-stranded ncRNAs; they are able to repress the translation or to cause degradation of the target mRNA or can regulate the gene expression at the post-transcriptional level. Each miRNA can regulate different genes. It is known to be involved in the regulation of various cell functions and their alterations are implicated in various pathological processes. The term 'micro RNA' was introduced in 2001 [31]. Thousands of miRNAs have been identified in humans and other species [32], and several databases which could be consulted now exist. Biogenesis of miRNA is a process that starts in the nucleus with the transcription by RNA polymerase II to form long primary transcripts called 'primiRNAs' that are capped (5'end) and polyadenylated (3'end), approximately of 70 nucleotides with the typical hairpin structure. PrimiRNAs are processed by a complex containing RNAse endonuclease III Drosha and DGCR8 (DiGeorge syndrome critical region gene 8). By processing, a 60–120-nucleotide long miRNA precursor (premiRNA) was obtained. Later, it is transferred from the nucleus to the cytoplasm by exportin-5 in the presence of a GTP-binding nuclear protein Ran (RanGTP). There, the final cleavage was made by the RNAse III enzyme Dicer that interacts with the 5'- and 3'ends of the hairpin and cuts the extremities, eliminating the stem-loop and producing a mature double stranded miRNA of about 22 nucleotides. Finally, one strand is normally deleted and the other is incorporated into the RNA-induced silencing complex (RISC). Here, miRNAs interact selectively with Argonaute proteins, a riboproteic complex responsible for the selection process of the filament guide and for the degradation of the complementary strand. Finally, within RISC, the mature single-stranded miRNA interacts with the target mRNA, most frequently with the 3' untranslated region [33–35]. The function of the miRNA is obtained when it finds its cognate RNA and pairs with it. This will involve a different action, depending on the degree of complementarity. In plants, there is a perfect pairing between miRNA and mRNA that promotes the degradation of the messenger; in animals, in turn, the pairing is imperfect and involves few nucleotides: 2–7 or 2–8 of the miRNA [36]; this can lead to mRNA cutting, degradation, or repression of translation. The miRNAs are able to bind not only 3'UTR of their target transcripts, but also other regions, like 5'UTR, promoter, and open reading frames [37]. Another important effect of the action of miRNAs is that one mRNA can be modulated by numerous miRNAs, suggesting the possibility of miRNA cooperative repression. The miRNAs regulate up to one-third of human genes participating in various biological processes, like cell proliferation, differentiation, cell cycle regulation, apoptosis, hematopoiesis, and hypoxia. The alterations of the normal pathway of miRNA expression may have consequences for normal cell physiology and lead to various types of diseases, including inflammatory conditions [35].

The siRNA

The siRNA is considered a double-stranded RNA of about 21–22 nucleotides, previously thought to be of exogenous origin, for example viruses. It typically binds perfectly to its mRNA target; it is a perfect match to the sequence like previously described in plants, and leads to mRNA degradation. The siRNA protects the integrity of the genome from nucleic acids strangers or invasion, and can be generated from long dsRNA molecules resulting from the replication of RNA viruses (vsiRNAs) [38] or from mobile transposable elements (endo-siRNAs) [39–42], or may be produced synthetically by transgenes expressing dsRNA molecules.

The piRNA

A major study is taking place on piRNA (Piwi-associated RNA) or non-coding strands of RNA consisting of 24–30 nucleotides, which, like miRNAs, interact with a different class of Argonaute proteins, Piwi, and, for their biogenesis, are independent of the Dicer. The piRNA primarily derives from transposons and other repetitive elements. Genetic studies indicate that piRNAs are very important in the development of the germ line, and proteins involved in the production of piRNA are employed in the regulation of gene expression in somatic cells, in learning and memory, suggesting that piRNA could have a very important role in different biological processes.

The altered expression of miRNAs is sometimes due to chromosomal rearrangements or epigenetic events, so it is essential to study miRNAs in the context of their genomic location so that one can find correlations between their aberrant expression and a specific disease [43, 44]. There are a number of activities underway to map all the miRNA and piRNA genes at fragile sites, in areas where there are repetitive elements and in CpG islands and SNP, to see the effect of the two groups of ncRNA in fragile sites and similarities or differences

in their distribution in the individual human chromosomes. Simultaneously, there is an analysis in progress to analyse their distribution in the human genome, and a public database is now present [45].

The circRNA

RNA versatility seems limitless, the latest surprise being the circular RNA (circRNA), which works by counteracting miRNAs; inhibition by miRNAs of the translation can be suppressed by circRNA that are able to bind many copies of a miRNA, allowing translation of mRNA. The circRNAs are very stable, quite conserved, and their genes are widely represented in the genome [46]; indeed, their number exceeds that of protein-coding genes. They are derived from the splicing of an exon and the subsequent fusion of the 5' with its 3' or that of a further upstream exon. The fact of being circular gives it a high stability because having no free ends, they cannot be degraded by the exonuclease [47, 48].

The circRNAs serve as reservoirs of miRNAs that bind with greater affinity than the mRNA, so they were defined as miRNA sponge [49]. Moreover, recent study hypothesize that they can also bind different proteins (RBP ribonucleoproteins) and sequester them in the cytoplasm [48]; prevent them from acting, perhaps provide a protective reservoir of these molecules; deliver factors to particular subcellular locations; or act as scaffolds for the assembly of other complexes [50].

Circulating miRNA

While the biogenesis of miRNA is an intracellular process, it is now clear that these molecules are released from cells in the environment and can be internalized from recipient cells where they can alter gene expression either in physiological or in pathological conditions [51].

The extracellular miRNAs represent a new form of intercellular communication through the transfer of genetic information. They are found in many biological fluids, such as plasma, serum, saliva, urine, tears, breast milk [52–55].

The extracellular miRNAs are unexpectedly stable; they must be protected from degradation because the naked RNA is readily targeted by exonuclease, abundantly present in many extracellular fluids. So, miRNAs are packed with different mechanisms: exosomes/microvesicles (MVs), apoptotic bodies, RNA binding proteins, HDL, and lesser, LDL [52].

Exosomes are extracellular vesicles (40–120 nm) that originate from multi-vesicular bodies (MVBs) and are released by exocytosis of these MVBs. They are produced by many cell types, have been identified in many circulating fluids, and may serve as a carrier of miRNAs [56].

MVs, another form of vesicles, are larger than exosomes (100 nm–1 μm) and derive from the plasma membrane of a large variety of cells. The presence of miRNAs in the MVs was described in 2008 [57].

Exosomes and MVs are now recognized as a fundamental way for cell-to-cell horizontal transfer of properties, in both physiological and pathological conditions. Cancer cells use extracellular vesicles (EV) to discard molecules which could be dangerous to them (e.g. differentiation-inducing proteins such as histone H1.0, or antitumor drugs [58]), to transfer molecules which, after entering the recipient cells, are able to transform their phenotype, and even to secrete factors (i.e. RNA, histones, RBPs) which allow escaping from immune surveillance [59].

Apoptotic cells release extracellular membrane vesicles into the environment; apoptotic bodies are large particles (1–5 μm) with a heterogeneous form. For instance, in atherosclerosis, endothelial cells produce apoptotic bodies enriched in mir-126 and containing other minor miRNAs. This brings the acceptor cells to produce a chemokine which limits atherosclerosis, leads to plaque stability, and causes recruitment and proliferation of endothelial progenitor cells [60].

A significant fraction of miRNAs is associated with extracellular proteins that bind RNA and protect it from degradation, like the family of Argonaute proteins, Ago 2 [61].

Finally, extracellular miRNA can also be carried by HDL and, in lesser amounts, by LDL, participating in the mechanism of intercellular communication [52, 62, 63].

Biomarkers for detection of doping in sport

For several years already, biomarkers have been used to detect biological disease; in the last years, a requirement appeared to find some of them to unearth the signs of doping. The potential of ncRNAs as a biological candidate is strongly debated and it seems to have become the right tool in the anti-doping hands. Although the way to act is similar, some may have effect on the expression of any genes by inducing interference, some other – on the degradation of mRNA (e.g. siRNA) [64]. An example of RNA interference aiming at performance enhancement in elite sports is the block of the myostatin gene: it has been demonstrated in mice that the latter is a muscle-growth inhibitor [65] and its down-regulation by an antisense oligonucleotide results in an increased growth of the leg muscle [66]. If myostatin is a negative regulator of proliferation, insulin growth factor-1 (IGF-1) induces satellite cells proliferation and is stimulated from growth hormone (GH), another widespread doping agent. Since the real problem is to discriminate between endogenous and exogenous GH, it is used to detect the abuse by an indirect method based on the evaluation of IGF-1 level [67]. All the strategies applied to characterize siRNA in biological fluids are comparable to an experimental 'RNomics' approach, which may help as

a potential link between genome and proteome in the next future [68]. Also called antagomirs, they represent a new strategy that could play an important role in doping control. The antagomirs block the action of endogenous miRNA, increasing the expression of certain target genes. One of the most prominent examples is represented by the myostatin gene, which currently has the highest potential for misuse in sport, given the numerous investigations in drug research. The downregulation of this gene has been shown to induce an improvement in performance and an increase in muscle mass [69].

Although most RNA molecules are unstable, circulating miRNAs are highly stable, probably because they are protected in exosomes and microparticles, and readily detectable in body fluids such as urine, saliva, and plasma [70, 71]; alternatively, circulating miRNAs may be associated with specific protective proteins. Many advanced technologies have to setup to miRNA-profiling and to detect any change in concentration [54]. The most widely used approach is the quantitative reverse transcription (qRT)-PCR, which remains the most sensitive and reliable method for detecting circulating miRNAs. Usually this method needs to validate the results from the first screening performed by microarray. Most of the existing tools (e.g., qRT-PCR and microarray) offer high sensitivity and specificity in ncRNA research: they are able to accurately detect a wide spectrum of selected ncRNAs and require prior knowledge of the sequence of interest to design primers. Among the various methods available to isolate exosomes before performing downstream analysis such as microarrays or qRT-PCR, the most innovative is the next-generation sequencing (NGS). The NGS technology does not require any prior knowledge of ncRNAs and thus it allows all ncRNA species to be sequenced in a high throughput manner [72]. In the last years, this approach was applied to draft the athlete biological passport (ABP), measuring the circulating miRNAs with the use of these new biomarkers in anti-doping [73]. The limit to employ this biomarker to detect performance-enhancing drug use must consider the intrinsic and extrinsic factors that might affect measurements. In addition, the influence of confounding factors such as gender, age, high altitude, or physical exercise remains to be determined [74]. In fact, a study reported the circulating miRNAs as biomarkers of exogenous testosterone administration; it showed a variation in the expression of two of the miRNAs analysed (miR-150 and miR-342, mainly expressed in white blood cells, and miR-150, particularly abundant in mature B and T lymphocytes) but when compared with the control samples, the change was testosterone-independent [75]. Thus, changes are not determinable and seem to be subject-dependent; this highlights a significant weakness of the method. With regard to one of the most widespread doping practices, blood doping, it represents one of the most effective methods used to improve the athletic performance in aerobic sports. The WADA has developed strategies for their detection methods based on both direct and indirect way. The direct method mainly concerns the identification of the use of recombinant human erythropoietin (rHuEPO), which stimulates red blood cell production. The rHuEPO detection methods currently exist, including measurement of hematologic parameters, use of peptide markers, isoelectric focusing (IEF) double immunoblotting, aptamer/antibody based methods [76]. The indirect methods are used in order to highlight e.g. the autologous blood transfusion. Schumacher and Pottgiesser [77] already revealed that the haemoglobin concentration had a significant variability, dependent on the different phases of the day or the exercise performed [78]. This is the principle of the ABP, which represents a milestone in the doping control. Actually other approaches of indirect methods are based on OMICS-sciences (in which ncRNA transcriptomics are the landmark) or on the detection of metabolites in urine samples, for example the 2-ethylhexylphthalate, a metabolite excreted as a result of autologous blood transfusion [79].

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