Seminars in Cell & Developmental Biology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Seminars in Cell & Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Non-coding RNAs as a new dawn in tumor diagnosis

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ARTICLE INFO

Article history:
Received 23 May 2017
Received in revised form 19 July 2017
Accepted 21 July 2017
Available online xxx

Keywords:
Non-coding RNA
Biomarker
Cancer
Diagnosis
Exosome
Delivery

ABSTRACT

The current knowledge about non-coding RNAs (ncRNAs) as important regulators of gene expression in both physiological and pathological conditions, has been the main engine for the design of innovative platforms to finalize the pharmacological application of ncRNAs as either therapeutic tools or as molecular biomarkers in cancer. Biochemical alterations of cancer cells are, in fact, largely supported by ncRNA disregulation in the tumor site, which, in turn, reflects the cancer-associated specific modification of circulating ncRNA expression pattern. The aim of this review is to describe the state of the art of pre-clinical and clinical studies that analyze the involvement of miRNAs and lncRNAs in cancer-related processes, such as proliferation, invasion and metastases, giving emphasis to their functional role. A central node of our work has been also the examination of advantages and criticisms correlated with the clinical use of ncRNAs, taking into account the pressing need to refine the profiling methods aimed at identify novel diagnostic and prognostic markers and the request to optimize the delivery of such nucleic acids for a therapeutic use in an imminent future.

Contents

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http://dx.doi.org/10.1016/j.semcdb.2017.07.035
1084-9521/

1. Introduction

In the last years it has become increasingly clear that the mammalian transcriptome is extremely complex due to the inclusion of a large number of small non-coding RNAs (sncRNAs) and long noncoding RNAs (lncRNAs). Non-coding RNAs (ncRNAs) are a heterogeneous class of transcribed RNA molecules from non-(protein)-coding regions, which lack an open reading frame and consequently have no apparent protein-coding ability. Based on the size of the functional RNA molecule, regulatory ncRNAs are classified analytically as long ncRNAs (lncRNAs, >200 nt), and small ncRNAs (sncRNAs, 18–200 nt) [1–3].

RNA biomolecules have been identified since the late 1800s, but their fundamental roles in cell functions have long been overshadowed by DNA and proteins. From 1950s, with the clarification of the molecular structure of DNA, it was proposed that RNA would be an intermediate molecule in the flow of genetic information from DNA to proteins, as assured in the central dogma of molecular biology. On the basis of the accepted importance of proteins in exerting biological functions, RNAs have been regarded for a long time as merely mediators in passing genetic codes to final protein molecules. As a consequence, the functional activities of RNAs themselves were largely neglected. Ribosome RNAs (rRNA) and transfer RNAs (tRNA) are among the early-discovered non-coding RNA transcripts. However, given their roles in facilitating protein translation, they are still considered part of the machinery translating genomic code into protein synthesis. Nonetheless, the discovery of these ncRNAs opened the field of regulatory RNAs with no or little protein-coding potential. Since then many new classes of regulatory non-coding RNAs have been identified and remarkable progresses have been made in elucidating their expression, biogenesis, mechanisms and function in many, if not all, biological processes, including cancer development and progression [4,5].

The idea that RNAs are much more than molecules involved in storage/transfer of information emerged since the discovery of ribozymes, endowed with active roles as catalysts of chemical reactions in cells. Indeed, RNA has been suggested to be the earliest molecule of life and thought to possess both informational and catalytic function [6–8]. These discoveries clearly encouraged a variety of studies to search for potential new roles of RNA molecules *in vivo*, and led to the re-evaluation of RNAs as crucial molecules in the evolution of life. Current advances on the sequencing technologies revealed that vast majority of the human genome is transcribed, while the protein-coding genes occupy only about 3% of the human genome [9]. Therefore, the widespread transcription of the genome into non-protein-coding RNAs strongly imply that RNAs are capable of process functions other than mere mediators between DNA and proteins, and emerging evidences from the last two decades have unambiguously proved the functional importance of non-coding RNAs in human biology and diseases. As a result, the involvement of RNAs in other critical molecular processes in eukaryotic cells was progressively revealed, as in the case of DNA replication, protein translation and RNA transcript maturation. With time, many small non-coding RNA molecules were isolated and characterized; among the first, U1, U2, U4, U5 and U6 small nuclear RNA (snRNA) as fundamental parts of ribonucleoproteic complexes (RNP), later identified as the components of the splicesome [10]. Thereafter, it was showed that RNA editing mechanisms, based on protein or protein-RNA complexes and regulating the information content of tRNA, rRNA and mRNA molecules, require a "guide RNA" molecule, which, through base-pairing with the target RNA molecule, determines the editing site [11]. In addition, post-transcriptional processing and modifications of rRNAs, essential for the production of efficient ribosomes, is directed by two large guide families of small nucleolar RNAs (snoRNA) [12].

In the wake of these findings, in the mid-1980s Blackburn and Greider came to the discovery of telomerase by demonstrating the existence of an enzymatic activity within cell extracts inserting tandem hexanucleotides to chromosome ends [13,14]. More recently it was hypothesized that telomerase arise by the association of an ancient ribozyme with the reverse-transcriptase subunit, showing a mechanism resembling that of pure ribozymes, and placing the telomerase as a missing link in the evolution from RNA enzymes to protein enzymes [15].

Already at this point, the growing descriptions of the importance of RNA molecules for cell life started to push them to public and scientific interest, but the complexity of their roles and the wide multiplicity of molecular mechanisms in which RNA molecules are critical players was still far from clear. The discovery of microRNAs (miRNAs) in early 1990s opened a new chapter of gene regulation by non-coding RNAs and represented a crucial boost for investigations on the RNA molecules not coding for proteins [16]. Concurrently, the observations that exogenously introduced double stranded RNA (dsRNA) and plasmids expressing short hair-pin RNA (shRNA) specifically base-pairing with target mRNA molecules were able to trigger mRNA degradation (RNA interference, RNAi) revealed, for the first time, that specific silencing pathways based on sncRNAs are operating in eukaryotic cells [17,18]. These observations led to the development of the RNA interference (RNAi) technique that has been extensively used in the study of gene function [19,20].

As mentioned before, in recent years the use of genome wide approaches and the large output of genome sequencing technologies have revealed that the mammalian transcriptome is much more complex than previously hypothesized, since it includes a large number of small non-coding RNAs (sncRNAs) and long non-coding RNAs (lncRNAs) [21,22]. miRNAs are an abundant class of endogenous non-coding small RNA molecules, 20÷25 nucleotides in length, which act as either oncogenes or tumor suppressors genes and thus have crucial roles in carcinogenesis [23,24]. Different other types of small non-coding RNAs have also been subsequently identified, including endogenous small interfering RNAs (endo-siRNAs), PIWI-associated small RNAs (piRNAs), small nucleolar RNAs (snoRNAs), sno-derived RNAs (sdRNAs), transcription initiation RNAs (tiRNAs), miRNA-offset RNAs (moRNAs), and others [25]. Conversely, the lncRNAs family contains multiple classes of RNAs, which are nuclear RNAs transcripts longer than 200 nucleotides, involved in the regulation of cellular processes such as apoptosis, proliferation and metastases development, thereby emerging as important regulators in a wide range of biological activities and human diseases [26]. Currently, a systematic classification of long non-coding RNA is missing; nevertheless, according to their genomic localization or other biological features, they are classified as natural antisense transcripts, long intergenic non-coding RNAs, transcribed ultraconserved regions (T-UCRs), circular RNAs, enhancer-associated RNAs, promoter-associated RNAs, and others [27]. Similar to miRNAs, the dysregulation of lncRNAs is associated with many human cancers and defines their phenotypes [28]. The expression profiling of both lncRNA and miRNA is deeply different depending upon both the histological type of the tissues and pathologic/not pathologic conditions as their expression is different in cancer tissues if compared to normal counterparts. Therefore, lncRNAs and sncRNAs, by means of their ability in post-transcriptional regulation of gene expression and in target gene translation, may become useful non-invasive diagnostic biomarkers and powerful tools in cancer prevention and treatment. Indeed, several emerging evidences have revealed for both lncRNAs and sncRNAs a close correlation with cancer development and progression, so that some ncRNAs have already been used as biomarkers and targets in cancer management, for diagnosis and targeted therapy, respectively [29]. Hence, the discovery of the biological functions related to ncRNAs

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have undoubtedly animated the scientific community and stimulated biomedical studies that are nowadays changing the way for cancer diagnosis and treatment.

2. Biogenesis and functional role of non-coding RNAs

The paradox that less than about 2% of the total human genome is recruited for protein expression, can be partly explained by the increase in diversity and functionality of the human proteome achieved through alternative pre-mRNA splicing, as well as through post-translational modifications of proteins [30]. In recent years, it has increasingly become more evident that the non protein-coding portion of the genome is of critical functional relevance in several mechanisms of gene regulation, both for normal development and physiology, and for human diseases [31]. In particular, the discovery that, in complex organisms many transcribed genome sequences are developmental- and tissue-regulated, has stimulated investigations aimed at the characterization of all the different types of non-coding regions, originating non-coding RNAs [32].

Depending on the ncRNA category, transcription can be driven by any of the three RNA polymerases (RNA Pol I, II, or III). Among sncRNAs, short interfering RNAs (siRNAs), miRNAs and piRNAs have been extensively studied so far and have been associated with pathways that lead ultimately to silencing of specific genes resulting in the protection of the cell/genome against viruses, mobile repetitive DNA sequences, retro-elements and transposons [33].

siRNAs and miRNAs (~20÷30 nucleotides long) derive from double-stranded RNA (dsRNA) precursors that are introduced into cells, or produced endogenously, by gene transcription of both sense and anti-sense DNA strands and of pseudogenes and inverted repeats. These molecules are critical in pathways engaged in mRNA degradation and translational repression, thereby regulating gene expression. In particular, miRNAs are constituted by about 22 nt and regulate hundreds to thousands of protein-coding and non-coding genes by post-translational gene silencing. It is believed that the human genome encodes thousands of miRNAs, but only 1100 have been described so far [34]. A number of evidence has revealed up- and/or down-modulation of miRNAs in human neoplasms, suggesting that miRNAs can act as canonical tumor suppressors and/or promoters [23–25]. Thus, miRNA expression profiles have been determined and employed for disease prognostication and early diagnosis. Furthermore, most recently, polymorphisms in both miRNAs and their binding sites have been related to pharmacogenomic differences that could explain changes in drug activation and metabolism [35]. More in detail, siRNAs are small RNA duplex molecules produced by the action of Dicer, a ribonuclease III (RNase III) enzyme that creates RNA duplexes with 2-nt overhangs at their 3′ ends and phosphate groups at their 5′ ends [36]. Conversely, miRNAs are mostly transcribed by RNA polymerase II as primary-miRNA (pri-miRNA) molecule precursors equipped with a characteristic stem loop structure, and are subsequently subjected to processing mechanisms [37].

In animals, the first step occurs in the nucleus where the RNase III Drosha acts over pri-mRNAs (long several hundreds nt still bound to the generating DNA) originating a pre-miRNA, a small RNA duplex of ~65÷70 nucleotides containing the hair pin. This step can be assisted by RNA processing proteins, such as hnRNP A1. The pre-miRNAs are then exported by a nuclear transport receptor complex, exportin-5-RanGTP, to the cytoplasm where they are processed by Dicer into ~22-nt mature miRNAs (miRNA-miRNA* duplexes, where miRNA is the antisense, or guide/mature strand, and miRNA* is the sense, or passenger strand) [38]. The next step, for both siRNA and miRNA production, is the subsequent association with members of the Argonaute protein family that diverge into specialized subfamilies, each recognizing different sncRNA types and conferring the specific features of the various silencing pathways operating in cells [39]. Argonaute loading occurs in the RNA-induced silencing complex (RISC)-loading complex, a ternary complex that consists of an Argonaute protein, Dicer and a dsRNA-binding protein (known as TRBP in humans). During loading, the non-guide strand is cleaved by an Argonaute protein. The selection of the different Argonaute proteins seems to be based on the small interfering RNA duplex structure [40]. The maturation and final function of certain miRNAs can be also associated to enzymatic post-transcriptional modifications, like mono-uridylation [41]. These modifications will increase the variety of miRNAs and their precursor pools allowing more complex schemes of regulation in different backgrounds. In both siRNAs and miRNAs the guide strands drives the RISCs to the target mRNAs that contain complementary sequences thereby causing their degradation or translation inhibition [42]. It has to be underlined that in humans and mammalians miRNAs are not able to induce degradation of the target mRNAs but they cause only its translational repression. In fact, in mammals they assemble only at the open reading frame at the 5′ of the mRNA target with partial complementarity inducing the lock of the translation of the target. In the lower animals and in plants, miRNAs can also bind to the target with a complete complementarity inducing its degradation by a RNAse named slicer. This mode of action of miRNAs in humans has an important effect: the amplification of the biological effects induced by a single miRNA due to the inhibition of multiple target mRNAs. This makes miRNA potent regulators of the cellular functions and potential useful targets in cancer therapy [23,24,34].

The piRNA is the largest class of small non-coding RNA molecules expressed in animal cells. The piRNAs (~24÷31 nucleotides), whose name derives from the ability to associate exclusively to the PIWI subfamily of the Argonaute protein family (Piwi proteins), usually have a uridine at the 5′ end, hold a 5′ monophosphate, and present a 2′-*O*-methyl (2′-*O*-Me) modification on the nucleotide at the 3′ end [43]. Compared to siRNAs and miRNAs, widely expressed in different tissues and cell types, piRNAs lack of sequence conservation and increased complexity, and have been essentially detected in germ cells of mammals, fish and *Drosophila melanogaster* [44]. This class of sncRNAs has been linked to both epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells, particularly those in spermatogenesis. Indeed, mutations that disrupt the piRNA biogenesis pathway are related to germline-specific cell death, and are also associated with increased transposon expression [45]. Although piRNAs biogenesis and transcription regulation still need to be clarified, it is now well documented that their origin differs from siRNAs and miRNAs, because piRNA are generated by RNase III-independent pathways without the involvement dsRNA precursors. In fact, these sncRNAs are generated from long single-stranded precursors that are preferentially cleaved at U residues and loaded onto Piwi proteins. In mammals, the majority of piRNAs are transcribed from discrete genomic *loci* that are clustered in large pericentromeric or subtelomeric domains, generally spanning from 100÷100 kb, that comprise mainly various transposable DNA elements and their remnants [46]. Interestingly, other studies have revealed that, besides being involved in keeping genome integrity, a subset of piRNA genes have been implicated in the assembly of the telomere protection complex. Recent investigations have further shown that, in addition to their role in germ line transposon regulation and genome stability, piRNAs have a broader function in heterochromatin formation and developmental gene regulation [47].

As previously discussed, the other prominent class of ncRNAs making up a wide portion of the mammalian non-coding transcriptome is represented by lncRNAs. The number of gene members

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integrating this class of ncRNAs is still under debate and ranges from 10,000 to >200,000.

Different mechanisms of transcriptional regulation of lncRNAs-mediated gene expression have been proposed. Among them, lncRNAs are well-known to be involved in epigenetic DNA modifications through the recruitment of chromatin remodelling complexes to specific loci [48,49].

Biogenesis of lncRNAs is relatively complex. Generally, lncRNA transcription and processing is extremely comparable to protein-coding RNAs. Although some lncRNAs could be transcribed by polymerase III, the majority of lncRNAs are transcribed by RNA polymerase II from intergenic regions, promoter regions or interleaved, overlapping or antisense to annotated protein-coding genes [50]. Moreover, there are growing evidences that lncRNAs molecules might be also produced by transcriptional active pseudogenes and, interestingly, it was demonstrated that some lncRNAs can be as well generated from mitochondrial genes [51]. Like coding genes, lncRNAs undergo post-transcriptional processing, including 5′capping, alternative splicing, RNA editing, and polyadenylation. The referred transcriptional origins have been used to establish classification classes for lncRNA, as for example promoter-associated long RNAs (lpaRNAs), natural antisense transcripts (NATs) or opposite-strand transcripts, large intervening noncoding RNA (lincRNA), and enhancer associated RNAs (eRNA) [52,53]. However, other criteria should probably be used since frequently one lncRNA molecule can be associated with more than one class. Interestingly, several lncRNAs are antisense transcripts, also named natural antisense transcripts (NATs) [54]. NATs can be classified in two subtypes: *cis*-NATs, which are transcribed from opposite DNA strands at the same genomic loci and *trans*-NATs, which are transcribed from distal loci [55]. Interestingly, numerous cancer genes, mainly of oncosuppressor type, produce long antisense ncRNAs, and despite mRNA and structural ncRNAs, many lncRNAs are situated in the nucleus, some others are localized in both cytoplasm and nucleus and others are exclusively located in cytoplasm [56].

3. Mechanism and clinical relevance of exosome-mediated miRNA secretion

About 15 years after the first identification of miRNAs, it has been discovered that they were detectable in body fluids encapsulated in lipid microvesicles [57]. Extracellular vesicles (EVs), characterized according to the size into exosomes (<100 nm), microvesicles (1000 nm) and apoptotic bodies (1–4 μm) [58], originate from cells and are able of transferring miRNAs, mRNAs and proteins in both paracrine (connecting cells belonging to the same tissue) and endocrine (to distant target cells) manner [58]. In addition to EV, circulating miRNAs can be also loaded into high-density lipoprotein (HDL) [59,60], or bound in a protein complex composed of Argonaute (AGO) family members outside of vesicles [61]. Collectively, each of these mechanisms is able to protect miRNAs from degradation ensuring their stability, since the naked RNA would be readily targeted by the endonucleases that are abundantly present in extracellular fluids. Among these mechanisms, exosomes have been defined as crucial mediators of the intercellular communication and promising vehicles for miRNA delivery and gene therapy. In fact, accumulating evidence suggest that miRNAs play an important role in exchanging information between cells and, therefore, they may serve as new and potentially useful markers and powerful targets for therapeutic interventions against various human diseases. The most abundant components of exosome membranes are represented by lipids and proteins [62–64], given their derivation from plasma membrane. As shown in Fig. 1, exosomes are firstly produced by endocytosis, when the cellular membrane is internalized to produce endosomes; thereafter, the invagination of endosome membranes originates many small vescicles within the same endosome, which is thus named multivescicular body (MVB). MVBs are then released from the cell into the extracellular space by fusing with the cell membrane, leading to the formation of exosomes [65]. All these steps of formation and secretion require the involvement of enzymes [66,67] and ATP [68]. Within the set of regulatory molecules implicated in the secretion phase, there are Rab27a and Rab27b, whose knockdown has been correlated with the inhibition of this process in HeLa cells [69]. Furthermore, the oncosupressor protein p53 and its downstream effector TSAP6 are also involved in this regulatory network being able to stimulate exosome production [70].

Concerning miRNA sorting into exosomes, current literature has described at least four potential mechanisms. The first mode of sorting to be discovered is based on the neural sphingomyelinase 2 (nSMase2)-dependent pathway; overexpression of nSMase2 was, in fact, put in correlation with the increased number of exosomal miRNAs and, on the other hand, the inhibition of nSMase2 was associated with the reduction of exosomal miRNA levels [67]. The second mechanisms, discovered by Villarroya-Beltri et al., view the involvement of the GGAG miRNA motif (in the 30 portion of miRNA sequence) that can be recognized by sumoylated heterogeneous nuclear ribonucleoproteins (hnRNPs) responsible for the specific packaging of miRNAs into exosomes [71]. The 30-end miRNA sequence-dependent pathway is the third mode of sorting, described by Koppers-Lalic et al.; in detail, 30 ends of uridylated miRNAs were mostly recognized in exosomes derived from B cells or urine, whereas the 30 ends of adenylated endogenous miRNAs were principally found in B cells [72]. The fourth mechanism involves the miRNA induced silencing complex (miRISC). In detail, miRISCs co-localize with the sites of exosome biogenesis (MVBs) and their components, such as AGO2 protein (which preferentially binds to U or A at the 50 end of miRNAs) and mRNA target, are also correlated with sorting of miRNAs into exosomes [73].

Exosomes present in body fluids can interact with recipient cells through three main mechanisms: i) the direct interaction between transmembrane proteins of exosomes and the signaling receptors of target cells [74]; ii) the exosomes fusion with the cellular membrane of recipient cells with the following delivery to the cytosol [75]; iii) the internalization of exosomes within the recipient cells. Subsequently, the fate of exosomes may follow two alternative routes: i) fusion with endosomes and transcytosis, with following release of the content, represented by miRNA, within the recipient cell or, otherwise, ii) maturation of endosomes, fused with exosomes, into lysosomes and subsequent degradation of their content [75,76]. Globally, the uptake of this lipid vescicles by recipient cells seems to involve clathrin- and caveolin-dependent mechanisms [77,78]. Anyhow, it has been proven that disruption of exosomal lipid rafts counteracts the internalization of exosomes and that annexins, which are associated with cell-to-cell adhesion, were crucial for exosomes uptake in breast cancer cells [79]. Moreover, in ovarian cancer cells, treatment with protease K determined a clear decrease of exosome uptake, thus confirming the involvement of surface proteins in the internalization of exosomes [80]. However, the detailed mechanism of exosome internalization still needs further clarification.

Clinical application of exosomes detectable in circulation consists in the analysis of their cargo, expressly represented by miRNAs, which may specifically reflect the pathogenesis of the cell of origin that can be transferred to recipient cells altering their molecular characteristics with inevitable effects on cell function. Circulating miRNAs stabilized in exosomes are, therefore, ideal candidates for the identification of non-invasive cancer biomarkers detectable in body fluids such as serum, blood, saliva, urine, tear fluid and breast milk [81,82]. A clear advantage in employing

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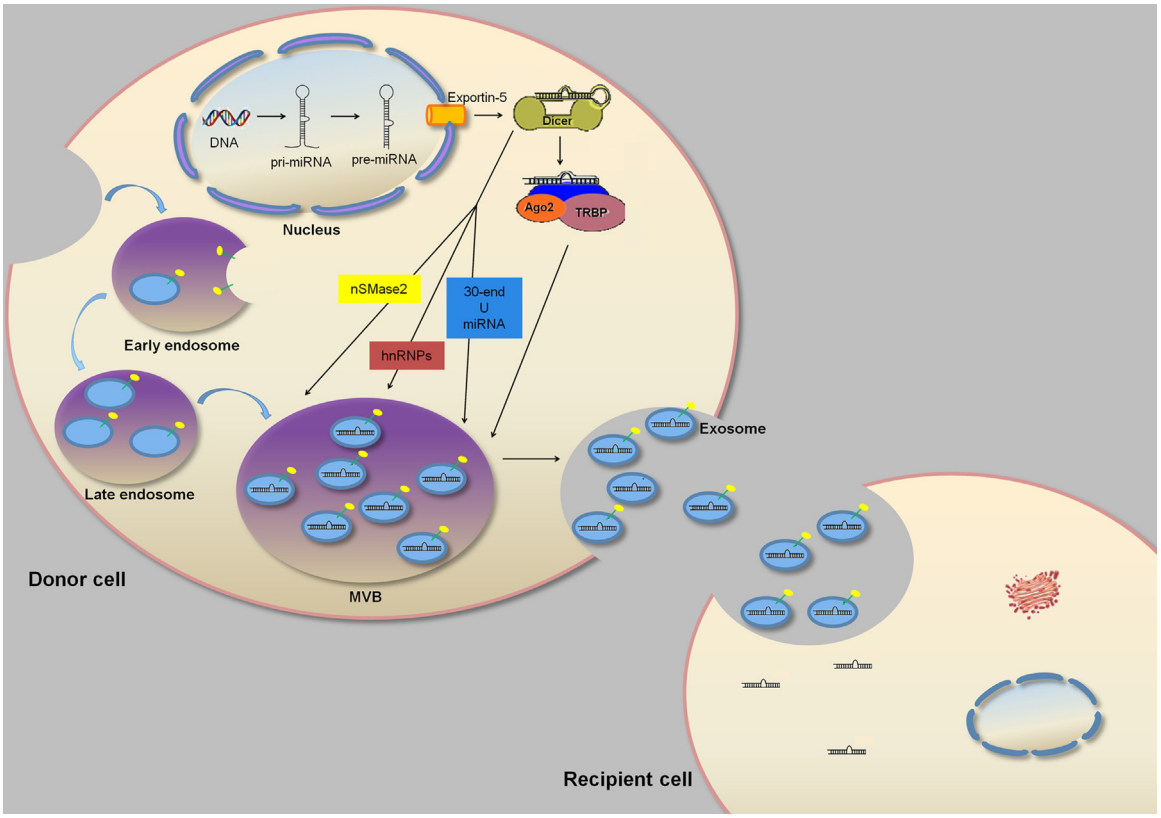

Fig. 1. Exosome formation and miRNA sorting.
Exosomes are firstly produced by endocytosis, when the cellular membrane is internalized to produce endosomes; thereafter, the invagination of endosome membranes originates many small vescicles within the same endosome, which is thus named multivescicular body (MVB). MVBs are then released from the cell into the extracellular space by fusing with the cell membrane, leading to the formation of exosomes. All these steps of formation and secretion require the involvement of proteins and ATP. miRNA sorting into exosomes can follow at least four potential mechanisms: 1) based on the neural sphingomyelinase 2 (nSMase2)-dependent pathway; 2) by sumoylated heterogeneous nuclear ribonucleoproteins (hnRNPs); 3) based on the recognizion of the 30-end of uridylated miRNA; 4) by miRNA induced silencing complex (miRISC). Exosomes released in body fluids can interact with recipient cells through three main mechanisms: the direct interaction between transmembrane proteins of exosomes and the signaling receptors of target cells; the exosomes fusion with the cellular membrane of recipient cells with the following delivery to the cytosol; the internalization of exosomes within the recipient cells.

exosomal miRNAs instead of AGO-miRNAs as biomarkers is represented by their stability during the processing of genetic material in circulation. In fact, these processes cause an extensive hemolysis that may induce the release of additional AGO-miRNAs from cells with subsequent contamination of blood with irrelevant miRNAs, thus complicating the analysis. Instead, these issues are reduced when the analysis is performed on miRNA internalized in exosomes [83]. Several exosomal miRNAs have been identified as biomarkers for cancer, proving their clinical utility and their potential in the field of personalized medicine and diagnostics. Particularly, exosomal miRNAs have several functions related with cell growth, migration, invasion, metastasis and impairment of the immune system response [84]. In some cases it has been identified a cell-independent miRNA biogenesis, associated with exosomes, able to alter the transcriptome of non-cancer cells transforming them in cancer cells in a Dicer-dependent mechanism [85]. The process of metastatic cascade activation during tumor progression can be coordinated by exosome-mediated secretion of miRNAs. Particularly, it was observed an augmented amount of the oncosuppressor miRNAs, such as miR-23b, secreted from metastatic cells derived from bladder cancer, suggesting that the release from exosomes may control the amount of intracellular miRNAs in donor cells and affect the levels of the same miRNAs in recipient cells [86]. Based on the central role of exosomal miRNAs in cell-to cell communication, they could drive the development of a novel clinical application founded on the employ of a new class of target-based anticancer agents directed versus miRNA pathways. Alongside the restoring of oncosuppressor miRNAs with synthetic miRNA mimics – an emblematic case is represented by miR-34a that is the first cancer-targeted miRNA drug (MRX34) entering Phase I clinical trials [87] – and the blockage of oncogenic miRNAs with antisense oligonucleotides, another therapeutic application might be the blockage of the secretion of tumor cell-derived exosomes. Nevertheless, this procedure could not be recommendable, since exosomes include a broad range of other molecules aside from oncogenic miRNAs. For that reason, a possible strategy could consist in the therapeutic use of cells that preferentially secrete a specific category of exosomal miRNA, such as mesenchymal stem cells (MSCs), whose secreted miRNAs are mainly involved in tissue repair [88]. Moreover, the relative abundance of circulating miRNAs with predominant tumor suppressor activity in the blood of healthy individuals, acting as a surveillance mechanism, and the specific alteration of miRNA signature in cancer patients represents an encouraging perspective in view of identifying stable and reliable biomarkers. Overall, the current scientific reports analyzing the exosomal miRNAs agree on their promising use for various clinical applications, including treatment and diagnosis of neoplasms, although it is clear that it is necessary an intensive investigation of exosomal miRNAs biology.

4. ncRNA dysregulation in cancer: preclinical studies and therapeutic implications

Recent studies indicate that miRNAs are deregulated in many types of tumors, being involved in several cancer processes, such

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as cell proliferation, invasion and metastasis. miRNAs can, in fact, inhibit the expression of genes involved in many cellular pathways regulating crucial mechanisms like cell cycle, cell death and cell migration. Numerous experiments and clinic analyses suggest that miRNAs may function as a novel class of oncogenes (oncomirs) or tumor suppressor genes. Oncomirs are overexpressed in tumors and act by promoting tumor development and by negatively inhibiting onco-suppressive genes and/or genes that control cell differentiation and cell death. A good example for an oncogenic miRNA is miR-21. It is overexpressed in most types of malignancies, including breast cancer, glioblastoma, colorectal cancer, lung cancer, pancreatic cancer, and leukemia [89–91]. Functional studies in cancer cell lines indicate that miR-21 plays an important role in the oncogenic process as indicated by its association with high proliferation, low apoptosis, high invasion, and metastatic potential [92–94]. A number of miR-21 target genes have been identified, including PTEN, PDCD4, and BTG2, which play important roles in the oncogenesis [95]. In glioblastoma, miR-21 was revealed to target several important components of the epidermal growth factor receptor (EGFR) and phosphatase and tensin homolog (PTEN) signaling pathways. Inhibition of miR-21 by specific antisense oligonucleotides in U251MG glioblastoma cells decreased the expression of EGFR and activated AKT, CYCLIN D, and BCL2 [96,97]. Another well known oncosuppressor miRNA is miR-34a, whose enforced expression in multiple myeloma (MM) cells and in MM mouse models, induces sequential inhibition of Erk and Akt activities and caspase-3 and -6 cleavage, as well as Bcl-2 and NOTCH1 downregulation [98]. Preclinical models of MM have also allowed to identify a tumor suppressor role also for miR-29b. It acts as epigenetic regulator through the inhibition of HDAC4, which is highly expressed in MM and, therefore, represents a relevant target for therapy [99]. Another oncogenic target of miR-29b is the transcription factor Sp1, which has been demonstrated to be, in turn, a negative regulator of miR-29b, thus establishing a negative feedback loop [100].

During tumorigenesis, the expression of some miRNAs is decreased, so these types of miRNAs are considered tumor suppressor genes. Tumor suppressor miRNAs negatively interfere with tumor development inhibiting oncogenes and/or genes that control cell differentiation or apoptosis. A miRNA considered as tumor suppressor gene is let-7. It plays a pivotal role in tumor suppression in many cancers, including esophageal squamous cell carcinoma, lung cancer, nasopharyngeal carcinoma, and prostate cancer [101–104]. Takamizawa et al. [105] found that let-7 was poorly expressed in lung cancers; moreover, reduced let-7 expression was significantly associated with shortened post-operative survival independent of disease stage. This suggested that let-7 may be a tumor suppressor gene. To confirm this conclusion, the authors overexpressed let-7 gene in A549 lung adenocarcinoma cell lines and induced the inhibition of lung cancer cell growth *in vitro* [105].

In addition to miRNAs, lncRNAs have also emerged as important regulators in both oncogenic and tumor suppressor pathways [106]. Some studies allowed the analysis of ncRNA role in cancer, evaluating the expression of more than 10,000 lncRNA genes in more than 1000 tumor samples. In these studies the authors identified the lncRNAs associated with specific tumors and their role in oncogenic cell growth [107]. Other studies have shown that some lnRNAs are more expressed and more active in tumors than in normal cells [108–110]. Accumulating evidence provides mechanistic insight demonstrating how lncRNAs regulate important cellular signaling pathways in cancer cells at transcriptional, post-transcriptional, and epigenetic levels [111]. In a recent study, the authors analyzed the expression and function of PCAT-14 in hepatocellular carcinoma HCC. LncRNA prostate cancer-associated transcripts (PCATs) were originally identified as biomarkers for prostate cancer [112]. In particular in this study the authors showed that the lncRNA PCAT-14 is overexpressed in patients with HCC, and is associated with a poor prognosis after surgery. PCAT-14 promotes proliferation, invasion, and cell cycle arrest in HCC cells and inhibits miR-372 expression by inducing methylation of the miR-372 promoter [113]. Other lncRNAs have been associated with tumor suppressor functions. In literature it is demonstrated that several lncRNAs are direct transcriptional targets of p53, and knockdown of specific lncRNAs modulates p53-induced apoptosis. In detail, a study revealed that the lncRNA NEAT1 is a direct transcriptional target of p53. The suppression of NEAT1 induction by p53 attenuates the inhibitory effect of p53 on cancer cell growth and also modulates gene transactivation, including that of many lncRNAs. Furthermore, low expression of NEAT1 is related to poor prognosis in several cancers. These results indicate that the induction of NEAT1 expression contributes to the tumor-suppressor function of p53 and suggest that p53 and NEAT1 form a transcriptional network contributing to various biological functions including tumor suppression [114].

Other ncRNA species such as piRNAs and snoRNAs are obtaining a greater appreciation for their role in carcinogenesis [115]. In detail, piRNA-651 was found to be up-regulated in several cancer cell lines including gastric, lung, mesothelial, breast, liver, and cervical cancer cells [116]; moreover, it was reported also for human snoRNAs an important role in tumorigenesis [117].

In the light of this evidence it can be stated that ncRNAs have been recognized as promising therapeutic targets for anticancer treatments. In particular a miRNA can simultaneously modulate its multiple target genes, altering oncogenic and tumor suppressor pathways. Cancer-associated upregulation of oncogenic miRNAs, can be counteracted by several means. Based on the evidence that miRNAs control their targets through base pair complementarity, antisense oligonucleotides (ASOs) have been developed to inhibit their function. In order to increase ASOs' stability and efficacy, different chemical modifications, such as locked nucleic acids (LNAs), anti-miRNA oligonucleotides (AMOs), and antagomirs, are introduced. For example, a specific antagomir was used to knockdown the oncogene miR-21 in breast cancer MCF-7 cells, resulting in significant inhibition of MCF-7 growth *in vitro* and in tumor xenografts through inhibiting cell proliferation and inducing apoptosis [118]. As far as it is concerned tumor suppressor miRNAs, miRNA mimics or lentiviral vectors can be used to restore their expression levels.

The request of long circulation in the blood and of preferential accumulation in the target site as a safety requirement has stimulated the exploration of nonviral vectors, such as nanocarriers. Nanocarriers are small particles (ranging from 1 to 300 nm) that can carry and deliver drugs, oligonucleotides, peptides or desired cargos to target tissues. Various nanocarriers have been used for ncRNA delivery in biomedical applications. Based on surface charge, size and hydrophobicity, they have unique tissue biodistribution, toxicity, and tumor cell uptake profiles [119]. Recently, it has been reported that direct conjugation of small drug molecules to ncRNAs can improve the *in vivo* pharmacokinetic behavior of ncRNAs. LncRNAs represent an important resource in terms of developing diagnostics and therapies because many of them are expressed in a tissue- and cancer-type specific manner and could became novel biomarkers. The discovery that lncRNA could be detected in the body fluid of cancer patients opened up a new and exciting possibility of using lncRNAs as non-invasive biomarkers. The development of more efficient and specific delivery system is necessary to achieve high therapeutic efficiency and target specificity. Also for the lncRNAs there are delivery systems targeting therapeutics: viral delivery and non-viral delivery. Viral delivery generally provides high gene transfer efficiency, but is deficient in biosafety and, therefore, not appropriate for human use despite the promising results in animal models. Currently, numerous synthetic delivery systems have been developed to manipulate the gene transfer effi-

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ciency, and some of them have demonstrated promising results on humans [120,121].

5. Potential of ncRNAs as novel biomarkers for solid and hematologic malignancies

Improved knowledge of ncRNAs' expression pattern and function may lead to a better understanding of the heterogeneity of malignancies and, most likely, also lead to their use as diagnostic, prognostic and therapeutic targets. The best-studied ncRNAs category is represented by miRNAs, which have emerged as suitable diagnostic and prognostic biomarkers with the capacity to drive treatment decisions in the clinical setting. Researchers have identified miRNA signatures in serum, plasma, peripheral blood mononuclear cells and whole blood, giving the opportunity to distinguish patients with different cancers, such as prostate (miR-141), lung (miR-21, miR-210 and miR-486-5 p), breast (miR-195 and let-7a) and lymphoma, from healthy individuals [122]. Some of the most deregulated miRNAs are listed in Table 1.

5.1. miR 155

Emerging evidence suggest that miR-155 is significantly upregulated in lung cancer tissues, plasma and sputum, and could serve as a promising marker for the diagnosis and poor prognosis of non-small cell lung cancer (NSCLC) [123]. On the other hand, overexpression of miR-155 in human breast cancer cells has shown a protective role in triple-negative breast cancers through RAD51 targeting, also affecting the cellular response to ionizing radiation. Furthermore, high miR-155 levels were associated with lower RAD51 expression and with better overall survival of patients in a large series of triple-negative breast cancers [124]. A high number of studies have investigated its potential as a biomarker in several B-cell malignancies, but conflicting results have been presented. Costinean et al., demonstrated that transgenic mice over-expressing miR-155 in B-cells, developed a pre-leukemic pre-B-cell proliferation, followed by a B-cell malignancy [125]. A study of miR-155 KO mice showed that miR-155 regulates germinal center reaction and T-helper cell differentiation by affecting cytokine production, and that BIC/miR-155 regulates the function of both lymphocytes and dendritic cells leading to defective immune response [126]. Therefore, in mice models, miR-155 deregulation in B cells causes acute lymphoblastic leukemia and high-grade lymphomas [126], whereas in myeloid cells it is associated with myeloproliferative disorders [127,128]. In addition, high levels of miR-155 were also found in newly diagnosed cytogenetically normal acute myeloid leukemia bearing FLT3-internal tandem duplications, associated with poor prognosis [129].

5.2. miR-29

The miR-29 family comprises of three members in humans, miR-29a, miR-29b and miR-29c, differently expressed in several solid or hematologic tumors, such as, nasopharyngeal carcinoma [130], non-small cell lung cancer [131], hepatocellular carcinoma [132], breast cancer [133], cutaneous melanoma [134] and diffuse large B cell lymphoma [135]. Performing a systematic review of the literature with a meta-analysis aimed to evaluate the prognostic value of the miR-29 family expression in different types of cancers, Qi et al. found that the low expression of miR-29 is associated with aggressiveness and poor prognosis of malignant neoplasms [136]. In 2005, Calin et al. [137] reported for the first time that the miR-29 family could discriminate good or poor prognosis between chronic lymphocytic leukemia samples. Also in mantle cell lymphoma miR-29 was identified as a prognostic marker and pathogenic factor that targeted cyclin-dependent kinase 6 (CDK6) [138]. Moreover, Garzon et al. [139] reported that the expression of miR-29 family was downregulated in acute myeloid leukemia (AML) patients with t(11q23) chromosome rearrangements *vs* all other AML patients, although miR-29 family expression was upregulated in AML with positive cytoplasmic nucleophosmin (NPMc + AML) [140]. Recently, several studies have identified miR-29 as a non-invasive diagnostic and prognostic tool for colorectal cancer (CRC). Serum levels of miR-29b and miR-194 were found to be significantly lower in CRC patients as compared with control subjects. Furthermore, serum levels of this miRNA were inversely correlated with the advanced tumour-node-metastasis (TNM) stages [141]. Inoue et al. evaluated the association between miR-29b expression and survival in 245 patients with CRC. They noticed lower miR-29b levels in serum of CRC patients, also finding further decreased expression in advanced clinical stages of tumor [142]. Regarding miR-29a, upregulated expression was detected in serum from colorectal liver metastastatic patients compared with non-metastasized CRC patients [143]. Moreover, reduced expression of miR-29a, with miR-223 and miR-224, was found in the feces from the CRC patients, thus providing informative biomarkers for both screening and early diagnosis of CRC [144].

5.3. miR-17-92 family

The oncogenic miR-17-92 family is composed of 3 related polycistronic miRNA gene clusters: miR-17-92 cluster and its paralogs miR-106b-25 and miR-106a-363 clusters [145]. The miR-17-92 cluster, transcriptionally activated by c-Myc and E2F, is upregulated in chronic lymphocytic leukemia cells and in various lymphoma cell lines [146]. In mantle cell lymphoma, upregulation of miR-17-92 antagonizes chemotherapy-induced apoptosis by inhibiting protein phosphatase PHLPP2, an additional target of miR-17-92 and an important negative regulator of the PI3K/AKT pathway [147]. The finding that miR-17-92 expression contributes to the signature of hematologic tumor cell lines provides evidence that miRNA expression patterns reflect the patterns observed in primary hematologic malignancies [148]. miR-17-92 cluster has also been demonstrated to play a crucial role in various other human cancers. High expression levels have been found in osteosarcoma tissues with a relationship between miR-17-92 cluster upregulation and advanced TNM stage, as well as poorer recurrence-free survival of osteosarcoma patients. [148]. Moreover, miR-17-92 cluster has been identified as potential serum biomarker for the early detection of both gastric cancer and intestinal metaplasia [150].

Along with miRNAs, also lncRNAs could act as non-invasive tumor markers in both diagnosis and prognosis prediction [151,152]. LncRNAs have been shown to regulate cellular processes that are pertinent to cancer development, including cell cycle progression [153], apoptosis [154] and metastasis [155] (Table 2).

5.4. NEAT1

Nuclear enriched abundant transcript 1 (NEAT1) is a novel lncRNA specifically localized to nuclear paraspeckles [156] that seems to be involved in regulating gene expression by retaining mRNAs for editing in the nucleus [157]. In NSCLC, NEAT1 expression was related to patient age, vascular invasion and clinical TNM staging [158]. Another study showed that circulating NEAT1 levels were significantly higher in NSCLC patients' plasma [159], suggesting that increased NEAT1 expression might be associated with progression of NSCLC, where circulating NEAT1 could be used as a diagnostic marker. In addition, NEAT1 could induce hsa-miR-377-3p to derepress E2F3, which is a key oncogene in promoting NSCLC [160]. In laryngeal squamous cell carcinoma (LSCC), NEAT1 overexpression was associated with advanced clinical stage and lymph node metastasis. Moreover, Wang et al., showed that NEAT1

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Table 1
miRNA deregulation in cancer diseases.

miRNA	Disease Association	Expression	Role	Biomarker Application	References
miR-155	Non-small cell lung cancer	upregulated	Oncogene	Diagnosis and poor prognosis	[123–129]
	Breast cancer		Oncosuppressor	Predicts better survival	
	B-cell malignancy		Oncogene	Myeloproliferative disorders	
	Acute myeloid leukemia		Oncogene	Predicts poor prognosis	
miR-29	Nasopharyngeal carcinoma	downregulated	Oncosuppressor	Aggressiveness and poor prognosis	[130–144]
	Non-small cell lung cancer				
	Hepatocellular carcinoma				
	Breast cancer				
	Cutaneous melanoma			Prognostic marker	
	B cell lymphoma				
	Chronic lymphocytic leukemia			Diagnosis and monitoring	
	Acute myeloid leukemia				
	Colorectal cancer				
miR-17-92	Chronic lymphocytic leukemia cells	upregulated	Oncogene	Risk factor	[145–155]
	Mantle cell lymphoma			Predicts therapeutic responsiveness	
	Osteosarcoma			Predicts advanced TNM stagea and poorer Recurrence-free survival.	
	Gastric cancer			Potential serum biomarker for the early detection	
	Intestinal metaplasia				

Table 2
lncRNA deregulation in cancer diseases.

LncRNA	Disease Association	Expression	Role	Biomarker Application	References
Neat-1	Non-small cell lung cancer	upregulated	Oncogene	Prognosis and clinical tumour-node-metastasis staging.	[156–170]
	Laryngeal squamous cell carcinoma			Poor differentiation, metastasis, invasion	
	Colorectal cancer, Hepatocellular carcinoma, Breast cancer, Oesophageal squamous cell				
	Prostate cancer			Predicts therapeutic responsiveness	
	Ovarian carcinoma			Diagnosis	
	Glioma			Shortened overall survival and tumour recurrence.	
Malat-1	Acute myeloid leukemia			Potential therapeutic use	
	Non-small cell lung cancer	upregulated	Oncogene	Metastases and prognosis in early stage. Predicts therapeutic responsiveness.	[171–180]
	Osteosarcoma			Recurrence after liver transplantation.	
	Hepatocellular carcinoma				
	Clear cell renal cell carcinoma Glioma			Tumor progression and poor prognosis.	
	Pancreatic cancer			Diagnosis and monitoring	
	Colorectal cancer				
	Multiple myeloma				
Hotair	Breast cancer	upregulated	Oncogene	Prognostic value for metastasis and survival	[181–186]
	Hepatocellular carcinoma			Stage marker and histological differentiation	
	Oral squamous cell carcinoma B			Poor overall survival and relapse-free survival times	
Cmultirow20.5inDLEU1/2	Acute myeloid leukemia Chronic lymphocytic leukemia B-cell lymphomas Multiple myeloma	downregulated	Oncosuppressor	Predicts poor prognosis	[187,188]

regulates CDK6 expression through modulating miR-107 and its knockdown induces the apoptosis of LSCC cells *in vivo* [161]. NEAT1 expression was associated with poor differentiation, metastasis, invasion, as well as TNM staging in colorectal cancer [162], hepatocellular carcinoma [163], breast cancer [164] and esophageal squamous cell carcinoma [165]. In prostate cancer it induces resistance to androgen receptor antagonists [166]. In addition, NEAT1 was significantly upregulated in stage III serous ovarian carcinoma [167]. Pils and colleagues performed genome-wide expression analysis in leucocytes isolated from 44 epithelial ovarian cancer patients and 19 normal controls, showing that NEAT1, in combination of 12 remaining genes and 6 plasma proteins, might discriminate patients with epithelial ovarian cancer from normal controls with a sensitivity of 95.6% and a specificity of 99.6% [168]. In glioma, high NEAT1 expression was also independently associated with shortened overall survival and tumor recurrence, as well as larger tumor size and WHO grade. Moreover, high NEAT1 expression in patients with stage III–IV glioma indicated poor prognosis [169]. NEAT1 expression level is significantly repressed by PML-RARα oncoprotein in de novo acute promyelocytic leukemia (APL). When all-trans retinoic acid (ATRA) has been used as a leukemia therapy to target the transcriptional repression mediated by the PML-RARα fusion protein, a significant NEAT1 upregulation was observed, in parallel with ATRA-induced NB4 cell differentiation; this effect was blocked by NEAT1 inhibition. These findings indicated that aberrant NEAT1 downregulation contributes to the blockade of differentiation in APL [170].

5.5. MALAT-1

MALAT-1 (metastasis associated lung adenocarcinoma transcript 1) is an intergenic transcript (7 kb) located on chromosome

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11 [171] implicated in several malignancies. It was firstly correlated with metastases and prognosis in early stage NSCLC [172] and subsequently with chemotherapeutic response in osteosarcoma [173] and recurrence of hepatocellular carcinoma in patients undergoing liver transplantation [174]. It is significantly overexpressed in many types of solid cancers in which it correlates with tumor progression and poor prognosis: in clear cell renal cell carcinoma [175] through the interaction with Ezh2 and miR-205 [176], in glioma [177] and pancreatic cancer [178], in CRC through the binding to SFPQ and the releasing of PTBP2 oncogene from SFPQ/PTBP2 complex [179]. MALAT-1 is also overexpressed in hematologic malignancies as in MM and may serve as a marker to predict disease progression [180].

5.6. HOTAIR

HOX transcript antisense RNA (HOTAIR) is a 2.2 kb, long intergenic non-coding RNA (lincRNA) localized to the HOXC locus (12q13.3) [181]. It functions as a molecular scaffold to link the polycomb repressive complexes 2 and the lysine specific demethylase 1 complexes, and regulates gene expression by mediating the modulation of chromatin structures in trans across the 40-kb HOXD locus [182], promoting cancer metastasis [183]. HOTAIR is systematically dysregulated in breast cancer [183] and in hepatocellular carcinoma [184], and it has prognostic value for metastasis and survival. HOTAIR expression increased also in oral squamous cell carcinoma (OSCC), where it is associated with metastasis, stage, histological differentiation, poor overall survival and poor disease-free survival in OSCC patients. Furthermore, this lncRNA promotes epithelial-mesenchymal transition (EMT) by repressing E-cadherin expression through the recruitment of EZH2 and H3K27me3 [185]. Moreover, HOTAIR may represent a biomarker for poor overall survival and poor relapse-free survival in AML patients with higher HOTAIR expression levels, with respect to patients with lesser expression levels of the lncRNA [186].

5.7. DLEU1/DLEU2

Deleted in leukemia 1 (DLEU1) and 2 (DLEU2) genes are transcribed in a 30-kb region situated in the long arm of chrormosome 13 (13q14), which is missing in more than 50% of patients with chronic lymphocytic leukemia (CLL) and can be associated with the prediction of poor prognosis [187] in this neoplasm, as well as in other B-cell malignancies, including de novo and transformed diffuse large B-cell lymphomas, and MM [188]. It has been demonstrated that intron 4 of DLEU2 encodes for miR-15a and miR-16-1 cluster that, as previously reported, can exert a crucial role in the tumorigenesis of CLL, in part through the regulation of the oncogene BCL2 [187].

6. miRNA detection and quantification in body fluids

miRNAs, unlike other biomarkers, are highly stable and can be isolated from tumor tissue samples after formalin and paraffin passages, and from serum and plasma samples after being at room temperature for 24 h and after freezing and de-freezing cycles [189]. In addition, miRNAs appear to be resistant to RNAses present in the plasma probably due to their small size or molecular structure, as well as in virtue of their loading in EV. The presence of circulating miRNAs has been demonstrated in several biological samples such as serum, tears, urine, amniotic and ascitic fluid [190]. The correlation between different profiles in body fluids and the tumor progression, the presence of metastases and, consequently, the prognosis, make it possible to use these molecules as biomarkers. The expression profile of circulating miRNAs is highly influenced by sample preparation methods [191,192]. The extraction of RNA is usually performed by using commercially available techniques including phenol/guanidinium products, such as TRIzol (Life Technologies), and column-based extraction kits, such as mirVana (Life Technologies) and miRNeasy (QIAGEN). In most studies, the extracted miRNAs are subjected to next-generation sequencing (NGS) or miRNA microarrays to obtain large-scale profiles of circulating miRNAs and to determine candidate miRNAs for further quantification. These methods have both advantages and limitations. NGS has a potential to identify novel miRNAs but it is less cost-effective and less efficient compared with microarrays. The candidate miRNAs are generally subjected to further validation by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in larger cohorts. To quantify it reliably it is necessary to identify endogenous controls. RNU-6B, RNU-48, and miR-16 are commonly used for this aim, but no definitive control gene has been established [193,194]. Recent studies have suggested more reliable endogenous controls for the quantification of circulating miRNAs. Chen et al. reported that a combination of let-7d, let-7g and let-7i serves as an endogenous control of serum miRNAs and it is superior to the commonly used reference genes [195]. Kok et al. suggested normalization panels for the better quantification of circulating microRNAs by RT-qPCR [196].

Currently there are several studies focusing on the use of miRNAs as diagnostic and prognostic biomarkers for different types of tumors, such as colon, prostate, breast, lung, and hematologic cancers. In the literature it has been reported that an aberrant alternation of miRNA was observed in the serum of NSCLC patients, as compared to healthy controls by using Solexia deep sequencing [197]; the authors affirm that miR-25 and miR-223 could be used as markers for diagnosing NSCLC. The currently known circulating miRNA signatures for NSCLC cancer include several miRNA panels and arrays [198]. Recent research has also shown that circulating miRNAs can play an important role also as prognostic markers for NSCLC patients. For instance, a four miRNA panel (miR-486, miR-30d, miR-1 and miR-499) was related to the overall survival of NSCLC patients undergoing surgery, as well as adjuvant chemotherapy [199], and increased serum levels of miR-125b in NSCLC patients were associated with non-responsive cisplatin-based chemotherapy [200].

Some studies have evaluated the use of circulating miRNAs as biomarkers for monitoring the response to therapy also for breast cancer cells; in fact, many studies confirmed that circulating miR-155 was elevated in the serum of breast cancer patients [201]. The altered levels of circulating miRNAs can also be used to predict the effect of chemotherapy; for example, elevated levels of miR-125b in breast cancer patients can indicate a lower therapeutic response to 5-Florouracil (5-FU), epirubucin or cyclophosphamide (FEC) [202].

Studies have also considered to employ circulating miRNAs as diagnostic and prognostic markers for CRC. miR-92 was found to be significantly elevated in the plasma of CRC patients and in tumor tissues, as compared to their normal counterparts, suggesting that this miRNA could be used as a potential non-invasive marker for CRC diagnosis [203]. Several circulating miRNAs have been shown to be related to CRC metastasis. A high level of serum miR-200c showed a significant correlation with the lymph node and distant metastasis, thereby suggesting that it could be used as an indicator for predicting CRC metastasis [204]. Overall, these studies allow to affirm that circulating miRNAs could serve as promising markers for cancer diagnosis and prognosis.

7. Challenges, opportunities and pitfalls of miRNA profiling

Tissue biopsies are still considered the gold standard for molecular evaluation of cancer, although this procedure is invasive and has several limits related to the low possibility of sampling and to the heterogeneity of tissues, the reason why biopsy only gives a

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small sampling of the entire tumor. These limitations account for the potentially missing clinical information coming from bioptic tissues. Moreover, another severe restriction is represented by the little opportunity to gain multiple samples following the various treatment cycles, thus precluding the possibility to analyze tumor evolution over time and in response to therapy. In addition, for certain malignancies it is impossible to access to the tissue due to the localization. This series of issues has generated the boost to find alternative methods for early detection, diagnosis and therapeutic monitoring of cancer.

miRNA profiling can provide a rich amount of biological information, in view of the multiplicity of messanger RNAs that may be modulated as a consequence of the precise alteration in miRNA expression pattern. The most important applications range from the analysis of a wide variety of physiological processes – such as organisms development, as well as establishment and preservation of tissue differentiation – to the monitoring of pathological conditions – as cancer, cardiovascular and autoimmune diseases – for which miRNAs are becoming helpful biomarkers and innovative reagents for re-programming cell fate in therapeutic applications [205]. A considerable interest in miRNA profiling has been developed also in the forensic field, where highly sensitive measurement of these molecules may provide information such as the cellular composition of inquired samples. A big source of information about gene regulation may derive from the combined analysis of miRNAs, mRNAs and protein profile on a genomic scale, although miRNAs are much more stable than mRNAs in a broad variety of specimens – including cell lines, blood, plasma, serum, urine, fresh tissues and formalin-fixed tissue blocks – and are also quantifiable with higher sensitivity than proteins, since they can be amplified by RT-PCR [206]. Sample processing and RNA extraction methods play a leading role in the successful outcome of miRNA profiling, especially for samples susceptible to degradation [207,208]. As previously said, miRNAs can be extracted with high quality from several cell and tissue sources [209]; the isolation methods are generally the same as for the isolation of total RNA, apart from that protocols used for miRNA isolation can be modified, to some extent, in order to retain and/or to enrich the small RNA fraction [209]; the global yield in miRNA from fresh tissues and cell lines is generally good and suitable for profiling studies. Instead, concerning miRNA coming from formalin-fixed paraffin embedded tissues (FFPE), it is surprisingly stable and intact independently from formalin fixation time and duration of tissue block storage [210], differently from mRNA, which is generally fragmented and less reliable in FFPE compared to fresh tissue. This stability confers to miRNAs a great advantage over mRNAs as tissue markers in the clinical setting, where FFPE is generally the only kind of sample available. Some issues may arise in the case of miRNA extraction from samples that require the tuning of protocols aimed at optimizing the efficiency and at increasing the quality of products. A challenging specimen is represented by body fluids, where there are high levels of endogenous RNase that may rapidly inactivate miRNAs in the case of extraction carried out with methods that fail to completely inactivate RNase [211]. Moreover, the quality and the amount of miRNA extracted from blood may also be influenced by other variables as centrifugation settings, white blood cell counts, and red blood cell hemolysis [212]. Therefore, it is strictly necessary, for reproducibility and accurateness of results, to assess both integrity and yield of extracted miRNA by means of spectrophotometry and/or automated capillary electrophoresis instruments; another strategy aimed at verify RNA extraction efficiency, consists in the addition, in the early stage of miRNA isolation, of a known amount of synthetic miRNAs unexpressed in the biological sample [213], since their measurement can give useful information about the accuracy of the extraction method and the preservation of miRNA integrity. Detection and quantification undergo also other issues correlated with the shortness of mature miRNA and the consequent difficulty encountered in the annealing to traditional primers designed for reverse transcription and PCR [214]; moreover, the absence of a common sequence, such as poli(A)-tail for mRNA, further complicates the possibility of a selective enrichment, that is necessary, given the scarce abundance of miRNA fraction (~0.01%) respect to the total amount of RNA [215] and the existence of miRNA families and variants whose members differ each other for single or few nucleotides. In spite of these issues, the exigency of profiling has given rise to the development of three main approaches – qRT-PCR, hybridisation-based methods (*e.g.*, DNA microarrays) and high-throughput sequencing (*i.e.*, RNAseq) – which allow the definition of tissue-based and circulating miRNA biomarkers with high accuracy [216,217].

8. Conclusions

The remarkable importance of an early cancer diagnosis, monitoring and treatment, in view of an efficient patient's management, has provided a strong boost to the recognition of tumor-specific, non invasive, and easy to detect and quantify biomarkers. Both blood-based and tissue biopsies-obtained protein markers, until now considered the gold standard for molecular evaluation of cancer, have shown limited specificity and sensitivity. In this review we have focused on the possibility to complement the existing biomarkers with the detection of specific ncRNAs from cancer tissues and, more importantly, we have analysed the opportunity to obtain a panel of clinical relevant circulating ncRNAs for a minimally invasive diagnosis and monitoring of neoplasms. Based on the functional role of non protein-coding portion of genome in a large number of mechanisms of gene regulation – from the transmission of transgenerational epigenetic information to the regulation of normal physiology and human diseases – we have herein reported a variety of miRNAs and lncRNAs particularly deregulated in preclinical and clinical cancer models, analysing their inhibitory or promoting role in the regulation of solid and hematologic malignancies. We have also described the most common methods employed for ncRNA detection and quantification in body fluids focusing on their advantages and limitations; the expression profile of circulating miRNAs is, in fact, highly influenced by sample preparation methods. Issues arising from ncRNA extraction require the fine-tuning of protocols for the optimization of the efficiency and for the improvement of both amount and quality of products. Anyway, a promising perspective comes from the employ of exosomal miRNAs for various clinical applications, including treatment and diagnosis of neoplasms; exosomal cargo may, in fact, specifically reflect the pathogenesis of the donor cell that can be transferred to recipient cells altering their molecular characteristics and it can also be easily detected in body fluids providing precious information with a non-invasive approach. Moreover, another topic here covered is the recognition of ncRNAs as promising therapeutic targets for anticancer treatments and the need to increase their time of circulation in the blood, as well as to potentiate their preferential accumulation in cancer tissues. Therapeutic strategies aimed at modulate miRNAs expression are recently emerging just in virtue of ncRNAs' ability to influence cellular behaviour [218]. miRNAs can contribute to tumor initiation and progression through several mechanisms and, therefore, multiple therapeutic approaches have been proposed to target these processes. In detail, the inhibition of oncogenic miRNAs can be obtained by using antisense oligonucleotides (ASOs) that are able to adhere to the miRNA target forming miRNA-anti-miRNA binding complexes. These molecules can be grouped into three major categories: antagomirs, locked nucleic acids (LNAs) and ASOs with chemical alterations to optimize efficacy. Another therapeutic approach for reducing the levels of oncogenic miRNAs consists in the use of competitive inhibitors,

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such as vectors bearing multiple artificial miRNA binding sites under the control of strong promoters. As regards oncosuppressor miRNAs, restoration of their basal levels is the most used strategy, which can be obtained either by using miRNA mimics, or availing of the so-called *epigenetic modifiers* -that act on hypermethylation and histone modification patterns, which have been linked to miRNA dysregulation - and *enhancers of the miRNA processing machinery* - another class of small molecules that has been developed to enhance RNAi and to induce miRNA processing [218,219]. These requirements have stimulated the development of both viral and non-viral delivery systems and some of them have demonstrated promising results on humans. Certainly, delivery strategies for the employ of ncRNA-based drugs in clinical practice and ncRNA profiling for the identification of novel biomarkers, still represent an important challenge for both clinicians and researchers and require further improvements for a successful clinical translation. However, the encouraging premises derived from the growing interest proven by scientific community offer an excellent perspective for the near future.

Conflict of interest

None.

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Please cite this article in press as: A. Grimaldi, et al., Non-coding RNAs as a new dawn in tumor diagnosis, Semin Cell Dev Biol (2017), http://dx.doi.org/10.1016/j.semcdb.2017.07.035

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Please cite this article in press as: A. Grimaldi, et al., Non-coding RNAs as a new dawn in tumor diagnosis, Semin Cell Dev Biol (2017), http://dx.doi.org/10.1016/j.semcdb.2017.07.035

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