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MINI REVIEW

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Summary Despite the intensive efforts to identify the molecular events responsible for the emergence of liver cancer, hepatocellular carcinoma (HCC) remains a major health problem in the world. Thus, the identification of new therapeutic opportunities is a short-term necessity. These last few decades, non-coding RNAs appeared as interesting therapeutic strategies with their pleiotropic inhibitory action in the cell itself but also in recipient cells via their secretion into extracellular vesicles. This short review recapitulates recent advancements concerning non-coding RNAs and their deregulations in liver cancer.

Introduction

For decades, proteins were believed to carry hereditary information, in particular during bacterial transformation described by the microbiologist Frederick Griffith in 1928. Nevertheless, fifteen years later, this precept was questioned by the experiment of Oswald Avery, Colin MacLeod, and Maclyn McCarty in 1944, which demonstrated that DNA was the hereditary genetic material controlling the pathogenicity of the bacteria [1]. After years of consolidating experiments, the scientific community assumed that DNA makes us who we are. Therefore, over several decades, proteins that result from DNA genetic information were credited to be the master regulators of cell behavior. It had to wait for the 2000s and the opportunity of whole exome sequencing to crush the concept of DNA world and open the new era of a RNA world. Indeed, the human genome only contains a tiny fraction of protein-coding genes, supporting that RNA play a more substantial role than originally believed [2]. The rest of the genome is nonetheless actively transcribed into RNAs without coding potential, and called non-coding RNAs (ncRNAs). NcRNAs are partitioned into repetitive DNA, which constitute the largest fraction (40-50%), including:

Abbreviations: circRNA, circular RNA; EMT, epithelial to mesenchymal transition; HCC, hepatocellular carcinoma; LINE, long interspersed nuclear elements; LNA, locked nucleic acid; lncRNA, long non-coding RNA; miRNA, microRNA; ncRNA, non-coding RNA; piRNA, piwiRNA; RISC, RNA-Induced silencing complex; RNP, ribonucleoprotein; P-bodies, processing bodies; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; PDX, patient-derived xenograft; SINE, short interspersed nuclear elements; snRNA, small nuclear RNA; SNAP, Soluble N-ethylmaleimide-sensitive-factor attachment protein; SNARE, SNAP Receptor; snoRNA, small nucleolar RNA.

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- telomeres;
- transposable elements (long (LINE) and short interspersed nuclear elements (SINE));
- introns (approximately 25%);
- miscellaneous sequences [3].

Non-protein-coding DNA sequences, once considered as junk DNAs, proportionally increase with evolution complexity from prokaryotes to mammals, reaching more than 98% of the human genome ([4] for a review). This discovery has shaken the conscience and largely modified our understanding of complex diseases like cancer. Scientists now face major issues to unveil the precise biological functions of these non-coding RNAs and the impact of their deregulations in pathological processes. And, even if crucial roles in gene regulations have been assigned to a great majority of these sequences, non-coding RNAs globally remain a mysterious world to explore.

Non-coding RNA biogenesis

NcRNAs are arbitrarily divided into two categories, small (< 200 nucleotides (nt)) and long non-coding RNAs (lncRNAs) (>200 nucleotides) [5]. Small ncRNAs notably include:

- Piwi-interacting RNAs (piRNAs), mainly identified in germ cells to silence genetic elements such as transposons [6];
- small nucleolar RNAs (snoRNAs), that guide chemical modifications of other RNAs [7];
- small nuclear RNAs (snRNAs), found within the spliceosome [8];
- the most widely characterized ncRNAs, the microRNAs (miRNAs), involved in post-transcriptional gene silencing [9].

Long non-coding RNAs are generally 1,000—10,000 residues in length and refer to natural antisense transcripts and other regulatory long non-coding RNAs [10]. Based on their proximity to protein-coding genes, miRNAs and lncRNAs have been classified into exonic, intronic and intergenic ncRNAs and quite commonly produced in a polycistronic manner. Although biogenesis and biological functions differ for all ncRNAs, and more notably for lncRNAs, small ncRNA biogenesis shares several similarities with RNA interference pathway [11] (Fig. 1). RNA-processing proteins usually cleave primary transcripts of small ncRNAs into small RNA fragments. Then, processed ncRNAs are loaded into the multi-protein machinery called RISC (*RNA-induced silencing complex*), which helps in Argonaute recruitment. Argonaute proteins are evolutionarily conserved and phylogenetically subdivided into the Ago subclass, for miRNA and siRNA processing, and the Piwi subfamily that binds to piRNAs [12]. Both Argonaute protein subfamilies have the common capacity to repress gene expression following binding of associated small guiding RNAs on complementary seed sequences in target genes.

In contrast, lncRNA biogenesis shares many features with protein-coding RNAs. LncRNAs are often 5′-capped, spliced and 3′ polyadenylated, but they are shorter in length, do not possess open-reading frames and are expressed in lower abundance. After processing, lncRNA play scaffolding roles to form ribonucleoprotein complexes (RNP) without processing activity. Growing evidence points that lncRNA functions are associated with their subcellular localization [13]. They function as signal, decoy, scaffold, guide or enhancer, to promote chromatin modification, transcription, translation, splicing, mRNA decay, protein transport and assembly [14].

Recently, the landscape of ncRNAs has been remarkably expanded with the emergence of next-generation sequencing and the development of robust bioinformatics tools [15]. In particular, the pattern of human piRNAs switched from about 32,000 identified piRNA sequences in 2014 to reach 8 millions in the second piRBase release published in 2018 [16]. Similarly, Lncipedia catalogs around 50,000 genes of LnRNAs in human [17], while miRBase refers 1,917 human miRNAs, a number in continuous increase [18]. Finally, another widespread and abundant class of non-coding RNAs has been identified in 2012 with the improvement of deep-sequencing, which is called circular RNAs (circRNAs). These RNAs transcriptionally produced from thousand of genes exhibit covalently linked 3′ and 5′ ends and frequently show a higher expression than their linear isoforms [19].

NcRNAs and liver cancer

These recent decades, researchers have paid growing interest for ncRNAs due to their pleiotropic regulating potentials and their ubiquitous distribution in mammalian cells. Now, ncRNAs are defined as versatile cellular switch for good or ill. Accordingly to several deregulations in ncRNA expression observed in various tumors, it is not surprising that the numbers of publications concerning ncRNAs and cancer are constantly increasing: slowly for snoRNAs and piRNAs, reported to be associated with cancer these last years (123 and 186 items in PUBMED respectively), but more intensively for LncRNA (5,182 items) and miRNAs (16,358 items). CircRNAs have already reached 84 items with cancer. Their potential as non-invasive prognostic and predictive biomarkers is substantial since ncRNAs have been detected in a great diversity of body fluids, such as blood, urine and saliva.

microRNAs

Since their discovery at the end of the 1990s, miRNAs are the best-characterized small ncRNAs. The transcription of primary transcripts of miRNAs (pri-miRNAs) is firstly driven by the RNA polymerase II. In about 15% of cases, pri-miRNAs derive from polycistronic miRNA transcripts. Pri-miRNAs are processed by specific complexes of proteins containing Drosha and Dicer, to become mature (pre-miRNAs). Then, they are integrated in RISC complexes to match with complementary sequences mainly located in the 3′UTR (untranslated transcribed region) of mRNAs, resulting in most cases in translation inhibition and accelerated mRNA degradation in subcellular compartments called processing bodies (P-bodies) [20,21]. MiRNAs are from 16 to 35 nucleotides in size. MiRNA expression levels are tissue-specific and finely regulate a precise pattern of genes during growth and development. A modification in cellular

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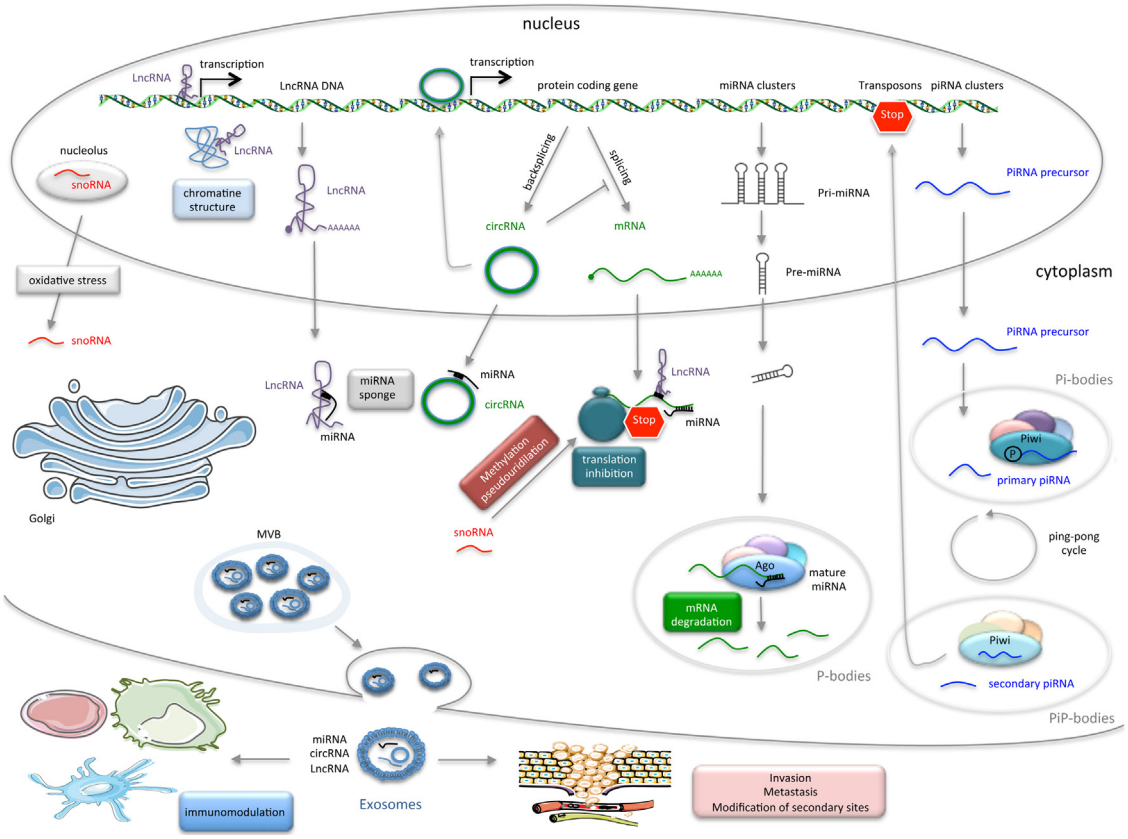

Figure 1 The great diversity of biogenesis and action of non-coding RNA world. NcRNAs are divided into small ncRNAs (< 200 nt) including piRNAs, snoRNAs and miRNAs, and long non-coding RNAs (> 200 nt) consisting of lncRNAs and circRNAs. Pre-miRNAs processed from pri-miRNAs are integrated in RISC complexes to impair translation and accelerate mRNA degradation. SnoRNAs serve as guides for the 2′-O-methylation chemical and pseudouridylation of other RNAs. piRNAs are produced from long single-stranded piRNA clusters and engaged into a ping-pong cycle to protect the genome from transposon invasion. LncRNAs control transcription, either positively or negatively, mRNA stability and translation and chromatin architecture. circRNAs generated by alternative splicing of pre-mRNAs act as microRNA sponges and transcriptional and translational modulators. This landscape of ncRNAs finely regulate gene program and thus cell function and fate. Disequilibrium in their expression could favor liver disease and promote liver tumorigenesis. NcRNAs could also be secreted from tumor and immune cells into exosomes to modulate cell fate in their neighborhood or at a distance to favor metastasis. CircRNA: circular RNA, LncRNA: long non-coding RNA, miRNA: microRNA, MVB: multivesicular bodies, P-bodies: processing bodies, Pi-P-bodies: processing bodies for piRNAs, piRNA: PiwiRNA, snoRNA: small nucleolar RNA.

miRNA expression is also largely described in a variety of tumors. MiRNA signature can be characteristic of each tumor type in line with its tissue origin and the driven molecular alterations [22]. By their multiplicity of action and their tissue-specific distribution, miRNAs have been pointed as robust therapeutic candidates, and potent biomarkers for cancer diagnosis.

MiRNA expression has been largely found disturbed during hepatocarcinogenesis [23], with specific miRNA deregulations at each step before HCC development, from liver disease to cirrhosis [24]. MiR-122 emerges as the most hopeful miRNA with diagnostic, prognostic and therapeutic potentials. It is the most abundant miRNA in the adult liver, whose expression is largely altered during non-alcoholic steatohepatitis [25] and in HCC, in the liver/tumor but also in the serum [26]. This year, Teufel and coworkers have identified a plasmatic signature of nine miRNAs including miR-122 (miR-122, miR-30a, miR-125b, miR-200a, miR-374b, miR-15b, miR-107, miR-320, and miR-645), which is significantly associated with patient overall survival during regorafenib treatment [27]. In addition, miR-135a, a miRNA upregulated in HCC [28], has been also described as a promising prognostic factor [29], since its expression is associated with recurrence after tumor resection [30].

In addition to their prognostic benefit, miRNAs could also be useful as therapeutic opportunities in HCC. Importantly, the first miRNA-based therapy to enter into clinical trials was an anti-miR-122 against hepatitis C virus based on the locked nucleic acid technology (LNA)—the virus perverting miR-122 in hepatocytes for its replication [31]. MiRNA-based therapies appear as promising therapeutic strategies as they simultaneously impact on proliferation, metabolic features and immune contexture of liver tumors. MiRNA-based therapies have been successfully tested in liver diseases and cancers (LNA against miR-34a, 2-O-metoxyethyl anti-miR-191) [24,32] — this type of molecules being preferentially delivered to the liver [33]. Mimic-based therapies have also been tested to increase the expression of tumor suppressive

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miRNAs. Mimics of miR-34a and Let-7 encapsulated into liposomes have been developed by the miRNAtherapeutics company and tested in HCC. Despite interesting results, their use in patients has been limited because of offside effects of mimics and/or toxicity of liposomes leading to immune dysfunction [34]. A number of challenges are yet to be addressed to improve the efficiency of miRNA-based therapies and reduce the current limitations concerning their stability, administration, specificity, distribution and secondary effects. The recent use of endogenous vesicles called exosomes could be a promising alternative, as developed at the end of this review.

Small nucleolar RNAs

SnoRNAs are usually localized in the nucleolus, a dense zone in the nucleus. They are from 60 to 300nt in length and constitute a conserved class of small non-coding RNAs anciently spread by retrotransposition [35]. SnoRNAs are expressed in all eukaryotes, incorporated into small nucleolar ribonucleoprotein complexes (snoRNPs) with specific conserved protein partners to usually serve as guides for the chemical modifications of other non-protein-coding RNAs: either 2′-O-methylation of target RNAs (box C/D snoRNAs), or pseudouridylation (box H/ACA snoRNAs). However, recent studies revealed that snoRNAs display common features with miRNAs for their genomic location, processing pathways and integration in RISC complexes [36]. Although most snoRNAs were originally thought able to modify ribosomal RNAs for translation, a subgroup of snoRNAs has been shown to target small nuclear RNAs, tRNAs and mRNAs or even to be involved in rRNA processing.

Until very recently, snoRNA dysfunction had not been studied in cancer. However, works performing genomic analysis of snoRNAs in tumors highlighted their aberrant expression levels in cancer, including HCC [37]. Su and coworkers were the first to report in tumor tissues a frequent overexpression of fibrillarin, a protein component of C/D box snoRNP [38], in correlation with elevated amount of snoRNA clusters. Last year, the snoRNA snoU2_19, which is upregulated in HCC, has been found to play an oncogenic role through Wnt/β-catenin pathway activation [39]. Another snoRNA regulating the Wnt/β-catenin pathway and promoting liver tumorigenesis is SNORD76, whose expression is increased in HCC tissues and associated with poorer patient survival [40]. Altogether, these observations support potent roles of snoRNA pathways in cancer. It remains to unravel how snoRNAs are deregulated and precise their roles during tumorigenesis to open new opportunities for therapeutic intervention.

PiwiRNAs

piRNAs are preferentially detected in germ line and are usually 25—31nt in length [41]. They are characterized by their outstanding diversity of millions of sequences, poorly conserved but with a genomic localization commonly shared among species. The best-characterized class of piRNAs is repeat-associated piRNAs, guardians of transposon invasion. Another class of piRNAs called pachytene piRNAs, originating from the 3′UTR regions of mRNAs or from intergenic lncRNAs, exhibits unknown targets and functions [42]. The mechanisms underlying piRNA biogenesis and functions are still elusive, due to the lack of easy-to-use *in vitro* models, and because little is common with the miRNA system. In contrast with miRNAs, repeat-associated piRNAs are produced from long single-stranded piRNA clusters independently to RNAse processing [43]. One mechanism of biogenesis is named ping-pong cycle creating secondary piRNAs, which target the initial piRNA clusters, amplifying the pattern of piRNAs. They bear 2′-O-methyl-modified 3′ termini recognized by the PIWI subfamily members of the Argonaute family, and prefer uracil in 5′-termini of their targets. By this repeated endonucleolytic cleavage, piRNAs guard the germline genome against detrimental DNA damage induced by multiplication and insertion of mobile transposons. Their major role aims at preserving spermatogenesis, a period of global epigenetic remodeling [44]. PiRNA-mediated transposon silencing could also be induced by epigenetic regulation during germline development by DNA and histone $H_3K_9me_3$ methylation [45]. PiRNAs are also required to silence the paternally imprinted gene *RASGFR1* during *de novo* methylation [46]. As a methylation inducer, the impact of piRNAs during cancer development could be a consistent question. These later years, some works have profiled piRNA expression and explored their biological functions in cancer, including liver cancer [47,48]. In particular, piR-Hep1 was found to be upregulated in half of HCC tumors and piR-Hep1 silencing inhibited cell viability, motility, and invasiveness *via* a reduction of AKT phosphorylation. The PIWI pathway also seems to be involved in the maintenance of cancer stemness and particularly in the resistance to chemotherapies [49]. These data gave new insights into liver tumorigenesis and support that piRNAs might be new disease biomarkers and could result in innovative treatment strategies.

Lon non-coding RNAs

LncRNAs are the class of ncRNAs, which currently knows the biggest attention from the scientific community. LncRNAs are transcribed by the RNA polymerase II from intergenic regions (lincRNAs), introns or enhancers in sense, antisense or bidirectional manner [50]. They are frequently polyadenylated. Their sequences are poorly conserved among species, challenging the comprehension of their underlying mechanisms. As for other ncRNAs, the number of lncRNAs is directly proportional to organism complexity. They exhibit a great diversity of sequences and structures and postulated to account for the majority of the non-coding transcriptome (50,000 lncRNAs are currently defined). LncRNAs display a tissue-specific pattern of expression and are finely regulated in time and space across the stages of development. In contrast to other ncRNAs, lncRNAs are predominantly nuclear and interact with ribonucleoproteins to control:

- transcription, either positively (ribosome guide, scaffold for transcriptional activators, chromatin remodelers, signal for transcription) or negatively (decoy, recruitment of transcriptional repressors);
- mRNA stability and translation (miRNA sponge);

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- chromatin architecture (chromosome loops and inter-chromosomal interactions) [51].

Panels of lncRNAs have been shown to play essential roles during embryogenesis [52] and tumorigenesis [53], due to their regulatory roles in proliferation, differentiation, stress response and survival. Numbers of LncRNAs have been recently associated with HCC, and particularly with epithelial-to-mesenchymal transition (EMT), stem cell features and tumor invasion: lncRNA DANCR [54], hPVT1 [55], lncTCF7 [56] or ATB [57]. Others have been associated with HCC development and progression such as MEG3 [58], H19 [59], MALAT-1 [60] or TUG1 [61]. The first lncRNA identified as overexpressed in HCC is HULC (highly upregulated in liver cancer) [62], and thus the most studied. It regulates HCC proliferation, angiogenesis, lipid metabolism, autophagy and sensitivity to chemotherapies [63]. HULC expression predicts better outcomes [64] and may functions as a non-invasive potent biomarker [65]. Another interesting lncRNA is CASC9, upregulated in half of HCCs and associated with lower recurrence after surgery [66]. This study also highlights CASC9 as a putative non-invasive prognostic biomarker in HCC since higher level of circulating CASC9 is correlated with tumor size and HCC recurrence. Regarding to the large amount of lncRNAs implicated in HCC, these RNAs represent rational candidates for innovative therapy using RNAi approach, small inhibitory molecules or modulators of their secondary structures. The last three months, a study obtained encouraging results with nanoparticles delivering an inhibitor of the LncRNA TUG1, which exhibit antitumour activity on pancreatic and brain PDX (*patient-derived xenograft*) models [40]. In addition, MALAT-1 appears as a potent druggable lncRNA using LNA-Gapmer in multiple myeloma and probably other tumors [67], opening potential new avenues in molecular medicine based on lncRNAs.

Circular RNAs

As previously mentioned, the landscape of circRNAs has rapidly grown with improvement of next-generation sequencing, to reach more than 100,000 circRNAs identified in humans [68]. In most cases, circRNAs are generated by alternative splicing of pre-mRNAs called backsplicing but expressed independently of their linear isoforms [69]. Thus, circRNA biogenesis competes with linear splicing because of overlapping dependence on the spliceosome. Accordingly to their recent discovery, the understanding of how circRNAs participate in biological processes is still preliminary. However, circRNAs have been extensively described as microRNA sponges [70] but also as transcriptional and translational modulators [71]. Hundreds of circRNAs have been described deregulated in human cancers, including in liver cancer [72], and thus may serve as potential biomarkers [73]. They could act either as oncogenes or tumor suppressors through regulatory actions on cell proliferation, invasion or EMT [74]. Circ_100338 [75] and circ_0078602 [76] are associated with poor prognosis in HCC patients. CircRNA-based research is still in its infancy. The main challenge in future to make circRNAs as powerful candidates for cancer therapy will be circRNA targeting without detrimental interfering with linear RNA expression.

Exosomal ncRNAs as therapeutic and diagnostic candidates

Exosomes are double membrane cell-derived microvesicles from 30 to 100 nm in diameter, containing nucleic acids (> 3,400 mRNAs and 2,800 miRNAs according to Exocarta) and proteins (> 9700) [77]. Most cells, and particularly immune cells, are physiologically secreting exosomes originated from multivesicular bodies (MVB), whose fusion with the plasma membrane is orchestrated by Rab, SNAP (*Soluble N-ethylmaleimide-sensitive-factor attachment protein*) and SNARE (*SNAP Receptor*) proteins [78]. During tumorigenesis, tumor cells aberrantly secrete exosomes to communicate with stromal cells, such as the lncRNA TUC339 secreted from HCC cells towards the surrounding macrophages to promote tumor growth and spreading [79]. Interestingly, a very recent work reciprocally showed that adipose tissue secrete exosomal circRNA circ-DB (hsa_circ_0025129) to favor HCC growth, providing new insights into the understanding of the association between adipose tissue and HCC [80]. Exosomes are also useful for tumor cells to modify secondary recipient sites and to subsequently favor metastasis [81]. The detection of exosomal ncRNAs in body fluids is thus a valuable non-invasive diagnosis tool for cancer, including HCC. This has been extensively described in literature for miRNAs [26], such as miR-221 [82] or miR-224 [83], but also miR-21 and other ncRNAs like LncRNA-ATB [84], and circSMARCA5 [85].

Additionally, exosomes offer an attractive modality for personalized cancer treatment [86]. They have been used as a targeted delivery system for chemotherapeutic agents, leading to cancer cell killing and promoting a domino effect through the release of secondary cytotoxic vesicles [87]. Importantly, new encouraging works have been obtained by injection of stellate cell-derived exosomes in mice developing liver cancer. Exosomes incorporating mimics of miRNAs have successfully impaired tumor cell proliferation and favored apoptosis *in vivo* [88,89]. To avoid limitations associated with patient-derived exosomes (expensive and complex production, time consuming, poorly reproducible), a promising alternative is the synthesis of bioengineered exosome mimetics, more suitable for clinical use [90].

Conclusion

In conclusion, this review describes how ncRNA could represent in future promising tools for the treatment of liver cancer. The current advances in the field of nanotechnologies might facilitate delivery of ncRNA-based therapeutics in favor of clinical studies. Further studies are thus critically needed to precisely unravel the impact of ncRNA deregulations in cancer and how they could be counteracted to finalize clinical trials in the coming years.

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Disclosure of interest

The authors declare that they have no competing interest.

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