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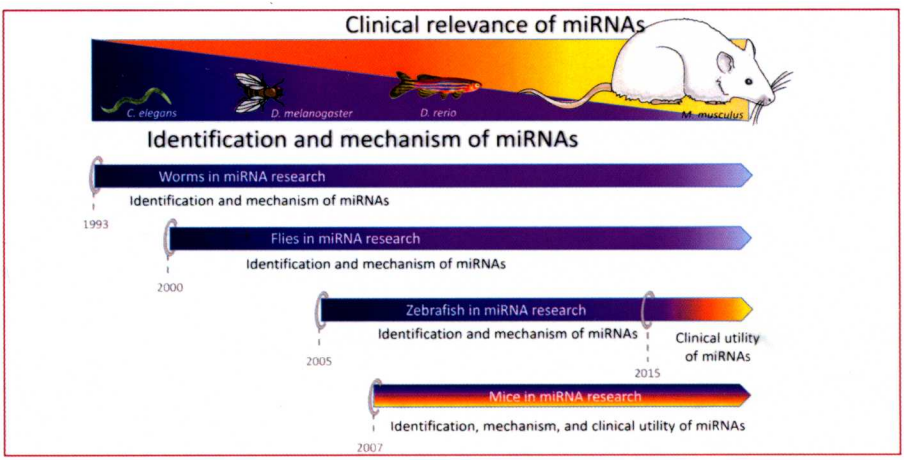

miRNA and Cancer

Edited by
Carlo M. Croce
Paul B. Fisher

VOLUME ONE HUNDRED AND THIRTY FIVE

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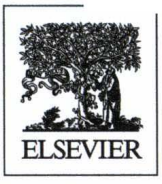

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PREFACE

It is well established that less than 2% of the human genome codes for proteins, while the vast majority of the genome is noncoding, although most of this noncoding genetic information is transcribed. For multiple reasons, it was thought that this noncoding part of the genome was just useless genetic information. Although it seemed quite unreasonable to think that nature designed a genome with most of its content unimportant, the relevance of the noncoding genome in all cellular functions, including normal physiology and in disease, was not evident until the last 15 years. The first suggestion that noncoding RNA could play a role in normal physiology was the discovery that mutations of a gene, lin 4, of the worm (*C. elegans*) affected its development. It was found that this gene encoded a very short noncoding RNA, a microRNA. After this discovery and that of another microRNA, let7, not much interest in microRNAs and noncoding RNA was expressed by the scientific community. Such short RNAs were found to bind, for the most part, to sites at the 3′ untranslated region of mRNA, causing a block of translation and/or degradation of the targeted mRNA. The situation dramatically changed with the discovery of siRNA in 1998, since microRNAs seemed to function in a way similar to that of siRNA. Accordingly, a renewed interest in microRNAs emerged and, as a result, it was found that Drosophila carried microRNA genes (2000), and in 2001 it was found that mice, rats, and human beings carried microRNA genes, which were seen as negative regulators of gene expression.

In 2002 investigation of the deletions at 13q14 in chronic lymphocytic leukemia (CLL), the most common human leukemia, led to a focus on two microRNA genes, miR-15a and miR-16-1, that map on the same polycistronic RNA. These studies indicated that in most CLL a loss of miR-15/16 occurs leading to the development of a malignancy. Since microRNAs have multiple targets, experiments focused on identifying a target of miR-15/16 that could explain the development of the malignant disease. In 2005 a specific target of miR-15/16, BCL2, a gene discovered in 1984 in the Croce laboratory that is involved in translocation with human immunoglobulin loci and responsible for follicular lymphoma was identified. Thus, loss of miR-15/16, two negative regulators of BCL2, results in constitutive overexpression of BCL2 and malignancy. The circle of discovery was closed when Abbott developed a small-molecule inhibitor of BCL2, ABT199 or

venetoclax. This drug is a phenomenal selective killer of leukemic cells with little side effects and has been approved by the FDA for the treatment of CLL in April 2016. A historical perspective on the discovery and development of the miRNA field can be found in Chapter 1 (Drusco and Croce: MicroRNAs and Cancer: A Long Story for Short RNAs) and a review of current therapeutics focused on miRNA-protein targets can be found in Chapter 4 (Van Roosbroeck and Calin: Cancer Hallmarks and MicroRNAs: The Therapeutic Connection).

During the past 10 years, it has become clear that microRNAs contribute to the function of each biochemical pathway and to all cellular functions. It has also become clear that dysregulation of microRNAs occurs in and contributes to every malignancy. Thus, we can exploit microRNA dysregulation for the diagnosis, prognosis, and monitoring of human cancer. MicroRNAs and antimicroRNAs could also be seen as drugs. MicroRNAs could be therapeutic in malignancies that depend on their loss for transformation and antimicroRNAs could be therapeutic for malignancies caused by overexpression of microRNAs, such as overexpression of miR-155 in aggressive lymphomas. In Chapter 5 (Ryan: microRNAs in Cancer Susceptibility) the role of miRNAs in cancer etiology and susceptibility is reviewed. This chapter also discusses the genotype–phenotype relationship between miRNA-SNPs (single nucleotide polymorphisms).

Animal models are essential to assess the role of genes in cancer, and animal models carrying dysregulated microRNA genes have been developed and have provided important validations for their role in cancer pathogenesis and progression. Chapter 3 (Pal and Kasinski: Animal Models to Study MicroRNA Function) discusses various strategies that have been used to develop transgenic organisms with a review of the application of transgenic mice for investigating preclinical efficacy of microRNA-based cancer therapeutics.

The major problem with the development of microRNAs and antimicroRNAs in cancer therapeutics is that at present it is extremely difficult to target enough microRNA/antimicroRNA into 100% of the cancer cells. Targeting technologies are, however, improving at a fast rate; thus, it is not difficult to predict that in the near future microRNAs and antimicroRNAs will be drugs. Insights into this area will benefit from a better understanding of the upstream regulatory molecules that regulate miRNAs and the role of epigenetic changes in regulating miRNA expression. These topics are discussed, respectively, in Chapter 2 (Pradhan et al.: The Enigma of miRNA

Regulation in Cancer) and in Chapter 7 (Moutinho and Esteller: MicroRNAs and Epigenetics).

Because of these extraordinary developments in studies of microRNA biology, function, and as biomarkers, it is extremely appropriate and timely to attempt to put together a volume that summarizes some of the most exciting developments in many of the aspects of microRNA biology and of the role of microRNA in disease, and of the use of microRNA dysregulation for the diagnosis, prognosis, and monitoring of cancer. Chapter 6 (Balatti et al.: Role of the tRNA-Derived Small RNAs in Cancer: New Potential Biomarkers and Target for Therapy) discusses the role of tRNA-derived small RNAs (tsRNAs) in cancer by regulating gene expression. We are optimistic that future developments in this exciting area of investigation will permit the efficient and successful entry of miRNA-targeted therapeutics and diagnostics into the clinic for the therapy of cancer and other pathological diseases.

Carlo M. Croce, MD
Paul B. Fisher, MPh, PhD

CONTENTS

CHAPTER ONE

MicroRNAs and Cancer: A Long Story for Short RNAs

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Abstract

More than six decades ago Watson and Crick published the chemical structure of DNA. This discovery revolutionized our approach to medical science and opened new perspectives for the diagnosis and treatment of many diseases including cancer.

Since then, progress in molecular biology, together with the rapid advance of technologies, allowed to clone hundreds of protein-coding genes that were found mutated in all types of cancer. Normal and aberrant gene functions, interactions, and mechanisms of mutations were studied to identify the intricate network of pathways leading to cancer. With the acknowledgment of the genetic nature of cancer, new diagnostic, prognostic, and therapeutic strategies have been attempted and developed, but very few have found their way in the clinical field. In an effort to identify new translational targets, another great discovery has changed our way to look at genes and their functions. MicroRNAs have been the first noncoding genes involved in cancer.

This review is a brief chronological history of microRNAs and cancer. Through the work of few of the greatest scientists of our times, this chapter describes the discovery of microRNAs from *C. elegans* to their debut in cancer and in the medical field, the concurrent development of technologies, and their future translational applications.

The purpose was to share the exciting path that lead to one of the most important discoveries in cancer genetics in the past 20 years.

Until the early 1990s, molecular genetic studies were based on hunting protein-coding DNA regions to understand their function, regulation, and role in physiologic and pathologic processes. The remaining DNA was considered by many as "junk." Complex protein networks were built to find targets for future applications, ignoring the silent presence of other players that, in the last 15 years, have become leading actors of the biomedical world.

It is in 1993 that Victor Ambros (Lee, Feinbaum, & Ambros, 1993) (Fig. 1) discovered the existence of a short noncoding region (lin-4) negatively regulating the expression of LIN-14 during the development of *C. elegans*.

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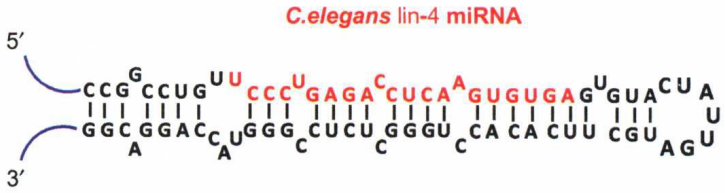

Fig. 1 *C. elegans* lin-4 miRNA is discovered by Ambros in 1993 (Lee et al., 1993).

In the same year and *Cell* volume, Gary Ruvkun (Wightman, Ha, & Ruvkun, 1993) showed that lin-4 binds to the lin-14 3′untranslated region (UTR) "that bears multiple conserved elements complementary to the lin-4 RNAs."

For several years, the scientific community regarded the lin-4 phenomenon with skepticism, but in 2000 Pasquinelli (Pasquinelli et al., 2000) and Reinhart (Reinhart et al., 2000) found another 21-nucleotide-long RNA, let-7, that regulates *C. elegans* development. It is in 2001 (Lagos-Quintana, Rauhut, Lendeckel, & Tuschl, 2001; Lau, Lim, Weinstein, & Bartel, 2001; Lee & Ambros, 2001) that these tiny RNAs were classified as a new class of genes and were named microRNAs, miRNAs, or miRs.

Hundreds of miRs were identified in worms, flies, mice, human cells, and plants (Lagos-Quintana et al., 2001, 2002; Lau et al., 2001; Lee & Ambros, 2001; Llave, Xie, Kasschau, & Carrington, 2002; Park, Li, Song, Messing, & Cheng, 2002; Reinhart, Weinstein, Rhoades, Bartel, & Bartel, 2002; Válóczi et al., 2004).

MicroRNA regulation is still debated because several exceptions to the general rules have been observed in the past few years. MicroRNAs are located by themselves or reside within intronic or exonic regions of coding and noncoding genes, can be single, bi- or polycistronic, are transcribed dependently and independently of other genes (Monteys et al., 2010; Veronese et al., 2015), but all of them derive from a larger double-stranded precursor of about 70 nucleotides (pre-miRNA), that originated from primary transcripts transcribed by RNA Polymerase II, named pri-miRNA. Once translocated to the cytoplasm by Exportin 5, their hairpin region is cleaved by the Dicer complex into 21–25-nucleotide RNA molecules that form the mature miRNA (Krol, Loedige, & Filipowicz, 2010; Li & Rana, 2012). Mature microRNAs function as guide RNAs to posttranscriptionally regulate mRNAs fate. The seed region is a sequence of 6–8 nucleotides at the 5′ end of microRNAs with partial or total complementarity to the

3′UTRs of target mRNAs. Partial or total microRNA::mRNA pairing results in repression of translation and/or in mRNA degradation (Oliveto, Mancino, Manfrini, & Biffo, 2017).

Each microRNA has dozens or hundreds of target mRNAs, while microRNAs with a common seed region regulate the same targets (Bartel, 2009; Di Leva, Garofalo, & Croce, 2014).

Recent reports have suggested that microRNAs also bind to the 5′UTR of mRNAs and instead of repressing, activate the translation of the target mRNA (Fabian, Sonenberg, & Filipowicz, 2010; Vasudevan, Tong, & Steitz, 2007, 2008).

It was only in 2002 that microRNAs made a debut in the medical research field. For years, several scientists looked for a coding gene on a region of the long arm of chromosome 13, at 13q14, that is frequently deleted in chronic lymphocytic leukemia (CLL) (Bullrich et al., 2001; Liu et al., 1997; Mabuchi et al., 2001; Migliazza et al., 2001; Rondeau et al., 2001; Rowntree et al., 2002; Wolf et al., 2001). In Croce's laboratory the deleted chromosome gap was narrowed down over and over until 2002, when George Calin et al. isolated the smallest deleted region, containing the precursor of a microRNA cluster, miR-15a/miR-16-1 (Calin et al., 2002) (Fig. 2). A chromosomal translocation t(2:13) within a breakpoint at 13q14 in a case of CLL involved the same precise region (Calin et al., 2002). Deletion or downregulation of miR-15 and miR-16 is found in approximately 70% of CLLs. Moreover, the latter is often associated with the indolent form of CLLs that frequently progresses into the aggressive form. Thus, the "junk" DNA is involved in cancer. This finding opened new perspectives in cancer research and became the milestone of a new era of biomedical science.

Other findings confirmed Calin et al. studies, showing that mutations affecting microRNAs protein processing complex can not only impair development and cell homeostasis, but can cause diseases and cancer (Carmell, Xuan, Zhang, & Hannon, 2002; McManus, 2003).

The following year Calin et al. found that 19% of 186 microRNAs are located at chromosomal fragile sites and 52.5% of 186 microRNAs are found in minimal regions of loss of heterozygosity or minimal regions of amplifications (Calin, Sevignani, et al., 2004). Interestingly, some microRNAs are organized in clusters of at least two microRNAs. These findings suggested that microRNAs might be located at chromosomal sites in which previous studies have failed to find deregulated coding genes, thus, behaving as tumor suppressor genes or oncogenes.

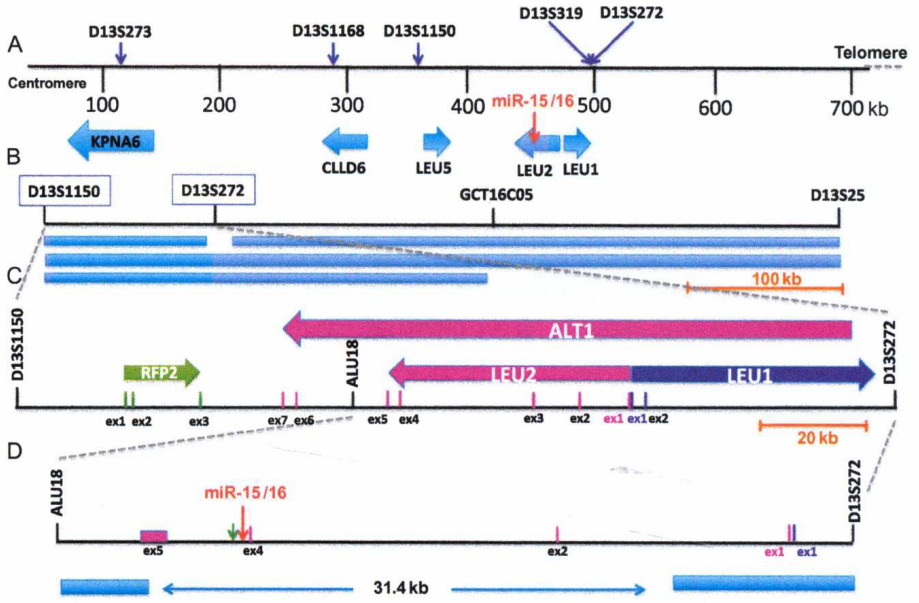

Fig. 2 (A) Map of the chromosomal region at 13q14 often deleted in CLL. Genes and genetic markers mapping locations are shown. (B) 13q14 locus between D13S1150 and D13S25 genetic markers. *Horizontal blue boxes* under the 13q14 locus represent reported deletions. (C) Area of the 13q14 locus delimited by D13S1150 and D13S272. Genes are represented by *arrows* that point the gene orientation. (D) Area of the 13q14 locus delimited by ALU18 and D13S272. The *green arrow* points at the breakpoint in CLL-B carrying a t(2;13)(q32;14) translocation. The *filled box* represents the portions of chromosome 13 found in the hybrid clone derived by a patient with retinoblastoma and CLL with a deletion of 31.4 kb. MiR-15/16 are highlighted in *red* in all maps. *Modified from Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., et al. (2002). Frequent deletions and down-regulation of micro-RNA genes miR-15 and miR-16 at 13q14 in chronic lymphocytic leukemia.* Proceedings of the National Academy of Sciences of the United States of America, *99(24), 15524–15529.*

Given these promising premises Chan-Gong Liu, in Croce's laboratory, developed the first oligonucleotide microchip array for the detection of microRNA expression patterns across human and mouse tissue samples (Liu et al., 2004; Liu, Calin, Volinia, & Croce, 2008), allowing to quantify the expression of 281 precursor microRNAs.

Thomson and Babak designed oligonucleotide microarrays to identify differentially expressed microRNAs in mouse tissues (Babak, Zhang, Morris, Blencowe, & Hughes, 2004; Thomson, Parker, Perou, & Hammond, 2004).

Meanwhile, Schmittgen set up the first experiments for microRNA detection and quantification by RT-PCRs (Schmittgen, Jiang, Liu, &

Yang, 2004) and Exiqon laboratories synthesized locked nucleic acid (LNA)-modified oligonucleotide probes to improve sensitivity and specificity of microRNA northern blot hybridization (Thomsen, Nielsen, & Jensen, 2005). The same probes will be used for in situ hybridization of paraffin-embedded tissues, assessing microRNAs localization and their targets (Kloosterman, Wienholds, de Bruijn, Kauppinen, & Plasterk, 2006; Nuovo et al., 2009).

The following years of cancer research were characterized by intense profiling of all kinds of tumor tissues (Fig. 3). The foremost articles remain the most exciting, each of them revealing a piece of the microRNA world that led to gradually foresee and understand the immense potentials of these small RNAs.

Chang-Gong microarray platform was first used by Calin (Calin, Liu, et al., 2004) in 2004. A total of 47 samples comprehensive of 42 samples of blood mononuclear cells derived by 38 CLL patients and 6 normal specimens (1 lymph node, 2 tonsillar CD5+ B cells, and 3 blood mononuclear cells) were processed and analyzed, defining clustering signatures that could differentiate between CLL and normal controls and, within the CLL cluster, between ZAP-70 and mutated IgV_H prognostic groups. Increased ZAP-70 levels in lymphoid cells were an early predictor of progression, while mutated IgV_H is an indicator of favorable outcome (Hamblin, Davis, Gardiner, Oscier, & Stevenson, 1999; Orchard et al., 2004; Oscier et al., 2002). Deletion at 13q14 strongly associated with IgV_H mutation and, given the new findings, with downregulation of miR-15 and miR-16.

Hence, microRNAs are involved in cancer pathogenesis as much as coding genes and, more importantly, seem to relate to patients' prognosis.

One year later, the same group published in the *New England Journal of Medicine* a profiling study of 94 CLL cases, confirming that loss of expression of miR-15 and miR-16 together with mutation of IgV_H and deletion at 13q14 is associated with a more favorable prognosis (Calin et al., 2005). They also found germ line or somatic mutations in 15% of CLL samples and in 11 miRs sequenced in 42 cases of CLL. In 8 of the 11 patients with microRNA abnormalities, 8 had a family history of cancer and, of the latter, 2 patients harbored a single nucleotide difference in the miR-15/16 pre-miR precursor. *In vitro* and *in vivo* experiments further showed that the abnormal base difference decreased the mature miR-15/16 expression levels and that, in both cases, the mutation was associated with deletion of the other allele at 13q14.

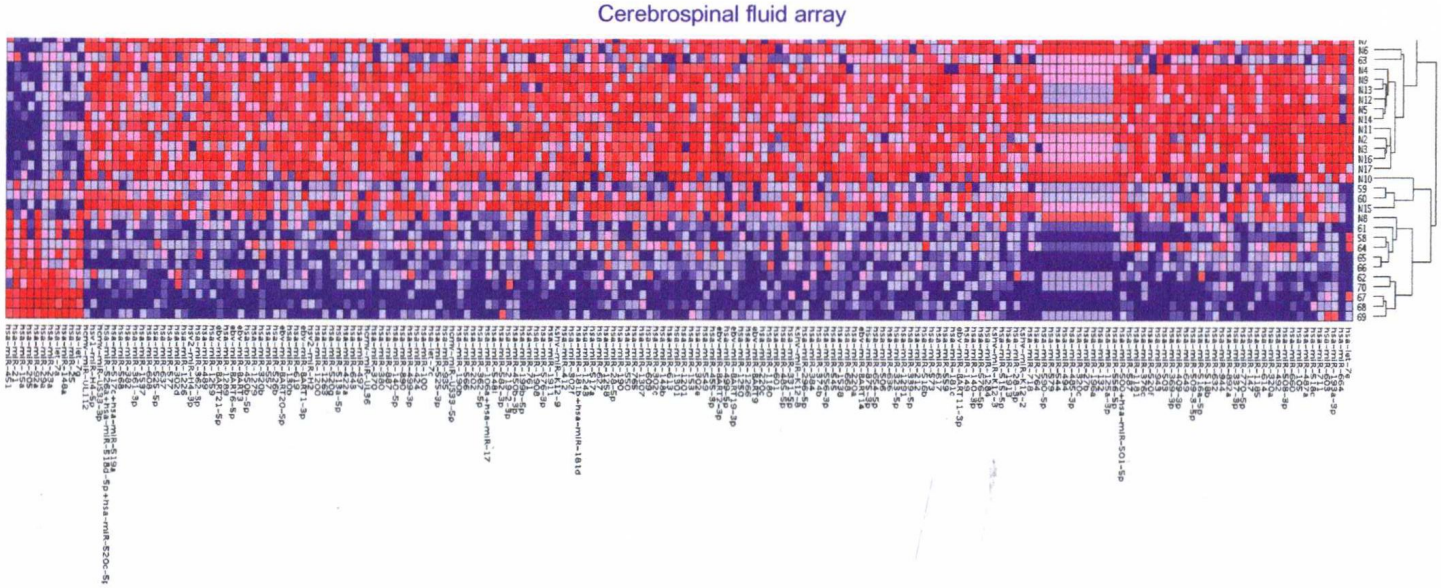

Fig. 3 Array profile of samples of cerebrospinal fluid coming from patients with brain metastasis from breast cancer vs controls.