DOMANDE ARGOMENTO SPECIFICO

AP BUR 77 – BANDO DELIBERA 787 DEL 6/9/23

DOMANDE ESTRATTE: n°1; n°6; n°4; n°3.

1) La/Il candidata/o esponga l'utilità clinica dello studio del ctDNA evidenziando le eventuali correlazioni con lo stadio di malattia

2) La/Il candidata/o esponga le differenze in termini di vantaggi e svantaggi di una analisi condotta su biopsia tissutale rispetto ad una biopsia liquida

3) La/Il candidata/o descriva un ideale workflow di lavoro per il processamento e studio del ctDNA da differenti fluidi biologici

4) La/Il candidata/o illustri le principali caratteristiche fisico-biologiche dei miRNA circolanti evidenziandone le differenze rispetto al ctDNA

5) La/Il candidata/o descriva come i miRNA circolanti possono essere misurati evidenziando le eventuali problematiche tecnico-biologiche correlate

6) La/Il candidata/o descriva le potenzialità dell'applicazione di miRNA circolanti nell'ambito di una patologia oncologica a scelta

DOMANDE INFORMATICA
AP BUR 77 – BANDO DELIBERA 787 DEL 6/9/23

DOMANDE ESTRATTE: m°1; n°6; n°4; n°3.

1. Cosa definisce la sovra-rappresentazione genica (enrichment analysis) in bioinformatica?
2. Cosa rappresenta un "heatmap" nell'analisi dei dati di espressione genica?
3. Cosa si intende con normalizzazione dei dati nella bioinformatica?
4. Cosa rappresenta il valore p (p-value) nell'analisi statistica dei dati di espressione genica?
5. Cosa rappresenta il valore di fold change?
6. Cos'è la bioinformatica?

Leading Edge
Review

Cell

The Role of Non-coding RNAs in Oncology

Frank J. Slack[1,2,3,*] and Arul M. Chinnaiyan[4,5,6,7,8,*]
[1]Harvard Medical School Initiative for RNA Medicine, Harvard Medical School, Boston, MA 02215, USA
[2]Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA
[3]Department of Medicine, Harvard Medical School, Boston, MA 02215, USA
[4]Michigan Center for Translational Pathology, University of Michigan, Ann Arbor, MI 48109, USA
[5]Department of Pathology, University of Michigan, Ann Arbor, MI 48109, USA
[6]Rogel Cancer Center, University of Michigan, Ann Arbor, MI 48109, USA
[7]Department of Urology, University of Michigan, Ann Arbor, MI 48109, USA
[8]Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109, USA
*Correspondence: fslack@bidmc.harvard.edu (F.J.S.), arul@umich.edu (A.M.C.)
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For decades, research into cancer biology focused on the involvement of protein-coding genes. Only recently was it discovered that an entire class of molecules, termed non-coding RNA (ncRNA), plays key regulatory roles in shaping cellular activity. An explosion of studies into ncRNA biology has since shown that they represent a diverse and prevalent group of RNAs, including both oncogenic molecules and those that work in a tumor suppressive manner. As a result, hundreds of cancer-focused clinical trials involving ncRNAs as novel biomarkers or therapies have begun and these are likely just the beginning.

Although now one of the hottest topics in biomedical science, the importance of non-coding RNA (ncRNA) was largely unrecognized until recently. RNA was once thought to mostly serve as a messenger that carried instructions encoded in DNA so that other molecules, like the ribosome, could use the code to make proteins. However, in the last 30 years, researchers have discovered that multiple types of RNA exist, and among the most important is ncRNA—the type that is not involved in producing proteins. The discovery of tens of thousands of ncRNA species has revolutionized the field, altering the way that researchers think about physiology and the development of disease (Adams et al., 2017; Bartel, 2018; Evans et al., 2016; Rupaimoole and Slack, 2017). ncRNAs constitute more than 90% of the RNAs made from the human genome, but most of the >50,000 known ncRNAs have been discovered only in the past 10 years and remain largely unstudied (Deveson et al., 2017; Esposito et al., 2019; Kopp and Mendell, 2018; Ransohoff et al., 2018).

Still, there are many ncRNAs that have since been shown to play key roles in both normal cellular function and disease, including cancer, and this information is being actively translated into the clinic. Some small ncRNAs are so stable that they survive in the bloodstream and could be the basis for accurate and sensitive screens for major human cancers in a few drops of blood (Yaman Agaoglu et al., 2011; Imaoka et al., 2016; Toiyama et al., 2013). Additionally, ncRNAs can be therapeutically targeted, and the delivery of ncRNAs can be based on an existing foundation of what has been learned regarding delivery of RNAi and oligonucleotides targeting protein-coding mRNAs (Levin, 2019; Pecot et al., 2011; Wu et al., 2014). In fact, the field of RNA medicine has seen a renaissance (Levin, 2019) with the recent approval of the first RNAi drug Onpattro (patisiran; reduces levels of *TTR* for treatment of the neurodegenerative disease hereditary transthyretin amyloidosis) (Adams et al., 2018) and the clinical success of the RNA-targeting oligonucleotide drug Spinraza (nusinersen; increases levels of full-length *SMN2* for treatment of the neuromuscular disease spinal muscular atrophy) (Wurster et al., 2019). In addition, clinical trials with drugs based on a class of ncRNAs called microRNA (miRNA), either therapies that increase or decrease the target miRNA, have begun for cancer (Beg et al., 2017; Seto et al., 2018; van Zandwijk et al., 2017).

In this review, we will discuss ncRNAs in relation to cancer cell biology and their relevance to current clinical practice. We first examine the intricacies of the different classes of ncRNAs (miRNAs, transfer RNA [tRNA]-derived small RNAs [tsRNAs], PIWI-interacting RNAs [piRNAs], long ncRNAs [lncRNAs], pseudogenes, and circular RNAs [circRNAs]) (Table S1) and provide fundamental examples of the far-reaching roles that these molecules have in affecting cancer processes. We then discuss how these basic science insights in ncRNA biology are being used to develop next-generation diagnostics and therapies in cancer. As the so-called "dark matter" of the genome continues to be brought into the light, it is evident that targeting ncRNA signaling has great potential to impact cancer patient care.

Overview of Classes of ncRNAs and Their Association with Cancer

For decades, the miniscule protein-coding portion of the genome was the primary focus of medical research. The sequencing of the human genome showed that only ~2% of our genes ultimately code for proteins, and many in the scientific community believed that the remaining 98% was simply nonfunctional "junk" (Mattick and Makunin, 2006; Slack, 2006). However, the ENCODE project revealed that the non-protein coding portion of the genome is copied into thousands of RNA molecules (Djebali et al., 2012; Gerstein et al., 2012) that not only regulate fundamental biological processes such as growth,

development, and organ function, but also appear to play a critical role in the whole spectrum of human disease, notably cancer (for recent reviews, see Adams et al., 2017; Deveson et al., 2017; Rupaimoole and Slack, 2017). Trailblazing research led to our understanding of how ncRNA molecules perform multiple vital roles in the coding, decoding, regulation, and expression of genes as well as how they communicate with each other (Anastasiadou et al., 2018b; Esquela-Kerscher and Slack, 2006; Gregory and Shiekhattar, 2005; Huarte and Rinn, 2010; Kasinski and Slack, 2011; Krichevsky et al., 2003; Rinn and Huarte, 2011; Tay et al., 2014). This knowledge of ncRNA regulatory roles revealed specific ncRNA networks based on complementary base pairing at work in different cancer types (Anastasiadou et al., 2018b), which in turn, opened up possibilities for scientists in this field to develop specific cancer therapeutic and preventive strategies focused on the ncRNAs made from the human genome (for recent reviews, see Cieślik and Chinnaiyan, 2018; Rupaimoole and Slack, 2017).

Cancer is characterized by cells that grow (proliferate) out of control, are able to spread to other tissues (metastasize), and lose the ability to die through the orderly process of cell death (apoptosis). The discovery of ncRNA has added a new dimension to the understanding of how cancer develops, and how it may be treated, by providing a window into the impact of the rest of the genome. Deregulated ncRNA expression, and subsequent downstream signaling processes that we detail in later sections, have been directly implicated in cancer development and progression. Genetic alterations in genes encoding ncRNAs have been found to be associated with cancer; however, compared to protein-coding genes, the list of genetic examples from studies thus far is considerably shorter. The most notable example is likely deletion of 13q14.3 in chronic lymphocytic leukemia (CLL) that deletes the miR-15/16 tumor suppressors (Calin et al., 2002). Conversely, amplification of chromosomal regions encoding oncogenic ncRNAs are also found in cancer, including amplification of lncRNAs *FAL1* (Hu et al., 2014) and *PVT1* (Tseng et al., 2014). Single nucleotide polymorphisms (SNPs) in the genes of lncRNAs *H19* (Hua et al., 2016), *ANRIL* (Pasmant et al., 2011), and *CCAT2* (Ling et al., 2013) have also been associated with varying risks of cancer development. In addition to genetic alterations within transcribed regions, mutations in the promoters of ncRNAs can lead to altered gene expression levels, such as recurrent driver mutations in the promoters of lncRNAs *NEAT1* and *RMRP* in breast cancer (Rheinbay et al., 2017). Besides aberrations in sequences encoding the ncRNA itself, mutations or dysregulation of enzymes involved in the biogenesis of ncRNAs are implicated in cancer, such as Drosha and Dicer involved in miRNA processing (Rupaimoole and Slack, 2017). In addition to these genetic mechanisms, up- or downregulation of ncRNA expression associated with cancer can occur through epigenetic, transcriptional, or post-transcriptional processes (see recent reviews Adams et al., 2014; Anastasiadou et al., 2018a; Rupaimoole and Slack, 2017).

ncRNAs can be divided into different classes, broadly based upon their size. The small ncRNAs important in cancer include miRNAs, tsRNAs, and piRNAs. At the opposite end of the size spectrum are the lncRNAs, which are characterized as untranslated RNAs greater than 200 nt in length, and include subclasses such as pseudogenes and circRNAs.

MicroRNAs

Near the turn of the millennium, the first miRNAs, *lin-4* and *let-7*, were identified through developmental studies in *C. elegans* (Lee et al., 1993; Reinhart et al., 2000). miRNAs are short ncRNAs of ~22 nt in length that regulate the expression of other RNAs, notably mRNAs through binding between the 5′ end (known as the "seed") of the miRNA with complementary sequences in target RNAs. Genes encoding miRNAs are transcribed by RNA polymerase II (Pol II) and processed through an evolutionarily conserved pathway. In the canonical processing pathway, this longer primary transcript, called the pri-miRNA, forms a characteristic hairpin structure that is recognized by the microprocessor complex (consisting of Drosha and DGCR8), cleaved into a pre-miRNA ~60 nt in length, and exported to the cytoplasm via an Exportin 5 and Ran-GTP complex. The ends of the pre-miRNA are then cleaved by Dicer to form a miRNA duplex, which consists of 5′ phosphates and 2 nt overhangs on each 3′ end. One strand of the miRNA duplex, the guide strand, is loaded onto an Argonaute protein and selected to form the RNA-induced silencing complex (RISC) containing the mature 22 nt miRNA (see recent reviews by Anastasiadou et al., 2018a; Bartel, 2018). The mature miRNA functions by binding to the 3′ untranslated regions (3′UTR) of mRNAs and inhibiting their use by either degradation or translational repression (Bartel, 2018; Esquela-Kerscher and Slack, 2006). Multiple studies have attempted to annotate the number of miRNAs in different species. For humans, higher estimates from miRBase v22 have placed the number of mature miRNAs at 2,654, but algorithms from other databases, such as MirGeneDB2.0, decrease this number to 588 high confidence miRNAs (Fromm et al., 2015; Kozomara et al., 2019). Despite discrepancies in their absolute numbers, it is evident that miRNAs have far-reaching effects on downstream processes, as more than 60% of coding genes are potential targets of miRNAs (Friedman et al., 2009). In addition, hundreds of miRNAs are conserved at their seed region across phylogeny (Bartel, 2018), suggesting key roles in developmental or physiological processes in animals.

Regarding cancer, miRNAs provide a powerful new avenue for the discovery of novel genetic risk factors (Ryan et al., 2010). Among the small ncRNA species, miRNAs are by far the most extensively studied in cancer compared to tsRNAs and piRNAs. miRNAs have been found to be altered in all cancer types studied (Volinia et al., 2006), and alterations in miRNAs have been demonstrated to play a crucial role in affecting molecular and cellular processes of the cancer state (Esquela-Kerscher and Slack, 2006; Nicoloso et al., 2009). Although researchers are still learning the extent of the contribution of miRNAs to cancer, these small ncRNAs seem to function in one of two ways—as tumor suppressors or oncogenes (commonly referred to as oncomiRs) that promote cancer growth or metastasis (Rupaimoole and Slack, 2017). Although small, miRNAs are powerful, with each molecule often able to regulate more than one target, and, vice versa, mRNAs are frequently targeted by several miRNAs (Bartel, 2018). As such, miRNAs function as master regulators that control the expression of thousands of coding and

Table 1. *Continued*

Name	ncRNA Class	Cancer Types Examined	*In Vivo* Experimental Techniques Used	Cancer-Related Mechanisms and/or Functions of ncRNA	References
NEAT1	lncRNA	prostate, skin, pancreatic	shRNA knockdown, overexpression in mouse xenografts; transgenic knockout mouse models	mediates oncogenic nuclear receptor (ER) signaling, prevents DNA damage and activation of p53 tumor suppressor, leading to proliferation, invasion, and decreased apoptosis; conversely, shown to also prevent transformation and proliferation in other settings	Chakravarty et al., 2014; Adriaens et al., 2016; Mello et al., 2017
NKILA	lncRNA	breast	shRNA knockdown, overexpression in mouse xenografts/PDX	binds/inhibits NF-κB and downstream inflammation, which increases apoptosis, reduces invasion; promotes activation-induced cell death in CTLs, T_H1 cells, leading to immune evasion	Liu et al., 2015; Huang et al., 2018

Abbreviations: AR, androgen receptor; ASO, antisense oligonucleotide; CDK, cyclin-dependent kinase; ceRNA, competitive endogenous RNA; circRNA; circular RNA, CTL, cytotoxic T lymphocyte; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; GR, glucocorticoid receptor; hnRNP, heterogeneous nuclear ribonucleoprotein; LNA, locked nucleic acid; lncRNA, long non-coding RNA; miRNA, microRNA; ncRNA; non-coding RNA, PDX, patient-derived xenograft; RCC, renal cell carcinoma; RTK, receptor tyrosine kinase; tsRNA, tRNA-derived small RNA.

non-coding genes, including most of the insidious oncogenes, such as *RAS*, *MYC*, and *EGFR*, and the critical tumor suppressors, including *TP53*, *PTEN*, and *BRCA1*. Table 1 provides a list of selected miRNAs (and examples from the other classes of ncRNAs) that have *in vivo* experimental evidence to either support an oncogenic, tumor suppressive, or context-dependent role. Many of these studies involved creation of transgenic mouse models and/or delivery of miRNA mimetics or antimiRs, synthetic oligoribonucleotides that either replenish or decrease levels of the target miRNA. Table 1 further provides instances of the cancer-related molecular mechanisms that each miRNA has been shown to regulate. Here, we highlight a few examples from each category, with Figures 1 and 2 also providing visual representations of one oncogenic and one tumor suppressive pathway, respectively.

Oncogenes

Research has shown that miRNAs can function as oncogenes, promoting abnormal cell growth and contributing to tumor formation. These miRNAs may directly inhibit the activity of tumor suppressors or work indirectly by removing the genetic brakes on oncogene activity. For example, miR-155 can promote abnormal B cell proliferation, setting in process a series of changes that eventually lead to the development of leukemia and lymphoma (Babar et al., 2012; O'Connell et al., 2009). Importantly, delivery of antimiRs targeting miR-155 can inhibit tumor growth (Babar et al., 2012; Cheng et al., 2015) (Figure 1A). Another miRNA, miR-21, is overexpressed in pre B cell lymphoma and has been implicated in other cancers, such as lung cancer, through targeting negative regulators of Ras signaling (Hatley et al., 2010; Medina et al., 2010). In glioblastomas, an oncogenic miRNA, miR-10b, is expressed at higher levels than in normal brain tissue and is required for tumor growth (El Fatimy et al., 2017). These oncogenic miRNAs exhibit the phenomenon of oncogene addiction (oncomiR addiction), because the tumors are dependent on the continued expression of the miRNAs for survival (Cheng and Slack, 2012), and are, thus, important potential targets in anti-cancer therapy. Recently, the potential for targeting oncomiRs uniquely overexpressed in an individual patient's tumors was demonstrated, opening the way to personalized miRNA therapy as we discuss at the end of the review (Gilles et al., 2018).

Tumor Suppressors

Research suggests that miRNAs can also act as tumor suppressors, and when their function is lost, so is their protective power. For instance, the conserved miRNA *let-7* represses *RAS*, a family of oncogenes implicated in approximately one-third of all human cancers (Johnson et al., 2005, 2007). Consequently, *let-7* expression can reduce levels of *RAS*, suggesting that it acts as a tumor suppressor and can be used as a new and promising therapeutic agent (Trang et al., 2010, 2011). As another example, miR-15a and miR-16-1 typically act as tumor suppressors but, when mutated or deleted, are associated with the development of chronic lymphocytic leukemia (CLL), the most common type of leukemia (Calin et al., 2002, 2005; Klein et al., 2010) (Figure 2A). Furthermore, miR-34a, a member of the conserved, redundant miR-34 family of miRNAs (Concepcion et al., 2012), regulates the expression of several oncogenes and is a direct downstream target of p53 (Adams et al., 2016a, 2016b; Kasinski and Slack, 2012; Liu et al., 2011). These miRNAs are also disabled in other types of cancer, such as multiple myeloma, mangle cell lymphoma, and prostate cancer (Volinia et al., 2006).

Context-Dependent

Some miRNAs function as either tumor suppressors or oncogenes, depending on the context. A notable example is miR-29,

2016). As noted above, to mediate their effects, miRNAs associate with the ubiquitously expressed AGO family of Argonaute proteins. In contrast, as their name implies, piRNAs are loaded onto proteins of the PIWI family of Argonaute proteins, which are often restricted to gonadal cells. The classic function of piRNAs is to silence transposons, and they do this in two ways. In the first mechanism, piRNAs guide PIWI proteins to nascent transposon transcripts and generate repressive chromatin states at target transposon promoters to silence their transcription. In the second scenario, piRNAs guide the PIWI complex to the transposon mRNA, where it cleaves the transcript (see recent reviews by Ng et al., 2016; Ozata et al., 2019). Although classically thought of as functioning only in gonadal cells, recent work shows that some of the tens of thousands of piRNAs are expressed in somatic tissues, albeit it at very low levels, and misexpressed in cancers, suggesting that these small ncRNAs might also be useful biomarkers (Martinez et al., 2015; Mei et al., 2015; Ng et al., 2016). However, the functional roles of piRNAs in somatic tissue and cancer are still being elucidated.

Long ncRNAs

Some of the earliest lncRNAs to be discovered were *XIST* and *H19* (Bartolomei et al., 1991; Brown et al., 1991). Although initially identified through studies characterizing X chromosome inactivation and embryonic development, respectively, these two genes are now among a long list of lncRNAs to be mechanistically linked to several types of cancers. lncRNAs are characterized as non-coding transcripts greater than 200 base pairs in length transcribed by RNA Pol II from independent promoters. Similar to protein-coding genes, their genomic locations are marked by enrichment of H3K4 trimethylation at the transcriptional start site and H3K36 trimethylation throughout the gene body. lncRNA transcripts consist of multiple exons that are spliced through canonical mechanisms into a mature transcript and usually include 5′caps and 3′poly(A) tails. However, lncRNAs have fewer exons and are expressed at lower levels overall compared to protein-coding transcripts (Cabili et al., 2011; Derrien et al., 2012; Iyer et al., 2015). Interestingly, lncRNAs are also not highly evolutionarily conserved, with only 5%–6% of lncRNAs harboring conserved sequences (Iyer et al., 2015). In regards to this, there is a line of thinking that highly conserved lncRNAs may be more likely to be functional. However, there are primate-specific lncRNAs that likely could be involved in disease processes. RNA-sequencing (RNA-seq) has shown that there are much higher absolute numbers of unique lncRNA transcripts compared to protein-coding genes. A recent extensive cancer-centric study, MiTranscriptome, analyzed 7,256 RNA-seq libraries primarily from human tissues (5,298 primary tumors, 281 metastases, and 701 normal/benign) and identified 58,648 lncRNAs. Furthermore, these datasets allowed for an in-depth exploration of the landscape of lncRNAs in cancer and led to the identification of 7,941 lncRNAs that were cancer- and/or lineage-specific (Iyer et al., 2015).

Compared to small ncRNAs, lncRNAs exhibit extensive mechanistic diversity to carry out their functional roles and, therefore, require a lengthier discussion in this area. lncRNAs can function either in *cis* or *trans*, meaning they mediate local effects near their own sites of transcription (*cis*), or they operate at distant genomic or cellular locations (*trans*). Different lncRNAs have also been shown to be able to influence gene expression at all levels- epigenetic, transcriptional, and post-transcriptional. Multiple lncRNAs bring other regulatory molecules (e.g., mRNAs, miRNAs, DNA) into proximity with one another and with proteins (e.g., chromatin modifying complexes, transcription factors, E3 ligases, RNA-binding proteins [RBPs]), essentially creating a flexible molecular scaffold that fosters the chemical interactions necessary to sustain cellular activity (see recent reviews by Anastasiadou et al., 2018b; Kopp and Mendell, 2018).

In terms of lncRNAs functioning by directly binding to protein complexes, one of the most well-characterized mechanisms is guiding of chromatin modifying complexes to target gene promoters to influence transcriptional repression/activation. Noted examples include the following: *HOTAIR* binds PRC2 and LSD1/CoREST/REST on its 5′ and 3′ ends, respectively, to target the complex to promoters and modulate histone methylation levels (Tsai et al., 2010); *SChLAP1*, an aggressive prostate cancer-specific lncRNA, interacts directly with the SWI/SNF nucleosome remodeling complex (Prensner et al., 2013); and *ANRIL* interacts with components of PRC1 and PRC2 to silence genes in the *CDKN2B/2A* tumor suppressor gene cluster (Yap et al., 2010). lncRNAs also commonly bind transcription factors, which can have broad downstream effects on cellular transcriptional programs. Intriguingly, the *GAS5* lncRNA acts as a mimic of a glucocorticoid response element (GRE), directly binding the DNA-binding domain of the glucocorticoid receptor (GR) and preventing it from activating its target genes, including those that prevent apoptosis (Hudson et al., 2014; Kino et al., 2010). Other direct interaction partners include *PANDA* and NF-YA transcription factors that are critical activators of p53-targeted cell death genes (Hung et al., 2011), *PCGEM1* enhancement of c-Myc activity as a master regulator of metabolism (Hung et al., 2014), *NKILA* directly binding to the NF-KB/IKB complex to block phosphorylation of IKB by IKK (Liu et al., 2015), and *DINO* interacting with and stabilizing p53 following DNA damage (Schmitt et al., 2016). lncRNAs are often also found in direct contact with RBPs that regulate mRNA processing and stability. The effects of *HOTAIR* are mediated by binding to hnRNPA2/B1, which function as "matchmakers" to target *HOTAIR*/PRC2 to mRNA transcripts (Meredith et al., 2016). Recently, the novel, ultraconserved lncRNA *THOR* was discovered and shown to function as an oncogene by stabilizing the binding of IGF2BP1 to target mRNAs (Hosono et al., 2017) (Figure 1C). Finally, lncRNAs can function as scaffolds for regulatory molecules found in nuclear speckles and paraspeckles, exemplified by lncRNAs *NEAT1* and *MALAT1* (Clemson et al., 2009; Tripathi et al., 2010).

In addition to proteins, lncRNAs can directly bind to nucleic acids to mediate their molecular mechanisms of action. Two interesting examples of this include the recently identified lncRNA *ARLNC1* that interacts with *AR* mRNA to regulate its cytoplasmic levels in prostate cancer (Zhang et al., 2018), and *LincRNA-p21*, which directly binds *JUNB* and *CTNNB1* transcripts to repress their translation (Yoon et al., 2012). Another common mechanism involves lncRNA acting as a competitive endogenous RNA (ceRNA) or "sponge" for miRNAs. One of