Long non-coding RNAs and their implications in cancer epigenetics

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Synopsis

LncRNAs (long non-coding RNAs) have emerged as key molecular players in the regulation of gene expression in different biological processes. Their involvement in epigenetic processes includes the recruitment of histone-modifying enzymes and DNA methyltransferases, leading to the establishment of chromatin conformation patterns that ultimately result in the fine control of genes. Some of these genes are related to tumorigenesis and it is well documented that the misregulation of epigenetic marks leads to cancer. In this review, we highlight how some of the IncRNAs implicated in cancer are involved in the epigenetic control of gene expression. While very few IncRNAs have already been identified as players in determining the cancer-survival outcome in a number of different cancer types, for most of the IncRNAs associated with epigenetic regulation only their altered pattern of expression in cancer is demonstrated. Thanks to their tissue-specificity features, IncRNAs have already been proposed as diagnostic markers in specific cancer types. We envision the discovery of a wealth of novel spliced and unspliced intronic IncRNAs involved in epigenetic networks or in highly location-specific epigenetic control, which might be predominantly altered in specific cancer subtypes. We expect that the characterization of new IncRNA (long non-coding RNA)–protein and IncRNA–DNA interactions will contribute to the discovery of potential IncRNA targets for use in therapies against cancer.

Key words: cancer epigenetics, intergenic IncRNA, intronic IncRNA, long non-coding RNAs, regulatory RNA, RNA-guided gene silencing.

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INTRODUCTION

According to the central dogma of molecular biology, the proteins are considered the main protagonists of cellular functions and RNAs are the intermediaries between the DNA sequences and their encoded proteins [1]. Most of the previously known classical ncRNAs (non-coding RNAs) had infrastructural functions, such as ribosomal RNAs, transporter RNAs and small RNAs involved in splicing. However, over the past decade, genome-wide transcriptional studies discovered that the eukaryotic genomes are pervasively transcribed in a large number and a wide variety of LncRNAs (long non-coding RNAs; >200 nt) and small ncRNAs (<200 nt), beyond the classical ncRNAs [2–5].

LncRNAs can be polyadenylated or non-polyadenylated and accumulate differentially in the nucleus and in the cytoplasm of cells [5,6]. They can be transcribed by RNAPII (RNA polymerase II) and have a 5’-cap [7]. The LncRNAs can be classified as intergenic LncRNA (long intergenic non-coding RNAs) that are transcribed adjacent to protein-coding genes, eRNAs (enhancer RNAs that are transcribed within the enhancer regions), intronic LncRNAs (transcribed within the introns of protein-coding genes) and...
LncRNAs in cancer

Considering the wide range of roles that lncRNAs play in cellular networks [11], it is not surprising that ncRNAs have been implicated in human diseases, especially in cancer [17]. The broader involvement of IncRNAs in cancer has been extensively reviewed elsewhere [18–20]. In particular, several large-scale studies have been performed to compare the expression of IncRNAs across non-tumour and tumour samples in the past 10 years. These studies have led to the identification of several IncRNAs-based expression signatures of malignancy [21–24]. Despite the fact that some of these expression changes can be related to secondary effects of the tumour progression, experimental approaches have suggested that various lncRNAs are indeed involved in cellular transformation, thus acting as potential tumour suppressors or oncogenes, and leading to tumorigenesis [19].

In contrast with protein-coding mRNAs, which are commonly used as diagnostic and prognostic markers and are frequently expressed from multiple tissue types and cancers, most IncRNAs have tissue-specific expression patterns [5]. This peculiar characteristic of lncRNAs can offer possible benefits in terms of specificity for clinical applications. An interesting example is the lncRNA PCA3 (prostate cancer antigen 3), which is a prostate-specific gene markedly overexpressed in prostate cancer and an established prognostic marker in prostate cancer [25]. The next expected step in clinical medicine is the use of lncRNAs in therapies, and this application may be possible in the future (reviewed in [18]).

LncRNAs that have been shown to be involved in cancer can act by diverse mechanisms, including the control of the mRNA splicing, of the mRNA translation and of the availability of miRNAs (microRNAs) to repress the mRNAs. In the present review, we will focus on those lncRNAs that act in cancer through epigenetic mechanisms.

DNA methylation

DNA methylation has been associated with both activation and repression of the gene expression. It is known to affect mainly the cytosine residues in CpG (cytosine-phosphodiester bond guanine) dinucleotides, which tend to concentrate within short CpG-rich DNA stretches called CpG islands, regions frequently located at the 5′-end of the genes that occupy approx. 60% of the human gene promoters [27]. DNA methylation can lead to gene silencing directly by preventing the recruitment of DNA-binding proteins or transcription factors to their target sites. Indirectly, it can facilitate the binding to methylated DNA of methyl-CpG-binding domain proteins, which can mediate gene repression through interaction with histone-modifying enzymes [28].

DNA methylation was one of the first epigenetic alterations identified in cancer [29]. The cancer epigenome is characterized by genome-wide hypomethylation, which can occur in several genomic sequences, such as retrotransposons, introns and repetitive elements, leading to genomic instability [30]. Additionally, hypermethylation of specific CpG islands can lead to the silencing of tumour suppressor genes involved in key cellular pathways,
Long non-coding RNAs and cancer epigenetics

Figure 1 Possible and known epigenetic roles played by lncRNAs in cancer

(A) A model of lncRNAs affecting DNA methylation. In this model, a lncRNA (red) interacts with a DNA methyltransferase and guides this protein to specific targets, leading to the methylation of the promoters and repression of tumour suppressor genes. A DNA-binding protein (dark blue) can mediate the interaction of the lncRNA with specific sites on DNA. (B) A model of lncRNAs changing the nucleosome positioning. A lncRNA can interact with a nucleosome remodelling complex, leading to the restructuring or dislocation of the nucleosome in specific genomic regions. An increase in the packing of the nucleosome in a region containing a tumour suppressor gene can lead to its repression. (C) A model of lncRNAs having in-cis function. In this model, an RNAPII transcribes an lncRNA (red) that can remain tethered to its transcriptional site and recruit a histone modifying enzyme (HME). This HME can lead to the methylation (left; small green circles) or to the deacetylation (right) of histones and to the subsequent silencing of tumour suppressor genes. (D) A model of lncRNAs acting on trans-regulation. In this model, an lncRNA (red) transcribed from a locus recruits a HME to a different, distant locus. This HME can lead to the methylation (left; small green circles) or to the deacetylation (right) of histones and to the subsequent silencing of tumour suppressor genes. Another possibility, not shown in the schemes, is that the lncRNAs recruit demethylases and/or acetylases to the promoter regions of oncogenes, and thus the lncRNAs might direct the transcriptional activation of such protein-coding genes.

such as DNA repair, apoptosis and cell cycle or to the silencing of transcription factors involved in the control of these genes [31].

A lncRNA named ASIDHRS4 (antisense 1 dehydrogenase/reductase SDR family member 4) is transcribed from the locus of the DHRS4 gene and recruits DNA methyltransferases and other factors to the DHRS4 gene cluster, inducing DNA methylation at the DHRS4L2 promoter region [32]. Two other lncRNAs have been shown to induce either DNA methylation at specific regions of the Kcnq1 locus [33] or demethylation at the Sphk1 CpG island [34]; these genes are known to be related...
to cancer. However, a direct participation of these lncRNAs in tumorigenesis remains to be determined. Figure 1(A) shows a possible mechanism in which a lncRNA could associate with a DNA methyltransferase and guide it to the promoter region of a tumour suppressor gene, leading to the transcriptional silencing of the latter.

Nucleosome positioning

Besides the covalent modifications printed on DNA and histones, non-covalent mechanisms play important roles in the control of gene expression by chromatin regulation. The dislocation, restructuring or destabilization of the nucleosomes are driven by ATP hydrolysis-dependent complexes that can be classified into four families: SWI/SNF, ISWI (imitation-SWI) protein, CHD (chromodomain helicase DNA-binding protein) and INO80 [35]. The positioning and remodelling of the nucleosomes are able to regulate the gene expression by altering the accessibility of regulatory DNA sequences to transcription factors and to the transcriptional machinery [36].

Unlike the roles of DNA methylation and histone modifications in the establishment and progression of cancer, the participation of nucleosome positioning in tumours is less well understood. It has been shown that mutations at and/or silencing of chromatin remodeler subunits, such as the BRG1 [also known as SMARCA4 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, type 4)], the BRM [also known as SMARCA2 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, type 2)] and the and SNF5 [also known as SMARCB1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily B, type 1)] subunits of the SWI/SNF complex [37] and the CHD complex [38], are present in diverse types of cancer. In plants, guiding of the ATP-dependent chromatin remodelling SWI/SNF complex by lncRNAs has been shown [39]; it is tempting to speculate that an analogous function of lncRNAs may be present in mammalians. Figure 1(B) shows the possible participation of an lncRNA in repositioning of the nucleosomes by associating with chromatin remodelling factors in the vicinity of a tumour suppressor gene.

In addition, histone variants, which are expressed outside of the S phase and are incorporated into chromatin independently from DNA replication, can influence the nucleosome occupancy and gene activity [40]. Up-regulation, down-regulation or mutations of histone variants have been associated with cancer [41]. The possible interplay between histone variants and their regulation by lncRNAs remains to be explored.

Histone modifications

Histone modifications are catalysed by a large variety of histone-modifying enzymes, which are able to read, add or remove covalent modifications to histone proteins [42]. The final effect of the histone modifications is a change in the accessibility of the chromatin or the recruitment and/or occlusion of non-histone effecter proteins, which decode the message stored in the modified histones [43].

Alterations in the expression of histone-modifying enzymes have been implicated in a wide variety of cancers. HDACs (histone deacetylases) were found to be mutated [44] or overexpressed [45] in different tumour types, which can contribute to a characteristic global loss of acetylated H4 Lys16 in cancer cells [46]. The balance in the acetylation levels in cancer cells is also affected by the altered expression of HATs (histone acetyl transferases), which genes were found to be mutated or deleted in several cancer types [47].

Aberrant expression of HMTs (histone methyltransferases) and HDMs (histone demethylases) has also been related to cancer by promoting an imbalance in the methylation patterns. Thus, deregulation of the HMT EZH2 (enhancer of zeste homologue 2) [48], responsible for the repressive mark H3K27me3 (histone 3 lysine 27 trimethylated), and of HMT G9a [also known as
EHMT2 (histone-lysine N-methyltransferase) [49], responsible for the repressive mark H3K9me3 (histone 3 lysine 9 trimethylated), leads to altered distribution of the methylation with consequent silencing of tumour suppressor genes. HDMs have also been found to be up-regulated in several cancers [50], and some of them have been proposed as targets for cancer therapy [51].

Most of the well-studied IncRNAs involved in cancer act through the recruitment of histone-modifying enzymes to target gene promoters (Table 1). Histone modification occurs in cis, when the IncRNA recruits the histone-modifying enzymes to the genes in the vicinity of the site of IncRNA transcription (Figure 1C). Other IncRNAs act in trans by recruiting the histone-modifying enzymes to different loci away from the IncRNA transcription locus (Figure 1D). Examples from the literature will be discussed in further detail below.

CTBP1-AS (C-terminal binding protein 1 antisense)
Recently, the identification of a new antisense IncRNA named CTBP1-AS has added further complexity to the regulatory epigenetic network in response to androgens in prostate cancer, in which this IncRNA acts in cis and in trans [52]. By searching for pairs sense–antisense situated in androgen-regulated tag clusters that possess AR (androgen receptor)-binding sites, the authors identified CTBP1-AS as an androgen-responsive IncRNA, whose expression is generally up-regulated in prostate cancer [52]. CTBP1-AS is located antisense to the CTBP1, a gene encoding a co-repressor for AR that inhibits cell growth. CTBP1-AS was shown to repress in cis the sense CTBP1 mRNA and therefore to be associated with stimulation of cell proliferation [52]. Low levels of CTBP1 mRNA are correlated to a poor cancer-specific survival [52]. CTBP1 mRNA repression is mediated by binding of CTBP1-AS to the HDAC-Sin3A together with the repressor PSF (phosphotyrosine-binding-associated splicing factor), leading to the chromatin modification by deacetylation of the CTBP1 promoter region in cis [52].

Additionally, CTBP1-AS acts in trans by participating in the recruitment and influencing the DNA-binding activity of PSF to genes involved in the cell cycle [52]. This work shows the versatility of antisense IncRNAs as players in the cell response to hormones, acting both in cis and in trans to regulate the transcriptional networks triggered by androgens. Other hormone-regulated IncRNAs have been identified [8,53], and many are likely to play a role in the regulation of expression of target genes in mammalian cells.

ANRIL (antisense non-coding RNA in the INK4 locus)
ANRIL is an antisense IncRNA that was initially described as part of the INK4b (inhibitor of cyclin-dependent kinase 4b)–ARF (ADP-ribosylation factor)–INK4a (inhibitor of cyclin-dependent kinase 4a) locus, which is deleted in the melanoma-neural system tumour syndrome [54]. Many studies show that the INK4b–ARF–INK4a locus has an important role in the regulation of the cell cycle, apoptosis and cell senescence [55,56]. Additionally, a wide range of human diseases has been associated with the aberrant expression and with SNPs (single nucleotide polymorphisms) within ANRIL, including several types of cancer [57].

ANRIL is transcribed by RNAPII and processed into alternatively spliced isoforms [58], including an unspliced transcript named p15AS (p15 antisense) that silences the tumour suppressor gene p15 [59]. However, the mechanisms underlying ANRIL-mediated repression of p15 were not elucidated [59]. More recently, it has been shown that ANRIL represses the INK4b/INK4a isoforms [60], and that this repression is mediated through the direct binding of ANRIL to CBX7 (chromobox homologue 7) [60], a component of the PRC1 (polycomb repressive complex 1), and to SUZ12 (suppressor of zeste 12 homologue) [61], a member of the PRC2, leading to the deposition of histone repressive marks at the locus. Together, these studies show a scenario in which ANRIL binds to two different PRCs and epigenetically represses the expression of genes in the INK4b–ARF–INK4a locus, leading to oncogenesis. Indeed, ANRIL has been proposed to be an oncogenic IncRNA [57].

HOTAIR (HOX antisense intergenic RNA)
Similar to ANRIL, the IncRNA HOTAIR is another example of possible oncogenic IncRNA. While ANRIL [60] regulates gene expression in cis, HOTAIR is located in the HOXc cluster and regulates human HOXD gene cluster expression in trans by epigenetic events [62]. HOTAIR was first described in fibroblasts, interacting and recruiting the PRC2, which leads to transcriptional silencing in the HOXD cluster through tri-methylation on H3K27 [62]. Subsequent studies demonstrated that HOTAIR interacts with another histone-modifying complex, the LSD1 (lysine (K)-specific demethylase 1)/CoREST (repressor element 1-silencing transcription factor corepressor)/REST (repressor element 1-silencing transcription factor), which demethylates the active histone mark H3K4me3 (histone 3 lysine 4 trimethylated) [63]. HOTAIR functions as a scaffold for PRC2 and LSD1 [63] as well as guiding these complexes to their endogenous targets that are widespread in the genome [64].

HOTAIR is up-regulated in breast, colorectal, hepatocellular, gastrointestinal and pancreatic carcinomas [65–67]. HOTAIR was shown to be associated with metastasis in breast cancer patients [65], is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer [67] and predicts tumour recurrence in hepatocellular carcinoma [66]. Importantly, it has been shown that BRCA1 (breast cancer early-onset 1) protein binding to PRC2 inhibits the binding of HOTAIR to the EZH2 component of PRC2, and abolishes HOTAIR-enhanced recruitment of PRC2 to its target HOX (homeobox) A9 gene promoter in human breast cancer cells and fibroblasts [68]. These findings establish HOTAIR as the most studied IncRNA in cancer.

PCAT-1 (prostate cancer-associated ncRNA transcript 1)
The concept of IncRNAs as disease markers has been strongly cemented by the notable discovery of an expression signature composed of 121 prostate-specific cancer-associated intergenic IncRNAs, identified by an unbiased high-throughput sequen-
cing of polyA+ RNA (RNA-Seq) [24]. In this work, the increased levels of lncRNA PCAT-1 were characterized as confirming poor prognosis to prostate cancer patients [24]. PCAT-1 is transcribed from chr8q24 and promotes prostate cancer cell proliferation by regulating target genes in trans, including BRCA2 (breast cancer early-onset 2), CENPF (centromere protein F) and CENPE (centromere protein E) [24]. Curiously, PCAT-1 defines a subset of aggressive cancers with low expression of the epigenetic regulator EZH2, a component of the PRC2. Besides pointing to PRC2-regulated selected target genes, ChIP (chromatin immunoprecipitation) revealed that the PRC2 complex directly binds to the promoter region and represses PCAT-1, and RIP (RNA immunoprecipitation) showed that PCAT-1 transcript reciprocally binds PRC2 in a feedback inhibition loop [24].

Recently, researchers explored the correlation between PCAT-1 expression and the progression of CRC (colorectal cancer) [69]. The authors described an increased expression of PCAT-1 in 64% of CRC tissues from 108 cases compared with matched 81 adjacent non-tumour tissues and found that the PCAT-1 gene copy number variation explains only a few percent of the observed PCAT-1 overexpression [69]. Overall, these data suggest that PCAT-1 can be used as a possible biomarker for clinical applications [24,69].

**ANRASSF1 (antisense non-coding RNA in the RASSF1A locus)**

An endogenous unspliced antisense IncRNA named ANRASSF1 has been detected as transcribed from intronic regions at the opposite strand in the tumour-suppressor RASSF1A [Ras association (RalGDS/AF-6) domain family member 1 isoform A] 3p21.3 gene locus in several human cell lines [70]. ANRASSF1 expression was higher in prostate and breast tumour cell lines compared with immortalized non-tumour lines, while the opposite pattern was found for the RASSF1A tumour suppressor gene [70].

ANRASSF1 is tethered to its transcriptional site forming an RNA/DNA hybrid, binds to PRC2, and recruits this complex only to the RASSF1A promoter, increasing H3K27me3 repressive histone mark. Interestingly, no ANRASSF1 effect on histone marks was detected either on the promoter of the RASSF1C isoform or on the promoters of four other genes in the tumour suppressor gene cluster at the 3p21.3 locus [70]. Typically, IncRNAs mediate the epigenetic modulation and silencing of imprinted gene clusters [15] or the silencing of gene clusters that are overlapped by a head-to-head natural antisense transcript [32]. ANRASSF1 exemplified a novel highly location-specific regulatory silencing mechanism involving an antisense unspliced IncRNA, in which ANRASSF1 repressed the expression of only the RASSF1A isoform overlapping the antisense transcript [70].

ANRASSF1 overexpression decreases RASSF1A expression levels and increases the cell proliferation rate, whereas its silencing causes opposite effects [70]. The involvement of this potentially oncogenic IncRNA with the modulation of RASSF1A and tumorigenesis in cancer patients has not been studied, and further work is warranted.

**XIST (X-inactive specific transcript)**

XIST is a spliced and polyadenylated lncRNA with a size of 17 kb. It is one of the first identified and best-studied IncRNAs. XIST is typically expressed in all female somatic cells and is involved in the initiation of XCI (X chromosome inactivation) in the female cells [71]. It has been shown that XIST is the key regulator that triggers the XCI by binding and recruiting PRC2 first in cis and then spreading to several binding sites across the X chromosome, leading to the deposition of histone repressive marks in one of the X chromosome copies (see details in [72]). The expression and function of XIST is controlled by other IncRNAs such as Jpx (X-inactive specific transcript activator), RepA (repeat A, activating XIST) and TSIX (inactivating XIST).

The loss of XCI and the down-regulation of XIST expression are frequently associated with cancers [72], but this association is strictly correlative. A recent work demonstrated that the loss of XIST results in X chromosome reactivation and can cause haematopoietic cancers in mice [71], presenting a direct link between X chromosome and cancer. Further studies are needed to characterize if changes in XIST and X chromosome are causative of cancer in humans.

**CONCLUDING REMARKS AND FUTURE PERSPECTIVES**

Conspicuous patterns of expression of IncRNAs have been so far identified, especially the tissue-specificity, the low copy numbers per cell and the wide diversity of transcripts per type of tissue. Given these characteristics, it becomes obvious that the development of new tools to detect IncRNAs with higher sensitivity and at lower costs will be essential to permit the identification of patterns of expression of IncRNAs in different types of cancer and in different patient cohorts. It is expected that this better identification be translated into clinical applications such as the use of IncRNAs as prognostic and/or predictors of cancer-specific survival, as already envisioned by integrative genomic analysis [73].

LncRNAs certainly possess structural versatility allowing them to bind and interact with a number of proteins, including epigenetic regulators. The field remains vastly open for the identification of additional IncRNA-protein partners implicated in cancer. In particular, there are tens of thousands of unspliced mono-exonic antisense IncRNAs expressed from intronic regions in the human genome [4,5] that remain to be explored. We believe that the recent identification of the unspliced antisense IncRNA ANRASSF1 as an in cis guide of PRC2 to a highly location-specific site [70] could be the tip of the iceberg of an epigenetic modulation mechanism driven through unspliced intronic IncRNAs that might act at highly gene-specific loci in
the human genome [70]. Most probably, the activity and regulation of many other epigenetic protein complexes, besides PRC1, PRC2, LSD1 and MLL (mixed-lineage leukaemia), will prove to be dependent on specific IncRNAs. We envision that this wealth of new knowledge will be used for the application of IncRNAs as targets for selective drugs that recognize the particular structural characteristics of the IncRNAs or the IncRNA-DNA hybrids and block the recruitment of the chromatin modifying and remodeler complexes at specific gene loci. Alternatively, those IncRNAs that repress specific protein-coding genes can be interesting candidates for selective targeting by strand-specific oligonucleotides, in order to therapeutically up-regulate the protein-coding gene expression [74]. This strategy has already been tested in vivo [74] and could be used for IncRNAs whose expression is increased in tumour compared with non-tumour tissues and that act on tumour suppressor genes or transcription factors.

Recently, increasing attention has been given to CpG island shores that are regions of lower CpG density localized at about 2 kb of CpG island [75]. These regions are responsible for most of the tissue-specific DNA methylation and their methylation is closely associated with transcriptional inactivation [76]. Interestingly, recent findings show that most of the aberrant DNA methylation in cancer occurs in CpG island shores instead of CpG islands located in gene promoters [76], suggesting that CpG island shores can contribute to the tumorigenic process. It remains to be determined if some IncRNAs detected as altered in tumours contribute to the deregulation of the methylation patterns of CpG island shores.

The present review is published as part of a special issue in honour of Professor Ricardo R. Brentani, who was essential in supporting the execution of, and in obtaining financial resources from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the LICR (Ludwig Institute for Cancer Research) for the high-throughput sequencing of over 1 million ESTs (expressed sequence tags) from tumour samples of patients with more than 20 different types of cancer [77]. The work was conducted in the years 1999–2001, through the FAPESP/LICR Human Cancer Genome Project at the Hospital A.C. Camargo and at 25 other laboratories in the State of São Paulo. Brentani realized the importance of the evidence of transcription outside of the protein-coding genes, which had accumulated in the project, and he rejected the proposal from some of the project participants to keep at Hospital A.C. Camargo a collection of only a sub-set of cDNA clones, from transcripts that would be pre-determined by bioinformatics analyses to represent the protein-coding genes of cDNA clones, from transcripts that would be pre-determined by bioinformatics analyses to represent the protein-coding genes of cDNA clones, from transcripts that would be pre-determined by bioinformatics analyses to represent the protein-coding genes. Rather, Brentani supported our alternative proposal, and he sponsored the establishment of a duplicate copy of the entire collection of physical cDNA clones from the project, which was located at our Department of Biochemistry, Institute of Chemistry at Universidade de São Paulo. This collection allowed us to build a custom-designed microarray enriched in cDNA probes for the putative IncRNA candidates, which was used in the pioneer work that has characterized the first intronic IncRNA expression signature correlated to the degree of tumour differentiation in prostate cancer [21].

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