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TOPIC HIGHLIGHT

2015 Advances in Colorectal Cancer

Non-coding landscapes of colorectal cancer

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Abstract

For two decades Vogelstein's model has been the paradigm for describing the sequence of molecular changes within protein-coding genes that would lead to overt colorectal cancer (CRC). This model is now too simplistic in the light of recent studies, which have shown that our genome is pervasively transcribed in RNAs other than mRNAs, denominated non-coding RNAs (ncRNAs). The discovery that mutations in genes encoding these RNAs [*i.e.*, microRNAs (miRNAs), long non-coding RNAs, and circular RNAs] are causally involved in cancer phenotypes has profoundly modified our vision of tumour molecular genetics and pathobiology. By exploiting a wide range of different mechanisms, ncRNAs control fundamental cellular processes, such as proliferation, differentiation, migration, angiogenesis and apoptosis: these data have also confirmed their role as oncogenes or tumor suppressors in cancer development and progression. The existence of a sophisticated RNA-based regulatory system, which dictates the correct functioning of protein-coding networks, has relevant biological and biomedical consequences. Different miRNAs involved in neoplastic and degenerative diseases exhibit potential predictive and prognostic properties. Furthermore, the key roles of ncRNAs make them very attractive targets for innovative therapeutic approaches. Several recent reports have shown that ncRNAs can be secreted by cells into the extracellular environment (*i.e.*, blood and other body fluids): this suggests the existence of extracellular signalling mechanisms, which may be exploited by cells in physiology and pathology. In this review, we will summarize the most relevant issues on the involvement of cellular and extracellular ncRNAs in disease. We will then specifically describe their involvement in CRC pathobiology and their translational applications to CRC diagnosis, prognosis and therapy.

Key words: Colorectal cancer; MicroRNA; Long non-coding RNAs; Circular RNAs; Diagnosis; Prognosis; Therapy

Core tip: For many decades the predominant view of molecular functioning of organisms stated that proteins represent the main regulators of genomes and their dysfunctions were the first cause of diseases. This protein-centred view was too simplistic to explain the complexity of cancer. In the last few years many studies have revealed that about 85% of our genome is pervasively transcribed, mainly as non-protein-coding RNAs (ncRNAs). The discovery of countless molecular alterations of ncRNAs related to cancer changed the paradigms of cancer biology. In this review, we report recent advances in the discovery of ncRNAs involved in Colorectal Cancer pathobiologies, and their potential applications in diagnosis, prognosis and therapy.

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INTRODUCTION

Although Jacob and Monod[1] had suggested in 1961 the centrality of RNA in the flow of genetic information, for many decades the most predominant view remained that proteins represent the main regulatory components of the genome. This "protein-centred" view is indeed simplistic and may be misleading when applied to higher organisms. Recent studies have suggested that there are about 20000 protein-coding genes in the human genome: this is very close to the number of protein-coding genes in *C. elegans*[2,3]. Such observations suggest that these genes alone are not sufficient to appropriately explain the complexity of higher eukaryotes such as mammals and primates[4,5]. An analogous remark may be made on the model proposed by Fearon and Vogelstein, which describes colorectal cancer (CRC) pathogenesis as a sequence of mutations in protein-coding genes: this model has been the paradigm of CRC pathological evolution and has provided a framework for many other cancer studies[6-8]. However, over the years many observations have shown that this model is not able to recapitulate the complexity and heterogeneity of CRC (*in vitro*, but especially *in vivo*)[9,10]. Recent high-throughput studies of the human transcriptome have revealed that about 85%-90% of our genome is dynamically and pervasively transcribed, mostly as non-protein-coding RNAs (ncRNAs)[5,11,12]. In the last decade, many observations have convincingly suggested that ncRNAs significantly contribute to the complex molecular signalling needed to regulate structures and functions in different cells and developmental contexts[13,14]. Accordingly, their dysregulation strongly contributes to the onset and progression of many pathological conditions[15-17]. The discovery of molecular alterations of ncRNAs, related to neoplastic phenotypes, has initiated a shift in the paradigms of cancer biology and has profoundly influenced our understanding of tumour genetics[18-20]. Moreover, several reports have shown that ncRNAs can be secreted by cancer cells into biological fluids, potentially spreading oncogenic signals to other cells: this suggests that cancers may exploit RNA-based, hormone-like mechanisms to advantageously mold their extracellular environment[21,22]. In this review, we will describe recent advances in the discovery of the involvement of ncRNAs in CRC pathobiology, analyzing the contribution of different species of ncRNAs (both cellular and extracellular), their participation to CRC progression and dissemination, and their applications in diagnosis, prognosis and therapy.

NON-CODING RNAS: MANY SIZES AND FEATURES TO PERFORM MULTIPLE FUNCTIONS

It is not precisely known how many ncRNA genes are present in the human genome. ncRNA genes are difficult to identify because of their structural heterogeneity: (1) extreme length variation from 20 nucleotides to > 100 kb; (2) absence of Open Reading Frames (ORFs); (3) no or low evolutionary conservation in many cases; (4) no preferential localization within the genome; and (5) relative tolerance to point mutations[23,24]. The most common (and approximate) classification of ncRNAs is based on their length[25,26]. It divides them into two classes: (1) long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides (nt); and (2) small non-coding RNAs, whose length is equal to or less than 200 nt [*i.e.*, microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs). Other classifications have been proposed to categorize ncRNAs. They can be divided into two classes according to functional features: (1) housekeeping ncRNAs; and (2) regulatory ncRNAs. Housekeeping ncRNAs are constitutively expressed in all cells for their physiological functioning: they include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and telomerase RNAs[27,28]. On the other hand, regulatory ncRNAs can be expressed in a cell-specific way, or during defined stages of development and cell differentiation, and finally in response to external *stimuli*[29,30]. This category comprises miRNAs, siRNAs, lncRNAs (*i.e.*, the RNA molecules more closely involved in cancer biology), and piRNAs[31,32]. It is remarkable that many ncRNAs share features that could allow their assignment to multiple categories, thus eluding systematic classification (for instance: trans-spliced transcripts encompassing huge genomic regions)[33].

SMALL NON-CODING RNAs

This class of ncRNAs includes different types of molecules involved in different steps of RNA synthesis, processing, translation, as well as modulation of transcription initiation [*i.e*., piRNAs, promoter-associated small RNAs (PASRs)], RNA degradation or protein synthesis block (*e.g*., miRNAs, siRNAs), RNA maturation (*e.g*., snoRNAs)[34,35]. Among these RNAs, miRNAs represent the most studied class of ncRNAs: they have also been shown to be tightly associated with neoplastic phenotypes, especially CRC[36,37].

MiRNAs

Originally discovered by Victor Ambros in *Caenorhabditis elegans*, miRNAs are 18-25 nucleotides long, evolutionary conserved, single-stranded RNAs[38]. They are processed from larger precursors through sequential cleavage by two RNase Ⅲ-like enzymes: *Drosha* (in the nucleus) and *Dicer* (in the cytoplasm). By interacting with the protein Ago2, one strand of the resulting duplex can associate with the RNA-induced silencing complex (RISC). In most cases, these miRNAs-RISC complexes target specific mRNA molecules binding to their 3' untranslated regions (UTRs), which may lead to translational repression or cleavage of the mRNAs[39,40]. The former effect may be due to interference with mRNA cap recognition, inhibition of mRNA interaction with the ribosomal subunit during translation initiation, or increased rate of ribosome drop-off during elongation. Degradation is instead mediated by mRNA decapping and deadenylation[41,42]. Currently, over 2000 human miRNAs have been identified by cloning and sequencing approaches. It is predicted that miRNA genes account for 1%-2% of the human genome and control the expression of at least 50% of all protein-coding genes[43]. MiRNAs regulate fundamental cellular processes, such as cell proliferation, differentiation, migration, angiogenesis and apoptosis: accordingly, they are considered potential oncogenes or tumor suppressors in cancer development and progression[44,45]. The discovery of miRNA dysregulation or mutations, etiopathogenetically related to neoplastic phenotypes, has provided new perspectives for the study of complex gene regulatory networks in CRC and other tumours[46-48].

LONG NON-CODING RNAs

Long non-coding RNAs are the broadest and most heterogeneous class of non-protein-coding RNAs: their length is greater than 200 nucleotides, frequently reaching up to 100 kb. They include transcripts that may: (1) be located in intergenic regions [long intergenic non-coding RNAs (lincRNAs)][49]; (2) lie within introns of protein coding genes[50]; (3) partially overlap UTRs or promoters of protein coding genes[51,52]; (4) be transcribed from pseudogenes and control the expression of their protein-coding functional paralogs[53]; and (5) be transcribed ultra-conserved regions (tUCRs), which are highly evolutionarily conserved and may be located in intra- or in intergenic regions[54]. Several thousand putative lncRNAs have been already identified and shown to be expressed in a developmental and tissue-specific manner[55,56]. Recent results have convincingly suggested the involvement of lncRNAs in a wide spectrum of biological processes, such as cell-cycle regulation, stemness, differentiation, and apoptosis[57-59]. Unlike miRNAs, which repress gene expression through a common mechanism involving RISC complexes, lncRNAs exhibit a broad range of mechanisms of action through which they are involved in the modulation of epigenetic regulation, alternative splicing, and protein localization and activity. It is probable that these functions are due to the ability of lncRNAs to bind DNA, other RNAs and proteins. LncRNAs can also serve as decoys, which preclude the access to the DNA of regulatory proteins and prevent transcription of specific genes (*e.g*., lncRNA Gas5, DHFR)[60,61]. Many lncRNAs are associated with polycomb repressive complex-2 (PRC2) or other chromatin-modifying complexes, which modulate epigenetic silencing of target genes (*e.g*., HOTAIR)[62,63]. LncRNAs can serve as scaffolds to bring two or more proteins into functional complexes (*i.e*., telomerase RNA TERC)[64], or are required to properly localize protein complexes[65]. A subset of ncRNAs, named Telomere-associated ncRNAs (telomeric repeat-containing RNA, TERRA), negatively regulates telomere length presumably by inhibiting telomerase activity[66]. LncRNAs may be processed into smaller ncRNAs (*e.g*., MALAT1)[67]; pseudogene transcripts can be processed into siRNAs that regulate protein coding genes through RNA interference (RNAi)[68]. By targeting RNAs through direct sequence complementarity, lncRNAs may also operate as antisense molecules against their targets and modulate alternative splicing events (*e.g*., antisense of ZEB2), or increase the stability of mRNAs by hiding their miRNA binding sites (*e.g*., BACEAS)[51,69]. Circular RNAs (circRNAs) belong to an odd, but extremely interesting class of lncRNA molecules, which has been recently described. CircRNAs can act as natural miRNA sponges to lower miRNA levels: accordingly, they perform a critical role modulating the connection between genotype and molecular phenotype[70]. Dysregulation of lncRNAs has been documented for many complex human diseases, including cancer. Dozens of lncRNAs have been reported to have altered expression in neoplasia and to be controlled by specific oncogenic and tumor suppressor pathways[71,72]. These observations strongly suggest that lncRNAs could be added to the list of proto-oncogenes and tumor suppressors, as suspects potentially involved in oncogenesis. Accordingly, they might also be considered as potential biomarkers and targets for novel therapeutic approaches in neoplastic diseases.

MULTIFACETED NON-CODING RNA LAYERS CONTROL THE COLORECTAL CANCER GENOME

MiRNAs perform key roles in CRC initiation and evolution

Since the original discovery connecting miRNAs to Chronic Lymphocytic Leukemia[73], researchers have convincingly demonstrated that miRNAs play a critical role in cancer. MiRNA oncogenic activity can be tissue-specific. Altered miRNA expression plays an etiological role in the initiation and progression of colon cancer: global miRNA expression patterns can discriminate between normal tissues and CRC tissues more efficiently than mRNA expression patterns. Furthermore, several investigations have shown the ability of miRNA expression patterns to improve diagnosis of poorly differentiated tumours and predict prognosis in CRC (see next paragraph in this review). In 2003, Michael *et al*[74] published the first study on miRNAs in CRC: they identified miR-143 and miR-145 as novel dysregulated miRNAs in colon cancer. Since then, the literature on miRNAs in CRC has grown considerably. This paragraph will provide an overview of the etiological connection between the molecular functions of miRNAs and CRC pathobiology. The first discovered CRC-related miRNAs, miR-143 and miR-145, act as tumor suppressor genes and are downregulated in CRC compared with normal colonocytes[74,75]. miR-143 targets KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog) and MACC1 (metastasis-associated in colon cancer-1), thus playing an important role in the regulation of EGFR (epidermal growth factor receptor) and HGFR (hepatocyte growth factor receptor) signalling[76,77]. Both miR-143 and miR-145 modulate CRC cell proliferation: transfection of DLD-1 and SW480 cells with premiR-143 and premiR-145 reduced cell proliferation[78]. MiR-145 indirectly promotes angiogenesis by binding p70S6K1 mRNA and inhibiting its translation: downregulation of p70S6K1 increases the levels of two proangiogenic factors, HIF1 (hypoxia-inducible factor 1) and VEGF (vascular endothelial growth factor)[79]. MiR-145 also targets the oncogene MYC (v-Myc avian myelocytomatosis viral oncogene homolog), reducing its expression: this explains mR-145-dependent inhibition of cell proliferation *in vivo* and *in vitro*[80]. MiR-21 is the most commonly upregulated miRNA in cancer, including CRC[81]. There are many notable downstream effects of elevated miR-21 levels: its genetic locus at 17q23 is amplified in many solid tumours[82,83]; its expression is stimulated by a variety of cancer-associated phenomena, such as inflammation and hypoxia[84-86]. MiR-21 targets various tumor suppressor genes, such as PDCD4 (programmed cell death 4), CCL20 [chemokine (C-C motif) ligand 20], CDC25A (cell division cycle 25 homolog A), PTEN (phosphatase and tensin homolog), thus promoting cell proliferation, invasion/intravasation/metastasis in CRC[87-90].

In addiction to miR-21, many other miRNAs can be induced in cancer cells under hypoxic conditions[91]. In this group of so called "hypoxamir" there is miR-210, which can mediate the hypoxia-induced metastasis of CRC cells[92]. MiR-210 is frequently up-regulated in CRC tissues, 2D, and 3D cultures[92,93]. Its enforced expression in CRC cells promotes the migration and invasion through the repression of its target VMP1 (vacuole membrane protein 1)[92]. Several investigations reported miR-31 upregulation in CRC[94,95]. Over-expression of miR-31 in CRC cells promotes cell proliferation, invasion, and migration in *in vivo* and *in vitro* models. Likely, its oncogenic functions are due to targeting of SATB2 (*SATB homeobox 2*) mRNA, which is followed by SATB2 mRNA and protein downregulation, and by targeting E2F2 (E2F Transcription Factor 2): in turn, E2F2 controls the expression of survivin and other cell cycle genes, such as CCNA2 (*Cyclin A2*), CDK2 (cyclin-dependent kinase 2), MCM4 (minichromosome maintenance complex component 4), and MYC[96,97]. Notably, members of the E2F family may have a dual function: it has been hypothesized that they may act as oncogenes when they are overexpressed and as tumor suppressors when they are downregulated[96]. MiR-31 also targets RASA1 (RAS p21 GTPase activating protein 1) and inhibits its translation, activating the KRAS signalling pathway[98]. One of the most known oncogenic miRNA clusters in CRC is the miR-17-92 cluster. It consists of six members (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) with oncogenic functions, all upregulated in CRC[99]. MiR-18a and miR-19 promote angiogenesis by targeting TSP-1 (thrombospondin-1) and CTGF (connective tissue growth factor) mRNAs, respectively[100,101]. Interestingly, different miR-17-92 cluster members can modulate cell proliferation in opposite ways. MiR-19a and miR-19b induce proliferation by acting on PTEN, whereas miR-18a has antiproliferative effects due to its target genes that activate proliferation, such as NEDD9 (neural precursor cell expressed, developmentally down-regulated 9) and CDK19 (cyclin-dependent kinase 19)[102]. MiR-17 and miR-20a target E2F1 (E2F transcription factor 1), whereas miR-20a represses E2F2 and E2F3 (E2F transcription factor 3)[103]. Among miR-17-92 cluster members, only miR-92a has antiapoptotic effects: a negative correlation has been described between miR-92a and BCL2L11 (BCL2-like 11), a pro-apoptotic BCL-2 (B-cell CLL/lymphoma *2*) protein family member[101]. Among miR-17-92 cluster members, miR-18a seems to act as a tumor suppressor in CRC: some studies have shown that this miRNA can affect cell proliferation by inhibiting CDC42 (cell division cycle 42)[103], or can promote apoptosis causing hnRNPA1 (heterogeneous nuclear ribonucleoprotein A1) autophagosomal degradation[104]. CDC42 is a small Ras-related GTPase involved in cell cycle progression[105], transendothelial migration through β1 integrin[106], cell motility and cytoskeletal remodelling[107]. The let-7 family is one of the most ancient and conserved group of miRNAs[108]: they act as tumor suppressors in various cancer models, including CRC[109-111]. The let-7 family owes its name to the *lethal-7* gene, identified for the

first time in *C. elegans*, where it is involved in development. Later, the same sequence was detected in the *genome* of *Drosophila melanogaster* and *Homo sapiens*, confirming that mature let-7 is highly conserved across animal species. However, the number of members of this family varies in different species: the let-7 family in humans includes 10 mature miRNAs produced from 13 precursor sequences[112]. Several studies have demonstrated that the let-7 family regulates KRAS expression in CRC and other cancer types[111-114]. KRAS is a small monomeric GTPase, involved in signal transduction of stimuli activating proliferation[115]. KRAS mutations or amplifications are frequently detected in CRC patients[116]: they are considered a key step in colorectal carcinogenesis according to the model proposed by Vogelstein[6]. Moreover, KRAS mutation status is a negative predictive factor of the response to anti-EGFR therapy (*i.e.*, Cetuximab)[117]. It has been reported that let-7b and let-7e were downregulated after Cetuximab treatment in a Cetuximab-resistant CRC cell line, suggesting their potential role in the resistance to anti-EGFR therapy[118]. Several reports debated the predictive utility of a let-7 microRNA-binding-site polymorphism in the 3'-UTR of KRAS for CRC outcome, although the results are conflicting[114,119-121]. Together with other let-7 family members, let-7c modulates cell cycle by targeting KRAS, and is also involved in suppressing metastasis *via* its targets MMP1(matrix metallopeptidase 1) and PBX3 (pre-b-cell leukemia homeobox 3). It has been demonstrated that ectopic expression in Lovo cells or inhibition of let-7c in HT29 cells reduces cell migration and invasion and increases cell motility and invasion, respectively[122]. Overactivation of KRAS signalling could induce the expression of oncogenic miRNAs in CRC. For instance, miR-372 expression is higher in KRAS-mutated CRC samples compared with wild type tumours[123]. miR-372 knockdown decreases cell proliferation and migration and increases apoptosis in CRC cell lines[123]. MiR-372 downregulation results in TXNIP (thioredoxin-interacting protein) overexpression[123]: TXNIP is a tumor suppressor gene involved in apoptosis induction and cell proliferation inhibition[124]. High miR-372 expression is significantly associated with liver metastasis: metastatic CRC samples show higher miR-372 expression than non-metastatic tumours; also, high miR-372 expression is associated with lower 5-year overall survival rate[125]. Similar to miR-372, also miR-720 was found to be more expressed in CRC with mutated KRAS than wild-type KRAS[123,126]. Its overexpression correlates with tumour size, spreading of metastases to distant sites and low 5-year overall survival[126]. MiR-720 knockdown reduces cell proliferation, migration and invasion, and induces apoptosis in CRC cell lines[123,126]. The same miR-720 regulates STARD13 [star-related lipid transfer (START) domain containing 13] expression[126], a GTPase-activating protein (GAP) for Rho and Cdc42. It has been demonstrated that STARD13 knockdown induces upregulation of the antiapoptotic protein BCL2, downregulation of proapoptotic BAX (BCL2-associated X protein), and promotes 3D motility[127]. The miR-200 family is one of the best-known miRNA families in mammals. It consists of five members, which are located at two different loci of chromosome 1 (miR-200a, miR-200b, miR-429) and 12 (miR-200c and miR-141)[128,129]. They are tumor suppressor miRNAs and are significantly involved in inhibition of epithelial-to-mesenchymal transition (EMT), repression of cancer stem cell self-renewal and differentiation, modulation of cell division and apoptosis, and reversal of chemoresistance[128-130]. MiR-200c is particularly interesting in CRC, due to its ability to regulate cell proliferation, invasion, migration, EMT and metastasis. MiR-200c expression is statistically lower in CRC clinical specimens and highly metastatic CRC cell lines. Transfection of CRC cell lines (RKO and SW620) with precursors of miR-200c induced cell proliferation, but reduced invasion and migration. Overexpression of miR-200c in CRC cell lines caused a reduced expression of its target ZEB1/2 (zinc finger E-box binding homeobox 1/2), and resulted in increased E-cadherin and reduced vimentin expression. The associations between miR-200c, its target genes, and EMT markers were validated in primary CRCs and matching liver metastasis tissues[131]. MiR-200c targets SOX2 [SRY (sex determining region Y)-box 2], a pivotal gene required for early development and propagation of undifferentiated embryonic stem cells. Knockdown of miR-200c increased the sphere-forming capacity of CRC cell lines and expression of CRC stem cell markers. MiR-200c suppresses the expression of SOX2, so repressing the activity of the PI3K (phosphoinositide 3-kinase)/AKT (v-Akt murine thymoma viral oncogene homolog 1) pathway[132]. Another tumor suppressor miRNA family controlling EMT in CRC is the miR-34 family. It includes three oncosuppressor members (miR-34a, miR-34b and miR-34c), which are regulated by p53; by promoting mesenchymal-to-epithelial transition (MET) *via* the inhibition of the EMT-inducing transcription factor SNAI1 (snail family zinc finger-*1*), they are involved in metastasis suppression. MiR-34a targets involved in CRC invasion and metastasis include IL6R (interleukin 6 receptor), ZNF281 (zinc finger protein 281), MET (MET proto-oncogene, receptor tyrosine kinase), SNAI1, CTNNB1 (β-catenin) and SNAI2 (snail family zinc finger 1)[133-136]. A common event in CRC carcinogenesis is the inactivation of APC (adenomatous polyposis coli), a negative regulator of Wnt signalling pathway through binding to β-catenin, together with Axin and Glycogen Synthase-3, followed by degradation through ubiquitination. Inactivated APC cannot interact with β-catenin, which accumulates in the cytoplasm and then translocates into the nucleus: here β-catenin binds the TCF/LEF (transcription factor/lymphoid enhancer-binding factor) transcription factors, thus leading to MYC and cyclin D transcription and induction of cell proliferation. An miRNA-based regulation of APC has been proposed: miR-135b binds APC mRNA regulating its expression; miR-135b upregulation in CRC cells

causes reduced APC protein levels, β-catenin accumulation and Wnt pathway activation[137]. Another study proved that miR-135b also affects apoptosis through its targets TGFβR2 (transforming growth factor β receptor 2) and DAPK1 (death-associated protein kinase 1), both frequently downregulated in CRC[138]. Similar to miR-135b, also miR-135a is frequently upregulated in CRC[139,140]. Acting as an oncomiR, miR-135a promotes cell proliferation, motility and invasion of CRC cells. A target of miR-135a is MTSS1 (metastasis suppressor-1), which is downregulated in CRC, promoting malignant phenotypes *in vitro*[141]. Interestingly, the oncogenic effects of miR-135b on the Wnt pathway can be counterbalanced by the tumor suppressive action of miR-320a on the same pathway[142-144]. Overexpression of miR-320a in CRC cell lines reduces cell proliferation, blocking cell cycle in G1. Cell cycle arrest is due to miR-320a and β-catenin mRNA interaction, which results in decreased levels of β-catenin protein, together with those of its transcriptional target genes: MYC, cyclin D, survivin[142]. MiR-320a expression inversely correlates with proliferation and migration of CRC cells and is significantly lower in metastatic compared with non-metastatic samples[143,144]. MiR-320a overexpression suppresses migration and invasion by targeting RAC1 (ras-related C3 botulinum toxin substrate-1). It has also been observed that miR-320a overexpression induces vimentin downregulation and E-cadherin upregulation, suggesting that miR-320a is also involved in EMT regulation[144]. Wnt signalling is also controlled by miR-181a. Its upregulation in CRC cell lines induces cell proliferation by means of its WIF1 target (WNT inhibitory factor-1), which is involved in apoptosis promotion, and PTEN, involved in the AKT signalling pathway[145,146]. Moreover, miR-181a overexpression causes downregulation of the epithelial markers E-cadherin and β-catenin and increased expression of vimentin, suggesting that miR-181a also promotes EMT in CRC[145]. MiR-106a acts as an oncogene in CRC, and its upregulation leads to decreased protein levels of RB1 (retinoblastoma protein-1)[81,147]. Active (unphosphorylated) RB1 binds transcription factors E2F and TFDP1 (transcription factor DP-1) at the promoter of the E2F-regulated genes, which are involved in cell cycle progression. The presence of the RB/TFDP1/E2F complex at the promoter inhibits transcription and recruits chromatin-remodelling complexes, inducing gene silencing and blocking cell cycle progression[148]. MiR-106a binds to the 3'UTR of the RB1 mRNA, inhibiting its translation and inducing cell cycle progression in CRC[147]. By targeting TGFβR2, a tumor suppressor commonly inactivated in CRC, miR-106a is also involved in migration and invasion *in vitro* and *in vivo*[149,150]. Upregulation of miR-155 in CRC has been shown by several studies[151-153]. It has been demonstrated that this oncomiR promotes migration and invasion by targeting the CLDN1 (claudin 1) protein, a component of tight junctions[152]. In turn, CLDN1 is involved in EMT, causing E-cadherin upregulation through ZEB1 downregulation[154]. Adrenaline-induced miR-155 upregulation modulates cell proliferation and chemoresistance in HT29 CRC cell line. Adrenaline increases miR-155 levels *via* NFκB (nuclear factor kappa-light-chain-enhancer in activated B cells), inducing cell proliferation and inhibiting cisplatin-induced apoptosis[153]. It has been recently demonstrated that cyclo-oxygenase 2/prostaglandin-endoperoxide synthase 2 (COX2/PTGS2) and prostaglandin E2 (PGE2), a COX2 metabolite, play an important role in colon cancer progression and are potential targets for prevention and therapeutic strategies[155,156]. COX2 is the inducible isoform of the key enzyme in prostaglandin biosynthesis and it has been associated with several malignancies, including CRC[157,158]. Its upregulation promotes cell proliferation *in vitro* and *in vivo*[159]. MiR-101 acts as a tumor suppressor in CRC patients and cell lines[158,160]. Among miR-101 targets are COX2 and PTGER4 (prostaglandin E receptor 4), a G protein-coupled cell surface receptor involved in PGE2 signal transduction[158,161]. MiR-101 negatively regulates PTGER4: both miR-101 overexpression and PTGER4 silencing reduce motility and colony formation *in vitro*[161]. MiR-101 overexpression also promotes cell adhesion and inhibits colonosphere formation, cell growth, invasiveness and survival in hypoxic conditions[160]. MiR-101 repression and Wnt signalling pathway activation show a strong association: miR-101 overexpression reduces β-catenin accumulation in the nucleus and transcriptional activity, leading to increased E-cadherin and decreased ZEB1 mRNA levels; this suggests miR-101 involvement in EMT regulation[160]. MiR-638 downregulation promotes EMT through its target SOX2[162]: it has been demonstrated that SOX2 overexpression induces dedifferentiation and EMT[163]. MiR-638 expression is reduced in CRC tissues and cell lines: its downregulation inversely correlates with tumour progression and predicts poor survival[162,164]. MiR-638 ectopic expression in CRC cell lines results in a reduction of migration, invasion and cell proliferation: this is due to overexpression of its target TSPAN1 (tetraspanin 1)[161,164], which is involved in CRC cell cycle and invasion regulation[165]. MiR-1 levels are reduced in CRC compared with normal tissues: this significantly correlates with MET gene overexpression. Moreover, miR-1 ectopic expression in CRC cell lines impairs MET-induced cell viability, migration and invasion[166]. MiR-23b acts as a tumor suppressor in CRC cell lines by performing a pleiotropic modulation of different cancer-related biological processes. Its ectopic expression strongly inhibits migration and invasion *in vitro* and primary tumour growth and metastasis *in vivo*. MiR-23b overexpression reduces cell resistance to *anoikis*, programmed cell death induced by detachment of anchorage-dependent cells from the extracellular matrix. MiR-23b may also promote mesenchymal-to-epithelial transition: it has been observed that its expression induces E-cadherin upregulation and vimentin reduction. These tumor suppressive effects are

due to miR-23b pro-metastatic targets, FZD7 (frizzled class 7 receptor), MAP3K1 (MEKK1, mitogen-activated protein kinase kinase 1, E3 ubiquitin protein ligase), PAK2 [p21 protein (Cdc42/Rac)-activated kinase 2], TGFβR2, RRAS2 [related RAS viral (r-Ras) oncogene homolog 2], and uPA (plasminogen activator, urokinase). Furthermore, miR-23b overexpression inhibits angiogenesis by indirectly suppressing VEGF through FZD7 and MAP3K1[167]. Several papers reported the downregulation of miR-126 in CRC tissues and cell lines[168-170]. Methylation studies suggest that miR-126 is epigenetically silenced by CpG methylation of the promoter of its host gene EGFL7 (EGF-like-domain multiple 7)[171]. Through its target PIK3R2 (phosphoinositide-3-kinase, regulatory subunit 2), a regulatory subunit involved in the PI3K signalling pathway, miR-126 ectopic expression results in cell proliferation impairment[168]. MiR-126 also binds the mRNAs encoding IRS1 (insulin receptor substrate 1) and CXCR4 [chemokine (C-X-C motif) receptor 4], thus regulating AKT and ERK1/2 (mitogen-activated protein kinase 3/1) activation. MiR-126 inhibits cell proliferation, inducing G0/G1 arrest, migration and invasion in CRC cell lines[168-171]. It has been shown that miR-126 ectopic expression in CRC cell lines impairs VEGF secretion in culture medium, suggesting that miR-126 affects angiogenesis[171]. Similarly, DNA hypermethylation of CpG islands seems to cause miR-148a downregulation in various cancers. In CRC cell lines, miR-148a upregulation promotes apoptosis through BCL2 inhibition[172]. Moreover, miR-148a downregulation in CRC correlates with increased tumour size[173]. All data reported in this paragraph (*i.e.*, CRC related miRNAs, their functions and targets) are summarized in Table 1.

The impact of lncRNAs on CRC pathobiology

Over the last few years, several papers have reported on the involvement of lncRNAs in CRC genesis and progression through a number of molecular mechanisms. LncRNAs impact on critical CRC signalling pathways by acting both as oncogenes and tumor suppressors through interactions with other regulatory molecules, such as DNA, RNA, and proteins. Although several lncRNAs have been reported to be dysregulated in CRC, which suggests their potential diagnostic/prognostic power (see paragraph "Clinicopathological significance of lncRNAs in CRC"), their molecular mechanisms of action in CRC biology were elucidated only for few of them. One of the most known cancer-related lncRNAs is Metastasis-Associated Lung Adenocarcinoma transcript 1 (MALAT1), located on chromosome 11q13.1 and 8000 nt long[174]. MALAT1 is highly expressed in metastases of various tumours, such as non-small cell lung cancer, hepatocellular carcinoma, and endometrial stromal sarcoma[174-176]. Several intracellular functions of MALAT1 have been proposed. It may have an important role in pre-mRNA metabolism: it is indeed associated with SC35 splicing domains within the nucleus[177]. It is concentrated in nucleoli as a "riboregulator", controlling expression of its target genes[178]. Moreover, MALAT1 is involved in the regulation of tumor suppressor proteins [*e.g.*, PTB-associated splicing factor (PSF)][179], and has also been found to regulate the activity of E2F1, a pivotal transcription factor for cell cycle progression[180]. *In vitro* silencing of MALAT1 has been shown to affect bladder cancer cell migration. MALAT1 acts as a negative regulator of EMT-associated ZEB1, ZEB2 and SNAI2, and positive regulator of E-cadherin[181]. Aberrant mitosis, with a large fraction of cells accumulating at the G2/M boundary and increased cell death, result from MALAT1 depletion[182]. Recently, a 3' end processing mechanism for MALAT1 has been identified: the primary transcription product of the MALAT1 locus is a 6.7 kb nuclear-retained lncRNA and a cytoplasmic 61-nt tRNA-like ncRNA, known as mascRNA (MALAT1-associated small cytoplasmic RNA)[67]. When the MALAT1 RNA fragment containing mascRNA was overexpressed in CRC cells, cell proliferation and invasion were induced. Point mutations of MALAT1 were detected in CRC cell lines and tissues[183]. BRAF-activated non-protein coding RNA (BANCR) seems to be closely associated with ^{V600E}BRAF, one of the most frequent mutation types of the BRAF (B-Raf proto-oncogene, serine/threonine kinase) gene in several tumours, including CRC[184]. BANCR is frequently overexpressed in CRC tissues: this overexpression significantly correlates with lymph node metastasis and tumour stage[185]. Enforced expression of BANCR increases cell migration of CRC cell lines, whereas its knockdown inhibits it. BANCR induces EMT by affecting the expression of epithelial and mesenchymal markers through a MEK (mitogen-activated protein kinase kinase 7)/ERK dependent mechanism, thus contributing to CRC migration[185]. Genome wide association studies (GWAS) have identified a set of risk *loci*, which are linked to susceptibility for different diseases (including CRC) on human chromosome 8q24[186]. Interestingly, this region (about 2 Mb) is within a large protein-coding-gene desert, but several non-coding genes map on it (*i.e.*, CCAT1, CCAT2, PCAT2, and PRNCR1)[187]. The rs6983267 SNP (single nucleotide polymorphism), mapping to the chromosomal region 8q24.21, has been strongly associated with an increased risk of CRC[188]. The genomic region spanning rs6983267 was found to contain DNA enhancer elements, and the allelic variants confer different binding affinity to TCF7L2 [transcription factor 7-like 2 (T-cell specific, HMG-box)], a transcription factor that has a central role in the transcriptional activation of Wnt target genes[189,190]. The SNP status of lncRNA colon cancer associated transcript 2 (CCAT2), which encompasses rs6983267, affects CCAT2 expression: the risk allele G produces more CCAT2 transcripts in CRC[191]. CCAT2 interacts with TCF7L2 and upregulates MYC, miR-17-5p and miR-20a; it also overactivates Wnt signalling[191]. Interestingly, CCAT2 is itself a Wnt downstream target, which suggests the existence of a positive feedback loop[191]. The long

Table 1 Functions of microRNAs deregulated in colorectal cancer

microRNA	Oncogene/tumor suppressor	Process in CRC	Targets	PMID
Let-7	Tumor suppressor	Cell proliferation, migration, invasion, metastasis	KRAS, MMP11, PBX3	23167843, 21984339, 16651716
miR-1	Tumor suppressor	Cell proliferation, invasion, migration	MET	22179665
miR-16	Tumor suppressor	Cell proliferation	PTGS2	22049153
miR-17-92 cluster	Oncogene	Angiogenesis, proliferation, metastasis	TSP-1, CTGF, PTEN, BCL2L11, E2F1, E2F2, E2F3, TGFBR2, CDKN1A, BIM	19460962, 16878133, 22308110, 24212931, 21883694
miR-18a	Tumor suppressor	Cell proliferation, migration	CDC42, HNRNPA1	25379703, 24166503
miR-21	Oncogene	Cell proliferation, migration, invasion, metastasis, stemness	PDCD4, CCL20, Cdc25A, TGFBR2, PTEN, RHOB, RASA1	22677902, 17968323, 22099878, 19826040, 22120473, 23788041, 23544170, 23174819, 22072622, 21872591, 25663768
miR-23b	Tumor suppressor	Cell migration, invasion, angiogenesis	FZD7, MEKK1, PAK2, TGFBR2, RRAS2, PLAU, VEGF	22109528
miR-31	Oncogene	Cell proliferation, invasion, migration	CDKN2B, RASA1	21062447, 25202407, 23322774
miR-34	Tumor suppressor	Migration, invasion, metastasis, EMT	IL6R, ZNF281, MET, SNAIL, CTNNB1, SLUG, ZEB1	24642471, 24185900, 23243217, 22134354
miR-101	Tumor suppressor	Cell proliferation, motility, invasion	EP4, PTGS2	22353936, 19133256
miR-103/miR-107	Oncogene	Invasion, migration, metastasis	DAPK, KLF4	22593189
miR-106a	Oncogene	Cell proliferation, migration, invasion, metastasis	RB1, TGFBR2	23178825, 22912877
miR-126	Tumor suppressor	Cell proliferation, migration, invasion, metastasis, angiogenesis	PIK3R2, IRS1, CXCR4, VEGF	18663744, 24312276, 24189753, 24653631, 23900443
miR-135a	Oncogene	Cell proliferation, migration, invasion	MTSS1	23017832
miR-135b	Oncogene	Cell proliferation, invasion	APC, TGFBR2, DAPK1	18632633, 24735923
miR-143	Tumor suppressor	Cell proliferation, metastasis	KRAS, ERK5, MACC1, HK2, IGF1R, DNMT3A	19137007, 16969504, 22533346, 22691140, 23574723, 19638978
miR-145	Tumor suppressor	Cell proliferation, angiogenesis	MYC, CDK6, E2F1, CCND2, p70S6K1, PAK4	21278451, 15944709, 19843336, 21917858, 22766504
miR-148a	Tumor suppressor	Cell proliferation	BCL2	21455217
miR-155	Oncogene	Cell proliferation, migration, invasion, chemoresistance	CLDN1	23588589, 23036199
miR-181a	Oncogene	Cell proliferation, invasion, metastasis, EMT	PTEN, WIF1	24685694, 24755295
miR-200c	Tumor suppressor	Cell proliferation, invasion, migration, EMT, metastasis	ZEB1, ETS1, FLT1, CDH1, VIM	22735571, 22407310
miR-210	Oncogene	Cell migration, invasion	VMP1	24632577
miR-320a	Tumor suppressor	Cell proliferation, migration, invasion, metastasis, EMT	CTNNB1, RAC1, NRP1	22459450, 24265291, 22134529
miR-372	Oncogene	Cell proliferation	TXNIP, LATS2	22660396, 22456107
miR-638	Tumor suppressor	Cell invasion, migration	SOX2, TSPAN1	24885288, 25301729
miR-720	Oncogene	Cell proliferation, invasion, migration	STARD13	25286763, 22660396

For all miRNAs are reported: (1) their function in CRC (oncogene, or tumor suppressor); (2) the biological processes they are involved in; (3) their mRNA validated targets; (4) bibliographic references reported as PubMed ID (PMID).

isoform of colon cancer associated transcript 1 (CCAT1-L) is upregulated and positively related to tumour stage and progression in CRC[192]. CCAT1-L is located in the nucleus, while the short isoform (CCAT1-S) is found in the cytoplasm. CCAT1-L can interact with a transcriptional enhancer of MYC (MYC-335) by chromatin looping, which in turn interacts with the MYC-promoter[193]. Knockdown of CCAT1-L leads to a decreased level of MYC mRNA, strongly suggesting that this lncRNA may regulate MYC expression in *cis*[193]. Another lncRNA locus mapping at 8q24 is Plasmacytoma Variant Translocation 1 (PVT1): PVT1 is located downstream of MYC on chromosome 8q24 and produces a wide variety of spliced RNAs, such as a cluster of six miRNAs (*i.e.*, miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p, and miR-1208)[194]. PVT1 is upregulated in CRC because of a copy number amplification of chromosome 8q24[195]. Knockdown of PVT1 in CRC cells leads to a significant reduction of proliferation and invasion through activation of TGF-β and apoptotic signalling. Increased PVT1 expression is required for high MYC protein levels in 8q24-amplified CRC cells. PVT1 and MYC protein expression correlate in primary tumours, while ablation of PVT1 from MYC-driven colon cancer line HCT116 diminished its tumorigenic potential[195]. Surprisingly, PVT1 is also a p53-inducible target gene: p53 binds and activates a canonical response element within the vicinity of miR-1204, and induces the endogenous PVT1 transcripts and consequent upregulation of miR-1204[196]. Ectopic expression of miR-1204 leads to increased p53 levels and causes cell death in a partially

p53-dependent manner[196]. The most intensively studied lncRNA in different neoplastic diseases is the Hox transcript antisense intergenic RNA (HOTAIR). It is located within the Homeobox C (HOXC) gene cluster on chromosome 12 and is coexpressed with HOXC genes[197]. HOTAIR interacts with PRC2; it functions as a scaffold to assemble PRC2 on the HOXD gene cluster, inducing the trimethylation of histone H3 lysine-27 (H3K27me3) of the HOXD locus[198]. By silencing multiple metastasis suppressor genes (such as HOXD10, PGR, and the protocadherin gene family), HOTAIR epigenetically regulates HOXD expression and promotes metastasis in breast cancer[199]. Expression analysis on CRC reveals a close correlation between HOTAIR expression and members of the PRC2 complex (*i.e.*, SUZ12, EZH2, and H3K27me3): this suggests that HOTAIR expression is associated with epigenetic functions of PRC2, which is involved in maintaining the mesenchymal and undifferentiated status in CRC cells[200]. Similarly to HOTAIR, also the lncRNA CRNDE (colorectal neoplasia differentially expressed) has been shown to be physically and functionally associated to PRC2[201]. Khalil *et al*[201] showed an overlap in the lists of genes affected by knockdown of CRNDE and PRC2. These data would suggest an involvement of CRNDE in the epigenetic remodelling of chromatin, and specifically in the downregulation of gene expression *via* targeted histone methylation by the PRC2 complex. The CRNDE promoter is bound by some pluripotency-related transcription factors [*i.e.*, MYC, MYCN (v-Myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog)] and CRNDE knockdown decreases the expression of several pluripotency markers (*i.e.*, SOX2, KLF4, NANOG, and OCT4)[202]. CRNDE expression appears highest in the early stages of mammalian development and progressively decreases thereafter. It is required for the maintenance of pluripotency in mouse embryonic stem cells and is potentially involved in tumorigenesis[202]. The tumor suppressor candidate 7 (TUSC7) is a p53-regulated tumor suppressor, which reduces tumour cell growth both *in vitro* as well as *in vivo* in CRC[203]. Specifically, its fourth exon (containing two miR-211 binding sites) is responsible for inhibition of tumour cell growth. *In vivo* studies confirmed that TUSC7 can bind to miR-211 inducing its down-regulation[203]. It has been shown that miR-211 promotes cell growth in CRC cell lines. Accordingly, TUSC7 works as an endogenous miRNA sponge. Interestingly, TUSC7 is a target of miR-211, showing the existence of a reciprocal negative feedback loop between TUSC7 and miR-211[203]. H19 is a paternally imprinted, maternally expressed, oncofetal gene[204]. It is highly expressed from the early stages of embryogenesis to fetal life in many organs, but is nearly completely downregulated postnatally[205]. H19 is upregulated in many cancers, including CRC[206], and acts as the primary miRNA precursor of miR-675 in mammals[207]. RB1 mRNA is a direct target of miR-675: in fact, knockdown of miR-675 increases RB1 expression and at the same time decreases cell growth in CRC. On the contrary, miR-675 gain-of-function causes RB1 downregulation and enhances tumour cell growth. Both TGF-β and hypoxia concomitantly induce H19 and miR-675, together with induction of EMT markers and suppression of E-cadherin protein expression[207]. Interestingly, H19 harbours both canonical and non-canonical binding sites for the let-7 miRNA family, whose critical tumor suppressive role in the development of CRC has already been discussed above. H19 is able to downmodulate let-7 availability by acting as a molecular sponge[208]. Recently, the role of another lncRNA, functionally linked to EMT, has been characterized in CRC[209]. N-BLR is an lncRNA involved in the apoptotic pathway: its inhibition leads to downregulation of XIAP (X-linked inhibitor of apoptosis protein) and subsequent increase of cell death. N-BLR also promotes invasion and migration by modulating vimentin and E-cadherin expression. Intriguingly, N-BLR seems to be regulated by members of the miR-200 family (*i.e.*, miR-141, and miR-200c). As previously mentioned, the miR-200 family has been strongly linked to EMT: indeed, its members target the ZEB1/ZEB2 transcription factors that are repressors of E-cadherin expression. According to the model proposed by Rigoutsos *et al*[209], the increase of N-BLR expression in CRC samples would attract the available endogenous miR-141/miR-200c and relieve their targeting ZEB1, thereby upregulating its levels. This would lead to a subsequent decrease of E-cadherin; it also would confer a mesenchymal phenotype to the cells, which is correlated with increased invasiveness and migratory potential of CRC cells. All data reported in this paragraph (*i.e.*, CRC related lncRNAs, their functions and targets) are summarized in Table 2.

Circular RNAs: New players in cancer regulation?

Recently, it has been discovered that hundreds of human genes are also expressed in a circular RNA isoform[210]. Initially, these circRNAs were thought to be rare RNA species representing just transcriptional noise. High-throughput sequencing of the RNase R treated, ribosomal-depleted fractions of RNAs showed a ubiquitous expression of circRNAs in human and mouse cells[211]. CircRNAs represent a class of little known post-transcriptional regulators, which compete with other RNAs for binding to miRNAs and RNA binding proteins (RBPs): they may have a role in modulating the local concentration of RBPs and RNAs, as part of the competing endogenous RNA network[211]. Moreover, in contrast with classical ceRNAs, circRNAs have no accessible termini, which makes them resistant to miRNA-mediated RNA degradation or other exonucleolytic activities. ciRS-7 [also termed CDR1as (cerebellar degeneration-related protein 1 antisense)], one of the most studied circRNAs, is a circular miR-7 inhibitor/sponge that binds miR-7, resulting in reduced

Table 2 Functions of long non-coding RNAs deregulated in colorectal cancer

lncRNA	Oncogene/tumor suppressor	Biological process	Target	PMID
AK123657	Tumor suppressor	Cell proliferation, invasion		24809982
BANCR	Oncogene	Cell migration, EMT		25013510
BX648207	Tumor suppressor	Cell proliferation, invasion		24809982
BX649059	Tumor suppressor	Cell proliferation, invasion		24809982
CCAT1-L	Oncogene	MYC expression regulation	MYC-335	24662484
CCAT2	Oncogene	WNT signalling pathway activation	TCF7L2, MYC, miR-17-5p, miR-20a	23796952
CRNDE	Oncogene	Epigenetic remodelling of chromatin		19571010
H19	Oncogene	miR-675 precursor, cell proliferation, EMT, miRNA sponge	let-7	17237358, 19926638
HOTAIR	Oncogene	HOXD expression regulation, metastasis	HOXD10, PGR	24075995, 20393566
MALAT1	Oncogene	pre-mRNA metabolism, target gene expression regulator, tumor suppressor protein regulation, cell cycle progression, cell migration, MET	PSF, E2F1	17270048, 16878148, 18067128, 22078878, 22722759
mascRNA	Oncogene	Cell proliferation, invasion		21503572
N-BLR	Oncogene	Apoptosis, cell migration, invasion		http://dx.doi.org/10.1101/004796
PVT1	Oncogene	miRNAs precursor, cell proliferation, invasion		18194563, 25043044
TUSC7	Tumor suppressor	Cell proliferation, miRNA sponge	miR-211	23558749

For all lncRNAs are reported: (1) their function in CRC (oncogene, and tumor suppressor); (2) the biological processes they are involved in; (3) their validated targets; (4) bibliographic references reported as PubMed ID (PMID). LncRNA: Long non-coding RNA.

miR-7 activity and increased levels of miR-7 targets[212]. As miR-7 negatively controls the expression of several oncogenes, impairing miR-7 activity would have an important impact on the cell phenotype. Recently, Bachmayr-Heyda *et al*[213] found a global reduction of circRNA abundance in CRC cell lines and CRC tissues compared with normal tissues and detected a negative correlation between circRNA expression and proliferation. The authors explained these findings by hypothesizing that the back-splice machinery, responsible for RNA circularization, is dysfunctional in tumoral cells; otherwise, downregulation of circRNAs could be due to increased degradation by oncomiRNAs which are deregulated in CRC[213].

NON-CODING RNAS AS DIAGNOSTIC AND PREDICTIVE TOOLS

miRNAs as diagnostic and prognostic CRC biomarkers

As previously reported, there is overwhelming evidence indicating that post-transcriptional and translational controls, mediated by various miRNAs, exert critical pleiotropic actions on different features of CRC evolution. Based on these premises, tremendous effort was made towards the discovery and characterization of miRNAs as predictive and prognostic biomarkers in CRC. Unsurprisingly, most miRNAs involved in CRC regulation also exhibit potential predictive/prognostic properties (Table 3). MiR-31 is the most frequently mentioned in miRNA-based biomarker discovery studies for CRC patients. MiR-31 is upregulated in colon cancer tissues, compared with adjacent non-neoplastic normal tissues, across all clinical stages; its expression correlates with clinical stages[94,97,214]. It was observed that upregulation of miR-31 in tumour samples is positively associated with advanced tumour-node-metastasis (TNM) stage, presence of lymph node metastasis, and distant metastases[94,97,214]; furthermore, high expression of miR-31 correlates with patients' short survival[97]. On the other hand, Slaby *et al*[95] detected upregulation of miR-31 in CRC tissues, but surprisingly found no association with tumour stage. They reported a low expression of miR-31 mainly in poorly differentiated tumours[95]. MiR-21 has been frequently reported as being involved in many neoplasias, including CRC. It has also been demonstrated that it possesses diagnostic and prognostic power. High miR-21 levels correlate with short disease-free survival[215], clinical stage and distant metastases[95]. Analyzing colon and rectal cancer tissues separately, overexpression of miR-21 was an independent prognostic factor of unfavourable recurrence-free survival only for T3-4a colon cancer patients[216]. MiR-21 levels increase, while miR-143 and miR-145 expression decrease, going from well (G1) to poorly (G3) differentiated tumours. Decreased miR-143 and miR-145 expression is also preferentially associated with increased tumour size and localization in the proximal colon[95]. Vickers *et al*[217] reported that miR-21, miR-135a and miR-335 were upregulated in CRC compared with normal adjacent tissues, in particular in metastatic primary tumours, whereas miR-206 levels inversely correlated with CRC progression. Moreover, let-7a showed elevated expression in metastatic CRC compared with normal mucosa or non-metastatic disease, but only in KRAS-mutated tumours. This prognostic signature of miR-21, miR-135a, miR-206, miR-335, and let-7a, used to detect the presence of metastases, had a specificity of 87% and sensitivity of 76%: these data suggest their application as a prognostic tool in CRC[217]. Díaz *et al*[218]

Table 3 Diagnostic and prognostic cellular microRNAs in colorectal cancer

Clinical feature	Oncogenes	Tumor suppressor genes	PMID
Fluorouracil based therapy positive response	let-7g, miR-181b, miR-26a-1 (SNP)		18172508, 20585341
Metastasis	miR-21, miR-372, miR-720, miR-181a, miR-135a, miR-335	miR-126, miR-34a, miR-27a (SNP)	22120473, 24653631, 22456107, 25286763, 23243217, 24755295, 25078482
Poor fluorouracil based therapeutic prognosis	miR-21		18230780
Progression		miR-100 (SNP)	20585341
Survival	miR-21, miR-17, miR-181a, miR-181b, miR-372, miR-720, miR-106a, miR-20a, miR-203, miR-423 (SNP), miR-196a-2 (SNP)	miR-200c, miR-320, miR-498, miR-608 (SNP), miR-219-1 (SNP)	18230780, 18079988, 22065543, 24098024, 18676867, 22456107, 25286763, 22028396, 22661538, 22161766
Tumour size	miR-720	miR-143, miR-145	18196926, 25286763
Undifferentiated phenotype	miR-21	miR-143, miR-145	18196926

Cancer-related phenotypes that may be predicted by expression analysis of oncomiRNAs and tumor suppressor miRNAs. miRNAs: MicroRNAs.

showed an association between downregulation of miR-126 and age under 50 on CRC diagnosis. miR-126 inversely correlated with metastasis and its expression levels were significantly lower in metastatic CRC than in localized tumours[219]. The same authors also observed a correlation between high miR-106a expression and 5-year disease-free survival and overall survival[218]. Recurrence-free survival of patients with stage Ⅱ CRC was also independently associated with high expression of miR-320 and miR-498[220]. MiR-181a is upregulated in CRC tissues compared with normal tissues and in liver-metastatic CRC: this suggests its correlation with liver metastasis and also with poor overall survival in EGFR-targeted therapy[145,221]. On the other hand, low miR-181b and let-7g expression correlates with a positive response to 5-fluorouracil-based antimetabolite S-1[222]. Overexpression of miR-15b, miR-181b, miR-191 and miR-200c in CRC, compared to normal colorectal tissues, was reported by Xi *et al*[223]. Kaplan-Meier survival analysis showed that patients with higher miR-200c expression had shorter survival time compared with patients with lower expression[223]. MiR-372 and miR-720 (both controlled by the KRAS pathway) showed high expression levels, which are significantly associated with CRC tumour size and distant metastases. Metastatic CRC samples showed higher miR-372 and miR-720 expression compared with the non-metastatic samples; moreover, their upregulation was found to be associated with lower 5-year overall survival[125,126]. The presence of metastases in CRC patients is also associated with reduced miR-34a expression, caused by high CpG methylation of miR-34a and miR-34b/c promoters[135]. Expression levels of the oncogenic miR-17-92 cluster and two of its paralogs (miR-106a and miR-106b) are significantly elevated in CRC. Although the authors observed no significant association between deregulation of these miRNAs and clinico-pathological features of patients, high levels of miR-17 were related to reduced overall survival of CRC patients[224]. A promising non invasive approach for CRC screening is to assay stools for molecular biomarkers that mirror the molecular alterations associated with cancer: colon cancer tissues consistently shed cancer cells into stools; accordingly, it would be an ideal substrate to detect specific CRC biomarkers. Several papers showed quantitative changes in the expression of some miRNAs in stools of CRC patients with respect to normal controls. Ahmed *et al*[225,226], reported 12 upregulated miRNAs (miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p and miR-214) and 8 downregulated miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222 and miR-938) in the stools of CRC patients; and these alterations were more pronounced in later carcinoma stages. MiR-21 and -106a upregulation in stools of adenoma and CRC patients was also reported in different papers[227,228]. Other studies on CRC stools suggested miR-18a, miR-31, miR-135b, and miR-221 as potential biomarkers of adenoma and carcinoma[229,230].

Pathological and clinical roles of lncRNAs in CRC

Several high-throughput profiling and reverse transcription polymerase chain reaction (RT-PCR) studies were published in the last few years that showed the potential diagnostic and prognostic power of lncRNAs in CRC (Table 4). By searching through previously published gene expression microarray data, Hu *et al*[231] analyzed lncRNA profiles of large cohorts of CRC patients and identified a prognostic six-lncRNA signature. These six lncRNAs (*i.e.*, AK024680, AK026784, AK123657, BX648207, BX649059, and CR622106) significantly correlated with disease-free survival: this signature was able to classify CRC patients into two subgroups: high-risk (shortened survival) and low risk (prolonged overall survival). Moreover, functional experiments demonstrated that repression of lncRNAs AK123657, BX648207 and BX649059 in CRC cell lines increased cell proliferation and invasion[231]. One of the most upregulated lncRNAs associated with CRC is CRNDE[232]. CRNDE exists in different splice variants that may have diagnostic usefulness: the most diagnostically

Table 4 Diagnostic and prognostic cellular long non-coding RNAs in colorectal cancer

Clinical feature	Oncogenes	Tumor suppressor genes	PMID
Disease-free survival	AK026784, AK024680, MALAT1	AK123657, CR622106, BX649059, BX648207, TUSC7, RPL34-AS1	24809982, 25031737, 23680400, 24908062
Distant metastasis	91H, PCAT1, CCAT1	TUSC7, RPL34-AS1	25058480, 23640607, 23594791, 23680400, 24908062
Liver metastasis	HOTAIR, HULC	ncRAN	21862635, 24519959, 19445043
Lymph node metastasis	PVT-1, AK021444, ENST00000425785, AK307796	ENST00000465846	24196785, 25009386
Metastasis risk	MALAT1		25031737
Overall survival	MALAT1, PVT-1, PCAT1	ncRAN, uc.73	25031737, 24196785, 24519959, 23640607, 22328099
Plasma diagnostic marker	CRNDE		22393467
Poor prognosis	HOTAIR, 91H, PVT-1		21862635, 25058480, 24196785
Tumour risk	PRNCR1 (SNP)		24330491
Tumour size	HOTAIR, PRNCR1 (SNP)	TUSC7	21862635, 23680400, 24330491
Tumour stage	CCAT1	lincRNA-p21, TUSC7, RPL34-AS1	24012455, 23594791, 23680400, 24908062
Undifferentiated phenotype	HOTAIR, PRNCR1 (SNP)	ncRAN	21862635, 24519959, 24330491
Venous invasion	PVT-1	lincRNA-p21	24196785, 24012455

Cancer-related phenotypes that may be predicted by expression analysis of oncogenic and tumor suppressive long non-coding RNAs.

relevant isoform CRNDE-h showed a sensitivity of 95% and a specificity of 96% for distinguishing adenomas from normal tissues and a sensitivity of 80% and a specificity of 96% for carcinoma *vs* normal tissues[233]. High expression levels of HOTAIR in CRC patients were closely related to poor prognosis[200]. Specifically, Kogo *et al*[200] divided 100 patients with CRC into two groups: a high HOTAIR expression group and a low expression group: their data show that CRC patients with the highest HOTAIR expression exhibit less differentiated histology, greater tumour size, and higher propensity to liver metastasis than CRC patients with low HOTAIR expression. Similar results were obtained for MALAT1 by Zheng *et al*[234], who statistically associated expression of MALAT1 to clinico-pathological parameters, disease-free survival, and overall survival. Stage Ⅱ/Ⅲ CRC patients with higher expression of MALAT1 showed a significantly higher risk of metastasis after radical surgery and significantly poorer overall survival[234]. 91H (also named LINC01219), an antisense RNA of lncRNA H19, is overexpressed in CRC tumour tissues with respect to adjacent normal tissues. Clinico-pathological factors were compared between CRC patients with high and low expression of 91H. Statistical analysis showed that high expression of 91H was significantly correlated with distant metastases and poorer prognosis[235]. LncRNA PVT1 is a precursor of a number of spliced ncRNAs and of six miRNAs. The biomolecular functions of PVT1 remain elusive, even though several studies were performed to determine them[236,237]. Takahashi *et al*[238] investigated the clinical significance of PVT1 expression on 164 CRC patients, showing that high PVT1 expression was positively related to the size of lymph node metastasis, venous invasion and poor prognosis. Furthermore, univariate and multivariate analyses showed that PVT1 expression was an independent risk factor for overall survival of CRC patients. NcRAN (non-coding RNA expressed in aggressive neuroblastoma) is an lncRNA whose expression is highly upregulated in neuroblastoma patients with poor prognosis; it could also have an important pathogenetic role in human bladder cancer[239,240]. Qi *et al*[241] demonstrated that ncRAN expression is significantly reduced in CRC tumour tissues and cell lines, compared with adjacent normal tissues and a normal intestinal mucous cell line. Downregulation of ncRAN was more evident in poorly differentiated or undifferentiated tumours and in CRC with liver metastases. Kaplan-Meier survival analysis showed that CRC patients with lower ncRAN expression had a worse overall survival. Overexpression of PCAT1 (prostate cancer associated transcript 1) was associated with distant metastases and poorer overall survival in CRC patients: in few cases, this was explained by gene copy number variation of the PCAT1 locus[242]. Long intergenic noncoding RNA-p21 (lincRNA-p21) is transcriptionally induced by p53 in CRC cell lines, following apoptosis induction by nutlin-3; it works as a repressor in p53-dependent transcriptional responses. Its expression levels were lower in CRC tumour tissues, when compared with adjacent normal tissues from the same patient. Expression of lincRNA-p21 was higher in rectum cancers with respect to colon cancers. Its overexpression was significantly correlated to higher primary tumour (pT) and vascular invasion[243]. Alaiyan *et al*[244] screened the expression of CCAT1 in normal colon tissues and in various stages of CRC development (*i.e.*, adenoma, invasive carcinoma, lymph nodes metastases, and distant metastases). By using quantitative RT-PCR (qRT-PCR) and *in situ* hybridization (ISH), they found: (1) increased CCAT1 expression in colon adenocarcinoma compared with normal tissues; and (2) heterogeneous upregulation across the colon adenoma-carcinoma sequence. CCAT1 upregulation was evident in premalignant conditions and through all disease stages, including advanced metastatic disease:

these data suggest its potential role both in transformation and in metastasis. Transcribed ultra-conserved regions are a recently discovered class of non-coding RNAs with a high degree of evolutionary conservation: this strongly suggests a critical role in the physiology of mammals and other vertebrates. T-UCRs mainly work as antisense molecules for protein-coding genes and miRNAs; their dysregulation has been reported for different types of tumours[245,246]. Sana *et al*[247] analyzed the expression in CRC tissues and adjacent normal tissues of t-UCR uc.43, uc.73, uc.134, uc.230, uc.339, uc.388, uc.399, which previously had been found to be associated with CRC. Among these t-UCRs, only uc.73 and uc.388 were significantly downregulated in CRC tissues; uc.73 showed a positive correlation with overall survival of CRC patients. The tumor suppressor lncRNA TUSC7 (LOC285194) is depleted in osteosarcoma, causing abnormal proliferation of osteoblasts; its deletions were associated with poor survival of osteosarcoma patients[248]. Qi *et al*[249] found that expression levels of TUSC7 significantly decreased also in CRC samples. In addition, this downregulation was correlated with larger tumour size, higher tumour stage, more distant metastases, and poor disease-free survival[249]. By microarray profiling of CRC samples and adjacent normal tissues from non-metastatic and metastatic patients, six aberrantly expressed lncRNAs (*i.e.*, AK097793, ENST00000423943, ENST00000393516, ENST00000428029, RP11-462C24.1, and uc002wvk.2) were identified[250]. Among these, RP11-462C24.1 (also called RPL34-AS1), whose functions are still unknown, exhibited interesting prognostic properties: its expression level decreased as the malignant degree of CRC increased. Furthermore, downregulation of RP11-462C24.1 significantly correlated with more distant metastases and a poor disease-free survival[250]. Some studies on the diagnostic and prognostic power of lncRNAs in CRC focused on their expression in metastatic sites rather than CRC tissues. LncRNA expression profiling in metastatic lymph nodes (MLN), normal lymph nodes (NLN), and tumour tissues from CRC patients showed that 14 lncRNAs were specifically upregulated (*e.g.*, AK021444, ENST00000425785, and AK307796) and 5 lncRNAs were specifically downregulated (*e.g.*, ENST00000465846) in the MLN group compared with the NLN group and tumour tissue group[251]. These data suggest that specific lncRNA dysregulation in MLN may have an important role in facilitating the occurrence of lymph node metastasis in CRC. However, the molecular functions of these lncRNAs are still unknown. The lncRNA highly upregulated in liver cancer (HULC) is usually upregulated in hepatocellular carcinoma and may perturb the circadian rhythm of hepatoma cells[252,253]. HULC is neither expressed in CRC tissues nor in normal colon mucosa; however, it was found to be significantly expressed in CRC with metastases in the liver, but not in lymph nodes[254]. These data suggest that HULC could play a role in the metastatic process of CRC tumours in the liver.

Non-coding RNAs with predictive power in CRC therapy response

One of the most challenging tasks in cancer treatment is to identify patient subpopulations who could benefit from chemotherapy and avoid over-treatment of chemorefractory patients. Discovery of predictive biomarkers would provide information on the likelihood of response to a given therapeutic agent and help to optimize therapy decisions. Much effort was made to find feasible predictive biomarkers in CRC, however, except for KRAS mutations, no clinical study provided markers which have entered into the clinical management of CRC. Recent studies revealed that expression levels of certain miRNAs could be associated with specific therapeutic outcomes in CRC, suggesting that miRNAs may be considered potential predictive biomarkers. Some *in vitro* studies on CRC cell lines showed that 5-fluorouracil can alter profoundly miRNA expression patterns and miRNAs modulate the expression of key proteins involved in the regulation of cell proliferation, apoptosis and drug response[255,256]. In one of the first studies on CRC patients, miRNA expression was evaluated in cancer biopsies before therapy and two weeks after starting preoperative capecitabine chemoradiotherapy: miR-137 and miR-125b were upregulated two weeks after starting therapy and higher levels of both of them were associated with worse response to the therapy[257]. Hansen *et al*[258], studied the predictive value of miR-126 in clinical response to capecitabine and oxaliplatin in CRC metastatic patients by *in situ* hybridization. MiR-126 expression levels were significantly higher in patients responding to therapy with respect to not responding patients: furthermore, its high expression was associated with a more prolonged free survival progression[258]. Overexpression of miR-145 was found in CRC patients who showed a good response to neoadjuvant chemoradiotherapy (5-FU and 50.4 Gy) and tumor regression[259], while miR-17-5p was found increased in chemoresistant patients[260]. Della Vittoria Scarpati *et al*[261] using microarray expression profiling detected specific miRNA signatures associated with complete response of CRC patients after neoadjuvant chemoradiotherapy. Specifically, they found a set of 13 miRNAs (*i.e.*, 11 upregulated and 2 downregulated miRNAs) strongly associated with good response to chemoradiotherapy. Among them, the upregulation of miR-622 and miR-630 showed 100% sensitivity and specificity in predicting the patients' response[261]. In a similar study, Svoboda *et al*[262] compared miRNA profiles of CRC patients classified as sensitive or resistant to neoadjuvant chemoradiotherapy. They identified an overexpression of miR-215, miR-190b,

and miR-29b-2* in non-responder patients, while the upregulation of let-7e, miR-196b, miR-450a, miR-450b-5p and miR-99a* was associated with good responders[262]. In another profiling study, Hotchi *et al*[263] analyzed miRNA expression in rectal cancer patients prior to pre-operative chemoradiotherapy and correlated them to different methods to evaluate the patients' response. They found different miRNA signatures that discriminated responders from non responders. MiR-223 was the only miRNA common to the different evaluation parameters: its expression was significantly higher in responders compared with non-responders and showed 100% and 78% sensitivity and specificity, respectively, in the prediction of response to pre-operative chemoradiotherapy[263]. In the last few years some evidence on the potential involvement of lncRNAs in molecular mechanisms controlling cancer drug resistance has been reported. Cancer Upregulated Drug Resistant (CUDR) is a lncRNA upregulated in several cancers, including CRC. Enforced overexpression of CUDR induced resistance to doxorubicin and etoposide, as well as drug-induced apoptosis in human squamous carcinoma A431 cells[264]. Lee *et al*[265], by analyzing the expression of 90 lncRNAs in 5-FU-resistant CRC cell lines, found that SnaR and BACE1AS were significantly downregulated in resistant cell lines; decreased expression of SnaR was responsible for increased cell viability after 5-FU treatment. Recently, colorectal cancer-associated lncRNA (CCAL) was found significantly upregulated in CRC patients with worse response to adjuvant chemotherapy[266]. CCAL regulates CRC progression and multidrug resistance (MDR) through activation of the Wnt pathway by suppressing AP-2α and leading to upregulation of MDR1/P-gp expression[266].

CRC associated single nucleotide polymorphisms in non-coding RNAs

According to the multigenic model of cancer development, combinations of polymorphic genetic variants in susceptibility genes could contribute to CRC risk. Recently, functional polymorphisms in miRNAs or their binding sites in mRNA targets have been discovered in association with pathological phenotypes. A SNP embedded in a miRNA sequence may alter miRNA maturation or its targeting and, accordingly, contribute to the onset and evolution of cancer. One of the first studies showing a prognostic role of miRNA SNPs in CRC reported a significant association of SNP rs7372209 in pri-miR26a-1 to positive chemotherapy response, and SNP rs1834306 in the pri-miR-100 to longer time to progression, suggesting that miRNA polymorphisms could be potential predictors of clinical CRC outcome[267]. Xing *et al*[268], by screening seven SNPs in 408 CRC patients of a Chinese population, identified two SNPs statistically associated with prognostic features: rs6505162 in pre-miR-423 was correlated to the overall survival and recurrence-free survival, while, rs4919510 in pre-miR-608 was correlated with recurrence-free survival. CRC patients with both SNPs had a 2.84-fold increased risk of recurrence and/or death.These associations were evident only in patients receiving chemotherapy[268]. In another study, performed on 1,097 CRC patients recruited at the University of Texas MD Anderson Cancer Center, rs4919510 in miR-608 associated with increased risk for recurrence and death, and rs213210 in miR-219-1 in association with death of patients with stage Ⅲ disease were found[269]. Patients carrying both SNPs showed a 5.6-fold increased risk of death. SNP rs11614913 in miR-196a-2 was associated to a significantly increased CRC risk in a Korean population[270]. Association between rs11614913 and CRC susceptibility was also valuated in two different Chinese studies, but the results were conflicting[271,272]. Recently, a study on SNP rs895819 in pre-miR-27a, previously associated with different cancers, was performed to valuate the potential association to CRC susceptibility in a Han Chinese population: GG genotype was significantly associated with risk of CRC and metastasis[273].Data on association to CRC risk of previously mentioned SNPs in miR-196a-2 and miR-27a was not confirmed in a Central-European Caucasian population[274]. The discrepancies in the diagnostic potential of miRNA SNPs in CRC above reported may be due to different molecular pathogenetic mechanisms that differently contribute to cancer or population-specific factors, such as the different genetic backgrounds of the studied population. In addiction to the above-mentioned SNP rs6983267 mapping to a genomic region abundant in lncRNAs, five SNPs in the lncRNA PRNCR1 (prostate cancer associated non-coding RNA 1) were investigated in a Chinese case-control study of 313 cases with CRC and 595 ethnicity-matched controls[275]. The final results of this study were that rs13252298 and rs1456315 are associated with significantly decreased risks of CRC. Tumours of patients expressing rs1456315G were larger than 5 cm. Patients expressing rs7007694C and rs16901946G had a decreased risk of developing poorly differentiated tumours; on the contrary, expression of rs1456315G was found to be associated with an increased risk[275].

NON-CODING RNAS IN BODY FLUIDS: BIOLOGICAL AND DIAGNOSTIC IMPLICATIONS

Among the most ambitious goals of oncology is the application of fast and non-invasive methods for the identification of specific biomarkers, to be applied to diagnosis and prognosis (*i.e*., liquid biopsies): these could be useful to discriminate between healthy individuals and cancer patients or as markers of specific responses to therapy. In this regard, blood is the

election sample: it contains a high amount of important biomolecules, such as proteins, hormones, metabolites, DNA and different RNAs such as mRNAs and miRNAs. The stoichiometric ratio of its components is highly dynamic and reflects the health status of patients. Blood sampling is fast and easy: serum and plasma have been used for a long time as a source of biomarkers for different conditions. MiRNA profiling is an established method for discriminating cancerous tissues from their healthy counterparts[276]. The discovery, in 2008, of miRNAs in human plasma opened new intriguing perspectives in cancer diagnosis[277,278]. An important requisite of biomarkers is stability in the medium where they are released: miRNAs have been shown to be present in serum in a stable form that prevents their digestion by RNases. Little is known about the origin and features of serum miRNAs, but it is known that they are cell-free molecules: therefore, there have to be other ways to protect them from degradation[277]. Currently, two major release mechanisms of circulating miRNAs have been proposed: (1) active secretion of miRNA-containing shedding microvesicles or exosomes[279]; and (2) active secretion of miRNAs in the form of ribonucleoprotein complexes[280]. Exosomes have been described as a new mechanism of cell-to-cell communication that has similar features to hormonal signalling[281]. Exosomes originate from inward budding of endosomal membranes forming multivesicular bodies, which are secreted into the extracellular environment when late endosomes fuse with the plasma membrane[282]. These nanovesicles are produced by several cell types, both in normal and pathological conditions, and were found in serum and other body fluids[283-286]. Interestingly, exosomes have been shown to carry a set of miRNAs that are specifically targeted to them[281,287-289]. The endosomal sorting complexes required for transport (ESCRT) has been hypothesized to be major regulators of types and amounts of biomolecules sorted into exosomes (*i.e.*, miRNAs, mRNAs, proteins)[290]. The molecular mechanisms involved were uncharacterized until recently. The identification of the role of sumoylated hnRNPA2B1 in transfering miRNAs to exosomes is the first evidence of a possible mechanism involved in the specific miRNA sorting into exosomes[291]. Despite the great interest and growing research in the exosome field, accumulating data suggest that these nanovesicles contain only 10% of circulating miRNAs, and that most of them are associated with RBPs[292]. In 2011, Arroyo *et al*[293] found that the majority of circulating miRNAs are destabilized by plasma digestion with proteinase K, demonstrating that a protein complex is responsible for their protection in the RNase-enriched plasma environment. Notably, for the miRNAs that are not carried by microvesicles or exosomes, a strong association with Argonaute-2 protein was detected. Another mechanism putatively involved in miRNA preservation in serum is the packaging of circulating miRNAs in high-density lipoproteins (HDL), which in turn can transfer them to recipient cells: this would identify a potential new role for HDL particles in cell communication[294]. Whatever the origin of circulating miRNAs, their expression profiling has proven to have prognostic relevance, as confirmed by several studies. For instance, in 2008 it was shown for the first time that miRNA profiling of circulating exosomes in ovarian cancer patients could potentially be used as a non-invasive diagnostic method of this malignancy. Eight miRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214), known to have a diagnostic value in ovarian cancer tissues, were shown to have the same expression pattern in the exosomes of cancer patients: this was different than that of healthy controls[295]. Moreover, it has been noted that tumour cells possess increased exosome-shedding properties compared with normal cells[295]. In 2009, Rabinowits *et al*[296] made similar observations in lung cancer patients; in addition, they showed that the serum of lung adenocarcinoma patients is highly enriched in exosomes compared with healthy patients. In 2014, Eichelser *et al*[297] showed that cell-free miR-101 and miR-373 can be used as breast cancer-specific markers, and that exosomal miR-373, whose expression is increased in Erb-b2 receptor tyrosine kinase 2 (ERBB2/HER2) - negative tumours, has a potential role as a blood-based biomarker for more aggressive tumours. High levels of exosomes in the plasma of CRC patients are secreted by poorly differentiated tumours and are associated with decreased overall survival[298]. Serum levels of exosomal let-7a, miR-21, miR-23a, miR-150, miR-223, miR-1229, and miR-1246 were significantly higher in primary CRC patients at any disease stage than in healthy controls; these miRNAs were significantly downregulated after surgical resection of tumours[299]. Interestingly, exosomes secreted by colon tumour cells play a key role in the recruitment of inflammatory $CCR6^{+}CD4^{+}Th17^{+}$ into tumour sites and subsequently promote tumour growth[300]. Ragusa *et al*[289,301] have demonstrated significant alterations of exosomal miRNA cargo in CRC cells following treatment with Cetuximab. The most upregulated miRNAs in exosomes from Caco-2 and HCT-116 cells perform a dual biological function: (1) negative regulation of proliferation or apoptosis induction in cancer cells; and (2) immunosuppression in B- and T- cells. In the last few years, several papers reported the discovery of circulating miRNAs with potential diagnostic or prognostic power in CRC patients (Table 5). The first study on plasma miRNA profiling, performed on 90 CRC patients and 50 healthy controls, showed the significant upregulation of miR-17-3p and miR-92a (both encoded by the miR-17-92 cluster). The authors suggested that elevated plasma levels of miR-92a were not associated with inflammation or other gastrointestinal cancers, but most likely were CRC-related[302]. Upregulation of miR-92a (together with miR-29a) in

Table 5 Diagnostic and prognostic circulating non-coding RNAs in colorectal cancer

Clinical feature	Oncogene ncRNAs	Tumor suppressor ncRNAs	PMID
Diagnostic	miR-17-3p, miR-92a, miR-29a, miR-18a, miR-19a, miR-19b, miR-15b, miR-335, miR-221, exo-let-7a, exo-miR-1229, exo-miR-1246, exo-miR-150, exo-miR-21, exo-miR-223, exo-miR-23a, miR-378, miR-21, CRNDE, miR-155, miR-200c, miR-210	miR-601, miR-760, miR-143	19201770, 19876917, 23267864, 22970209, 20880178, 24705249, 25547322, 22868372, 22393467, 24310813
Metastasis detection	miR-29a, miR-21		22018950, 23704278
Survival	miR-141, miR-221, miR-21		21445232, 20880178, 23704278
Tumour size	miR-21		23704278
Tumour stage	miR-141		21445232

Cancer-related phenotypes that may be predicted by expression analysis of plasma/serum circulating oncogenic and tumor suppressive non-coding RNAs (ncRNAs). Exo-miR: MiRNAs purified from exosomes.

plasma was also reported by Huang *et al*[303]. They strengthened these data showing that levels of both miR-29a and miR-92a were significantly reduced in post-operative plasma samples when compared with pre-operative samples. In a different study, the same authors found that the levels of plasma miR-601 and miR-760 were significantly decreased in CRC compared with healthy controls[304]. It is interesting to note that by adding expression data of miR-29a and miR-92a for combined receiver operating characteristic (ROC) curve analysis with miR-760, the resulting AUC (Area Under Curve) increased to 0.943 with 83.3% sensitivity and 93.1% specificity in discriminating CRC from controls: this suggests the additive effect in diagnostic power of these 3 miRNAs[304]. Overexpression of miR-29a was also detected in the serum of CRC patients with liver metastases, but not in CRC patients without metastases: this suggests its potential as a novel non-invasive biomarker for early detection of CRC with liver metastasis[305]. By genome-wide miRNA expression profiling on 196 plasma samples from 123 patients with sporadic CRC and 73 healthy individuals, Giráldez *et al*[306] found significant upregulation of 6 miRNAs (*i.e.*, miR-15b, miR-18a, miR-19a, miR-19b, miR-29a, and miR-335), suggesting specific expression patterns to be considered as biomarkers for CRC. Upregulation of miR-141 levels was demonstrated in plasma of CRC patients with stage Ⅳ with respect to patients with stages Ⅰ-Ⅱ and unaffected controls; they were also associated with shorter survival. These expression differences of miR-141 may be related to a differential inflammatory response in CRC patients[307]. Similarly, the expression levels of miR-221 in plasma of CRC patients are significantly higher than in the healthy controls. According to the Kaplan-Meier analysis, upregulation of miR-221 is a significant prognostic factor for poor overall survival in CRC patients: patients with higher miR-221 plasma levels had dramatically lower survival rates than those belonging to the low expression group[308]. Upregulation of miR-155, miR-200c, and miR-210 from serum of CRC patients was reported by Chen *et al*[309] Expression levels of these miRNAs returned to normal levels in patients with good prognosis after surgery and chemotherapy. Unsurprisingly, several authors reported high levels of miR-21 in plasma and serum of CRC. Upregulation of plasma miR-21 discriminates CRC patients from controls with 90% specificity and sensitivity[310]. MiR-21 levels are significantly elevated in preoperative serum from patients with adenomas and CRCs. High miR-21 expression in serum is statistically associated with tumour size, distant metastases, and poor survival[311]. Recently, Monzo *et al*[312] showed that miR-21 expression was higher in plasma from blood drawn from the mesenteric veins (MV), near the site of primary CRC than in that drawn from the peripheral veins (PV) of the same CRC patients. Moreover, high levels of miR-21 in MV plasma were associated with shorter disease-free survival of patients. These data suggested that CRC cells release high concentrations of miR-21 in the MV, but that these concentrations are progressively diluted in the peripheral circulatory system. Accordingly, miR-21 dosage from MV would be a stronger prognostic CRC marker than from PV. Recently, Clancy *et al*[313] analyzed miRNA expression in serum of CRC patients in a literature review on about twenty different published studies: they reported that the 6 circulating miRNAs most frequently found to be dysregulated in CRC are miR-18a-5p, miR-21-5p, miR-29a-5p, miR-92a-5p, miR-143-5p and miR-378-5p. Recently, there has been a notable increase of reports on serum or plasma lncRNAs, potentially useful as biomarkers for different types of tumours. One of the first studies associating circulating lncRNA expression to specific cancers reports the upregulation of lncRNA HULC in blood samples of hepatocellular carcinoma patients[252]. The lncRNA H19 has been found to be highly expressed in the plasma of gastric cancer patients compared with healthy controls, and its levels significantly decrease after tumour resection[314]. Recently, a three lncRNA [urothelial cancer associated 1 (UCA1/CUDR), long stress-induced non-coding transcript 5 (LSINCT-5), phosphatase and tensin homolog pseudogene 1 (PTENP1)] signature in serum was identified as a diagnostic marker for gastric cancers[315]. Tong *et al*[316] identified the lncRNA POU3F3 (POU class 3 homeobox

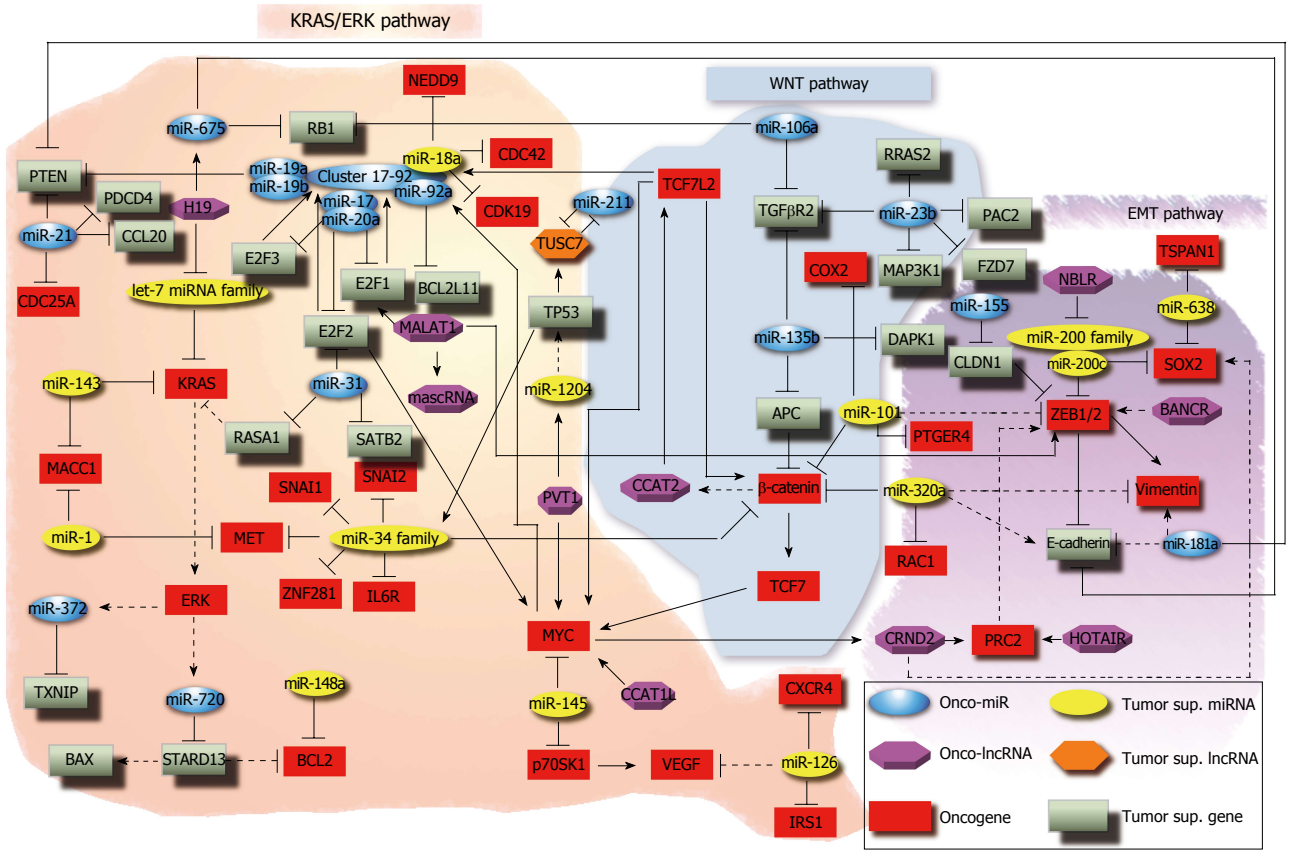

Figure 1 Non-coding network of colorectal cancer. Molecular signalling of non-coding RNAs [*i.e.*, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs)] in most common cancer-related pathways involved in CRC etiopathogenesis (*i.e.*, KRAS/ERK, WNT, and EMT). RNA-RNA and RNA-protein interactions were retrieved from literature cited in this review. Lines with arrowheads represent expression activation, those with bars represent expression inhibition. Dotted lines show indirect interactions.

3) as a novel serum biomarker for esophageal squamous cell carcinoma, showing that combining POU3F3 expression with plasma levels of the squamous cell carcinoma antigen notably improves efficiency of screening for early detection. Quite surprisingly, only one report has been published to date on the expression of circulating lncRNAs as potential non-invasive diagnostic markers in CRC. The expression level of CRNDE-h transcript in plasma of CRC patients was significantly higher than that of healthy controls, with a sensitivity of 87% and specificity of 93% for diagnosing CRC[233] (Table 5).

FROM A COMPLEX NON-CODING RNA NETWORK TO NEW THERAPEUTIC TARGETS

We have discussed the multiple lines of evidence that pinpoint ncRNAs as key regulators of global gene expression for cancer-related processes. NcRNAs appear to constitute multiple control layers that define the correct functioning of classically known coding layers in cells: they perform this job by modulating chromatin architecture and genome expression (RNA synthesis, stability, processing and translation). The existence of this sophisticated RNA-based regulatory system has important biological and biomedical consequences. Indeed, it could appropriately explain the diversity of phenotypes of mammals, despite their relatively similar proteomes. On the other hand, it is becoming increasingly clear that alterations of these regulatory RNA signals may cause cells to acquire a pathological phenotype, including cancer, as reported for CRC in this review. Both miRNAs and lncRNAs contribute to bring about dysfunctions of critical CRC signalling pathways, such as KRAS/ERK, WNT, and EMT (Figure 1). RNA-based regulation in CRC constitutes a complex, and only partially explored, network of RNA-RNA, RNA-protein interactions that performs a multilayered control on proliferation, invasion, and differentiation in CRC cells. The complexity of this scenario is increased by the presence of several positive and negative regulatory loops. For instance, the miRNA cluster 17-92 represses E2F2, which in turn transcriptionally controls MYC. On the other hand, MYC acts as a transcriptional activator for the cluster 17-92[317]. Moreover, several ncRNAs act as mediators in the cross-talk between different molecular processes in CRC. For instance, MALAT1 induces the expression of E2F2, thus altering MYC signalling and increasing proliferation; it also activates the expression of ZEB1,

causing the impairment of epithelial differentiation. Indeed, these apparently different non-coding landscapes converge into a unique RNA network that pervades and controls protein-coding patterns. This key role of ncRNAs makes them very attractive and promising targets for innovative therapeutic approaches. Compared with other strategies, RNA-based therapeutics have several advantages. RNAs are molecules more "druggable" than proteins, because their targeting is mainly based on nucleic acid complementarity, and therefore easy to design and inexpensive to synthesize. RNA-based therapeutic approaches can be divided into two different categories: (1) RNA inhibition therapy, when target RNA is overexpressed; and (2) RNA replacement therapy, when the ncRNA is repressed (this is more feasible for miRNAs). A widely used method for miRNA inhibition is the use of chemically modified oligonucleotides complementary to mature miRNAs (antagomiRs). AntagomiRs quench target miRNAs, which may induce miRISC disruption and miRNA degradation[318]. MiRNA sponges with multiple complementary 3'UTR mRNA sites have been developed to inhibit the activity of miRNA families sharing a common target sequence[319]. Another approach to specifically inhibit miRNA function is to exploit miRNA masks (miR-Masks): these are complementary to the binding sites in the 3'UTR of the target mRNA, so that the miRNA is no longer able to perform its repressive activity[320]. MiRNA replacement therapy is based on the restoration of tumour suppressive miRNAs, expressed at lower levels in cancer. This can be obtained by inserting oligonucleotide mimics, containing the same sequence as the mature endogenous miRNAs, into the cells; alternatively, synthetic pre-miRNA mimics can be used[321,322]. The depletion of oncogenic lncRNAs could be obtained by using similar approaches to those applied to miRNAs: siRNAs and shRNAs (small hairpin RNAs) exhibit great RNA selectivity and knockdown efficiency and utilize a similar silencing molecular mechanism (RISC)[323]. However, in the last few years new appealing methods have been developed, which have the potential to substitute RNAi-based approaches [*i.e.*, antisense oligonucleotides (ASOs), ribozymes and aptamers]. ASOs are single stranded DNAs or RNAs (8-50 nt) with sequence complementary to their target RNAs[324]. When ASOs bind to target RNAs, RNase H1 recognizes the DNA:RNA/RNA:RNA heteroduplexes and catalyzes the cleavage of the RNA molecules[324]. Because ASO-mediated silencing is independent from the RISC machinery, this technology produces less off-targeting effects than siRNAs. As a consequence of their double-stranded structure, siRNAs and shRNAs may trigger an innate immune response and induce high levels of inflammatory cytokines and a toll-like receptor (TLR)-mediated response; such effects are less evident for single-stranded molecules[325]. Ribozymes are naturally synthesized RNA molecules that are able to degrade other RNAs by their intrinsic catalytic functions: their potential application as a therapeutic cancer tool has been already mentioned[326]. Ribozymes bind their targets by complementarity and catalyze cleavage of target RNAs, destabilizing their phosphodiester backbone[326]. An important hurdle to antisense-based lncRNA modulation is the presence of extensive secondary structures along these RNA molecules. Indeed, the effectiveness of siRNAs/shRNAs, ASOs and ribozymes could be seriously impaired by secondary structures in target RNAs[327]. This obstacle could be overcome by aptamers as their targeting mechanism is independent from sequence complementarity[328]. Aptamers are short DNA or RNA oligonucleotides or peptides with a stable three-dimensional structure that specifically bind to their targets (*i.e.*, RNAs, proteins, and small molecules) by fitting the three-dimensional shape of their ligands[329]. Aptamers would be able to recognize distinct RNA structures through tertiary interactions and alter their functions by sequestering them or masking potential binding sites. These RNA-based therapeutic strategies applied to ncRNAs have already shown very promising results in cancer, including CRC. The reintroduction of miR-145 has been demonstrated to perform an antitumoral role in a mouse model of colon cancer[321]. *In vivo* depletion of miR-21 by ASOs strongly inhibits pancreatic ductal adenocarcinoma tumour growth by inducing cell death[330]. Targeting MALAT1 and HOTAIR with ASOs in mouse xenograft models prevented lung metastasis and inhibited pancreatic tumour growth, respectively[331,332]. However, the main issue related to ncRNA therapeutics is to find a delivery mechanism that would allow their stability in the circulation and efficient tissue-specific uptake, at the same time minimizing off-target side effects[333]. Rapid progress in drug delivery systems has provided optimistic perspectives for advances in this field[334]. Different chemical groups, such as steroids and cholesterol, can be covalently added to miRNAs or ASOs to improve intracellular uptake and extend circulation time[335]. Further delivery mechanisms are the use of adenoviral vectors as carriers of RNA therapeutics, cationic liposomes, or polymer-based nanoparticles, which are able to form micelle-like structures[336]. Recently, an exosomal-based miRNA delivery has been reported to be an effective therapeutic delivery system because these nanoparticles are less toxic and better tolerated by the organism. Moreover, they are naturally used by cells for intercellular communication, and have been proven to protect their molecular cargo in the circulation[337]. For the development of RNA therapeutic strategies, it is interesting to consider that several biological functions of miRNAs and lncRNAs may be partially redundant (Figure 1): accordingly, it may be quite difficult to modulate them by targeting a single ncRNA molecule[338]. This should lead researchers to develop multitargeted RNA therapies in order to increase their impact on oncogenic molecular signalling. For instance, to attenuate KRAS overfunctioning in CRC, exogenous

let-7 miRNAs could be introduced to repress KRAS expression; concomitantly, ASOs against lncRNA H19 could be applied to deplete its action as a molecular sponge of the let-7 family. This would strengthen the antiproliferative effects triggered by KRAS repression. Such synergic approaches have already provided very encouraging results by simultaneously administrating miRNA mimics, siRNAs and shRNAs against mRNAs in *in vitro* and *in vivo* models[339]. Finally, despite the high number of putatively "*druggable*" ncRNAs, it is clear that researchers still have to overcome many conceptual and technical challenges to develop effective anticancer strategies that can be applied to patients. However, the results obtained so far are promising and provide optimistic perspectives for the future of anticancer treatments.

FUTURE PERSPECTIVES ON THE CLINICAL UTILITY OF NON-CODING RNAS

Experimental evidence of the potential predictive and prognostic value of cellular and circulating ncRNAs strongly suggests the possibility of exploiting them as useful clinical tools for cancer management. Expression changes of specific ncRNAs can be detected in CRC tissues, utilizing slide-based staining assays: these are standard diagnostic procedures in clinical laboratories. Analysis of plasma or serum ncRNAs could detect tumour biomarkers through PCR at the preoperative stage, indicating their value as early-diagnostic tools. Notwithstanding this, only few reliable diagnostic tests, based on dosage of ncRNAs, have been developed and commercialized to date (*e.g.*, the miRNA-based diagnostic tests of Rosetta Genomics). This slow clinical translatability could be due to different biological and technical factors. Many pre-analytical and analytical factors, including those that are donor/patient-related, can interfere with accurate ncRNA quantification. Due to non homogeneous technical approaches or to real biological heterogeneity of different patient cohorts, expression profiles obtained by various research groups may be different. The real predictive power of these profiles has to be carefully investigated on extended cohorts of patients to understand their actual clinical significance; for instance, data reported on the prognostic value of cellular miR-31 in CRC seem to be very promising, but no univocal results have been obtained to date. Many researchers have compared different protocols for plasma/serum preparation and analyzed the differences between the two biological fluids in terms of amount of circulating miRNAs, but contrasting results have been obtained[277,340,341]. This inconsistency could be explained by the different methods used for the separation of plasma and serum from whole blood (such as different centrifugation protocols): these could lead to different amounts of blood cell contamination[342,343]. However, the effective tumour-specificity of ncRNAs as blood biomarkers remains to be substantiated. Many circulating ncRNAs, whose increased expression had been associated to CRC, could also be related to the presence of other neoplastic diseases. In three independent meta-analysis studies, the diagnostic value of circulating miR-21 in CRC was verified: the authors reported that miR-21 has a moderate potential diagnostic value for CRC[344-346]. However, several papers on different types of carcinoma have also proposed circulating miR-21 as a diagnostic marker of cancer[347-349]. Accordingly, the common upregulation of miR-21 in the blood of patients with different types of cancers makes it a poorly specific diagnostic marker. In fact, Wang *et al*[350] analyzed the value of circulating miR-21 and reached the conclusion that it might be considered a relevant prognostic biomarker in general carcinomas, but not a sensitive specific diagnostic biomarker. The same observations could also be applied to other CRC-related circulating RNA molecules, commonly found in patients with different tumours or other pathological conditions (*e.g.*, miR-17, miR-18a, miR-20a, miR-29a, and miR-92a)[351-354]. Definitively, all these considerations would suggest that ncRNA-based diagnostic approaches have to be greatly improved. CRC screening should be based on a complex panel of different RNA species (both miRNAs and lncRNAs), rather than on a single or few RNAs. Simultaneous dosage of multiple RNAs having complementary discriminatory and prognostic power could represent a powerful predictive tool. This approach could be improved by analyzing the ratio of expression levels among different ncRNAs (as combined biomarkers), rather than evaluating the concentrations of single RNAs[348,355,356]. Finally, the use of ncRNA-based assays will open new and interesting fields in the screening and monitoring of cancers, including CRC. However, many issues must be addressed before these findings can be translated into a clinically useful screening strategy for CRC patients.

CONCLUSION

The literature on ncRNAs in CRC has grown considerably over the last decade, confirming their role in this neoplasia. Based on this huge amount of data, there is no doubt that ncRNAs will be important biomarkers in cancer diagnosis and prognosis, as well as therapeutic targets for the treatment of CRC[357]. However, before ncRNAs are routinely applied to clinical settings, it will be critically important to activate large collaborative efforts to fully realize the clinical potential of this approach. Moreover, a deeper knowledge on ncRNA molecular targets, together with new selective methods for RNA delivery to cancer cells, would lead to great benefits for the treatment and management of CRC. NcRNA biomarkers and RNA-based therapies are very promising tools and approaches to supplement current

diagnostic/prognostic and therapeutic strategies for CRC.

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