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Review
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Review

Exosomal microRNAs and other non-coding RNAs as colorectal cancer biomarkers: a review

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Abstract

The circulating human transcriptome, which includes both coding and non-coding RNA (ncRNA) molecules, represents a rich source of potential biomarkers for colorectal cancer (CRC) that has only recently been explored. In particular, the release of RNA-containing extracellular vesicles (EVs), in a multitude of different *in vitro* cell systems and in a variety of body fluids, has attracted wide interest. The role of RNA species in EVs is still not fully understood, but their capacity to act as a form of distant communication between cells and their higher abundance in association with cancer demonstrated their relevance. In this review, we report the evidence from both *in vitro* and human studies on microRNAs (miRNAs) and other ncRNA profiles analysed in EVs in relation to CRC as diagnostic, prognostic and predictive markers. The studies so far highlighted that, in exosomes, the most studied category of EVs, several miRNAs are able to accurately discriminate CRC cases from controls as well as to describe the progression of the disease and its prognosis. Most of the time, the *in vitro* findings support the miRNA profiles detected in human exosomes. The expression profiles measured in exosomes and other EVs differ and, interestingly, there is a variability of expression also among different subsets of exosomes according to their proteic profile. On the other hand, evidence is still limited for what concerns exosome miRNAs as early diagnostic and predictive markers of treatment. Several other ncRNAs that are carried by exosomes, mostly long ncRNAs and circular RNAs, seem also to be dysregulated in CRC. Besides various technical challenges, such as the standardisation of EVs isolation methods and the optimisation of methodologies to characterise the whole spectrum of RNA molecules in exosomes, further studies are needed in order to elucidate their relevance as CRC markers.

Introduction

Colorectal cancer (CRC) is a worldwide health care problem, being the second and third most common cancer type in females and males, respectively, with over 1.3 million new cases annually (1). In the last years, the application of national screening programs has consistently helped to reduce the incidence rate of this cancer (2). Screening programs are based on endoscopic methods, such as the colonoscopy, which remains the gold standard, despite being invasive and expensive to be performed on a large scale for the national health systems (3). Other tests include non-invasive methods, such as faecal occult blood test (FOBT) and faecal immunochemical occult blood test (FIT), which detect hidden blood in stool (4). Although both these tests are largely applied and are contributing to the detection of CRC in the general population screened, they suffer from low specificity and sensitivity in identifying pre-neoplastic lesions. This is of high relevance because, despite its high prevalence and mortality rate, CRC is one of the few cancers in which early detection, especially of precancerous lesions, may have a strong impact on the life of patients (5).

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Editor's choice

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New molecular CRC biomarkers are constantly searched with particular attention to those potentially detectable in body fluids or easily collectable biospecimens (including plasma, stool, urine and saliva) by non-invasive or minimally invasive approaches. Proteins, metabolites and nucleic acid-based markers, including DNA mutations and methylation, are among the most explored (6–9). Thanks to the extensive application of next-generation sequencing (NGS) approaches, an increasing number of studies also investigate non-coding RNAs (ncRNAs), considering their major involvement in cancer development and progression (10). ncRNAs include a large group of RNA molecules that are not transcribed into proteins but are mainly involved in gene expression regulation and modulation (11). Conventionally, they are classified into two main categories based on their length: small ncRNAs (sncRNAs) and long ncRNAs (lncRNAs) with a length range of 18–200 nucleotides (nt) and >200 nt, respectively.

sncRNAs include several species, such as microRNAs (miRNAs), P-element-induced wimpy testis (PIWI) interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and tRNAs. sncRNAs have emerged as key post-transcriptional regulators of gene expression in many cellular pathways and systems (12). In particular, miRNAs, the most famous and studied category of sncRNAs, are about 22-nt-long molecules that target a large number of mRNAs involved in many physiological and developmental processes, as well as in diseases (13). An altered expression of several miRNAs has been associated with many tumour types, including CRC. For instance, they have been thoroughly studied in the context of CRC initiation and progression, as well as in the cellular pathways involved in carcinogenesis (14). miRNAs can act as oncogenes or tumour suppressors depending on the tumour environment. As an example, miR-21 (more correctly miR-21-5p), considered as an oncogenic miRNA, is frequently described being upregulated in CRC, having an active role in cell proliferation, invasion and migration through targeting genes, such as *PDCD4, SPRY2* and *PTEN* (15–17). On the contrary, miR-143 and miR-145 are considered tumour suppressor miRNAs, modulating the expression of important oncogenes, such as *KRAS, MYC, CD44* and *BRAF*, which promote chronic inflammation and neoplastic progression. Both miRNAs have been found downregulated in CRC (18).

The group of lncRNAs includes intervening/intergenic ncRNAs (lincRNAs), promoter upstream transcripts (PROMPTs), enhancer RNAs and natural antisense transcripts (NATs), located either in the nucleus or in the cytoplasm (19). These recently discovered ncRNAs are involved in several epigenetic processes, such as the chromatin state and post-transcriptional gene regulation (20). Dysregulation of lncRNAs has been largely observed in cancers, including CRC (21,22). The most studied lncRNAs, HOX transcript antisense RNAs (*HOTAIR*), colon cancer-associated transcripts (*CCAT*), metastasis-associated lung adenocarcinoma transcript 1 (*MALAT-1*) and *H19* have been repeatedly associated with CRC development, invasion and metastasis and proposed as potential biomarkers for early cancer diagnosis or prognosis (23).

Circular RNAs (circRNAs) represent a special class of ncRNAs characterised by the bond between the 3' and 5' ends. They have a relevant role in the regulation of gene expression acting as sponges for cytoplasmic miRNAs and RNA-binding proteins such as nuclear transcriptional regulators (24). Dysregulation of circRNAs has also been correlated with CRC (25,26).

The attention on miRNAs and other ncRNAs as biomarkers has exponentially increased since their discovery in the blood of healthy individuals and their subsequent correlation with neurodegenerative, cardiovascular, metabolic diseases and cancer (27–29). This strong interest has been mainly supported by the high stability of circulating miRNAs in body fluids (30) but also by their resistance against unfavourable conditions, such as extreme pH levels and temperature, extended storage time and repeated freeze-thaw cycles (30). miRNA resistance to degradation can be attributed to different reasons beyond their small size. For example, miRNAs are synthetised in the nucleus and then exported to the cytoplasm where the mature miRNA is loaded into an Argonaute (AGO) protein, which protects it from RNase activity (31,32). Circulating miRNAs can also be found in association with high-density lipoproteins and nucleophosmins, which also improve their stability (33,34). Finally, as it has been confirmed in the past few years, miRNAs can be released by cells in membrane-derived extracellular vesicles (EVs) (35) for cell-to-cell communication (36). EVs can be considered as a form of extracellular communication through the transfer of DNA, RNAs, proteins and lipids, regulating numerous processes even at long distances from the cells of origin. EVs have been generally classified into three major categories based on their size: microvesicles, exosomes and apoptotic bodies (37). Exosomes are small phospholipid bilayer vesicles derived from the cell membrane with a diameter range of 30–100 nm. They are released into extracellular fluids, such as blood, urine, cerebrospinal fluid and saliva, by different types of cells, irrespective of their condition (healthy or damaged) and then recycled through endocytosis process (38). Tumour-derived exosomes have been shown to be involved in different processes, including cancer invasion, angiogenesis, chemoresistance, immune evasion and cell death (39). Their role has also been clearly assessed in biological pathways involved in CRC onset and progression (40). Based on the fact that exosomes are released in a larger amount from tumour cells than from non-malignant ones and that these EVs are relatively stable, they have attracted increasing attention as suitable/potential biomarkers in the past few years. Bernard *et al*. observed for the first time in LIM1215 human colon cancer cell line the presence and secretion of full-length cadherin-17, an exosomal surface protein for immunocapture of specific CRC-derived exosomes (41). Other studies have focussed on exosomal DNA, considering its stability and its potential reflection of the mutational status of the tumour. For instance, mutant *KRAS*, which is found in 30–40% of tumours, including CRC, is preferably encapsulated in exosomes compared to its wild-type form (42). However, thanks to the advantageous properties of exosomes and the progress both in their isolation techniques and in the analyses of different ncRNA species, there has been a particular interest in exploring exosomal miRNAs as potential non-invasive cancer biomarkers (Figure 1).

The aim of this work is to present the current knowledge on the expression of exosomal miRNAs in relation to CRC. In particular, we report the main outcomes coming from *in vitro* and human studies and provide evidence for the potential use of exosomal miRNAs as diagnostic and prognostic biomarkers. Despite the very recent interest, in the present review, we have also included the available studies investigating in exosome other ncRNAs than miRNAs in relation to CRC.

The starting point for the preparation of this work was to create a list of abstracts resulting from a query in PubMed using the following keywords: 'microRNAs', 'miRNAs', 'exosomes,' 'non-coding RNAs' and 'colorectal cancer', published between 2014 (corresponding to the date of the first report) and August 2019. All publications in this initial set were manually reviewed to ensure they focussed on exosomal miRNA or ncRNA profiles in CRC. In total,

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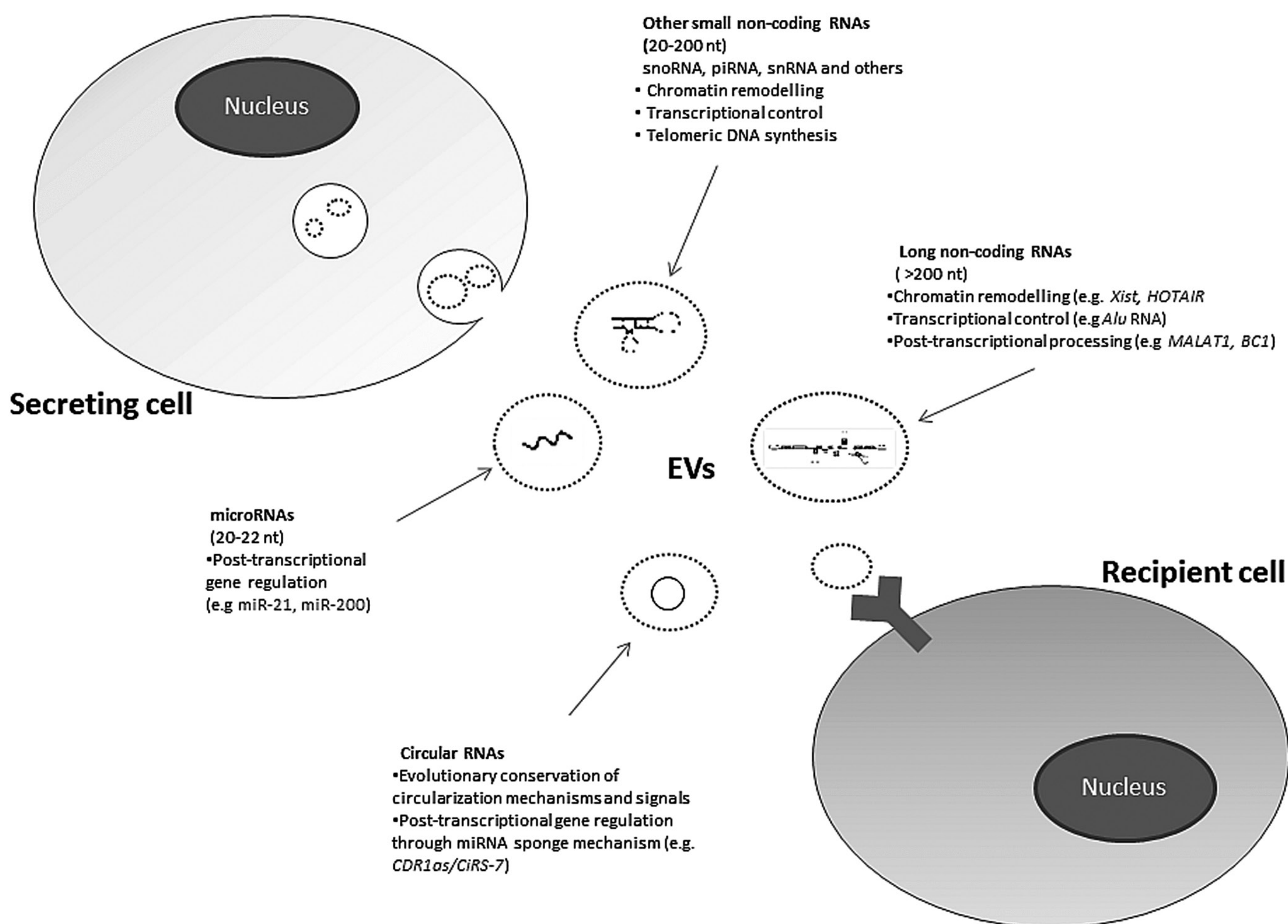

Figure 1. A simplified scheme showing the ncRNA molecules carried in EVs and their exchange between secreting and recipient cells.

40 research papers were deemed to be sufficiently topical, information rich or compelling to be included in this review.

In vitro evidence on exosomal miRNAs and CRC

Many studies investigated miRNA expression levels in exosomes derived from CRC cell lines (43–59), as reported in Table 1. In this respect, several cell lines representing different CRC stages were examined, such as LoVo, HCT-116, HT-29, Caco, SW480 and the metastatic SW620. Most of the works analysed individual miRNA expression levels by quantitative reverse transcription PCR (RT-qPCR), few inquired the whole miRNome by small RNA sequencing, while array analysis was performed in a couple of studies. Some of the studies tested specific miRNAs *in vitro* that were selected in preliminary analyses in human biospecimens. In contrast, others performed an untargeted/unspecific miRNA profiling in cell lines or tested specific miRNAs in relation to functional investigations.

In general, exosomal miRNA profiles obtained from CRC cell lines correlate with those observed in CRC tissue or plasma/serum exosomes. For instance, Ogata-Kawata *et al.* found that levels of exosomal let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223 and miR-23a were higher in several tumour cell lines with different characteristics in comparison with non-malignant cell lines in accordance with their findings in exosomes from serum samples of CRC patients and healthy individuals (59). Zeng *et al.* observed an upregulation of miR-25-3p in SW480, LS174T, SW620, LoVo and HCT-116 when compared to non-malignant NCM460 cell lines, as well as in tumour tissue and serum exosomes of CRC patients (49).

Two studies investigated miRNA expression levels by small RNA sequencing in exosomes derived from SW620 and SW480 cell lines (44,47). Interestingly, in both studies, only a few hundred known miRNAs were found expressed in exosomes derived from these cell lines. Several miRNAs, including the miR-17-92a cluster, were upregulated in exosomes derived from SW620 cells when compared to SW480 cells, while a smaller group downregulated (Table 1). Moreover, a salient finding of the study of Chen *et al.* was that, in exosomes from each cell line, many miRNAs were uniquely expressed with respect to the cell lysate. Specifically, 26 and 18 miRNAs were detectable only in exosomes of SW480 and SW620, respectively, compared to cells of origin. Furthermore, authors found seven miRNAs absent in SW480 cells but detected in SW480-derived exosomes (miR-1224-5p, miR-125b-5p, miR-7641, miR-99a-5p, miR-1266-5p, miR-194-3p and miR-125b-2-3p) to be upregulated in SW620 cells, with miR-125b-5p, miR-7641 and miR-99a-5p also enriched in SW620 exosomes. Moreover, other seven highly expressed miRNAs in SW480 cells but not detected in SW620 cells (miR-6716-3p, miR-26a-1-3p, miR-203b-3p, miR-891a-5p, miR-143-3p, miR-371a-5p and miR-509-3p) were significantly enriched in SW620-derived exosomes. These results demonstrated not only that specific exosomal miRNAs are detectable in different CRC cell lines (from early to metastatic) but also that their inclusion in EVs is precisely regulated and independent from their cellular

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Table 1. Overview of the studies investigating exosomal miRNA expression levels in different CRC cell lines

Reference	Year	Country	CRC cell lines	Cell lines control	Technique	*n* of miRNAs analysed (detected)	Upregulated miRNAs	Downregulated miRNAs
Bjørnetrøm *et al.* [43]	2019	Norway	HCT-116, HCC2998, KM20L2, RKO and LoVo cultured in hypoxia and normoxia conditions		Array 'Discovery phase'	372 (of which 211 were detected and 119 were expressed in all samples)	16 miRNAs in hypoxia compared to normoxia-cultured cells	20 miRNAs in hypoxia compared to normoxia-cultured cells
					RT-qPCR 'Validation phase'	6	miR-194-5p miR-194-5p	miR-30d-5p miR-30e-5p miR-31-5p
Chen *et al.* [44]	2019	Australia	SW480, SW620		Small RNA-seq 'Discovery phase'	Whole miRNome (292 and 315 identified in SW480 and SW620 cells, respectively)	61 miRNAs in SW620 cells compared to SW480 cells	73 miRNAs in SW620 cells compared to SW480 cells
					RT-qPCR 'Validation phase'	11	miR-7641 in SW620-cells compared to SW480 cells	
Hu *et al.* [45]	2019	China	DLD-1, HCT-116, HT-29, LoVo, HUVECs, SW480	NCM460	RT-qPCR	1 (selected from Ogata-Kawata H)	miR-1229 (all CRC cell lines)	
Jin *et al.* [46]	2019	China	HCT-8, HCT-116, SW480 oxaliplatin/5-fluorouracil resistant	FHC	RT-qPCR	30 (selected by Huang)	miR-21-5p, miR-96-5p, miR-135b miR-425, miR-1229-5p and miR-1246 in exosomes of resistant cells vs. control cells	
Fu *et al.* [47]	2018	China	SW480/ SW620	NCM460	Small RNA-seq	All known miRNAs (361 and 306 detected in SW620 and SW480 exosomes, respectively)	miR-17–92 cluster, miR-7, miR-192, miR-200b, miR-375, miR-549 and other 25 miRNAs in SW620 vs. SW480	miR-29a, miR-146, miR-372, miR-3910, miR-5787 and miR-7704 in SW620 compared to SW480
Herrera *et al.* [48]	2018	China	9 patients paired CAF and NOF cells		Small RNA-seq	Whole miRNome	mir-26b, mir-98, mir-126, mir-146b, mir-224, mir-335, mir-379, mir-487a, mir-6087 in CAFs vs. NOFs	
Zeng *et al.* [49]	2018	China	HCT-116, LoVo, LS174T, SW480 and SW620	NCM460	RT-qPCR	1	miR-25-3p (all CRC cell lines)	

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Table 1. Continued

Reference	Year	Country	CRC cell lines	Cell lines control	Technique	*n* of miRNAs analysed (detected)	Upregulated miRNAs	Downregulated miRNAs
Gu *et al.* [50]	2018	China	SW480 and SW480 treated with oxaliplatin		RT-qPCR	10		miR-19b-3p in SW480 treated cell line
Bhome *et al.* [51]	2017	UK	3 patients paired CAF and NOF cells		'Nanostring miRNA assay' RT-qPCR 'Validation phase'	801 6	miR-21, miR-181a, miR-199b, miR-215, miR-329 and miR-382 in CAFs vs. NOFs	
Dai *et al.* [52]	2017	China	HCT-116	CRL1554, 293T cells	RT-qPCR	1	miR-10b	
Yu *et al.* [53]	2017	China	Caco-2, DLD-1, HT-15, HT-29, HCT-116, LoVo, SW48, SW480 and SW620	NCM460	RT-qPCR	1		miR-217 (all CRC cell lines)
Tanaka *et al.* [54]	2017	Japan	SW480 SW620 and SW620/OxR		RT-qPCR	4	Let-7b in SW620/OxR treated with zebularine vs. not treated SW620/OxR cell lines exosomes	
Tanaka *et al.* [55]	2015	Japan	SW480 SW620 and SW620/OxR		RT-qPCR	2	miR-200c, miR-141 in SW620/OxR treated with decitabine vs. not treated SW620/OxR cell lines exosomes	
Senfter *et al.* [56]	2015	Austria	CCL227 and CCL227-RH (5-FU)		RT-qPCR	4		miR-200a miR-200b miR-200c and miR-141 in CCL227-RH exosomes
Ji *et al.* [57]	2014	China	LIM1863		RT-qPCR	Whole miRNome	miR-200c-3p miR-221-3p and miR-320a/b/c/d in exosomes vs. sMVs	
Moshammer *et al.* [58]	2014	Austria	Caco-2, HT-29 and SW480		RT-qPCR		miR-21, miR-200c-3p in exosomes compared to cell lysate	
Ogata-Kawata *et al.* [59]	2014	Japan	HCT-116, HT-29, RKO, SW48, SW480		Array	754	let-7a, miR-21, miR-23a, miR-150, miR-223, miR-1229, miR-1246,	

CAF = cancer fibroblasts; NOF, normal fibroblasts.

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concentration. In particular, two studies originally reported the specific encapsulation of some miRNAs in exosomes (60,61). These findings are also in agreement with those of Moshammer *et al.*, reporting that miR-21 and miR-200c-3p were enriched in exosomes of all the investigated CRC cell lines. On the contrary, other miRNAs, although being part of the miR-200 family, were barely transferred into exosomes, confirming that miRNA encapsulation into exosomes is individually regulated (58).

Ji *et al.* demonstrated that exosome miRNA content and expression profiles are different from those of other types of EVs. They observed that unique detection of six miRNAs (miR-320a/b/c/d, miR-221-3p and miR-200c-3p) discerned exosomes from shed microvesicles (sMVs) released from organoids derived from LIM1863 colon carcinoma cells, while miR-98-5p encapsulation was a typical feature of sMVs (57). Moreover, authors investigated miRNA content in two different types of exosomes characterised by specific protein profiles (A33 and EpCAM exosomes). In fact, in a previous study, they identified by an immunocapture purification method that A33-exosomes contain proteins consistent with a release from the basolateral cell surface while EpCAM exosomes proteins from the apical cell surface (62). In a more recent work, they also observed that EpCAM exosomes contained two exclusively enriched miRNAs, while A33 exosomes 32. Notably, 14 out of these 32 miRNAs were not previously associated with CRC.

In addition to the above-reported evidence, the study by Bjørnetrø *et al.* analysed miRNA expression levels in exosome from HCT-116, HCC2998, KM20L2, RKO and LoVo cell lines cultured under normoxic (21% O_2) and hypoxic (0.2% O_2) conditions. Interestingly, authors observed dysregulated expression levels of 36 miRNAs when comparing hypoxia with normoxia within each of the studied cell lines, with 16 and 20 upregulated and downregulated miRNAs, respectively. Subsequently, they selected six of the dysregulated miRNAs and validated the results for miR-30d-5p, miR-30e-5p, miR-31-5p and miR-194-5p. In this respect, tumour hypoxia is involved in poor treatment outcome in locally advanced rectal cancer and circulating EVs have not been fully explored as potential biomarkers of tumour hypoxia and adverse prognosis (43).

miRNA expression levels in exosomes have also been studied in relation to CRC chemoresistance in *in vitro* experiments. Two studies by the same group (54,55) investigated the effects of two DNA methyltransferase inhibitors, decitabine and zebularine, in SW620 cell lines with acquired resistance to oxaliplatin (SW620/OxR) by profiling released exosomal miRNAs and evaluating tumour progression. Authors found that miR-200c and miR-141 were upregulated by decitabine and let-7b by zebularine and they stated that these miRNAs may serve as useful indicators of mesenchymal-epithelial transition of CRC cells. In agreement with these findings, Senfter *et al.* found that miRNAs of the miR-200 family were upregulated in exosomes of naïve CCL227, while they were dramatically downregulated in exosomes released by CCL227 high-resistant cells (56).

Jin *et al.* (46) selected 30 miRNAs based on their previous microarray analysis and assessed their expression levels in three oxaliplatin/5-fluorouracil-resistant cell lines, HCT116, SW480 and HCT-8 CRC, and in the corresponding secreted exosomes. They observed upregulated miR-21-5p, miR-1246, miR-1229-5p, miR-135b, miR-425 and miR-96-5p levels in exosomes from culture media of resistant cells compared to those of their parent cells. Interestingly, higher expression levels of miR-21-5p, miR-1246, miR-1229-5p and miR-96-5p were also observed in serum exosomes of oxaliplatin/5-fluorouracil chemoresistant compared to chemosensitive patients. Another study on the field is the one of Gu *et al.* in which authors observed that suppressing the secretion of exosomal miR-19b by exosomal inhibitor GW4869 promotes oxaliplatin sensitivity of SW480 cells (50). All these studies suggest a promising application for exosomal miRNAs to monitor oxaliplatin-resistant patients. Few studies on the field are also reported in the chapter below.

Exosomal miRNAs and CRC in human studies

Several works carried out miRNA profiling in exosomes isolated from human biospecimens in relation to CRC diagnosis, progression and prognosis (43,45-47,49,59,63-79). In the following sections, a brief overview of each of these aspects is presented and Table 2 summarises all the retrieved studies. miRNA expression levels in exosomes were mainly assessed in serum and plasma samples and, in some instances, they were compared with those measured in the primary tissues of the same CRC patients. In few cases, a comparison between exosomal and free-circulating miRNAs has also been provided. Human studies have been conducted on cohorts of patients and healthy controls of variable size, so far: 12 included less than 100 subjects, while 9 described more. From a methodological point of view, most of the studies performed exosome isolation by precipitation, typically using the Exoquick kit, which has been reported as an efficient and reproducible method (80). A smaller number of studies employed ultracentrifugation (UC) as an alternative methodology for exosome separation. However, only small volumes of biological samples are usually available and they are often qualitatively heterogeneous. miRNA expression level analyses were mainly performed by arrays and RT-qPCR. For most of the investigated works, initially, a discovery phase by array experiments was performed and, then, a subset of miRNAs was validated by RT-qPCR. Importantly, in the majority of cases, only a subset of miRNAs were detected in exosomes in agreement with *in vitro* studies, proving that only specifically selected miRNAs are encapsulated in exosomes.

Exosomal miRNAs as diagnostic biomarkers

Research of biomarkers for the early detection/screening of CRC is still a field that attracts a high interest (81). In this respect, many studies have focussed on the role of miRNAs and their potential as accurate markers (82–84). Most of them have investigated circulating miRNA levels in plasma/serum or other biospecimens. In recent years, an increasing number of studies have also examined miRNAs in exosomes or other EVs. Among the first works on this field, Ogata-Kawata *et al.* compared miRNA expression levels in exosomes derived from serum of 88 CRC patients with those of 11 healthy individuals and confronted the outcomes with those from CRC cell lines as previously discussed. They found and validated by RT-qPCR that miR-1229, miR-1246, miR-223, let-7a, miR-150, and miR-21 levels were significantly higher in CRC patients compared to controls. Interestingly, after surgical tumour resection, the expression levels of these miRNAs decreased to values comparable to those observed in controls (59). Other similar works have been published since this study. Matsumara *et al.* by comparing miRNA expression levels in serum exosomes of multi-stage CRC patients versus healthy controls noticed that miR-19a and miR-92a were significantly upregulated in both early and advanced tumour stages (79). Similarly, the latter miRNA together with miR-17-5p were found significantly upregulated by Fu *et al.*, who tested miRNA expression levels in exosomes from serum in a case-control study (47). Authors also evaluated the accuracy of miR-92a-3p and miR-17-5p to classify CRC patients from healthy subjects and reported an area under the curve (AUC) of 0.845 and 0.897, respectively. Ren *et al.* studied miR-196b-5p expression in CRC cells and in tumour tissues, observing an overexpression of this miRNA in

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Table 2. Overview of the human studies investigating exosomal miRNA expression levels in serum/plasma samples in relation to CRC (highlighted in bold)

Reference	Year	Country	*n* of cases and specimens	*n* of healthy controls and specimens	Technique	*n* of miRNAs analysed in exosomes	Main outcome		
							Cases vs. controls	Prognosis	Sensitivity/ specificity for CRC
Meltzer *et al.* [63]	2019	Norway	29 locally advanced rectal cancer **plasma exosomes** 'Discovery phase'		Array 'Discovery phase'	372		↑ miR-141-3p miR-375 in patients with synchronous liver metastasis	
			64 locally advanced rectal cancer **plasma exosomes** 'Validation phase'		RT-qPCR 'Validation phase'	6			
Yagi *et al.* [64]	2019	Japan	6 (3 stable and 3 progressive) CRC **plasma exosomes**	3 **plasma exosomes**	Array 'Discovery phase'	2578	↑ miR-125b	↑miR-125b in CRC progressive patients after mFOLFOX6 treatment	
			55 advanced/ recurrent CRC **plasma exosomes**	30 **plasma exosomes**	RT-qPCR 'Validation phase'	1			
Hu *et al.* [45]	2019	China	97 CRC **serum exosomes**	30 **serum exosomes**	RT-qPCR	1 (selected from [53])	↑ miR-1229	↑ miR-1229 associated with tumour size, lymphatic metastasis and TMN stage as well as poorer overall survival	
Bjørnetrø *et al.* [43]	2019	Norway	24 locally advanced rectal cancer **plasma exosomes**	–	RT-qPCR	372 (previously analysed *in vitro*) 138 detected		↑ miR-30d-5p with metastatic progression ↓ miR-486-5p, miR-181a-5p associated with organ-invasive primary tumour and lymph node metastases, respectively.	
Jin *et al.* [46]	2019	China	43 CRC Stages III and IV (of which 25 and 18 chemoresistant and chemosensitive, respectively) **serum exosomes**		RT-qPCR	6		↑ miR-21-5p, miR-96-5p, miR-1229-5p, miR-1246 in chemoresistent vs. chemosensitive	
Liu *et al.* [65]	2018	China	40 CRC Stage I **plasma exosomes,** 'Training set'	40 **plasma exosomes** 'Training set'	RT-qPCR 'Discovery', 'Validation' and 'External validation phase'	2	↑ miR-27a, miR-130a	↑ miR-27a or miR-130a associated with poorer prognosis	Combining both the two miRNA (AUCs = 0.898 and 0.801 for the discovery and validation phase, respectively)
			40 CRC Stage I, 20 CRC Stage II, 14 CRC Stage III, 6 CRC Stage IV, **plasma exosomes** 'Validation phase'	40 **plasma exosomes** 'Validation phase'					
			50 CRC Stage I, 50 adenomas **plasma exosomes** 'External validation phase'	50 **plasma exosomes** 'External validation phase'					

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Table 2. Continued

Reference	Year	Country	*n* of cases and specimens	*n* of healthy controls and specimens	Technique	*n* of miRNAs analysed in exosomes	Main outcome		
							Cases vs. controls	Prognosis	Sensitivity/ specificity for CRC
Zeng *et al.* [49]	2018	China	57 CRC pairs of tumours and matched non-malignant tissues 'Discovery phase'	–	Array 'Discovery phase'	All mature miRNAs in mirBase 15 1	↑ miR-25-3p, miR-92a, miR-92b, miR-221, miR-371-5p, miR-1246 (tissues)		
			75 CRC **serum exosomes** 'Validation phase'	12 **serum exosomes** 'Validation phase'	RT-qPCR 'Validation phase'		↑ miR-25-3p in CRC compared to controls and in metastatic vs. non-metastatic patients (serum exosmes)		
Kral *et al.* [66]	2018	Czech Republic	20 pairs of rectal tumours and matched non-malignant tissues 'Discovery phase' 100 paired rectal tissues, whole plasma	31 whole plasma and **plasma exosomes**	Array 'Discovery phase'	2555	↑ miR-17, miR-18a, miR-106a ↓ miR-18b, miR-19a, miR-19b, miR-20a, miR-20b	↑ miR-17/92 cluster correlated with treatment response.	
			102 paired colon tumours and non-tumour tissues 100 rectal tissue **plasma exosomes** 'Validation phase'		RT-qPCR 'Validation phase'	13			
Fu *et al.* [47]	2018	China	29 CRC (11 metastatic) **serum exosomes**	10 **serum exosomes**	RT-qPCR	11	↑ miR-17-5p, miR-92a-3p		miR-17-5p AUC = 0.897 for CRC, AUC = 0.841 for metastasis. miR-92a-3p AUC = 0.845 for CRC, AUC = 0.854 for metastasis
Santasusagna *et al.* [67]	2018	Spain	50 CRC patients (Stages I–III) paired MV and PV blood Among them, 33 (25 metastasis-free and eight with liver metastases) **plasma exosomes**		RT-qPCR	5	↑ miR-141, miR-200a, miR-200b, miR-200c, miR-429 in MV compared to PV (whole plasma)	↓ miR-200c, miR-141 (plasma exosomes) with longer survival ↑ miR-200c, miR-141 in MV plasma with poor prognosis	

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Table 2. Continued

Reference	Year	Country	*n* of cases and specimens	*n* of healthy controls and specimens	Technique	*n* of miRNAs analysed in exosomes	Main outcome		
							Cases vs. controls	Prognosis	Sensitivity/ specificity for CRC
Yan *et al.* [68]	2018	China	3 primary CRC **serum exosomes** 77 CRC **serum exosomes**	3 **serum exosomes** 20 **serum exosomes**	Array 'Discovery phase' RT-qPCR 'Validation phase'	754 39	↑ miR-486-5p, miR-3180-5p ↓miR-548c-5p, miR-638, miR-5787, miR-6869-5p, miR-8075	↓ miR-638 associated with increased risk of liver metastasis and later TNM stage	
Takano *et al.* [69]	2017	Japan	240 CRC **serum exosomes**		RT-qPCR	2	↑ miR-203 with tumour progression	↑ miR-203 with poor prognosis	
Tsukamoto *et al.* [70]	2017	Japan	3 CRC plasma exosomes and paired primary tumour and liver metastasis tissues 'Discovery phase'	3 **plasma exosomes** 'Discovery phase'	Array 'Discovery phase'	2578	↑ miR-21, miR-23a, miR-224, miR-92a, miR-155 (plasma exosome, pri-mary and liver metastasis tumour tissues)	↑ miR-21 with poor prognosis	
			326 CRC **plasma exosomes** 'Validation phase'		RT-qPCR 'Validation phase'	1	↑ miR-21 (plasma exosomes)		
Teng *et al.* [71]	2017	China/ USA	25 CRC paired tumour and non-malignant tissues **plasma exosomes** 15 CRC with liver metastasis **plasma exosomes**	Not specified	RT-qPCR	4 (selected after mouse colon can-cer investigation)	↑ miR-126, miR-148a, miR-193a, ↓ miR-196b (plasma exosomes) ↓ miR-193a, miR-126 (tissue)	↑ miR-126, miR-148a, miR-193a, ↓ miR-196b (plasma exosomes) in metastatic patients	
Yan *et al.* [72]	2017	China	168 CRC (Stages I–IV) **serum exosomes**	20 **serum exosomes**	RT-qPCR	1	↑ miR-6803-5p	↑ miR-6803-5p (exosome) associated with poorer prognosis	AUC = 0.7399 for CRC
Wang *et al.* [73]	2017	China	50 CRC (Stages I and II) **plasma exosomes**	50 **plasma exosomes**	RT-qPCR	9	↑ miR-125a-3p, miR-320c		miR-125a-3p AUC = 0.685 Combination of miR-125a-3p and CEA AUC = 0. 855
Ren *et al.* [74]	2017	China	150 CRC **serum exosomes** 20 paired CRC and non-malignant tissues 90 CRC tissues	90 **serum exosomes**	RT-qPCR	1	↑ miR-196b-5p (serum exosomes and tissues)		AUC = 0.88 for CRC

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Table 2. Continued

Reference	Year	Country	*n* of cases and specimens	*n* of healthy controls and specimens	Technique	*n* of miRNAs analysed in exosomes	Main outcome: Cases vs. controls	Main outcome: Prognosis	Main outcome: Sensitivity/ specificity for CRC
Monzo *et al.* [75]	2017	Spain	50 CRC patients (Stages I–III) paired MV and PV blood Among them, 33 (25 metastasis free and eight with liver metastases) **plasma exosomes**		Array RT-qPCR	754 4	↑ let-7g, miR-15b, miR-155, miR-328 in MV compared to PV	↑ same miRNAs in MV related to poor prognosis, ↑ in PV related to good prognosis. ↑ let-7g in MV associated with pre-existing polyps, ↑ miR-328 in MV with K-ras mutations	
Peng *et al.* [76]	2017	China	108 CRC (25 Stage I, 21 Stage II, 43 Stage III, 19 Stage IV) **serum exosomes**		RT-qPCR	1 (selected from their previous work)	↓ miR-548c-5p	↑ in CRC patients with liver metastasis and III/IV tumour stages	
Li *et al.* [77]	2016	China	102 CRC (I and II stages) tissues (89 paired tumour and non-malignant tissues) whole plasma and **plasma exosomes** (before and after surgical treatment)	80 whole plasma and **plasma exosomes**	RT-qPCR	3	↑ miR-182-5p ↓ miR-96-5p, miR-149	↓ miR-96-5p, miR-149, (exosomes) after surgery	
Uratani *et al.* [78]	2016	Japan	19 CRC and 27 adenomas tissues 'Discovery phase' 26 adenoma whole serum and **serum exosomes** 'Validation phase'	20 normal mucosa 'Discovery phase' 47 whole serum **and serum exosomes** 'Validation phase'	RT-qPCR	4	↑ miR-21, miR-29a, miR-92a, miR-135b (tissues) ↑ miR-21, miR-29a, miR-92a, (serum) ↑ miR-21, miR-29a (exosomes)	↑ miR-21 (exosomes) correlated with adenoma size and total adenoma number and discriminate patients with high-risk adenomas	
Matsumura *et al.* [79]	2015	Japan	2 CRC (repeated pre-/post-operative) **serum exosome** 'Discovery phase' 221 CRC **serum exosomes** 'Validation phase'	– 28 **serum exosomes** 'Validation phase'	Array 'Discovery phase' RT-qPCR 'Validation phase'	417 6	↑ miR-17-92a cluster (tissues) ↑ miR-19a, miR-92a (serum)	↑ miR-19a (exosomes) with poorer overall survival	
Ogata-Kawata *et al.* [59]	2014	Japan	88 CRC **serum exosomes** 'Discovery phase' 13 CRC **serum exosomes** 'Validation phase'	11 **serum exosomes** 'Discovery phase' 8 **serum exosomes** 'Validation phase'	Array 'Discovery phase' RT-qPCR 'Validation phase'	754 8	↑ let-7a, miR-21, miR-23a, miR-150, miR-223, miR-1229, miR-1246	↓ same miRNAs after surgical resection	

Other investigated biospecimens are eventually included. In bold are highlighted the samples from which exosomes were isolated and miRNA analysed.
CEA, Carcinoembryonic antigen marker; MV, mesenteric vein; PV, peripheral vein; TNM, tumour, node, metastasis.

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exosomes isolated from serum of CRC patients compared to those of controls (74). Interestingly, they reported a high AUC value (0.88) for this miRNA to discriminate CRC cases from controls. Other relevant evidence comes from the works of Yan *et al*. In their first study on this topic, authors found miR-638, miR-5787, miR-8075, miR-6869-5p and miR-548c-5p downregulated, while miR-486-5p and miR-3180-5p were upregulated in serum exosomes of CRC subjects compared to controls (72). In their following work, they also found miR-6803-5p upregulated in serum exosomes of CRC subjects in comparison with controls, reporting an AUC value of 0.74 for CRC (68).

An interesting finding coming from such studies is the fact that serum exosomal miRNAs are not only able to discriminate CRC patients from healthy controls but they can also distinguish metastatic patients from those without. In fact, exosomal miR-1229 and miR-25-3p were more expressed in metastatic patients compared to non-metastatic ones and so were miR-17-5p and miR-92a-3p, with high AUC value of 0.841 and 0.854 for metastatic discrimination, respectively (45,47). Likewise, miR-548c-5p and miR-638 showed lower expression levels in CRC metastatic patients compared to non-metastatic ones (72,76).

Despite substantial interest in studying miRNA profiles in exosomes in relation to the early detection of CRC, these have not been thoroughly investigated in subjects with precancerous lesions. To the best of our knowledge, only Uratani *et al.* studied four serum exosomal miRNAs in both CRC and advanced adenoma patients (78). They reported that the levels of miR-29a and miR-21 were increased in exosomes of subjects with adenomas compared to healthy individuals and, in particular, exosomal miR-21 reached an AUC value of 0.77 with a sensitivity of 69.8% and a specificity of 80.0% to discriminate adenoma patients from healthy controls. miR-21 expression levels also correlated with size and counts of polyps. However, no data about the discrimination power of miRNAs between adenomatous polyps and healthy subjects were provided.

In parallel to serum samples, miRNAs expression levels were also investigated in plasma-derived exosomes in several studies in humans. In the work of Xiangxiang *et al.*, miR-27a and miR-130a, which were previously selected from Gene Expression Omnibus (GEO) database repository and The Cancer Genome Atlas (TCGA) data sets, were upregulated in CRC and their combination reached AUCs of 0.85, 0.90 and 0.80 for the training, validation and external validation phases, respectively (65). Similarly, in the work of Wang *et al.*, miR-125a-3p and miR-320c showed increased expression levels in plasma exosomes of CRC compared to healthy subjects (73). For miR-125a-3p, authors obtained AUC values of 68.5%, which increased to 85.5% when this miRNA was combined with levels of carcino-embryonic antigen (CEA), a known serological tumour marker. In the study of Li *et al.* (77), investigating CRC patients at stage I or II, researchers found three miRNAs dysregulated in cancer compared to healthy subjects. They also noted that the decreased expression of miR-149 and miR-96-5p in plasma exosomes of CRC patients significantly increased after surgery, becoming comparable to those of healthy subjects. Of note, as previously reported by (59) in serum, also for miRNAs in plasma exosomes, it was possible to record in CRC patients after surgery/treatment a return to expression levels similar to those of healthy subjects.

miRNA profiles in plasma exosomes in relation to CRC metastasis have also been assessed. Monzo *et al.* explored miRNAs in plasma exosomes isolated from mesenteric vein (MV) and peripheral vein (PV) blood. Authors assumed that blood of the tumour-draining vein could contain all the molecules released by the tumour before they reach a potential metastatic site and, overall, provide more general information than blood from PV blood. They found that miR-328, let-7g, miR-15b and miR-155 were upregulated in MV compared to PV. In patients with liver metastasis, miR-328 was particularly overexpressed in MV compared to PV, indicating an active role in the spreading of the malignancy (75). Teng *et al.* also observed an increase in the levels of miR-193a, miR-126 and miR-148a in metastatic patients compared to non-metastatic ones while miR-196b was downregulated (71). Recently, Bjørnetrø *et al.* reported that miR-30d-5p was upregulated in metastatic patients while miR-486-5p and miR-181a-5p were downregulated (43). In addition, authors observed the upregulation of miR-141-3p and miR-375 in subjects with synchronous liver metastasis from the same cohort of patients (63).

Exosomal miRNAs as prognostic biomarkers

Exosomal miRNA profiles may also provide useful insights in CRC monitoring and surveillance. Seven of the works selected for the preparation of the present manuscript also assessed the prognostic value of exosomal miRNAs, most of which have already been proved to be useful in other biospecimens, such as tissues or whole plasma/serum. In fact, Liu *et al.* found that upregulation of both miR-27a and miR-130a in plasma exosomes was also associated with poor prognosis (Table 2) and these results were consistent with previous findings on overall survival analysing the same miRNAs in tissue samples from CRC patients (65,85,86). Santasusagna *et al.* investigated miR-200c and miR-141 in MV and PV plasma exosomes from CRC patients. They reported that miR-141 upregulation in MV plasma exosomes was associated with poor overall survival. The effect was even more pronounced when this miRNA was studied in combination with miR-200c (67). Also, in this case, the results were in line with those coming from CRC whole plasma examination (87). Other miRNAs associated with survival and previously studied in CRC tissues and biospecimens other than exosomes include miR-203, miR-19a and the ubiquitous miR-21 (88). Interestingly, when investigated in exosomes, even these miRNAs mirrored their circulating counterparts. In the study of Takano *et al.*, authors noted the upregulation of serum exosome miR-203 in relation to CRC progression and poor prognosis (69). Similar findings come from the work by Matsumura *et al.*, which described exosomal miR-17–92 cluster upregulation in CRC serum and also noticed that CRC patients with high exosomal miR-19a expression showed poorer prognosis compared to the low expression group (79). Tsukamoto *et al.* found miR-21 upregulation in CRC plasma exosomes as well as in primary tumour tissues and liver metastasis, with high levels of this miRNA associated with poor overall survival (70).

Two newly discovered serum exosomal miRNAs, miR-1229 and miR-6803-5p, have also been shown to have a prognostic value. The former miRNA was upregulated in CRC patients and associated with tumour size, stage, the presence of lymphatic metastasis and overall survival (72). Similarly, the latter was increased in CRC and related to overall and disease-free survival of patients (45).

Exosomal miRNA profiles may also represent a useful marker for monitoring treatment response, although there is scanty information. In this context, Kral *et al.* analysed the expression levels of the miR-17–92 cluster in whole plasma and plasma exosomes of 100 rectal cancer patients at the moment of the diagnosis at the termination of the adjuvant therapy and 1 year after diagnosis. The authors found that, in exosomes derived from plasma of CRC patients, but not in the whole plasma, miRNA levels differed from those of controls in the presence of the tumour, diminishing 1 year after diagnosis with the resolution of the malignancy. These results showed

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that exosomal miRNA expression levels can reflect disease stage and could function as prognostic factors of treatment response (66). Yagi *et al.* investigated the potentiality of plasma exosomal miR-125b as biomarker for the detection of resistance to 5-fluorouracil, leucovorin and oxaliplatin (mFOLFOX6) based chemotherapy. After the treatment, they observed significantly higher levels of this miRNA in patients with progressive disease, while no differences in expression levels were observed in subjects with stable disease before and after chemotherapy. Interestingly, authors observed that the differences of expression for this miRNA were significant even 1 month after the chemotherapy initiation, thus purposing this miRNA as potential biomarker for early detection of chemotherapy response (64). Finally, the study of Jin *et al.*, previously discussed for testing exosomal miRNA expression in chemoresistant cell lines, also investigated same miRNAs in patients. Interestingly, higher expression levels of miR-21-5p, miR-1246, miR-1229-5p and miR-96-5p were also observed in serum exosomes of chemoresistant compared to chemosensitive oxaliplatin/5-fluorouracil patients (46).

Other ncRNAs in exosomes: a possible role as CRC biomarkers

Several other RNA species, besides miRNAs, are known to be encapsulated in exosomes and for this reason might serve as cancer biomarkers (89). Huang *et al.* were the first to investigate human plasma-derived exosome RNA species by deep sequencing. They found that despite miRNAs being the most representative RNA type (76.0% of all mapped reads), significant fractions of other RNA species could be detected, such as ribosomal RNAs (rRNAs) (9.16%), lncRNAs (3.36%), piRNAs (1.31%), tRNAs (1.24%), snRNAs (0.18%) and snoRNAs (0.01%), fragments of coding sequences (1.36%) and 80 untranslated regions (0.75%) (90). Similarly, Miranda *et al.* investigated human urinary exosome/microvesicle RNA content by NGS and observed a substantial proportion of reads (87%) aligned to rRNAs. They also found that 60% of non-ribosomal RNA sequences aligned to ncRNAs and repeated sequences, including LINE, SINE, satellite repeats and RNA repeats [tRNAs, snRNAs, small conditional RNAs (scRNAs) and signal recognition particle (srpRNAs)] (91). Both evidence demonstrated that a wide variety of RNA species can be found in exosomes justifying the increased interest of the last years to study them in EVs in relation to cancers and diseases.

Twelve studies (48,53,92-101) have focussed on profiling ncRNAs in exosomes so far, mainly lncRNAs and circRNAs, in relation to CRC, as shown in Table 3. In general, ncRNA expression levels were measured by RT-qPCR or NGS, mostly in serum samples. In some of the retrieved studies, a comparison with RNA tissue profiles was also accomplished (93,94,100). Furthermore, five of the studies were performed *in vitro* (or both *in vitro* and *in vivo*) exploring exosome ncRNA content in CRC cell lines. Among them, three papers based their analyses on cancer-associated fibroblasts (CAFs) and non-malignant mucosa derived fibroblasts (NFs), while others tested different CRC cell lines, including HT-29, SW480, HCT-116, SW620, LoVo, SW48, DLD-1, Caco2 and HT-15.

It is noteworthy that several *in vitro* experiments found different ncRNA content in exosomal fraction compared to cell lysates, confirming the hypothesis/assumption that ncRNAs are selectively sorted in EVs. In fact, according to Herrera *et al.*, the cellular fraction of CAFs was significantly enriched in snoRNAs relative to its exosomal fraction, which in turn was rich in YRNAs. Moreover, 52 ncRNAs were differentially expressed between exosomes isolated from CAFs and NFs cells. Among these dysregulated ncRNAs, authors found two upregulated lncRNAs (*LINC00326* and *WEE2-AS1*), as well as 8 and 42 sncRNAs (mainly piRNAs, miRNAs and snRNAs) downregulated and upregulated, respectively (48). Chen *et al.* investigated the ncRNA content in EVs released by CRC cell lines finding that 2389 mRNAs, 317 pseudogene transcripts, 1028 lncRNAs and 206 sncRNAs were selectively distributed to LIM1863 EVs, with respect to their cells of origin (98). Similarly, Ren *et al.* detected an increased lncRNA *H19* expression in CAFs compared to NFs cells and their derived exosomes (96). Analyzing the same cell types, Deng *et al.* observed increased exosomal *CCAL* levels in CAFs compared to NFs (92). Yu *et al.* explored *CRNDE-p* expression levels in exosomes from different CRC cell lines (HT-29, SW480, HCT-116, SW620, LoVo, SW48, DLD-1, Caco2 and HT-15). Authors noted an upregulation of this lncRNA in exosomes of all CRC cell lines compared to the non-malignant NCM460 (53). Moreover, the expression of a splicing variant of this lncRNA, *CRNDE-h*, was also studied by Liu *et al.* who observed its upregulation in exosomes from HCT116, SW620, SW480, HT29 and LoVo cells compared to normal human intestinal epithelial cell line (FHC). Finally, they discovered that after 3 days of incubation, exosomal *CRNDE-h* could enter into the cells at a detectable level and the expression level steadily increased over time in all the investigated CRC cell lines compared to the FHC cell line (100). Overall, even if authors did not investigate *CRNDE-h* expression levels in cell exosomes, their results together with those of Yu *et al.* suggest an active role of this lncRNA in CRC formation and development as well as its potential diagnostic use.

The exosomal ncRNA dysregulation in CRC and its potential diagnostic and prognostic application has been studied in humans as well (Table 3). For this purpose, Li *et al.* saw for the first time enrichment of circRNAs in exosomes compared to the cells releasing them. Authors found that 67 circRNAs were missing and 257 new circRNA species were present in cancer patients. In addition, circRNA *KLDHC10* was upregulated in CRC serum exosomes compared to controls (101). Similarly, Dong *et al.* performing a differential expression analysis on TCGA RNA-seq data from CRC tissues vs. non-malignant adjacent mucosa selected 79 lncRNAs to be measured in serum exosomes of a case-control study. They found 24 of them downregulated in exosomes of cancer subjects compared to healthy control (99). Barbagallo *et al.* measured 17 lncRNAs and other 31 circRNAs in the same CRC biospecimens and validated the downregulation of one lncRNA, *UCA1*, and the upregulation of other two lncRNAs, *circHIPK3* and *TUG1*. Receiver operating curve analyses provided an AUC of 0.814 for the combination of *circHIPK3* and *UCA1* with 93% sensitivity and 64% specificity and an AUC of 0.9 with 100% sensitivity and 70% specificity combining *TUG1* and *UCA1* (94). The potentiality of *UCA1* in exosomes has also been assessed in relation to cetuximab resistance both *in vitro* and *in vivo*. Yang *et al.* observed higher *UCA1* expression levels in exosomes of Caco-2 cetuximab-resistant cells compared to the sensitive ones and in exosomes of the two cell lines compared to their parental cells. In agreement with these results, they also observed higher levels of *UCA1* in patients who did not respond to the treatment than in those responding, suggesting a potential role of this lncRNA in the prediction of resistance to cetuximab therapy (97).

The studies of Yu and Liu, previously cited in the *in vitro* experiments, also assessed *CRNDE-p* and *CRNDE-h* expression levels, respectively, as diagnostic/prognostic markers. They both observed an upregulation of these lncRNA splicing variants in exosomes of CRC patients in comparison with healthy controls. In addition, higher levels were associated with advanced cancer stages (T3 and T4), lymph node metastasis (53) and with reduced overall survival (100).

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Table 3. Overview of the studies investigating other ncRNAs than miRNAs in exosomes in relation to CRC

Reference	Year	Country	*n* of cases and specimens	*n* of healthy controls and specimens	Technique	ncRNAs investigated	Main outcome		
							Case control	Prognosis	Sensitivity/ specificity for CRC
Deng *et al.* [92]	2019	China	15 paired CAFs and NFs and **cell-derived exosomes**		RT-qPCR	1 lncRNA	↑ *CCAL*		
Wang *et al.* [93]	2019	China	75 CRC paired tumour and adjacent non-malignant tissues 'Discovery phase' 10 paired pre-operative and post-operative serum samples Independent cohort CRC **serum exosomes**	Not specified **serum exosomes**	RT-qPCR	1 lncRNA	↑ *CCAT2* (CRC tissues, whole serum and serum exosomes)	↓ *CCAT2* after surgical resection (whole serum) ↑ *CCAT2* in lymph node metastasis (tissue)	
Barbagallo *et al.* [94]	2018	Italy	20 CRC pairs of tumours and matched non-malignant tissues 20 CRC **serum exosomes**	20 **serum exosomes**	RT-qPCR	17 lncRNAs 31 circRNAs	↑ *CCAT1, CCAT2, HOTAIR,* and *UCA1* ↓ *CDR1AS, MALAT1, TUG1* (tissue) ↓ *UCA1* ↑ *circHIPK3, TUG1* (serum exosomes)		Combining *TUG1-UCA1* AUC = 0.81 with 93% sensitivity and 64% specificity Combining *circHIPK3:UCA1* AUC = 0.90 with 100% sensitivity and 70% specificity
Herrera *et al.* [48]	2018	China	9 paired CAFs and NFs cells and **cell-derived exosomes**		Small RNA-seq	All detectable ncRNAs	↑ *LINC00326, WEE2-AS1* (as lncRNAs) ↓ 8 sncRNAs ↑ 42 sncRNAs among these: 7 piRNAs, 9 miRNAs (see Table 1) and 26 snRNAs		
Hu *et al.* [95]	2018	China	10 CRC **plasma exosomes** 'Discovery phase' 50 CRC **plasma exosomes** 'Validation phase'	10 **plasma exosomes** 'Discovery phase' 50 **plasma exosomes** 'Validation phase'	Microarray 'Discovery phase' RT-qPCR 'Validation phase'	lncRNAs spotted on the array (number not specified) 6 selected lncRNAs	↑ *LNCV6_116109, LNCV6_98390, LNCV6_38772, LNCV6_108266, LNCV6_84003, LNCV6_98602*		*LNCV6_116109* AUC = 0.77, *LNCV6_98390* AUC = 0.75, *LNCV6_38772* AUC = 0.65, *LNCV_108266* AUC = 0.69, *LNCV6_84003* AUC = 0.75, *LNCV6_98602* AUC = 0.72
Ren *et al.* [96]	2018	China	10 paired CAFs and NFs and **cell-derived exosomes**		RT-qPCR	1 lncRNA	↑ *H19* (CAFs and CAFs derived exosomes)		
Yang *et al.* [97]	2018	China	Caco2 cetuximab-resistant (CR) and sensitive (CS) **cell-derived exosomes** 30 CRC responding to cetuximab therapy and 23 sensitive **serum exosomes**		RT-qPCR	1 lncRNA	↑ *UCA1* (exosomes from resistant cells and patients serum)		

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Table 3. Continued

Reference	Year	Country	*n* of cases and specimens	*n* of healthy controls and specimens	Technique	ncRNAs investigated	Main outcome		
							Case control	Prognosis	Sensitivity/ specificity for CRC
Yu *et al.* [53]	2017	China	Caco2,DLD-1, HT-15, HT-29, HCT-116, LoVo, SW480, SW620, SW48 **exosomes**	NCM460 **exosomes**	RT-qPCR	1 lncRNA	↑ *CRNDE-p* (cell exosomes and serum exosomes)	↑ *CRNDE-p* with advanced T-stages (T3 and T4), lymph node metastasis and clinical stages (III and IV)	*CRNDE-p* AUC = 0.85 Combining *CRNDE-p* and miR-217 AUC = 0.93
			411 CRC, 58 adenoma **serum exosomes**	175 **serum exosomes**					
Chen *et al.* [98]	2016	Japan	LIM1863 CRC cell lines		RNA-seq 'Discovery phase'	lncRNAs, sncRNAs	↑ 1028 lncRNAs, 206 sncRNAs, in EVs relative to cells (1717 sMVs, 2543 A33-Exos and 2565 EpCAM-Exos transcripts)		
Dong *et al.* [99]	2016	China	8 CRC **serum exosomes**	8 **serum exosomes**	RT-qPCR	79 RNAs (mRNAs and lncRNAs selected from TCGA)	↑ 20 mRNAs and 24 lncRNAs in exosome vs. ABs and MVs		*KRTAP5-4, BCAR4 MAGEA3* AUC = 0.936 (exosomal), AUC = 0.857 (whole serum)
			30 CRC **serum exosomes** 'Training set'	30 **serum exosomes** 'Training set'			16 mRNAs, 21 lncRNAs		
			30 CRC **serum exosomes** 'Test set'	30 **serum exosomes** 'Test set'			↓ *KRTAP5-4, BCAR4, MAGEA3* in CRC and adenoma		
			20 adenomas **serum exosomes**	76 **serum exosomes**					
Liu *et al.* [100]	2016	China	HCT116, SW480 SW620, HT-29 and LoVo **cell-derived exosomes**	FHC **exosomes**	RT-qPCR	1 lncRNA	↑ *CRNDE-h* in CRC cells exosomes, tissue and serum exosomes	↑ *CRNDE-h* low OS	AUC = 0.892 in CRC vs. adenoma, IBD and HP patients and controls, with 70.3% sensitivity and 94.4% specificity
			148 CRC, 80 adenomas, 80 HP, 80 IBD **serum exosomes**	80 **serum exosomes**			↑ *CRNDE-h* in CRC vs. with adenoma, HP, IBD and controls serum exosomes	↑ *CRNDE-h* ↑ CEA (combined) lowest OS vs. ↓ *CRNDE-h* and ↑ CEA (combined) and vs. ↑ *CRNDE-h* alone	
			50 CRC paired tumour and adjacent non-malignant tissue				↑ *CRNDE-h* in adenomas vs. HP, IBD and controls		
Li *et al.* [101]	2015	China	11 CRC **serum exosomes**	3 **serum exosomes**	RNA-seq 'Discovery phase'	All detectable circRNAs	↑ 53 circRNAs		
					RT-qPCR 'Validation phase'	1 circRNA	↑ *KLDHC10*		

In bold are highlighted the samples from which exosomes were isolated and ncRNAs analysed.
AB, apoptotic bodies; HP, hyperplastic polyps; IBD, inflammatory bowel disease; NFs, normal mucosa derived fibroblasts; OS, overall survival.

In contrast with multiple reports on serum exosomes, only one study investigated ncRNAs in human plasma so far. Hu *et al.* compared plasma exosome lncRNAs between CRC patients and healthy controls and described six competing endogenous RNAs (ceRNAs), *LNCV6_116109, LNCV6_98390, LNCV6_38772, LNCV_108266, LNCV6_84003* and *LNCV6_98602*, upregulated in CRC and with an AUC value of about 0.7 (95). Although more evidence is needed, these results are consistent with those coming from miRNAs analysed in serum exosomes (Table 3).

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Conclusions

Increasing evidence reports that exosomes released from cancer cells play a major role in CRC oncogenesis. Exosomes can reach different types of cells also at distant locations, influencing the biological activities of tumours, such as proliferation, invasion and metastasis, immunoregulation, generation of a pre-metastatic niche and stimulation of angiogenesis (102). Recent findings indicate that exosomes exhibit specific characteristics in educating macrophages, alter dysbiosis and exacerbate the progression of CRC. Unfortunately, at present, we are still far from clearly understanding how exosomes are recognised by macrophages, how they can interact and influence the microbiome, as well as their interactions with CRC cells (103). However, their functions are evidently affected by their cargo, which includes, besides other molecules, many RNA species, including miRNAs and other ncRNAs. These molecules are known to be involved in CRC oncogenesis and also in the regulation of host-microbiome gene expression at post-transcriptional level (104).

The molecular characterization of exosomes can help not only to understand their role and function better but may also provide new non-invasive, blood-based biomarkers for CRC detection or prognosis. Notably, considering that free-circulating miRNAs and other ncRNA species are emerging as very attractive biomarkers, to study their expression profiles specifically in exosomes can hopefully, improve their future clinical significance. Moreover, the research of new microvesicle populations other than exosomes can add interesting future perspectives in this area. As an example, recently, Zhang *et al.* discovered a class of non-membranous nanoparticles, which authors termed 'exomeres' (~35 nm). Profiling their content revealed a selective enrichment of proteins involved in metabolism and associated with coagulation and hypoxia (105).

The evidence from the collected studies may bolster further interest to investigate this area. Exosomal miRNA profiles observed in both serum and plasma samples accurately discriminated CRC from healthy subjects as was also confirmed by sensitivity and specificity analyses. In particular, miR-92a-3p, miR-17-5p and miR-196b-5p showed the best performances in this context and, together with miR-21, were repeatedly reported dysregulated in the examined studies. Exosomal miRNA profiles may also be able to detect precancerous lesions, such as different types of adenomas. Indeed, findings from Uratani *et al.* are promising as expression levels of miR-29a and miR-21 in serum exosomes of adenoma subjects differed from those of healthy individuals (38). However, this work is, so far, the only available on this topic, highlighting the need for further investigations.

Exosomal miRNA signatures could help monitor CRC progression since they can discriminate early from advanced stages and metastatic from non-metastatic CRC subjects (38) although more evidence is strongly required. Based on the few available studies, exosomal miRNAs may also provide useful information on CRC patients prognosis. Indeed, some miRNAs, including miR-21 and those of the miR-17–92 cluster, show good performance in predicting CRC patients long or poor survival (38). From *in vitro* studies, the outcomes generally mirror those coming from human studies since a consistent group of miRNAs showed similar dysregulation in cancer. Interestingly, miRNAs are selectively encapsulated or they are differentially expressed among different types of EVs released by the cells. Overall, the evidence from the still limited number of studies on expression profiles in exosomes of other ncRNAs is in line with those of miRNAs. Indeed, also other ncRNAs, mainly lncRNA and circRNAs are selectively distributed in EVs. Moreover, also their diagnostic power is worth and, in this sense, *CCAT2*, *CRNDE-h*/*-p* showed interesting results (38).

In general, the following points should be taken into considerations for future investigations on the field. First, an application of miRNAs and other ncRNAs as non-invasive biomarkers requires to accurately define the precise and specific dysregulation of these molecules in various diseases and according to their severity. As an example, the aforementioned miR-21, together with other miRNAs, is upregulated in different tumours besides CRC, such as lung, breast and cervical ones, as well as in immune and neurodegenerative diseases (106–110). This implies that its dysregulation cannot be uniquely related to a specific disease and applicable as a specific biomarker (111). Thus, it is important to study the whole miRNome and ncRNA spectra focussing on single molecules or their combinations, which can be more specifically related to the diseases.

As it can be observed from the hereby reviewed studies, exosomal miRNAs and other ncRNAs are mainly assessed individually or in small groups by RT-qPCR or array. This aspect highlights the need of further investigations based on omic scale, such as NGS, to compare study populations from different parts of the world. Genetics, geography, ethnicity and dietary habits may differently affect ncRNA expression in CRC patients. To this aspect, it is noteworthy to say that the majority of the studies retrieved in this work were performed in China and Japan, with few others from different countries. Then, we need to understand better if exosomal ncRNA profiles can be more specific and sensitive as CRC biomarkers than those of the circulating counterpart. Indeed, some dysregulated exosomal ncRNAs, in particular miRNAs, mirror the expression profiles of the free circulating forms (38).

Importantly, the knowledge on the exosomal RNA field is constantly expanding as witnessed by the release of databases and data repositories available online (https://www.exosome-rna.com/tag/database/). Efforts to standardise methodologies for EVs isolation and content analyses have also been done, such as those from the International Society for Extracellular Vesicles (112).

To conclude, this can be a fruitful area for further work, especially considering that, so far, we might have just seen the tip of the iceberg. However, more investigations are needed to clearly establish the feasibility of exosomal ncRNAs application in CRC diagnosis and prognosis.

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