Cytokine and Growth Factor Reviews 51 (2020) 84–91

Contents lists available at ScienceDirect

Cytokine and Growth Factor Reviews

journal homepage: www.elsevier.com/locate/cytogfr

The role of exosomes in colorectal cancer disease progression and response to therapy

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ARTICLE INFO

Keywords:
Colorectal cancer
Exosomes
Biomarkers
Tumor microenvironment
Pre-metastatic niche
Immune cells

ABSTRACT

Colorectal cancer (CRC) is the second leading cause of cancer mortality in both men and women worldwide. Survival of patients is significantly associated with disease stage at diagnosis. Recent studies highlighted a role of exosomes in CRC development and progression, thus raising the interest on these nanosized vesicular structures as possible biomarkers. Exosomes contain a large variety of molecules, including proteins, lipids and nucleic acids, that are exchanged between cells either within tumor microenvironment or at distant sites from the primary tumor, where they prepare a suitable soil for tumor metastases. The present review summarizes the principal effects of exosomes on CRC development, progression, and provides an update of the most recent findings on the use of exosomal molecules as diagnostic, prognostic and predictive biomarkers in CRC.

1. Introduction

Colorectal cancer (CRC) is the third most common malignancy globally, and the second deadliest neoplasm among both men and women [1]. Survival of patients affected by this cancer is significantly associated with disease staging at diagnosis. The five-year survival rate for CRC patients diagnosed in early stage is > 90 %, while for those diagnosed in late stage is approximately 7 %.

Currently, there are several screening approaches, mainly based on endoscopic analysis of the mucosae followed by biopsy and fecal occult blood test (FOBT) for colon cancer. These techniques have inherent limitations, especially in early diagnostic settings: endoscopic exams are invasive, costly and associated with patients discomfort and procedural risks, while FOBT, although non-invasive and affordable, has inadequate sensitivity and specificity to stand alone as a diagnostic test [2,3]. Serum biomarkers such as CEA (carcinoembryonic antigen) and CA 19-9 (Carbohydrate antigen 19-9) are considered among the best available prognostic markers for CRC [3,4]. However, low sensitivity and low specificity of these markers affects their use as biomarkers for early diagnosis, and their expression level is only considered for post-resection monitoring of patients with already diagnosed cancer.

Several studies propose circulating tumor cells (CTCs) as valuable indicators of cancer progression and predictors of progression-free survival and overall survival of CRC patients [5,6]. Nevertheless, they are extremely rare in blood and have a short lifetime, issues which make them hardly useable in clinical practice. Studies have shown that potential biomarkers (e.g. micro-RNA), previously detected in liquid biopsy of serum and urine, are indeed concentrated in the exosomes [7]. Therefore, isolating these exosomes from biological fluids and analyzing their content may represent a promising strategy for cancer diagnosis and prognosis.

However, it must be kept in mind that exosomes belong to a heterogeneous population of extracellular vesicles (EVs) released by cells that is difficult to sub-fractionate with the current technical procedures. In this respect, a recent consensus paper [8] has established guidelines for EVs field of study, although it does not provide a standardized method for isolation of a pure exosomal population.

In the present review, we will provide an overview of the studies regarding *bona fide* exosomes in CRC development, progression and as a source of potential tumor biomarkers suitable for liquid biopsy approaches.

Abbreviations: circ-RNA, circular RNA; miRNA, microRNA; lnc-RNA, long non-coding RNA; CC, cancer cells; CSC, cancer stem cells; CAF, cancer associated fibroblast; ECM, extracellular matrix; M1-mac, macrophages M1; M2-mac, macrophages M2; DC, dendritic cells; MDSC, myeloid-derived suppressor cells; Tregs, T regulatory T cells; Teff, T effector T cells; EC, endothelial cells; TDE, tumor-derived exosome; TME, tumor microenvironment; PMN, pre-metastatic niche; EVs, extracellular vesicles; CTCs, circulating tumor cells; EMT, epithelial to mesenchymal transition; WT, wild-type; PTEN, phosphatase and tensin homolog; PDCD4, programmed cell death 4; WIF, WNT inhibitor factor; SNT, supernatant; 5-FU, 5-fluorouracil; OXA, oxaliplatin

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https://doi.org/10.1016/j.cytogfr.2019.12.004
Received 21 November 2019; Received in revised form 17 December 2019; Accepted 17 December 2019
Available online 18 December 2019
1359-6101/

Table 1
List of representative exosomal molecules involved in CRC tumor progression and drug resistance.

Exosome Content	Origin of Exosome	Effects and Molecular Mechanisms	Ref. n°
non coding RNAs			
miR-196b-5p	HCT116, SW480 cell lines snt; patients serum	Induction of stemness and chemioresistance through STAT3	[23]
miR-203	Patients' serum; primary tumor tissue	Tumor-promoting M2 polarization of monocytes	[48]
miR-25-3p	SW480, LS174 T, HCT116, SW620, LOVO cell lines snt; patients' serum	Vascular permeability and angiogenesis in endothelial cells	[47]
miR-1246	HCT116, HT29 cell lines snt	Macrophage reprogramming	[26]
lncRNA UCA1	Cetuximab-resistant Caco-2 snt; patients serum	Cetuximab resistance	[86]
lncRNA H91	Patients' and healthy donors' plasma; HCT116, HCT8 cell lines snt	Alters tumor-cell migration and invasion in tumor development by modifying HNRNPK expression	[76]
miR-21	Patients' plasma	Liver metastases and PMN	[64]
miR-17-5p, miR-92a-3p	Patients' serum	Tumor progression	[79]
miR-146a-5p	HT29, HCT15 - organoids snt	Tumorigenesis and immune-suppression	[35]
miR-92a-3p	CAF from patients	Stemness; EMT; 5-FU and OXA resistance; activation of β-catenin pathway	[45]
miR-199b-5p, miR-150-5p, miR-29c-5p, miR-218-5p, miR-99a-3p, miR-383-5p, miR-199a-3p, miR-193a-5p, miR-10b-5p, miR-181c-5p	Peritoneal lavage of patients vs ascitic fluids of healthy donors	Diagnostic biomarker from peritoneal lavage	[80]
miR-320d	Patients' serum	Discriminate between metastatic and non metastatic disease	[78]
miR-10a, miR-16-5p, miR-21-5p, miR-26a-5p, miR-146a-5p, miR-210-3p, miR-222-3p	Mouse organoids; SW1222, SW620, HT29, HCT116 -organoids; patient-derived organoids	Fibroblast mediated growth of tumor cells	[29]
circFMN2	Patients' serum	Tumor progression via miR-1182/hTERT axis	[81]
lncRNA RPPH1	Patients' plasma; SW620; HCT8 cell lines snt	Tumor cells proliferation and metastasis; inhibits TUBB3 ubiquitination; enhances exosomes-mediated M2-macrophage polarization; influences tumor microenvironment	[71]
CCAL	CAFs and normal fibroblasts	Tumor progression, resistance to OXA; activation of wnt/β-catenin pathway	[87]
CCAT2	Patients' plasma	Not described	[72]
lncRNA H19	CAFs from patients	Stemness and chemoresistance; activation of β-catenin pathway, endogenous sponge for miR-141	[44]
miR-1249-5p, miR-6737-5p, miR-6819-5p	HCT116, HT29 cell lines snt	Fibroblast-mediated growth of tumor cells; TP53 expression in fibroblasts and proliferation	[46]
HOTTIP	Patients' and healthy donors' plasma	Inversely correlated with poor overall survival	[77]
proteins			
KRAS,EGFR, SRC family, integrins	DKs-8, DLD-1, DKO-1 and RIE-1 cell lines snt	Growth in 3D; carcinogenesis	[15]
scr, GRB2, Traf2, TNIK, RAP2A, EPCAM, CD44, CLDN7	SW480, SW 620 cell lines snt	Tumor growth, cytoskeletal rearrangement and metastasis	[16]
HLA-A29.1, A, B, C, E; Rab13; EEA1; A33; EpCAM; CLDN7; CD44	LIM1863 cell line snt	Basolateral sorting process; antigenic information to lamina propria or immune cells; tumor progression	[27]
ABCG1	Colon26, NM11, LuM1 - organoids snt	Increased expression in 3D; ABCG1 depletion induces EVs intracellular accumulation; malignancy regression	[33]
IRF-2	Serum from patients with or withour LN metastases	Macrophage increase at secondary lympoid organs and angiogenesis; metastatization	[63]
WNT-1	HCT116, SW480, DLD-1, Lovo, SW116 cell lines snt	Proliferation and migration	[18]

2. Biological functions of exosomes in cancer

Exosomes are nanosized (30–120 nm) vesicular structures with membrane-bound phospholipids that belong to the large EVs population constitutively released by cells to enable intercellular communication. Exosomal cargo, which includes proteins, lipids and nucleic acids (DNA, mRNA, cirRNA, miRNAs and lncRNAs), can be exchanged between neighboring cells within tumor microenvironment or travel across the circulation to reach distant targets sites, altering the physiological functions of recipient cells. Table 1 summarizes the molecules delivered by exosomes and the modifications induced in the target cells here reviewed.

Exosomes are involved in a variety of biological processes, including immune response, gene expression regulation and signal transduction, and play an active role in carcinogenesis [9]. Cancer cells release a larger amount of exosomes as compared to non-transformed cells, and their composition and content reflects that of the cells of origin. Several papers suggest that tumor-derived exosomes (TDE) are involved in tumor stroma cross-talk, microenvironment remodeling, angiogenesis, induction of epithelial to mesenchymal transition (EMT), tumor growth and progression, immune escape, angiogenesis, invasion, and drug resistance [10,11]. For this reason, several studies have aimed at the quantitative and qualitative analysis of these nanovescicles collected from blood, urine or saliva for early diagnosis of tumors and monitoring of disease progression.

3. Role of exosomes in CRC development: lessons from *in vitro* studies

CRC arises in the normal colonic mucosa as a consequence of a multi-step transformation process that involves one or more of the following mechanisms: i) chromosomal instability, ii) hypermethylation of promoter CpG island sites, iii) activation of proto-oncogene KRAS, iv) inactivation of tumor suppressor genes (Apc, p53), v) microsatellite instability (rev in [12]). In this scenario, it is possible to envisage that shuttling of genetic material through exosomes from precancerous lesions or tumor-initiating cells may contribute to CRC development and progression, similarly to what has already been proposed for breast and gastric cancer [13,14].

Many studies have tried to address the role of TDE in CRC biology

and progression by using traditional 2D cultures employing cell lines with single gene mutations on key signaling pathways. Thus, the parallel proteomic analysis of exosomes released from the KRAS mutant versus p53 mutant CRC cells demonstrated in the former an enrichment in proteins (KRAS, EGFR, SRC family kinases, and integrins) associated with cellular adhesion, cytoskeletal rearrangement, and migration, pathways that are all related to tumor progression [15]. When these exosomal proteins were transferred to wild-type (WT) KRAS non-transformed cells, they stimulated the growth of KRAS WT cells in collagen matrix and soft agar, thus indicating a direct effect of exosomal cargo transfer on neoplastic transformation.

Other studies carried out on isogenic CRC cells from a primary tumor (SW480) and from its lymph node metastatic variant (SW620), demonstrated the existence of a tumor stage-specific exosomal cargo, with a molecular profile likely responsible for the molecular events of CRC progression [16]. The proteome profile identified an enrichment of metastatic factors (S100A8, S100A9, TNC), signal transduction molecules (EFNB2, EGFR, JAG1, SRC, TNIK), and a unique expression of Met signal transduction components (Met, Src, and GRB2, TNIK-RAP2A complex) in exosomes from metastatic CRC, as compared to exosomes from primary CRC. This observation indicates the involvement of pathways related to cytoskeletal rearrangement and cell spreading in CRC metastasis.

Almost all CRCs demonstrate hyperactivation of the WNT pathway, which is believed to be a driving event in tumor onset, by regulating β-catenin–dependent signaling cascade. This is a multistep process that involves the relocalization, phosphorylation, and degradation of multiple proteins, culminating in a coordinated transcriptional response [17]. A role of exosomal WNT was described in CRC by Wang et al., who demonstrated that exosomal WNT1, acting in an autocrine manner through a non-canonical WNT signaling involving RHO, JNK, and AKT, enhanced the proliferative and migratory activity by tumor cells [18].

CRC-derived exosomes may also deliver mRNAs, lncRNAs, miRNAs (e.g. miR-21, miR-192 and miR-221) and natural antisense RNAs (inhibitors of LRRC24, MDM2 and CDKN1A genes) to target cells, thus participating in the reprogramming of the target cell transcriptome [13,19]. In this respect, a recent study uncovered that lncRNA-APC1 is an important mediator of APC function through the direct regulation of Rab5b mRNA stability, thereby reducing exosome production in CRC cells and, consequently, angiogenesis and tumor growth [20].

Among miRNA, miR-100, miR-25-3p, miR-196b-5p and miR-1246 were also identified in CRC progression. MiR-100 was found selectively packaged in exosomes derived from mutant KRAS cells [21]. This allowed the downregulation of LGR5 with the inhibition of migration and invasion of CRC cells [22]. Serum exosomal miR-196b-5p was elevated in CRC patients and associated with liver metastases. As described, it modulates IL-6/JAK/STAT3 signaling, one of the most important pathways involved in initiation, development and formation in CRC [23,24]. In this study, exosome-associated miR-196b-5p promoted CSC properties and chemoresistance via activating STAT3 signaling pathway *in vivo* and *in vitro*, thus uncovering a novel mechanism contributing to the activation of STAT3 signaling in CRC [21]. miR-1246 has been linked to invasiveness and stemness, and highly expressed in metastases and may target WT p53 in hepatocellular carcinoma where it inhibits cell growth [25]. Moreover, mutant p53 CRC were found to reprogram macrophages to tumor supporting macrophages via exosomal miR-1246 [26].

Studies on 3D organoids allowed the analysis of exosomes in a tumor complex morphology and cellular heterogeneity close to *in vivo* condition. An example is represented by the LIM1863 cell line, which grows as an organoid composed of polarized epithelial cells that spontaneously differentiate into crypt-like structures *in vitro*. This complex morphology allowed the identification of two distinct populations of exosomes, A33+ basolateral exosomes and EpCAM+ apical exosomes, reflecting functional specialization of the intestinal epithelium. The analysis of their content demonstrated that the former are uniquely endowed with proteins belonging to basolateral sorting process (Rab13 and EEA1), and antigenic information to lamina propria or systemic immune cells (HLA-A29.1, A, B, C, E); the latter contain CLDN7 and CD44, known to complex together to promote tumor progression [27]. Interestingly, the discovery of these distinct sets of vesicles furthermore revealed the complexity of functional networks existing in CRC development.

3D organoid technology proved very useful to evaluate the production and role of exosomes in CRC as a function of extracellular matrix composition, whose changes came out as major drivers of tumor progression and metastasis formation [28]. It was demonstrated that collagen I deposition increased exosome release from tumor organoids via inducing the WNT pathway [29]. Also, at the early event of adenocarcinoma development, i.e. at APC mutation stage, an increased exosome release was described, thus indicating a precise role for these vesicles as mediators of cancer signals starting from the first expansion of the tumor [29].

In 3D cultures, it was also possible to address the role of exosomes under hypoxic and acidic conditions, typical of a tumor microenvironment. The acidic milieu of tumors increases malignancy in several tumors through an enhanced release of exosomes [30–32]. In CRC, a recent study demonstrated that the 3D hypoxic tumoroids grow very slowly, but produce robust levels of ABCG1, a molecule mediating efflux of cholesterol and phospholipids, and preventing cellular lipid accumulation [33]. The depletion of the ABCG1 pump increased the accumulation of exosomes, lipoproteins, and their potential cargos in the metastatic cancer tumoroids, leading to reduced viability and tumor regression.

Finally, a 3D model was also applied to evaluate the role of CSCs in CRC development. The presence of colorectal cancer stem cells (CRCSCs) has been associated with tumor initiation, propagation and with a more aggressive tumor type [34]. A recent study demonstrated that the uptake of CRCSC-derived exosomal miR-146a into CRC cells promoted the development of stem-like properties, thus supporting the continuous expansion of tumor [35].

Overall, these findings highlight the multifaceted molecular mechanisms exploited by TDE to alter the functions of target cells in the process of CRC carcinogenesis and progression.

4. Multiple roles of tumor-derived exosomes in cancer spread

Emerging evidence suggests that tumor cells exchange information with other cells within the tumor microenvironment (TME) or at distant sites by releasing exosomes. In this way, TDE reprogram the tumor neighboring stromal cells [36–38], and direct themselves to specific target tissues and organs to prepare a suitable soil for tumor implant, i.e. a pre-metastatic niche (PMN) [39]. In fact, -TDE can educate stromal cells and macrophages towards a tumor-promoting state, facilitate angiogenesis and induce vascular leakiness [39]. All these modifications contribute to cancer progression and spread.

4.1. Reprogramming of stromal non-immune cells

Cancer-associated fibroblasts (CAFs) are the main component of tumor stroma within TME. The interplay between these cells and tumor cells is a critical step in tumor development and progression [40,41]. CAFs display a plasticity that support the continuous changes occurring in multistage carcinogenesis. In fact, when exposed to signals deriving from primary CRC they acquire pro-proliferative and pro-angiogenic functions, whereas when activated by exosomes from metastatic CRC, they display a striking ability to invade through the extracellular matrix [42] (Fig. 1).

CAFs secrete diverse miRNAs, growth factors, chemokines, and cytokines (TGF-β, VEGF) that directly or indirectly affect the proliferation, metastasis and angiogenesis of surrounding cancer cells. They can also remodel the extracellular matrix structure, thus contributing to the

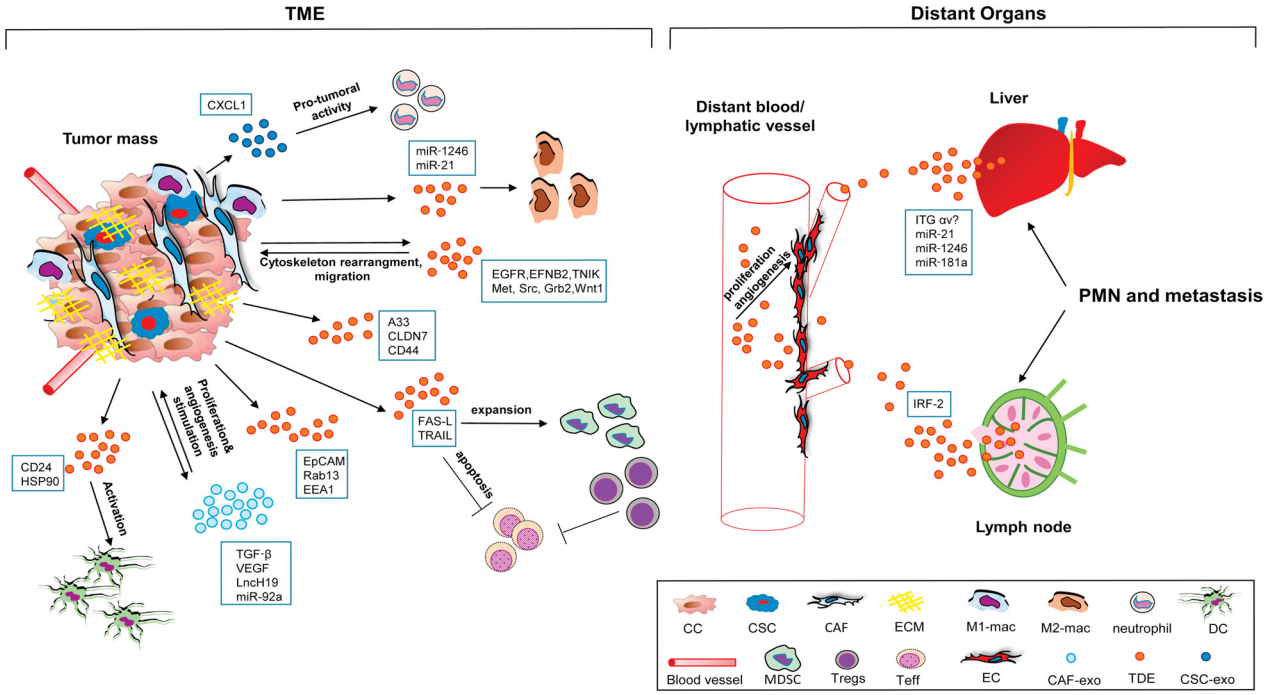

Fig. 1. Schematic representation of the multiple roles of exosomes in tumor microenvironment and in tumor cell spreading to distant organs.
CRC tumor releases different exosome populations that modulate the response of non-immune (CAF, EC, CSC) and immune (Neutrophil, M1-mac, MDSC, DC, T regs, Teff) cells within TME. The complex interplay of exosomes amongst different cell populations supports tumor growth and the creation of a PMN in the liver and lymph nodes.

generation of the CSCniche [43]. Moreover exosomal lncRNA-H19 and miR-92a-3p from CAFs can be transferred to tumor cells, where they promote stemness and drug-resistance via the activation of the Wnt/β-catenin pathway [44,45]. Other miRNAs such as miR-1249-5p, miR-6737-5p, and miR-6819-5p contained in TDE have been shown to influence tumor progression by suppression of TP53 expression in recipient fibroblasts and deregulation of their growth [46].

An effect of TDE on endothelial cells was also reported. Zeng and coworkers, demonstrated that CRC-derived exosomal miR-25-3p can be transferred to vascular endothelial cells, promoting vascular permeability and angiogenesis by targeting Krüppel-like factor 2 (KLF2) and Krüppel-like factor 4 (KLF4). This in turn enhanced CRC metastasis in liver and lung [47]. Altogether, these data evidence the essential role of exosomes in tumor-stroma interplay and suggest that targeting these entities or their content (e.g. miRNAs) may be envisaged as a possible promising therapeutic strategy.

4.2. Effects of tumor-derived exosomes on immune cells

Exosomes released from tumor cells or from non-immune stromal cells within the tumor microenvironment have the potential to hijack key components of the immune system, such as APCs and effector T cells, thus indirectly contributing to tumor progression.

Exchange of genetic material or proteins between macrophages and CRC cells via exosomes is one mechanism. For example, uptake of miR-1246-enriched exosomes released from mutant-p53 cancer cells by neighboring macrophages triggers their reprogramming into a cancer promoting state [26] (Fig. 1). Accordingly, exosomal miR-203 isolated from sera of CRC patients was associated with tumor pathological aggressiveness and metastasis and has been shown to promote the differentiation of monocytes to protumoral M2 macrophages *in vitro* [48].

The impact of exosomes from CRCSC on bone marrow-derived neutrophils has been examined both *in vitro* and *in vivo*. Upon administration of exosomes derived from CRC spheroids, bone marrow-derived neutrophils showed prolonged survival and acquired a protumoral phenotype [49]. In CRC patients, abundant exosomal miR-146a expression in serum was associated with higher CSC traits, an increased number of tumor-filtrating CD66+ neutrophils as well as a reduced number of tumor-infiltrating CD8+ T cells, suggesting a role for miR-146a-5p in tumorigenesis and immunosuppression [35].

Exosomes released from tumor cells can also interfere directly with T cell effector functions. Analysis conducted on CRC cell lines, as well as in the blood and tumor specimens from advanced CRC patients, indicate that tumor cells exploit microvesicle release to deliver FasL- and TRAIL-mediated apoptotic signals to antitumor T cells, thus promoting immune evasion [50]. Accordingly, it has been reported that exosomes from different cancer cell lines, including bladder, prostate and CRC, express CD39 and CD73 ecto-nucleotidases [51] that are involved in the adenosine triphosphate (ATP)-dephosphorylation to adenosine [52] and negatively regulate T cell functions [51]. On the other hand, tumor-derived exosomes have been shown to modulate T cell activity through the expansion of immunosuppressive cell subsets such as myeloid-derived suppressor cells (MDSC) and Regulatory T cells (Tregs) (Fig. 1).

CRC-derived extracellular vesicles have been shown to suppress the proliferation of T cells through the perturbation of intracellular signaling, including MAPK, AKT and TGF-β/Smad signaling [53]. As a consequence, these cells acquire several characteristics of Tregs and display a remarkable tumor-growth promoting activity *in vitro* and *in vivo*.

In a recent paper, the ability of a tumor to incorporate circulating exosomes was investigated *in vivo*. Exosomes obtained from CT26 (autologous) or from B16 (allogeneic) cultures were injected in CT26-bearing mice and their fate was monitored over time. Autologous exosomes preferentially accumulated in the tumor mass with respect to allogeneic exosomes, thus suggesting the existence of a cancer cell-type specific accumulation of exosomes. Most importantly, infused exosomes were taken up not only by cancer cells, but also by specific tumor-associated immune cells (i.e. predominantly TAM and T cells, and not by DC and B cells). This result indicated that uptake of exosomes by certain immune cells also plays a major role in the intratumoral accumulation

of exosomes [55].

Exosomes may also stimulate antitumor activities, depending on the tumor type and disease stage. For example, exosomes isolated from ascites of T cell lymphoma-bearing mice were shown to express surface CD24, heat shock protein (HSP90) and the tumor antigen, and were capable of activating DC, thereby priming the immune system to recognize and kill cancer cells [56]. Likewise, exosomes from heat-stressed tumor cells, derived either *in vitro* or recovered from ascites of CRC patients undergoing hyperthermia treatment, possessed the ability to convert immunosuppressive Tregs into Th17 and stimulate powerful antitumor responses [57].

4.3. Role of tumor-derived exosomes in the pre-metastatic niche formation and in disease progression

Some recent studies indicate pre-metastatic niche formation (PMN) as a mechanism facilitating CRC organ metastasis (Fig. 1). In some tumors exosomal integrins (ITGs) direct exosomes to specific organs where they fuse with target cells, in turn inducing inflammation and increment of vascular permeability through Src activation and S100 expression [58]. In CRC, ITG $\alpha v\beta 5$ binds to Kupffer cells and is associated with liver metastasis, thus it is conceivable that CRC-exosomes expressing ITG αv may contribute to PMN formation in liver. It has been shown that TDE within liver induce a macrophage pro-inflammatory phenotype through miR-21-TLR7-IL-6 axis, ultimately leading to liver metastases [58,59].

Several reports support the role of exosomal miRNAs in metastatic progression of CRC [60]. miR-21 and miR-181a have been proposed to mediate liver metastases of CRC (Fig. 1), by suppressing their target gene phosphatase and tensin homolog (PTEN), programmed cell death 4 (PDCD4) or WNT inhibitor factor (WIF)-1 in hepatocytes [61,62]. In another report, the administration of exosomes from HT-29 cells (highly metastatic to the liver) to the poorly metastatic Caco-2 inoculated in nude mice led to a pronounced enhancement of metastasis of Caco-2 cells to the liver [60]. The authors propose that TDE may promote CRC metastasis by recruiting CXCR4-expressing stromal cells to develop a permissive metastatic microenvironment in the liver. In a similar way, exosomes derived from CT26 tumor cells promote the proliferation of lymphatic endothelial cells and the formation of lymphatic network in the secondary lymphoid organs, facilitating the formation of metastasis of CRC.

In another study, knocking-down exosomal IRF-2 attenuated the lymphatic network remodeling and the dissemination of metastasis [63]. Exosomal IRF-2 was highly expressed in serum exosomes isolated from CRC patients with LN metastasis as compared to non-metastatic or healthy controls. This finding suggests that exosomal IRF-2 remodels the lymphatic network *in vivo* and may predict the development of LN metastases in CRC patients (Fig. 1). Along these lines, miR-21 expression in plasma-derived exosomes was positively correlated with liver metastasis in CRC patients [64].

Overall, these findings have important implications for the progression of CRC, suggesting a possible mechanism by which other non-tumor cells within tumor microenvironment (fibroblasts, endothelial cells, bone marrow–derived cells, alveolar epithelial cells, and Kupffer cells) may be reprogrammed by exosomes released by mutant KRAS tumor cells to support development of the pre-metastatic niche, and metastasis spread [65,66].

5. Exosomal biomarkers in CRC patients: recent advances in the field

Due to their abundance and high half-life in all biological fluids, exosomes represent ideal candidate biomarkers for the early diagnosis and monitoring of disease progression in cancer patients. The role of exosomal molecules, in particular lncRNA and miRNA, as biomarkers in CRC has recently been extensively reviewed [67–69]. Here, we will provide an update that substantiates recent advancements in the field.

LncRNAs are transcripts longer than 200 nucleotides, located within the intergenic stretches or overlapping antisense transcripts of protein coding genes. The secondary structure of lncRNAs confers to these RNA species the capacity to specifically bind to a variety of proteins and nucleic acids, and act as competitive endogenous RNAs (ceRNAs) or RNA sponges to regulate gene expression at translational and/or transcriptional levels [70]. The upregulation of lncRNA RPPH1 in CRC tissues is associated with advanced TNM stages and poor prognosis. Exosomal RPPH1 levels in blood plasma turned out to be higher in treatment-naive CRC patients and lower after tumor resection. Compared to CEA and CA199, exosomal RPPH1 in CRC plasma displayed a better diagnostic value (AUC = 0.86) [71].

Colon cancer-associated transcript 2 (CCAT2) plays a crucial role in several cancers. The analysis by qRT-PCR of CCAT2 expression in plasma exosomes of CRC patients revealed that its levels of expression were associated with tumor progression and were consistent with the analysis of tumor tissues where CCAT2 levels were higher in advanced CRC patients [72].

Previous studies have reported that lncRNA H19 maintains the pluripotent capacity of hematopoietic stem cells through a miR-675-Igf1r signaling circuit that is highly expressed in several cancers [73]. In CRC, high H19 expression correlated with poor prognosis and was involved in cell proliferation and migration [74,75]. In addition, Ren et al., have recently found that exosome-enriched H19 promoted stemness of CSCs and chemoresistance of CRC cells *in vitro* and *in vivo*, by activating the β-catenin pathway and acting as a competing endogenous RNA sponge for miR-141, an inhibitor of stemness of CRC cells [44]. Another lncRNA 91H is known to play a critical role in tumor development, in enhancing tumor-cell migration and invasion by modifying HNRNPK expression. Gao and co-workers have analyzed the pathological significance of exosomal lncRNA 91H observing that CRC patients with high lncRNA 91H expression demonstrated a higher risk of tumor recurrence and metastasis [76]. Conversely, exosomes from healthy donors carried a significant amount of HOTTIP transcripts compared to CRC patients, with a significant statistical correlation between low levels of exosomal HOTTIP and poor overall survival [77].

MicroRNAs (miRNAs) are small noncoding, double-stranded RNA molecules that can inhibit protein translation by degradation of complementary mRNA sequences in the target cell. Their involvement in tumor pathogenesis and progression has been addressed in a variety of cancer types. Recently, exosomal miR-320d was identified as promising diagnostic biomarker capable to discriminate between metastatic and non-metastatic disease in serum samples of CRC patients [78]. miR-21 contributed to the induction of proinflammatory macrophages in the liver by inducing an inflammatory PMN through the TLR7–IL-6 axis, leading to liver metastasis. Accordingly, miR-21 expression in plasma–derived EVs was positively correlated with liver metastasis in CRC patients, thus representing a promising prognostic marker [59]. In addition, miR-17-5p and miR-92a-3p, belonging to the miR-17-92 cluster, were found to be up-regulated in the circulating exosomes of CRC patients. Their expression levels were significantly correlated with the pathological stage and grade of the patients [79].

Another source of vesicle-associated biomarkers in CRC is represented by tumor proximal fluid, i.e. peritoneal lavage, a fluid offering an improved representation of the molecular alterations that take place in the tumor. A study unveiled the promising use of 10 dysregulated miRNAs (miR-199b-5p, miR-150-5p, miR-29c-5p, miR-218-5p, miR-99a-3p, miR-383-5p, miR-199a-3p, miR-193a-5p, miR-10b-5p and miR-181c-5p) as diagnostic biomarkers [80].

CircRNAs are a class of non-coding RNAs that can participate in exosome-based intercellular communication. However, the molecular mechanisms that link circRNAs with CRC are still poorly understood. A recent report demonstrates that circFMN2 is upregulated in serum exosomes from CRC patients, thus linking circFMN2 to CRC progression via a miR-1182/hTERT axis [81].

One of the roles of circFMN2 in non-transformed cells is the removal of unfavorable molecules such as misfolded proteins, nucleic acids and molecules that are potentially lethal for the cell. The same mechanism is exploited by cancer cells to extrude cytotoxic drugs, leading to drug resistance. Furthermore, exosomes can induce a drug-resistant phenotype in surrounding cells by transferring drug-resistance-related molecules or activating these molecules in host cells [36,82–85]. In this respect, defined exosomal molecules have been associated with therapy resistance and may be exploited to tailor tumor treatments. For example, it has recently been shown that circulating exosomes containing lncRNA urothelial carcinoma-associated 1 (UCA1) could predict the clinical outcome of cetuximab therapy in CRC patients.

Furthermore, UCA1 expression was considerably higher in the progressive disease/stable disease patients, than in the partial response/complete response patients. Interestingly, exosomes derived from cetuximab-resistant cells could themselves alter UCA1 expression, and transmit cetuximab resistance to sensitive cells [86]. Along these lines, CRC-associated lncRNA (CCAL) transferred from CAFs to cancer cells via exosomes, suppressed CRC cell apoptosis, and activated the wnt/β-catenin pathway, thus promoting the resistance to oxaliplatin [87]. Several exosomal miRNAs have also been correlated with therapy resistance in CRC patients. High expression of exosomal miR-92a-3p in serum was highly linked with metastasis and chemotherapy resistance in CRC patients [79].

Overall, the analysis of exosomal cargo from plasma of CRC patients reveals an affordable, poorly invasive, highly specific and sensitive method to identify biomarkers for the diagnosis, prognosis and clinical outcomes.

6. Conclusions

CRC development takes advantage of exosomes during disease progression. In fact, CRC-exosomes can act simultaneously at intratumoral level by: i) enhancing cell proliferation, ii) inducing molecular transformation, leading to a pro-invasive/pro-angiogenetic phenotype, and iii) reprogramming a plethora of tumor surrounding immune and non-immune cells in TME. These combined effects strongly cooperate in CRC progression, culminating in liver and lymph node metastases. At each stage of tumor development, exosomes with different molecular cargoes are released into the circulation, thus providing a potential disease-stage molecular signature.

Because of their abundance in blood and in other biological fluids (saliva, urine, peritoneal lavage), as well as their high stability, exosomes represent a highly promising biomarker source for diagnosis and prognosis of CRC.

However, a main obstacle to the development of exosomes as biomarkers in CRC has been the lack of an elective procedure to separate exosomes from other EVs that are abundantly released by tumor cells. In fact, to date the combination of ultracentrifugation and gradient techniques ensures the separation based on size and density only, regardless of their endosomal or plasma membrane origin. Further efforts are therefore needed to develop new protocols and techniques that would ensure the isolation of a pure exosomal fraction from the bulky vesicular population.

Further validation of vesicular biomarkers in large clinical trials is also required to support their application as standard liquid biopsy biomarkers for monitoring cancer progression and driving treatment decisions. Despite of these considerations, the study of exosomes holds promise for understanding the biology CRC, as well as for the identification of circulating biomarkers and/or new molecular targets exploitable for the development of novel diagnostic/prognostic/therapeutic programs.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgement

We are grateful to Dr Katia Fecchi for support in artwork creation. We acknowledge the Italian Ministry of Health for funding (Ricerca Corrente).

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