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REVIEW

The role of exosomes on colorectal cancer: A review

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Abstract

Exosomes are extracellular microvesicles released from cells, which are involved in many biological and pathological processes, mainly because of their role in intercellular communication. Exosomes derived from colorectal cancer (CRC) cells are related to oncogenesis, tumor cell survival, chemo-resistance, and metastasis. The role of the exosomes in these processes involves the transfer of proteins, RNAs, or mutant versions of proto-oncogenes to the target cells. In recent years, great efforts have been made to identify useful biomarkers in CRC exosomes for diagnosis, prediction of prognosis, and treatment response. This review focuses on recent studies on CRC exosomes, considering isolation, cargo, biomarkers, and the effects of exosomes on the development and progression of CRC, including resistance to antitumor therapy.

Introduction

In 2012, colorectal cancer (CRC) was the most common type of cancer of the digestive system in the world. In terms of gender, CRC was the third most common tumor in men and the second in women worldwide. Countries with a high human development index are the most affected by CRC. Considering demographic factors, the global burden of CRC is expected to increase by 60% up to 2030.[1,2]

According to the literature, cancer cells release different types of extracellular vesicles that can travel through body fluids such as plasma, cerebrospinal fluid, urine, breast milk, and exudates.[3] A type of microvesicles called exosomes have been reported to be involved in cancer progression.[4]

Exosomes can act in a paracrine or endocrine manner to affect the behavior of cells. A combination of specific exosomal cell surface molecules is necessary for cell targeting, adhesion, and vesicle internalization.[5] Exosomes also may contain proteins that were originally in the endosome membrane, such as annexins and flotilins. These proteins facilitate the transport and fusion of exosomes with the membrane. Furthermore, tetraspanins are involved in adhesion of the exovesicles with the target cell, like other proteins such as Alix and TSG101.[3]

Furthermore, exosomes derived from tumor cells seem to be implicated in different processes such as tumor invasion, angiogenesis, chemo-resistance, immune evasion, and cell death.[6] These aspects suggest the importance of the study of exosomes and, in our area of interest, their role in the development and progression of CRC. Moreover, several studies have suggested that exosomes could be useful as biomarkers and as drug carriers, in order to achieve greater specificity and efficacy of current therapies.[3,7] This paper aims to review the actual knowledge of exosomes regarding their nature, isolation, mechanisms of action, content, and especially their role in CRC.

What are exosomes and how can they be isolated?

Exosomes are small vesicles enveloped by a lipid bilayer. However, there is no consensus about the size of these particles. Indeed, in the literature, variations in the definition of exosome range from 30–100, 40–100, 50–150, to 40–200 nm.[8–11] In addition, some authors call exosomes "communicasomes" because they are involved in intercellular communication processes.[12]

Exosomes originate from plasma membrane during endocytic internalization. First, an early endosome is formed, which matures progressively into late endosomes or multivesicular bodies (MVBs). The membrane of MVBs forms intraluminal vesicles (ILVS), which are called exosomes. Exosomes incorporate specific domains of the membrane, mRNA, miRNA, proteins, and DNA. Finally, MVBs fuse with the plasma membrane resulting in the release of exosomes. However, more studies are needed in order to better understand the mechanism underlying the biogenesis of exosomes.[3,13]

To identify, isolate, and characterize exosomes, various aspects are considered, such as their density, which is about 1.13 to 1.19 g/mL,[14] and especially the presence of specific proteins used as exosome markers. Thus, CRC-derived exosomes express tetraspanins such as CD9, CD63, CD81, Alix, and TDG101.[8,9,15–18] Moreover, it is known that exosomes membrane contains microdomains rich in sphingolipids and cholesterol, known as lipid rafts.[19]

There are several methods of exosome isolation from plasma, serum, culture medium, among others, based on some of their own characteristics, such as size, density, content, markers, or electrokinetic potential. The first method described was ultracentrifugation, which was noted for its high performance, simplicity, and accessibility.[20] For exosome isolation from cell cultures, serum-free medium or culture medium supplemented with exosome-depleted serum must be used to avoid unwanted serum exosomes that could interfere with the results. A possible procedure is an initial centrifugation of the supernatant at 480 g for 5 min and then a second centrifugation at 2000 g for 10 min,[16,17] thus removing the cells and cellular debris. The supernatant can be filtered using a 0.2 μ pore to remove large vesicles and then centrifuged at 100.000 g for 60 min at 4 °C. The pellet resuspended in phosphate-buffered saline (PBS) can be purified by a sucrose gradient centrifugation at 100.000 g for 60 min at 4 °C, followed by a centrifugation for 60 min at 100.000 g. The resulting pellet containing exosomes must be washed with PBS and stored at −80 °C.[21] Liquid samples from patients with a higher viscosity might be diluted with PBS (1:2) before ultracentrifugation.[17] Upon completion of the procedure, it is convenient to verify the correct isolation by electron microscopy.

During recent years, different isolation methods have proliferated as alternatives to ultracentrifugation, including ultrafiltration.[22] Bioo Scientific (www.biooscientific.com) provides a kit called "the ExoMir extraction kit" where cells and cellular debris are retained by a microfilter, using positive pressure to direct the flow and finally capturing the exosomes.[23] Similarly, there are affinity methods, which allow the isolation of exosomes by using antibodies against membrane proteins such as CD9, CD63, CD81, CD82, Alix, annexin, EpCAM, and Rab5. These antibodies are immobilized on magnetic beads, chromatography matrices or plates.[24] An example of this type of method is ExoQuick from System Biosciences (www.systembio.com), which is able to precipitate exosomes from plasma samples, serum, or culture medium, based on the recognition of specific surface markers.[24,25]

It is worth highlighting that there is not a universal method for the isolation, purification, quantification, or storage of exosomes. Considering the heterogeneity of EVs, it would be interesting to develop a consensual method to compare all results of all the studies that will be carried out from now on.

Once isolated, exosomes can be studied by different techniques such as ELISA, Western-Blot, flow cytometry, qPCR, proteomic, or NanoSight, in order to analyze their composition or for quantification. In this regard, it has been reported that pH would affect the detection of exosome membrane proteins analyzed by these techniques, because protein detection may increase after incubating the samples in acidic medium.[26]

What do colorectal cancer exosomes contain?

Exosomes contain part of the genome, transcriptome, and secretome of tumor-derived cells. It has been stated that exosomes can carry oncoproteins, tumor suppressor proteins, transcriptional regulators, splicing factors, and RNAs (mRNAs, microRNAs, and other non-coding RNAs).[17] Moreover, exosomes can contain fragments of DNA, which may provide information about the origin of cancer cells.[5,27] Furthermore, proteins related to cytoskeleton, apoptosis, cell cycle, cell signaling, oxidative stress, focal adhesions, and cellular mobility have been identified[3], as well as mutant versions of tumor suppressor genes[28] and microRNAs associated with metastasis in CRC exosomes.[29]

Several cell lines are used for the study of CRC, such as LIM1215, LIM1863, HCT-15, SW480, SW620, DiFi, and WiDr. Molecular studies have shown some similarities in the content of the exosomes derived from these cell lines. Generally, these vesicles contain high concentrations of cell adhesion proteins, proteins involved in molecular transport and extracellular matrix proteins. Ji *et al*., by using immunoaffinity methods, found two populations of exosomes (E-A33 and E-EpCAM) in the LIM1863 cell line. These populations showed significant differences regarding the content of miRNAs, 14 of which had not been previously associated with CRC, a fact that suggests that these miRNAs could be useful as biomarkers of disease.[17] Similarly, it has been reported that exosomes derived from HCT-15, SW480, and WiDr cell lines contain different antisense RNAs involved in cell cycle progression, such as MDM2 and CDKN1A. It should be highlighted that these exosomes could be incorporated by cells of different lineage as both HepG2 liver and A549 lung cell lines.[9] In fact, it was found that exosomes originating from the CRC cell line SW480 promote cell migration in the HepG2 liver cell line.[30]

miR-21 is overexpressed in some CRC cell lines. This miRNA was one of the first called "oncomiRs," because its targets are tumor suppressors. It has been described that exosomes derived from SW480 and WiDr cell lines contain miR-21, which has the potential to inhibit the translation of PDCD4 protein involved in cell apoptosis.[9,31] Table 1 shows a relation of miRNAs identified in CRC exosomes and their functions.

An interesting assay compared the content of exosomes derived from the SW480 cell line (E-SW480) with its metastatic variant, SW620 cell line (E-SW620). The study found significant differences in metastatic factors (MET, S100A8, S100A9, TNC), molecules involved in signal transduction (EFNB2, JAG1, SRC, TNIK), and the presence of lipid rafts or their associated components (CAV1, FLOT1, FLOT2, PROM1). In addition, high levels of Src family kinases were also found in E-SW620. These proteins are involved in the mechanisms of signal transduction, endocytosis, and cytoskeleton remodeling through interactions with CAV1,

Table 1 Colorectal cancer (CRC)-derived exosomal microRNAs

microRNA	Description/function
miR-21	miR-21 is an oncomiR elevated in exosomes derived from SW480 and WiDr cell lines and has the potential to inhibit the translation of PDCD4 protein, involved in cell apoptosis.[9,31]
miR-145	miR-145 and miR-34a were associated with 5-FU resistance, because of the secretion of
miR-34a	these miRNA via exosomes in DLD-1 CRC cell line.[23]
miR-200	miR-200 is a pleiotropic miRNA. The loss of miR-200 produces chemo-resistance to 5-FU and could lead to an aggressive migratory phenotype *in vitro*.[25]
miR-210	miR-210 is upregulated in exosomes derived from HCT-8 cell line, and it would lead cells in to a metastatic phenotype; this miRNA participates in the development of the epithelial-mesenchymal transition process.[29]
miR-100	miR-100 is upregulated in exosomes derived from mutant KRAS cell lines and it is proposed as a potential biomarker.[32]
miR-192	Both miRNAs are downregulated in CRC. miR-192 is capable of inducing cell-cycle arrest.
miR-215	Moreover, it is known that miR-192 and miR-215 contribute directly to chemo-resistance mechanisms of fluoropyrimidine and antifolates.[33]
miR-1229, miR-1224-5p, miR-223, let-7a, miR-150, miR-21 and miR18a	All these miRNAs are proposed as useful biomarkers for detection of CRC[34,35]
miR-19a	miR-19a could be a useful biomarker for CRC recurrence. [36]

FLOT1, and FLOT2. The increased presence of both lipid rafts and their associated molecules in E-SW620 suggests that they could play an important role in signal transduction, which could promote cell transformation and tumor progression.[15,16] In addition, it has been reported that seven phosphorylated tyrosine (pTyr) kinases encapsulated in the E-SW620 could be involved in the regulation of the life cycle of exosomes.[37] Moreover, another study demonstrated that pTyr fraction in exosomes was expressed more than 10 times than in pTyr cellular sites, so tyrosine kinases and RTKs might play a crucial role in oncogenesis, especially in CRC.[38]

The KRAS gene is involved in many cases of CRC, and it appears mutated in 30–40% of tumors. Several studies have been conducted to analyze the relation between KRAS status and the content of exosomes. It has been found that exosomes derived from cells with KRAS mutations contain more proteins involved in tumor progression, epidermal growth factor receptor (EGFR), kinases from Src family, integrins, and the mutant KRAS. In addition, it has been reported that mutant KRAS is loaded preferably in exosomes *versus* its wild type (KRASwt), and exosomes with mutant KRAS induce a higher rate of proliferation in KRASwt cells. KRAS could participate in selective loading of miRNAs. In fact, it has been shown that miR-100 was upregulated in exosomes from mutant KRAS.[8,32]

A recent study made a comparison of lipid profiles between LIM1215 CRC cell line and LIM1215-derived exosomes. The results revealed a higher presence of certain lipids in the exovesicles, such as sphingolipids or sterol lipids.[39]

Because of the large amount of information generated in the field of exosomes, some tools have been developed, highlighting databases that display the molecules identified in exosomes from multiple organisms, including humans. Two representative examples are ExoCarta (http://www.exocarta.org) and EVpedia (http://evpedia.info).[40]

In this section, we have shown the importance of the exosomes and their cargo in cell–cell communication. As previously discussed, exosomes may influence proliferation, tumor microenvironment, cell-matrix interactions, immune response, and angiogenesis, through the transference of components derived from CRC cells. In addition, exosomes may promote the malignancy of tumor cells by transferring proteins involved in cell survival, tumor growth, and metastasis.

Mechanisms of loading in exosomes

While the content of exosomes is increasingly known, regarding the selective recruitment in these vesicles, much remains to be investigated. However, a recent study has highlighted the critical role of the intracellular adaptor protein syntenin in the biogenesis of exosomes and its loading of cargo.[41]

Recent evidences have shown the key role of the endosomal sorting complex required for transport (ESCRT), heparanase and tetraspanins in the loading of exosomas. Moreover, components of the ESCRT have been related to this process. It has been suggested, both *in vitro* and *in vivo*, that ESCRT-0 binds ubiquitinated molecules for subsequent loading into exosomes.[42] Then, other members of the complex would act sequentially, such as ESCRT-I, -II, and -III, during the formation of intraluminal vesicles in MVBs.[13] Although it is not known with certainty, ubiquitination could be the preferred mechanism by which mutant KRAS is added to exosomes.[8]

Heparanase is the only mammalian enzyme capable of cleaving heparan sulfate inside the cell. It has been demonstrated that this enzyme stimulates loading of some exosomal markers, such as CD63, in a dose-dependent manner. Furthermore, high expression of heparanase in tumor cells is associated with an increased angiogenesis, invasiveness, and metastasis. Independently of its enzymatic activity, heparanase is also able to activate some signal transduction pathways, thus altering gene expression and increasing cell survival and migration.[43]

Tetraspanins also have an important role in selective cargo of exosomes. For example, tetraspanin 8 (Tspan8) leads to a selective recruitment of CD49d, allowing the interaction of exosomes with endothelial cells and finally, their internalization.[44] Indeed, exosomes that express certain tetraspanins and target molecules of endothelial cells may contribute to angiogenesis and vasculogenesis. It is believed that tetraspanins are internalized into

exosomes through a tyrosine motif YXXϕ. However, this motif is not present in all tetraspanins, for example, in CD9, but it is present in CD151 and CD49d.[45]

Sumoylation is a mechanism involved in the loading of miRNAs into exosomes. A2B1 (hnRNPA2B1) is a heteronuclear ribonucleoprotein, which, when it is sumoylated, mediates the loading of specific miRNAs into exosomes through the recognition of short sequences of four nucleotides (GGAG). For this reason, it has been reported that the loading of miRNAs into exosomes could be modulated by mutagenesis of these sequences or by changes in the expression levels of A2B1.[46]

According to the literature reviewed, there are different mechanisms of exosomal loading. The variety of these mechanisms could be related to the diversity of the content of exosomas. Exosome biogenesis opens a wide field of study where therapeutic targets could be found that may increase the effectiveness of cancer treatment.

The role of exosomes in colorectal cancer

Cell transformation and proliferation. It is believed that the first mechanism required for tumor spreading is the epithelial-mesenchymal transition (EMT). This process is associated with changes in the expression of intercellular proteins, cell matrix components, elements involved in cell polarity, cytoskeleton, remodeling, and proteases, including matrix metalloproteinases.[47]

Recent studies have shown that tumor-derived exosomes can contribute to the EMT process in recipient cells.[48] In fact, the presence of EpCAM in CRC, which is frequently associated with Claudine-7 (cld7), seems to contribute to tumor progression by EMT.[49] In a recent study conducted by Bigagli *et al*., it was found that after 7 days of culture of the CRC cell line HCT-8, some cells started to grow in suspension, suggesting metastatic potential that was confirmed after vimentin and E-cadherin expression analysis. Using electron microscopy, they found that exosomes secreted by adherent cells were taken up by metastatic cells. Because the miR-210 level was higher in exosomes derived from adherent cells than in their cytoplasm, they suggest that this miRNA may be the trigger of the EMT process, which would lead cells to a metastatic phenotype.[29]

Besides the EMT process, exosomes derived from SW640 and SW680 cell lines are able to significantly increase the proliferation of 2F2B mouse endothelial cells *in vitro*, which could stimulate the development of tumor vasculature.[16] This effect on endothelial cells may be related to the high content of transcripts of genes involved in the cell cycle.[50] CRC exosomes can also produce important changes in colonic mesenchymal cells (MSCs). Induced MSCs acquire an atypical morphology and high proliferation, migration, and invasion rates, characteristics that seem to reflect a transformed phenotype.[51] Similar results were found when inducing CCD-18Co normal colon cells with exosomes from HCT116 CRC, which increase their clonogenicity.[52]

It has also been reported that exosomes from the CRC mouse cell line CT26 significantly promote tumor growth and reduce the animal survival rate. The tumor-associated macrophages exhibited an M2 phenotype and were able to interact with cytokines and chemokines of the tumor microenvironment. In addition, it was found that exosomes were capable of subverting macrophages from M1 to M2, thus promoting tumor growth.[53]

Migration, invasion, and metastasis. Numerous evidences indicate that tumor cells influence other organs and tissues before invasion. These new locations are known as pre-metastatic niches. It has been suggested that exosomes derived from tumor cells play an important role in this conditioning process, which involves specific integrin expression on exosomes.[54]

Exosomes contain active proteases capable of extracellular matrix degradation and remodeling. Exosomes from pancreatic tumor cell line ASML express CD44v, a marker involved in tumor-stroma interactions that could contribute to establishing the niche.[55] CXCR4, present in exosomes of HT-29 cells, may also contribute to the metastatic microenvironment by recruiting stromal cells,[56] and as stated before, exosomes derived from SW480 and SW620 CRC increase the capacity of migration and invasion of MSCs.[29,51]

There are numerous cellular components in exosomes, which may be involved in CRC metastasis through diverse mechanisms. miR-21, highly expressed in SW480 and WiDr cell lines, inhibits the protein translation of PDCD4, promoting invasiveness and metastasis.[9,31] However, the loss of expression of members of the miR-200 family may contribute to lymphendothelial invasiveness *in vitro*.[25] Cld7 may also influence the EMT process, mobility, and invasiveness of CRC cells, especially when it is colocalized with MMP14 in the exosomal membrane. Furthermore, the recruitment of MMP2 and MMP9 by MMP14 also contributes to invasiveness.[49] And exosomes secreted by CRC cell lines (HCT116 and DLD-1) carry high levels of tissue factor, which is involved in blood coagulation, and it is a modulator of angiogenesis and metastasis processes.[57]

Tspan8 contributes to selective recruitment of CD106 and CD49d in exosomes, which are involved in binding and internalization of exosomes by endothelial cells. In addition, an abundance of MMPs and CD13 proteases in exosomes, which contain Tspan8, could facilitate the integration process. Other exosomal proteins, such as VAULT, CD71, and HSP70, seem to provide advantages for cell survival.[44] Moreover, exosomes released by hypoxic CRC cells promote the proliferation and migration of endothelial cells. These exosomes were enriched with Wnt4 that increased β-catenin nuclear translocation in endothelial cells. *In vivo* studies confirmed an enhanced tumor growth and angiogenesis.[58] The overexpression of Wnt5b is associated with cancer aggressiveness. Wntb5 is secreted within exosomes from Caco-2 cells, one of the mostly used human CRC cell lines, and stimulates cell migration and proliferation of A549 cells.[59] Mutations in adenomatous polyposis coli gene are common in CRC patients and are associated with the deregulation in Wnt signaling. When the expression of adenomatous polyposis coli is restored in SW480, cells release DKK4 through exosomes, which could be a mechanism to regulate the Wnt signaling pathway that may be lost during the progression of CRC.[60]

Interestingly, other studies, both *in vitro* and *in vivo*, have shown that exosomes from CRC cells were able to induce apoptosis in T cells when TRAIL and FasL were present, contributing to the evasion of the immune system.[61]

All the data presented earlier suggest the importance of exosomes in the development and survival of CRC cells, including cellular transformation, proliferation, preparation of pre-metastatic niches, apoptosis, invasion, and metastasis. Figure 1 illustrates some processes in which CRC exosomes could be involved in relation to metastasis.[62]

Resistance to antitumor therapy

Resistance to chemotherapy is common in patients with CRC and is usually related to the presence of cancer stem cells, but underlying mechanisms are still to be clarified. Some studies link deregulation of cellular components, such as cld7, with chemoresistance.[49]

Cetuximab (Erbitux) is a monoclonal antibody, which acts as an EGFR competitor, and it is one of the most used drugs for the treatment of metastatic advanced CRC. As a result of the interaction of Cetuximab with EGFR, there occurs a blocking of the KRAS signaling, thus reducing the proliferation of tumor cells. However, mutations in the KRAS gene in patients with CRC may lead to a permanent state of mitogenic activation independent of EGFR signaling, thus reducing the effectiveness of therapy.[63]

As mentioned earlier, exosomes also play an important role in resistance to chemotherapy. An interesting study showed that miR-145 and miR-34a were released in response to treatment with 5-fluorouracil (5-FU) into exosomes in DLD-1 cells. This mechanism would maintain low intracellular levels of both miRNAs. The authors proposed that these miRNAs may participate as tumor suppressors, contributing to uncontrolled cell growth and resistance against antitumor drugs.[23]

A correlation between the effectiveness of Trastuzumab and exosomes has been found. Trastuzumab is a monoclonal antibody that binds to HER2, a receptor that is overexpressed in some cancers, such as some subtypes of breast cancer. Exosomes are secreted by breast tumor cells over-expressing HER2. It has been found that HER2 was expressed on the exosome surface and was able to bind to Trastuzumab, reducing its therapeutic efficacy.[63] Similar results were obtained *in vitro* with Cetuximab and Caco2 and HCT-116 human CRC cell lines, because of the EGFR expression in exosomes.[64]

Drug treatment significantly alters the content of miRNAs and proteins in exosomes. MiR-192 is regulated by p53, and it is able to induce cell cycle arrest. It has been shown that miR-192 and miR-215 contribute directly to chemoresistance mechanisms of fluoropyrimidine and antifolates in CRC.[33] In addition, it has been observed that chemoresistance to 5-FU could lead to a very aggressive migratory phenotype associated with loss of miR-200.[25] Moreover, both *in vivo* and *in vitro* studies have reported that ΔNP73, an isoform of a homolog of p53 called p73, is transferred

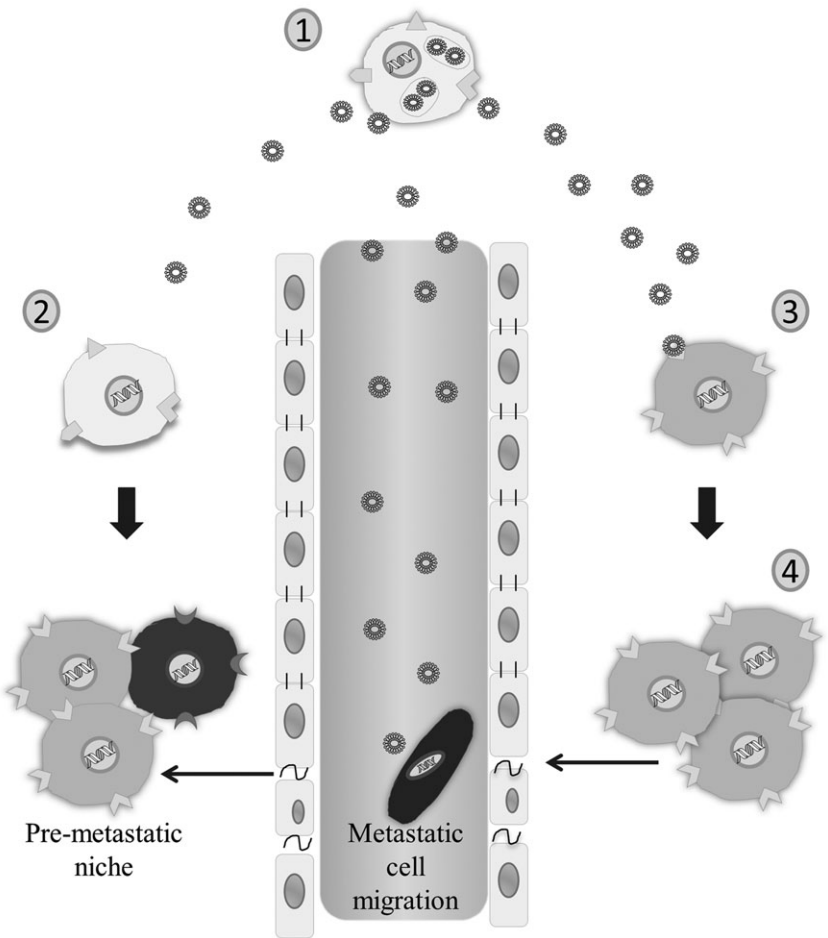

Figure 1 Suggested role of colorectal cancer (CRC) exosomes in metastasis. Exosomes produced by CRC cells (1) could affect the behavior of distant normal cells contributing to the development of pre-metastatic niches (2). Tumor metastatic cells are able to break endothelial gap-junctions, enter into the circulation, and reach pre-metastatic niches. In addition, CRC exosomes could be internalized by other tumor cells (3) and thus acquire molecular components (oncoproteins, miRNAs …) that may accelerate the disease progression and the acquisition of a metastatic phenotype by epithelial mesenchymal transition (4). Figure modified from Tovar-Camargo *et al.*[62]

by exosomes increasing CRC cell proliferation and conferring resistance to oxaliplatin.[18]

Other assays have shown that carcinoma-associated fibroblasts are intimately involved in tumor recurrence. There is evidence that exosomes derived from carcinoma-associated fibroblasts promote clonogenicity and CRC tumor growth after treatment with 5-FU or oxaliplatin.[65]

Several studies relate exosomes to antitumor therapy resistance either by the action of the cargo miRNAs on the recipient cells or by the incorporation of the antitumor drug into the exovesicle.

Exosomes as biomarkers in diagnostics

In recent years, the search for biomarkers in order to detect diseases has increased exponentially. Several studies have included miRNAs-bearing exosomes as potential biomarkers for the detection of different malignancies, including CRC.[10]

Some trials have focused on amplifying miRNAs directly from plasma or serum samples from patients,[66] although there are questions concerning their stability. Other studies have focused on isolating miRNAs from tumor exosomes, which are considered more stable, and therefore could be more reliable for use as biomarkers.[67]

An interesting study has shown that serum levels of exosomal miRNAs (miR-1229, miR-1224-5p, miR-223, let-7a, miR-150, and miR-21) were significantly higher in CRC patients compared with controls, and those levels fell after surgical resection of the tumor. The positive rates for detection of CRC were 22.7%, 31.8%, 46.6%, 50%, 55.7%, and 61.4%, respectively, and the false positive rates of these miRNAs ranged from 0% to 9%. The sensitivity of CEA and CA19.9 was 30.7% and 16%, respectively, so these miRNA might be useful as new biomarkers. Similarly, it has been found *in vitro* that these miRNAs were secreted at higher levels in CRC cell lines compared with non-tumor lines, which could make them potential biomarkers for the early detection of the disease.[34] In addition, CRC exosomes may be useful biomarkers for the detection of disease recurrence. It was found that there were significant differences in the expression of miR-17-92 cluster in poor prognosis patients with CRC in comparison with healthy patients, and among them, miR-19a has been proposed as a biomarker of CRC recurrence.[36] Other studies have focused on miR-18a, which is a highly expressed microRNAs in several types of cancers, and it is also located in the potentially oncogenic miR-17-92 cluster. It has been observed that the concentration of miR-18a is higher in plasma/serum of patients with CRC than in healthy volunteers.[35]

Other molecules have also attracted attention as CRC biomarkers. Bernhard *et al*. showed for the first time the presence of the full-length cadherin-17 in exosomes from the human colon carcinoma cell line LIM1215. They confirmed the exosomal secretion of this cadherin, which was proposed as an exosomal surface protein for immunocapture of specific CRC-derived exosomes.[68] Other researchers suggest the utility of the exosomal DNA genome because it may reflect the mutational status of tumors and because it is more stable than the aforementioned miRNAs.[27]

All the evidences described earlier endorse the importance of conducting studies aimed at identifying the content of exosomes, as this is consolidating into a very promising field for finding CRC biomarkers. Moreover, some authors suggest the analysis of the combination of the most promising biomarkers to improve specificity and sensitivity for CRC detection and prognostic evaluation.

Clinical trials

The importance of exosomes in cancer may be reflected through their appearance in recent and ongoing clinical trials. We searched in the database www.clinicaltrials.gov using the terms "exosome + cancer" those clinical trials focused on the study of exosomes and gastrointestinal tract. Interestingly, we only found one trial directed toward the analysis of the role of tumor-derived exosomes in CRC, probably due to the novelty of this field.

The Centre Oscar Lambret (Lille, France) is recruiting patients for the NCT02439008 clinical trial (March 2016). It is focused on the study of released exosomes and factors and the immune cell populations in blood before, during, and after hypofractionated high dose radiation therapy ($\geq$ 3 fractions, dose $\geq$ 9 Gy per fraction). Inclusion criteria of patients are hepatic lesion of metastatic CRC, hepatocellular carcinoma, and metastasis from melanoma or renal cancer.

Another interesting trial is related to CRC immunotherapy. It evaluated the use of exosomes derived from ascites in combination with granulocyte colony-stimulating factor in advanced CRC patients. It was found that the therapy produced a beneficial specific antitumor response of cytotoxic T lymphocytes. A similar immunotherapy approach is being assessed for non-small-cell lung cancer (NCT01159288). This trial evaluated the administration of metronomic cyclophosphamide for 3 weeks followed by vaccination with exosomes derived from tumor antigen-loaded dendritic cells.

Regarding advanced gastric cancer, the trial NCT01779583 is designed to characterize the molecular signature of tumor-derived exosomes and to correlate the level of circulating exosomes with prognosis (overall survival, progression-free survival, and overall response rate). Similarly, the pilot study NCT01344109 was aimed at identifying breast tumor-exosome markers that correlate with neoadjuvant chemotherapy response and prognosis. Unfortunately, this study was withdrawn before enrollment.

The field of study of exosomes is growing. However, we currently do not have much information of clinical utility. The running of clinical trials aimed at clarifying the practical value of the discoveries made in basic and preclinical studies is necessary.

Conclusions

Exosomes, as vehicles of intercellular communication, are increasingly close to being recognized as useful biomarkers for diagnosis and prognosis of CRC. Nucleic acids and other components of CRC exosomes isolated from non-invasive liquid biopsies could be useful markers for early CRC diagnosis and for prognostic monitoring. CRC exosomes also have potential utility as vehicles to carry genetic modulators, RNAs, proteins, and drugs to target cells for clinical application. Once secreted, exosomes can deliver their content to adjacent or distant cells and might modify gene expression, signaling, and overall functions in recipient cells. Some reports have shown that exosomes seem to be actively involved in CRC and even that inhibiting the tumor exosome release could represent a way to interfere with cancer progression

and chemo-resistance. In fact, there have been attempts to remove tumor exosomes from the bloodstream, an interesting strategy not assessed yet in a clinical context.[51] In this sense, novel technologies directed to remove or specifically block tumor exosomes from patient fluids may offer new opportunities to control the disease. In the majority of studies analyzed, exosomes are extracted from *in vitro* cultures and are used at concentrations that may be far from physiological conditions. *In vitro* models provide useful tools to achieve rapid and valuable experimental results. However, to deepen the knowledge of CRC exosomes and their clinical implications, more *in vivo* and patient studies are needed to evaluate the role of CRC exosomes in tumor propagation, interaction with the host immune system, angiogenesis and response to treatment, among other aspects.

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References

1 Ferlay J, Soerjomataram I, Dikshit R *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* 2015; **136**: E359–86.
2 Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. *Gut* 2017; **66**: 683–91.
3 Kourembanas S. Exosomes: vehicles of intercellular signaling, biomarkers, and vectors of cell therapy. *Annu. Rev. Physiol.* 2015; **77**: 13–27.
4 Carvalho J, Oliveira C. Extracellular vesicles—powerful markers of cancer evolution. *Front. Immunol.* 2015; **5**: 685.
5 Kahlert C, Kalluri R. Exosomes in tumor microenvironment influence cancer progression and metastasis. *J. Mol. Med. (Berl)* 2013; **91**: 431–7.
6 Ogorevc E, Kralj-Iglic V, Veranic P. The role of extracellular vesicles in phenotypic cancer transformation. *Radiol. Oncol.* 2013; **47**: 197–205.
7 Lässer C. Exosomes in diagnostic and therapeutic applications: biomarker, vaccine and RNA interference delivery vehicle. *Expert Opin. Biol. Ther.* 2015; **15**: 103–17.
8 Demory Beckler M, Higginbotham JN, Franklin JL *et al*. Proteomic analysis of exosomes from mutant KRAS colon cancer cells identifies intercellular transfer of mutant KRAS. *Mol. Cell. Proteomics* 2013; **12**: 343–55.
9 Chiba M, Kimura M, Asari S. Exosomes secreted from human colorectal cancer cell lines contain mRNAs, microRNAs and natural antisense RNAs, that can transfer into the human hepatoma HepG2 and lung cancer A549 cell lines. *Oncol. Rep.* 2012; **28**: 1551–8.
10 Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. *Expert Rev. Proteomics* 2009; **6**: 267–83.
11 Choi D-S, Park JO, Jang SC *et al*. Proteomic analysis of microvesicles derived from human colorectal cancer ascites. *Proteomics* 2011; **11**: 2745–51.
12 Yoon YJ, Kim OY, Gho YS. Extracellular vesicles as emerging intercellular communicasomes. *BMB Rep.* 2014; **47**: 531–9.
13 Bellingham SA, Guo BB, Coleman BM, Hill AF. Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? *Front. Physiol.* 2012; **3**: 124.
14 Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* 2002; **2**: 569–79.
15 Choi D-S, Choi D-Y, Hong BS *et al*. Quantitative proteomics of extracellular vesicles derived from human primary and metastatic colorectal cancer cells. *J. Extracell. Vesicles* 2012; **1**: 18704.
16 Ji H, Greening DW, Barnes TW *et al*. Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components. *Proteomics* 2013; **13**: 1672–86.
17 Ji H, Chen M, Greening DW *et al*. Deep sequencing of RNA from three different extracellular vesicle (EV) subtypes released from the human LIM1863 colon cancer cell line uncovers distinct miRNA-enrichment signatures. *PLoS One* 2014; **9**: e110314.
18 Soldevilla B, Rodríguez M, San Millán C *et al*. Tumor-derived exosomes are enriched in ΔNp73, which promotes oncogenic potential in acceptor cells and correlates with patient survival. *Hum. Mol. Genet.* 2014; **23**: 467–78.
19 de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M. Lipid raft-associated protein sorting in exosomes. *Blood* 2003; **102**: 4336–44.
20 Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol. Biol.* 2015; **1295**: 179–209.
21 Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. Cell Biol.* 2006 Chapter 3:Unit 3.22.
22 Cheruvanky A, Zhou H, Pisitkun T *et al*. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *Am. J. Physiol. Renal Physiol.* 2007; **292**: F1657–61.
23 Akao Y, Khoo F, Kumazaki M, Shinohara H, Miki K, Yamada N. Extracellular disposal of tumor-suppressor miRs-145 and -34a via microvesicles and 5-FU resistance of human colon cancer cells. *Int. J. Mol. Sci.* 2014; **15**: 1392–401.
24 Zeringer E, Barta T, Li M, Vlassov AV. Strategies for isolation of exosomes. *Cold Spring Harb. Protoc.* 2015; **2015**: 319–23.
25 Senfter D, Holzner S, Kalipciyan M *et al*. Loss of miR-200 family in 5-fluorouracil resistant colon cancer drives lymphendothelial invasiveness in vitro. *Hum. Mol. Genet.* 2015; **24**: 3689–98.
26 Ban J-J, Lee M, Im W, Kim M. Low pH increases the yield of exosome isolation. *Biochem. Biophys. Res. Commun.* 2015; **461**: 76–9.
27 Thakur BK, Zhang H, Becker A *et al*. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res.* 2014; **24**: 766–9.
28 Kahlert C, Melo SA, Protopopov A *et al*. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *J. Biol. Chem.* 2014; **289**: 3869–75.
29 Bigagli E, Luceri C, Guasti D, Cinci L. Exosomes secreted from human colon cancer cells influence the adhesion of neighboring metastatic cells: role of microRNA-210. *Cancer Biol. Ther.* 2016; **17**: 1062–9.
30 Chiba M, Watanabe N, Watanabe M *et al*. Exosomes derived from SW480 colorectal cancer cells promote cell migration in HepG2 hepatocellular cancer cells via the mitogen-activated protein kinase pathway. *Int. J. Oncol.* 2016; **48**: 305–12.
31 Asangani IA, Rasheed SAK, Nikolova DA *et al*. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* 2008; **27**: 2128–36.
32 Cha DJ, Franklin JL, Dou Y *et al*. KRAS-dependent sorting of miRNA to exosomes. *Elife* 2015; **4**: e07197.

33 Ju J. Implications of miRNAs in colorectal cancer chemoresistance. *Int. Drug. Discov.* 2011; **2011**: 2063.
34 Ogata-Kawata H, Izumiya M, Kurioka D *et al*. Circulating exosomal microRNAs as biomarkers of colon cancer. *PLoS One* 2014; **9**: e92921.
35 Komatsu S, Ichikawa D, Takeshita H *et al*. Circulating miR-18a: a sensitive cancer screening biomarker in human cancer. *In Vivo* 2014; **28**: 293–7.
36 Matsumura T, Sugimachi K, Iinuma H *et al*. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. *Br. J. Cancer* 2015; **113**: 275–81.
37 Guo J, Cui Y, Yan Z *et al*. Phosphoproteome characterization of human colorectal cancer SW620 cell-derived exosomes and new phosphosite discovery for C-HPP. *J. Proteome Res.* 2016; **15**: 4060–72.
38 Morgan B, Thomas AL, Drevs J *et al*. Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. *J. Clin. Oncol.* 2003; **21**: 3955–64.
39 Lydic TA, Townsend S, Adda CG, Collins C, Mathivanan S, Reid GE. Rapid and comprehensive "shotgun" lipidome profiling of colorectal cancer cell derived exosomes. *Methods* 2015; **87**: 83–95.
40 Simpson RJ, Kalra H, Mathivanan S. ExoCarta as a resource for exosomal research. *J. Extracell. Vesicles* 2012; **1**: 18374.
41 Fares J, Kashyap R, Zimmermann P. Syntenin: key player in cancer exosome biogenesis and uptake? *Cell Adh. Migr.* 2017; **11**: 124–6.
42 Clague MJ, Liu H, Urbé S. Governance of endocytic trafficking and signaling by reversible ubiquitylation. *Dev. Cell* 2012; **23**: 457–67.
43 Roucourt B, Meeussen S, Bao J, Zimmermann P, David G. Heparanase activates the syndecan-syntenin-ALIX exosome pathway. *Cell Res.* 2015; **25**: 412–28.
44 Nazarenko I, Rana S, Baumann A *et al*. Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. *Cancer Res.* 2010; **70**: 1668–78.
45 Rana S, Yue S, Stadel D, Zöller M. Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. *Int. J. Biochem. Cell Biol.* 2012; **44**: 1574–84.
46 Villarroya-Beltri C, Gutiérrez-Vázquez C, Sánchez-Cabo F *et al*. Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat. Commun.* 2013; **4**: 2980.
47 De Wever O, Pauwels P, De Craene B *et al*. Molecular and pathological signatures of epithelial-mesenchymal transitions at the cancer invasion front. *Histochem. Cell Biol.* 2008; **130**: 481–94.
48 Greening DW, Gopal SK, Mathias RA *et al*. Emerging roles of exosomes during epithelial-mesenchymal transition and cancer progression. *Semin. Cell Dev. Biol.* 2015; **40**: 60–71.
49 Philip R, Heiler S, Mu W, Büchler MW, Zöller M, Thuma F. Claudin-7 promotes the epithelial-mesenchymal transition in human colorectal cancer. *Oncotarget* 2015; **6**: 2046–63.
50 Hong BS, Cho J-H, Kim H *et al*. Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells. *BMC Genomics* 2009; **10**: 556.
51 Lugini L, Valtieri M, Federici C *et al*. Exosomes from human colorectal cancer induce a tumor-like behavior in colonic mesenchymal stromal cells. *Oncotarget* 2016; **7**: 50086–98.
52 Mulvey HE, Chang A, Adler J *et al*. Extracellular vesicle-mediated phenotype switching in malignant and non-malignant colon cells. *BMC Cancer* 2015; **15**: 571.
53 Peng B, Hu J, Zhang H, Xi J. Exosomes from CT26 cells promote tumor progression by subverting macrophage from M1 to M2 mediated partially by interleukin-4. *Biomedica* 2014; **30**: 1–7.
54 Pancione M, Giordano G, Remo A *et al*. Immune escape mechanisms in colorectal cancer pathogenesis and liver metastasis. *J. Immunol. Res.* 2014; **2014**: 686879.
55 Mu W, Rana S, Zöller M. Host matrix modulation by tumor exosomes promotes motility and invasiveness. *Neoplasia* 2013; **15**: 875–587.
56 Wang X, Ding X, Nan L *et al*. Investigation of the roles of exosomes in colorectal cancer liver metastasis. *Oncol. Rep.* 2015; **33**: 2445–53.
57 Yu JL, May L, Lhotak V *et al*. Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. *Blood* 2005; **105**: 1734–41.
58 Huang Z, Feng Y. Exosomes derived from hypoxic colorectal cancer cells promotes angiogenesis through Wnt4 induced β-catenin signaling in endothelial cells. *Oncol. Res.* 2017; **25**: 651–61.
59 Harada T, Yamamoto H, Kishida S *et al*. Wnt5b-associated exosomes promote cancer cell migration and proliferation. *Cancer Sci.* 2017; **108**: 42–52.
60 Lim JWE, Mathias RA, Kapp EA *et al*. Restoration of full-length APC protein in SW480 colon cancer cells induces exosome-mediated secretion of DKK-4. *Electrophoresis* 2012; **33**: 1873–80.
61 Huber V, Fais S, Iero M *et al*. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology* 2005; **128**: 1796–804.
62 Tovar-Camargo OA, Toden S, Goel A. Exosomal microRNA biomarkers: emerging frontiers in colorectal and other human cancers. *Expert Rev. Mol. Diagn.* 2016; **16**: 553–67.
63 Villagrasa A, Álvarez PJ, Osuna A, Garrido JM, Aránega A, Rodríguez-Serrano F. Exosomes derived from breast cancer cells, small trojan horses? *J. Mammary Gland Biol. Neoplasia* 2014; **19**: 303–13.
64 Ragusa M, Statello L, Maugeri M *et al*. Highly skewed distribution of miRNAs and proteins between colorectal cancer cells and their exosomes following Cetuximab treatment: biomolecular, genetic and translational implications. *Oncoscience* 2014; **1**: 132–57.
65 Hu Y, Yan C, Mu L *et al*. Fibroblast-derived exosomes contribute to chemoresistance through priming cancer stem cells in colorectal cancer. *PLoS One* 2015; **10**: e0125625.
66 Menéndez P, Villarejo P, Padilla D, Menéndez JM, Montes JAR. Diagnostic and prognostic significance of serum microRNAs in colorectal cancer. *J. Surg. Oncol.* 2013; **107**: 217–20.
67 Köberle V, Pleli T, Schmithals C *et al*. Differential stability of cell-free circulating microRNAs: implications for their utilization as biomarkers. *PLoS One* 2013; **8**: e75184.
68 Bernhard OK, Greening DW, Barnes TW, Ji H, Simpson RJ. Detection of cadherin-17 in human colon cancer LIM1215 cell secretome and tumour xenograft-derived interstitial fluid and plasma. *Biochim. Biophys. Acta* 1834; **2013**: 2372–9.