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REVIEW

Common molecular markers between circulating tumor cells and blood exosomes in colorectal cancer: a systematic and analytical review

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Abstract: Nearly half of patients with colorectal cancer (CRC), the third leading cause of cancer deaths worldwide, are diagnosed in the late stages of the disease. Appropriate treatment is not applied in a timely manner and nearly 90% of the patients who experience metastasis ultimately die. Timely detection of CRC can increase the five-year survival rate of patients. Existing histopathological and molecular classifications are insufficient for prediction of metastasis, which limits approaches to treatment. Detection of reliable cancer-related biomarkers can improve early diagnosis, prognosis, and treatment response prediction and recurrence risk. Circulating tumor cells (CTCs) and exosomes in peripheral blood can be used in a liquid biopsy to assess the status of a tumor. Exosomes are abundant and available in all fluids of the body, have a high half-life and are released by most cells. Tumor-derived exosomes are released from primary tumors or CTCs with selective cargo that represents the overall tumor. The current systematic review highlights new trends and approaches in the detection of CRC biomarkers to determine tumor signatures using CTC and exosomes. When these are combined, they could be used to guide molecular pathology and can revolutionize detection tools. Relevant observational studies published until July 24, 2019 which evaluated the expression of tumor markers in CTCs and exosomes were searched in PubMed, Scopus, Embase, and ISI Web of Science databases. The extracted biomarkers were analyzed using String and EnrichR tools.

Keywords: colorectal cancer, circulating tumor cell, CTC, exosomes, diagnosis, prognosis, biomarker, systematic review

Introduction

Colorectal cancer (CRC) is the third highest cause of cancer deaths worldwide.[1,2] The time of diagnosis directly influences the overall survival rate of patients. The five-year survival rates are estimated to decrease 12.5% after the occurrence of metastasis vs for localized cancer. Histological examination of tumor tissue is the gold standard for diagnosis, but is invasive, time-consuming, and nonrepeatable over time. There is a need for new methods that are simple, non-invasive, and inexpensive to provide clear clinical evidence and improve early detection or predict a response to treatment.[3,4]

Serum biomarkers such as carcinoembryonic antigens (CEAs) and carbohydrate antigen 19-9 (CA19-9) along with multi-target stool DNA tests represent the concrete implementation of non-invasive methods for CRC screening[5,6] There is urgent need for more reliable molecular markers that demonstrate the heterogeneity of cancer

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cells during progression. The use of biological fluids as sources of nucleic acid-biomarkers for liquid biopsies in oncology has clinical promise[7,8] Molecular characterization of cancer signatures also can provide relevant information for personalized treatment of tumors.[9,10] Circulating tumor cells (CTCs) and exosomes are shed from a tumor mass and enter the bloodstream. They can provide a metastatic niche for the invasion and migration of a tumor, so detection of their markers is critical.[11]

Ashworth et al, first identified CTCs as valuable indicators of cancer progression.[12] CTCs detach from the primary tumor, intravasate into the bloodstream, evade immune detection, survive and extravasate into the microvessels of target tissue to establish a micro-metastatic niche.[13] They have been identified in many cancers, including colon cancer. CTCs in the bloodstream may exist as single cells with a different EMT phenotypes or as clusters that bind to platelets or macrophages or are reactivated as stromal cells.[14,15] The presence and number of CTCs before and during treatment are a strong independent predictor of shorter progression-free survival and overall survival of CRC patients.[16] In spite of their advantages, researchers believe that the most challenging obstacles related to research on CTCs are their extremely low numbers, short lifetimes, fragility, and their heterogeneity and plasticity. The investigation of specific and reliable markers for their detection or isolation is an undeniable issue.[17]

Extracellular vesicles (EVs) generally include microvesicles (100–350 nm), apoptotic bodies (500–1000 nm), and exosomes (30–150 nm).[18] Exosomes are nanovesicles with membrane-bound phospholipids which introduced and confirmed by Pan et al,[19] and are actively secreted by mammalian cells into body fluids such as urine, plasma, and saliva. Exosomal cargo includes lipids, proteins, DNA, and RNA (mRNA, miRNA, long non-coding RNA) that are selected according to their roles. Exosomes involved in many biological processes, especially intercellular communication, establish a premetastatic niche by carrying oncogenic elements that suppress host immune responses.[20]

Exosomes are abundant, have high half-lives and are released by most cells. This is in contrast with CTCs, which are tumor specific, rare, fragile, have a short life and are difficult to isolate. It is possible to design a molecular marker common between the exosomes and CTCs for better understanding of the metastasis process. American Society of Clinical Oncology suggests circulating exosomes may provide an alternative platform for monitoring disease progression as opposed to CTCs.[21] Several ongoing studies have aimed at quantifying a stress protein or other biomarkers in the blood and urine for monitoring and early diagnosis of malignant solid tumors (https://clinicaltrials.gov). The current analytical review is the first to explore similar molecular mechanisms and pathways between CTCs and Exosomes. In this systematic review, all molecular mechanisms that can potentially apply to the diagnosis and prognosis of CRC using CTCs and exosomes are discussed.

Materials and methods

Search strategy for literature mining

Observational studies evaluating the expression of circulating CRC cells and exosomes markers from 1980 to July 24, 2019 were electronically searched for in the PubMed, Scopus, Embase, and ISI Web of Science databases. The search syntax was modified for each database in accordance with their rules, the Mesh terms and keywords as listed in detail in Table 1.

The authors (S. Vafaei and F. Fattahi) searched and identified eligible studies and excluded all irrelevant articles after reviewing the publication titles and abstracts. Duplicate publications were excluded. Discrepancies were resolved between the two reviewers by consensus and by consulting the other authors. Next, the full text of the selected publications was retrieved and fully reviewed. This systematic review has been carried out in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses.[22]

Publication inclusion criteria

The inclusion criteria for this systematic review followed the criteria of population, intervention, control, and outcomes. Observational studies (case-control) investigating CTC and exosomes mRNA and gene markers for the diagnosis and prognosis of CRC patient samples were included if they met the following criteria:

1. The article must be published in English and the full text must be available.
2. Studies included those on CRC patient blood samples and human blood for CTC, although tissue or cell lines for exosomes were done because exosomes research is rare and in its initial stages.
3. Expression of mRNA and gene markers in patient specimens or cell lines was detected by established molecular methods.

Table 1 Search strategy of CTC and exosome in colorectal cancer

Search strategy		No. of papers
		2019 24 July
SCOPUS		
1	(TITLE-ABS-KEY (cecum OR colon OR sigmoid OR rectum OR anal)) AND (TITLE-ABS-KEY ((neoplasm OR cancer OR tumor OR tumors OR carcinoma))) OR (TITLE-ABS-KEY ((colorectal AND neoplasms OR crc)))	258,569
2	(TITLE-ABS-KEY (circulating AND tumor AND cell)) OR (TITLE-ABS-KEY (circulating AND neoplastic AND cells)) OR (TITLE-ABS-KEY (neoplasm AND micro-metastasis)) OR (TITLE-ABS-KEY (ctc OR ctm OR dtc))	60,012
3	((TITLE-ABS-KEY (gene AND expression AND profiling)) OR (TITLE-ABS-KEY (messenger AND rna)) OR (TITLE-ABS-KEY (rna OR transcriptome OR mrna))) AND ((TITLE-ABS-KEY (early AND diagnosis)) OR (TITLE-ABS-KEY (early AND detection)) OR (TITLE-ABS-KEY (prognosis OR diagnosis OR biomarkers OR screening OR diagnostic OR prognosis OR prognostic)))	209,207
4	(TITLE-ABS-KEY (extracellular AND vesicle)) OR (TITLE-ABS-KEY (cell-derived AND microparticles)) OR (TITLE-ABS-KEY (extracellular AND vesicles)) OR (TITLE-ABS-KEY (ev OR microvesicle OR exosomes))	274,615
1 & 2 & 3	(((TITLE-ABS-KEY (gene AND expression AND profiling)) OR (TITLE-ABS-KEY (messenger AND rna)) OR (TITLE-ABS-KEY (rna OR transcriptome OR mrna))) AND ((TITLE-ABS-KEY (early AND diagnosis)) OR (TITLE-ABS-KEY (early AND detection)) OR (TITLE-ABS-KEY (prognosis OR diagnosis OR biomarkers OR screening OR diagnostic OR prognosis OR prognostic)))) AND ((TITLE-ABS-KEY (circulating AND tumor AND cell)) OR (TITLE-ABS-KEY (circulating AND neoplastic AND cells)) OR (TITLE-ABS-KEY (neoplasm AND micrometastasis)) OR (TITLE-ABS-KEY (ctc OR ctm OR dtc))) AND ((TITLE-ABS-KEY (cecum OR colon OR sigmoid OR rectum OR anal)) AND (TITLE-ABS-KEY ((neoplasm OR cancer OR tumor OR tumors OR carcinoma))) OR (TITLE-ABS-KEY ((colorectal AND neoplasms OR crc)))) AND (LIMIT-TO (LANGUAGE, "English")) AND (LIMIT-TO (SRCTYPE, "j")) AND (LIMIT-TO (DOCTYPE, "ar") OR LIMIT-TO (DOCTYPE, "no") OR LIMIT-TO (DOCTYPE, "le")) AND (LIMIT-TO (EXACTKEYWORD, "Human"))	118
1 & 2 & 4	(((TITLE-ABS-KEY (gene AND expression AND profiling)) OR (TITLE-ABS-KEY (messenger AND rna)) OR (TITLE-ABS-KEY (rna OR transcriptome OR mrna))) AND ((TITLE-ABS-KEY (early AND diagnosis)) OR (TITLE-ABS-KEY (early AND detection)) OR (TITLE-ABS-KEY (prognosis OR diagnosis OR biomarkers OR screening OR diagnostic OR prognosis OR prognostic)))) AND ((TITLE-ABS-KEY (extracellular AND vesicle)) OR (TITLE-ABS-KEY (cell-derived AND microparticles)) OR (TITLE-ABS-KEY (extracellular AND vesicles)) OR (TITLE-ABS-KEY (ev OR microvesicle OR exosomes))) AND ((TITLE-ABS-KEY (cecum OR colon OR sigmoid OR rectum OR anal)) AND (TITLE-ABS-KEY ((neoplasm OR cancer OR tumor OR tumors OR carcinoma))) OR (TITLE-ABS-KEY ((colorectal AND neoplasms OR crc)))) AND (LIMIT-TO (DOCTYPE, "ar") OR LIMIT-TO (DOCTYPE, "ip")) AND (LIMIT-TO (EXACTKEYWORD, "Human")) AND (LIMIT-TO (LANGUAGE, "English")) AND (LIMIT-TO (SRCTYPE, "j"))	37
PUBMED		
1	((Colorectal Neoplasms[Title/Abstract] OR "Colorectal Neoplasms"[Mesh] OR CRC[Title/Abstract]) OR (("Cecum"[Mesh] OR "Colon"[Mesh] OR "Colon, Sigmoid"[Mesh] OR "Rectum"[Mesh] OR "Anal Canal"[Mesh]) AND ("Neoplasms"[Mesh] OR "Carcinoma"[Mesh])) OR ((cecum[Title/Abstract] OR colon[Title/Abstract] OR sigmoid[Title/Abstract] OR rectum[Title/Abstract] OR anus[Title/Abstract]) AND (neoplasm[Title/Abstract] OR cancer[Title/Abstract] OR tumor[Title/Abstract] OR tumors[Title/Abstract] OR carcinoma[Title/Abstract]))	251,819
2	("Neoplastic Cells, Circulating"[Mesh] OR Circulating Tumor Cell[Title/Abstract] OR "Neoplasm Micrometastasis"[Mesh] OR CTC[Title/Abstract] OR CTM[Title/Abstract] OR DTC[Title/Abstract]	20,001
3	("Prognosis"[Mesh] OR "Diagnosis"[Mesh] OR "Early Diagnosis"[Mesh] OR "Early Detection of Cancer"[Mesh] OR "Biomarkers, Tumor"[Mesh]) OR ("screening"[Title/Abstract] OR "early detection"[Title/Abstract] OR "Diagnosis"[Title/Abstract] OR "Diagnostic"[Title/Abstract] OR "Prognosis"[Title/Abstract] OR "Prognostic"[Title/Abstract]) AND ("RNA, Messenger"[Mesh] OR "RNA"[Mesh] OR "Transcriptome"[Mesh] OR "Gene Expression Profiling"[Mesh] OR "mRNA" OR "RNA" OR "Transcriptome" OR "gene expression profiling")	376,269
4	("extracellular vesicles"[Mesh] OR "Cell-Derived Microparticles"[Mesh] OR "EV" OR "microvesicle" OR "extracellular vesicle" OR "Exosomes"[Mesh] OR Exosome)	41,831

(*Continued*)

Table 1 (Continued).

Search strategy		No. of papers
		2019 24 July
1 & 2 & 3	Search ((((((Colorectal Neoplasms[Title/Abstract] OR "Colorectal Neoplasms"[Mesh] OR CRC[Title/Abstract]) OR (("Cecum"[Mesh] OR "Colon"[Mesh] OR "Colon, Sigmoid"[Mesh] OR "Rectum"[Mesh] OR "Anal Canal"[Mesh]) AND ("Neoplasms"[Mesh] OR "Carcinoma"[Mesh])) OR ((cecum[Title/Abstract] OR colon[Title/Abstract] OR sigmoid[Title/Abstract] OR rectum[Title/Abstract] OR anus[Title/Abstract]) AND (neoplasm[Title/Abstract] OR cancer[Title/Abstract] OR tumor[Title/Abstract] OR tumors[Title/Abstract] OR carcinoma[Title/Abstract])))) AND (("Neoplastic Cells, Circulating"[Mesh] OR Circulating Tumor Cell[Title/Abstract] OR "Neoplasm Micrometastasis"[Mesh] OR CTC[Title/Abstract] OR CTM[Title/Abstract] OR DTC[Title/Abstract])) AND ((("Prognosis"[Mesh] OR "Diagnosis"[Mesh] OR "Early Diagnosis"[Mesh] OR "Early Detection of Cancer"[Mesh] OR "Biomarkers, Tumor"[Mesh]) OR ("screening"[Title/Abstract] OR "early detection"[Title/Abstract] OR "Diagnosis"[Title/Abstract] OR "Diagnostic"[Title/Abstract] OR "Prognosis"[Title/Abstract] OR "Prognostic"[Title/Abstract]) AND ("RNA, Messenger"[Mesh] OR "RNA"[Mesh] OR "Transcriptome"[Mesh] OR "Gene Expression Profiling"[Mesh] OR "mRNA" OR "RNA" OR "Transcriptome" OR "gene expression profiling")))) Filters: Humans; English	164
1 & 2 &4	Search (((((((Colorectal Neoplasms[Title/Abstract] OR "Colorectal Neoplasms"[Mesh] OR CRC[Title/Abstract]) OR (("Cecum"[Mesh] OR "Colon"[Mesh] OR "Colon, Sigmoid"[Mesh] OR "Rectum"[Mesh] OR "Anal Canal"[Mesh]) AND ("Neoplasms"[Mesh] OR "Carcinoma"[Mesh])) OR ((cecum[Title/Abstract] OR colon[Title/Abstract] OR sigmoid[Title/Abstract] OR rectum[Title/Abstract] OR anus[Title/Abstract]) AND (neoplasm[Title/Abstract] OR cancer[Title/Abstract] OR tumor[Title/Abstract] OR tumors[Title/Abstract] OR carcinoma[Title/Abstract])))) AND ((("Prognosis"[Mesh] OR "Diagnosis"[Mesh] OR "Early Diagnosis"[Mesh] OR "Early Detection of Cancer"[Mesh] OR "Biomarkers, Tumor"[Mesh]) OR ("screening"[Title/Abstract] OR "early detection"[Title/Abstract] OR "Diagnosis"[Title/Abstract] OR "Diagnostic"[Title/Abstract] OR "Prognosis"[Title/Abstract] OR "Prognostic"[Title/Abstract]) AND ("RNA, Messenger"[Mesh] OR "RNA"[Mesh] OR "Transcriptome"[Mesh] OR "Gene Expression Profiling"[Mesh] OR "mRNA" OR "RNA" OR "Transcriptome" OR "gene expression profiling")))) AND ((("extracellular vesicles"[Mesh] OR "Cell-Derived Microparticles"[Mesh] OR "EV" OR "microvesicle" OR "extracellular vesicle" OR "Exosomes"[Mesh] OR Exosome))))) Filters: Humans; English	66
Embase		
1	(cecum OR sigmoid OR rectum OR anal) AND (neoplasm OR cancer OR tumor OR tumors OR carcinoma) OR "colorectal cancer" OR crc	323,384
2	ctc OR ctm OR dtc OR (circulating AND neoplastic AND cells) OR (circulating AND tumor AND cell) OR (neoplasm AND "micro-metastasis")	54,423
3	(early AND diagnosis) OR (early AND detection) OR biomarkers OR screening OR diagnostic OR prognosis OR prognostic) AND (messenger AND rna) OR (gene AND expression AND profiling) OR mrna OR transcriptome	101,305
4	"membrane microparticle" OR "exosome"	25,614
1 & 2 & 3	#1 AND #2 AND #3 AND ([article]/lim OR [article in press]/lim OR [letter]/lim OR [note]/lim) AND [english]/lim AND [humans]/lim AND [embase]/lim	135
1 & 2 & 4	#1 AND #2 AND #4 AND ([article]/lim OR [article in press]/lim OR [letter]/lim OR [note]/lim) AND [english]/lim AND [humans]/lim AND [embase]/lim	52
Web of Science		
1	TI=(Cecum OR Colon OR Colon Sigmoid OR Rectum OR Anal) AND (neoplasm OR cancer OR tumor OR tumors OR carcinoma) OR TI=(Colorectal Neoplasms OR CRC)	43,039
2	TS=(Circulating Neoplastic Cells OR Circulating Tumor Cell OR Neoplasm Micrometastasis OR CTC OR CTM OR DTC)	44,339
3	TS=(Prognosis OR Diagnosis OR Early Diagnosis OR Early Detection OR Biomarkers OR screening OR Diagnostic OR Prognosis OR Prognostic) AND TS=(Messenger RNA OR RNA OR Transcriptome OR Gene Expression Profiling OR mRNA)	138,133
4	TS=(extracellular vesicles OR Cell-Derived Microparticles OR EV OR microvesicle OR extracellular vesicle OR Exosomes)	212,089

(*Continued*)

Table 1 (Continued).

Search strategy		No. of papers
		2019 24 July
1 & 2 & 3	#1 AND #2 AND #3 (#8 AND #7 AND #3) AND LANGUAGE: (English) AND DOCUMENT TYPES: (Article)	19
1 & 2 & 4	#1 AND #2 AND #4 AND (#8 AND #7 AND #3) AND LANGUAGE: (English)	15

Abbreviation: CTC, Circulating tumor cells.

4. Studies demonstrated the correlation between mRNA profiling using isolation, detection, or validation methods, included sample type and size and other clinical parameters of diagnosis and prognosis, tumor stage and the frequency of estimated marker expression.
5. Study characteristics (first author surname, publication year, and study design) were included.

Publication exclusion criteria

Exclusion criteria included:

1. Evidence and article on CTC and exosomes covering review articles, seminars, letters, expert opinions, book chapters, meeting records, commentaries, and clinical guidelines.
2. In-vitro or in-vivo experimental studies.
3. Articles that were not published in English.
4. Full text of the article not available.

Exclusion criteria for CTC articles were:

1. Studies performed only on cell lines or tissue samples.
2. Studied housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase, actin beta, β2-microglobulin, as they are not specific markers for CTC detection and expressed in all cells.
3. Bioinformatics analysis or data mining without experimental confirmation of the introduced biomarkers.
4. Therapy gaudiness based on the CTC results (perioperative and postoperative) in predicting the clinical outcome, not counting for drug effect on the expression of CTC genes.
5. The study only tested the spiked cell lines in human blood donors and not the actual patients.

In exosome studies, because of the limited data, we reviewed all articles on all markers that were introduced using the cell lines, tissue, or blood, even those only introduced through bioinformatics means without experimental confirmation.

Risk of bias (quality) assessment

The quality of each study was assessed using the Newcastle–Ottawa Scale (NOS), a well-known scale for assessing the quality and risk of bias in observational studies.[23] NOS gives a score between 0 (minimum) and 9 (maximum). Studies with a NOS score >6 were considered to be of high quality, making them possible for use as potential moderators in meta-regression analysis.

Statistical analysis

Because the studies included were not sufficiently similar in terms of study design, experimental techniques, and heterogeneity of genetic variants, a meta-analysis was not performed.

Bioinformatics approach to systematic search

Molecular pathology is a valuable tool in the development of a cancer signature. The initially extracted markers in this article were subjected to STRING (https://string-db.org/) for better understanding of the significantly related pathway and secondary data were enriched using the EnrichR (amp.pharm.mssm.edu/Enrichr/) web tool. The GO project provided ontologies to describe the attributes of the gene products in the non-overlapping domains of molecular biology. Molecular function describes activities (such as catalytic or binding activities) at the molecular level. Biological processes describe biological goals accomplished by one or more ordered assemblies of molecular functions. Cellular component describes the locations of subcellular structures and macromolecular complexes.[24]

Results

Literature

The initial search retrieved a total of 607 studies using the search strategy. After primary selection, 497 papers were

excluded because they were duplicates, had irrelevant titles or were paper abstracts. Eventually, 110 studies were selected for further evaluation. The schematic of the design and the reasons for exclusions are summarized in Figures 1 and 2 for CTC and exosomes, respectively.

Clinical applications of CTCs and exosomes in CRC as diagnostic markers

CTCs

Antigen expression of circulating cells and their specific phenotypes affects the progression of cancer and patient survival; thus, the focus was on CTC molecular markers that could lead to the detection of CTC rather than isolation in blood samples. CTC detection methods included real-time polymerase chain reaction (RT-PCR), flow cytometry, fluorescence in situ hybridization, and immunocytochemistry. Isolation methods included Cellsearch, OncoQuick, Filration, magnetic-activated cell sorting, fluorescence-activated cell sorting, Adnatest Colon Cancer Select and Detect, CELLection electrophoresis assay, and microfluidic devices.

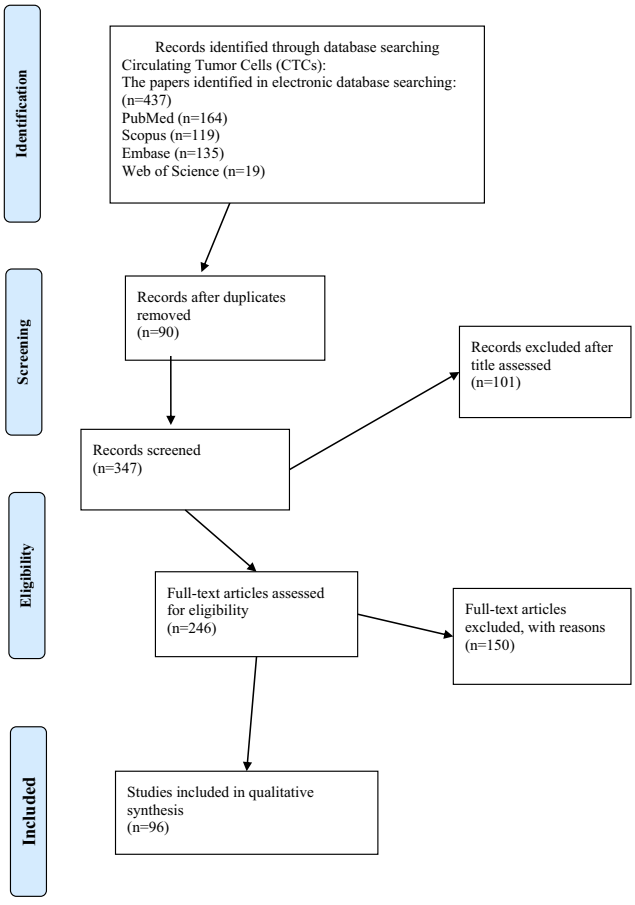

Figure 1 Design of PRISMA flow diagram explaining details of our search process was applied during the article selection for circulating tumor cell.

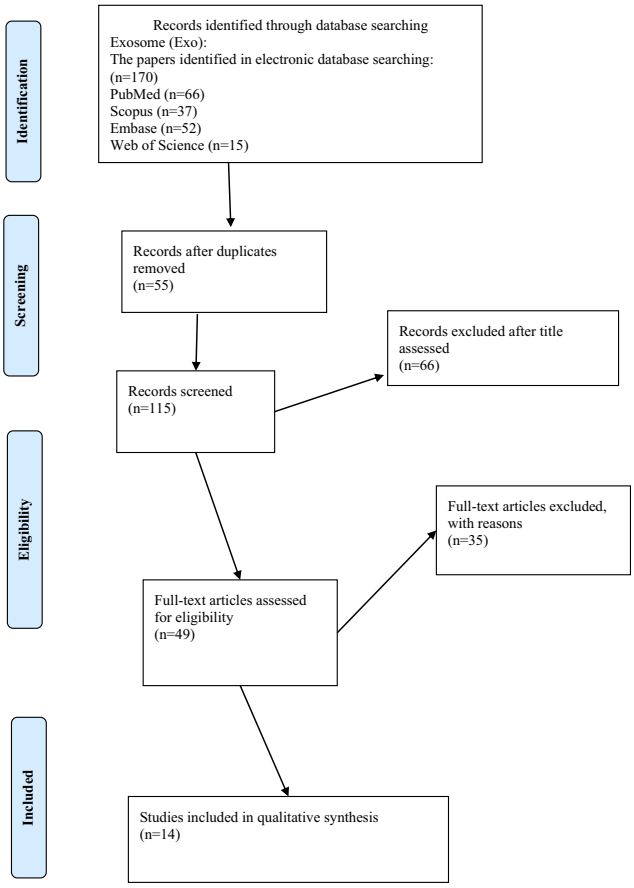

Figure 2 Design of PRISMA flow diagram explained details of our search process that applied during the article selection for Exosome.

When attempting to find more reliable markers for CTCs in CRC cases, 6 out of 39 articles described only CK20 mRNA as the target gene, which is not transcribed in normal hematopoietic cells. It has previously been reported through immunohistochemistry by Moll et al,[25–30] and has been seen in control blood samples through sensitivity assay and sampling,[29] in addition to CK20, CA19-9, and CEA, which is used in clinics routinely for CRC detection, also has been introduced as a marker of CTC in CRC. Six of 39 studied examined CEA alone[31–36] or in association with markers such as CK19,[37,38] anti-epithelial cell adhesion molecule (EPCAM),[39–43] and transmission electron microscopy (TEM)-8.[44]

Wong et al, used a sensitivity assay for the detection of CTCs and nodal metastases using CD44 splice variants as a tumor marker.[45] It has been proven that RT-PCR in combination with positive isolation of epithelial tumor cells (addition of Ber-EP4

immunomagnetic) and negative isolation of non-epithelial cells (CD45 immunomagnetic beads used to deplete leukocytes from MNC) could improve detection.[30,36] Guanylyl-cyclase C (GCC) is another marker introduced to detect rare epithelial circulating metastatic cancer cells.[46–48]

After 2004, researchers focused on multi-marker panels in literature or data mining as listed in Table 2.[49–56] Besides these, novel markers such as serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), SERPINB5,[57] epidermal growth factor receptor (EGFR),[58–60] epithelial cell transforming sequence 2 oncogene (ECT2)[61] FAM172A,[62] A3 receptor[63] have been examined as well as other markers, especially through bioinformatics analysis.[64]

Exosomes

Exosome isolation methods consisted of ultracentrifugation, commercial kits, and a combination of several methods based on their physical, chemical, immunological, and molecular markers. Characterization of exosomes was also achieved based on morphology, such as with scanning electron microscopy and TEM, based on size, such as with dynamic light-scattering and nanoparticle tracking assay or based on molecular profiling through conventional enzyme-linked immunosorbent assay, polymerase chainreaction, and Western blotting.

Exosomes carry molecular markers such as DNA, RNA, and proteins. Many reports indicate that exosomes contain miRNAs;[65–68] moreover, blood EVs contain a substantial fraction of intact mRNAs[69–72] and a large number of assembling spliced junctions-circRNAs[73] and long non-coding RNAs.[72,74,75] Exosomal proteins belong to the following functional groups: tetraspanins, including CD63 antigen (CD63), CD9 antigen (CD9), CD81 antigen (CD81), heat shock proteins (HSC70 and HSC90), and endosomal sorting complexes required for transport proteins such as Alix and TSG101, found in a wide range of exosomes.[76] The size of the extra vesicles varied and could influence gene expression. Larger vesicles (<100 nm) exhibited the greatest amount of EPCAM in extracted exosomes of HCT116 (CRC cell line) cells.[77] The level of glypican-1 was evaluated in exosomes of patients before and after surgical treatment.[78]

KRTAP5-4 and MAGEA3 mRNA in the serum of patients could be used as diagnostic biomarkers to detect CRC.[79] Ct-OATP1B3 mRNA was present in EVs derived from HCT116, HT-29, and SW480 cells that were declared to be serum-based CRC biomarkers,[80] Huang et al, introduced UBC, H3F3A, HIST2H2AA3, AKT3, and HSPA1B as hub genes in bioinformatic analysis to serve as diagnostic markers and therapeutic targets of CRC in the future.[81] Table 3 shows all of these results.

Clinical applications of CTCs and exosomes in CRC as prognostic markers

CTCs

Many researchers had discovered prognostic markers related to CRC as a beneficial tool for the detection of CTC. Five papers reported only CK20-positive as a prognostic marker. It caused significantly shorter survival in patients than the CK 20-negative marker.[82–86] However, some studies emphasized only on CEA as a marker (five articles)[87–91] and several studies also introduced both CK20 and CEA as prognostic markers.[92–96] In most articles, CK20 and/or CEA were accompanied by markers such as CK19,[97–102] GCC,[96,103,104] Prominin 1 (CD133),[95,100,105,106] EPCAM,[107–109] survivin,[110,111] ProtM,[112] mucin 1 (MUC 1),[105] and mucin 2 (MUC 2),[99] and telomerase reverse transcriptase (hTERT).[101,113,114]

Douard et al, showed that the expression of carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5; formerly CEA)[102,115] and CEACAM7 (formerly CGM2)[115,116] was more sensitive than use of a single marker in detecting CTCs, in contrast to the other studies, Bessa et al, showed that assessment of CTCs using RT-PCR CEA before surgery does not have prognostic value for CRC patients.[117]

Some articles examined markers that had been investigated previously, such as EGFR,[107,118–121] Plastin3),[122,123] anterior gradient-2,[102,124,125] leucine-rich repeat-containing-G-protein-coupled receptor 5,[102,109,126–128] double cortin-like kinase 1,[109,127] twist family bHLH transcription factor 1,[110,129] and aldehyde dehydrogenase 1[105,129] as prognostic markers in CRC through CTC.

Gradilone et al, assessed CK19 (75%), CK20 (8%), and EGFR (25%) expression in CTCs of some malignant tumors, including CRC samples, by RT-PCR followed by southern blot hybridization. They reported no correlation between prognostic values of CTCs and clinical manifestations of CRC.[130]

Histone-like protein (HLM),[120] tenascin C,[121] aquaporin (AQP5),[131] plakophilin 3, tyrosinase, prostate-specific antigen),[132] universal MAGE-A,[133] disheveled segment polarity protein 1 (DVL1),[134] CD47,[135] and

Table 2 The biomarkers which worked for diagnostic of CRC in circulating tumor cells

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/ year	CTCs positive rate	PMID
CK20	Nested RT-PCR[1]	SW1 116, HT29 cell spiking	–	5 mL	57 patients, 2 controls[2]/Blood	I–IV[3]	Soeth, 1996.[28]	35%	8,797,868
	Nested RT-PCR	A818-4 cell spiking	–	5 mL	39 patients, 12 controls/Blood	I–IV	Soeth, 1997.[27]	24%	9,242,433
	RT-PCR	HT29 cell spiking, Immunohistochemistry	PBGD	5–10 m	30 patients, 16 controls/Blood	I–IV	Vlems, 2002.[29]	30%	12,032,226
	CD45 Immune magnetic beads,/or Ber-EP4 immuno magnetic beads	LS174T cell spiking		5 mL	40 patients, 10 controls/Blood	A–D Dukes[3]	Guo, 2005.[30]	80.0%, 82.5%, 72.5%	16,048,578
	RT-PCR	–	–	5–10 mL	58 patients, 12 controls (abnormal)/ Blood	A–C Dukes	Zhang, 2005.[25]	44.8% to 69.0%	15,637,763
	RT-PCR		CEA, CK19	15 mL	57/Blood	A–D Dukes	Katsumata, 2006.[26]	42.1%	17,058,136
CEA	CD45 Immune magnetic beads and/ or Ber-EP4 immuno magnetic beads	LS174T cell spiking		5 mL	25 patients, 10 controls/Blood	A–D Dukes	Guo, 2004.[36]	25.0%, 83.3%, 88.9%	15,490,093
	RT-PCR	Southern blotting, Colo201, HT1 16, HT29, and HT115 cell spiking	–	14 mL	31 patients, 22 controls/Blood	Liver metastasis	Jonas, 1996.[31]	58%	9,014,772
	RT-PCR	Cell spiking	–	10 mL	95 patients, 11 controls/Blood	I–IV	Castells, 1998.[32]	41%	9,823,981
	RT-PCR	Colo201 cell spiking	–	14 mL	24 patients, 9 controls/Blood	B, C, D Dukes	Noh, 1999.[33]	41.1%	10,642,939

(*Continued*)

Table 2 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/ year	CTCs positive rate	PMID
	Nested RT-PCR	In-vivo assay	CA19.9, CA72-4	7 mL	51 patients, 40 controls, 18 patients with benign colorectal disease/Blood	A–D Dukes	Guadagni, 2001.[34]	67%	11,289,125
	RT-PCR	HT29 and LS147T cell spiking, Sequence analysis	CK20	20 mL	32 patients, 17controls/Blood	–	Hampton, 2002.[35]	36%	12,420,218
CEA, CK19	Semi-quantitative RT-PCR	Southern blotting, SK-BR-3 cell spiking	–	20 mL	33 patients, 26 controls/Blood	B–D Dukes	Wong, 2001.[38]	64%, 88%	11,121,864
	RT-PCR	-	–	3 mL	53 patients, 25 controls/Blood	I–III	Silva, 2002.[37]	73.6% 32%	11,889,075
CEA, EPCAM.	Adnatest ColonCancerSelect & Detect.	Multiplex RT-PCR	–	–	50 patients, 40 controls/Blood	I–III	Mourtzikou, 2012.[41]	66%, 6%	10.6051/j.issn.2224–3992.2012.01.070
EPCAM	Multigene qRT-PCR, flow cytometry		CK19, CK20, CEA, EGFR.	7.5 mL	49 patients/Blood	I–IV	Cohen, 2006.[39]	80%	16,945,168
EPCAM	Microfluidic device, FISH, Cellsearch		Pan CK, EPCAM	2 mL	5 patients, 200 controls/blood	With metastasis	Gogoi, 2016.[40]	100%	26,808,060
EPCAM	CTC-chip	NCI-H1650 cell spiking	–	2.7 mL	10 patents/Blood	Advanced	Nagrath, 2007.[43]	67%	18,097,410
CEA, TEM-8	RT-PCR	MAD-MB231 and HT29 cell spiking		5 mL	40 patients, 40 controls/Blood	I–III	Raeisossadati, 2011.[44]	55%, 22.5%	21,573,768
CD44	RT-PCR	Southern blotting, HCT116 cell spiking, Restriction enzyme analysis	–	15 mL	24 patients, 8 controls/Blood	B, C Dukes	Wong, 1997.[45]	16%	10.1046/j.1365–2168.1997.02685

(*Continued*)

Table 2 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/ year	CTCs positive rate	PMID
GCC	Nested RT-PCR	–	PSA, PSMA, CEA, CK-19, CK-20, mucin 1, GA733.2.	-	24 patients, 20 controls/Blood	D Dukes	Fava, 2001.[46]	100%	11,579,116
	Nested Duplex RT-PCR	Immuno histochemistry	CD31	10 mL	58 patients, 11controls/Blood	B–D Dukes	Tien, 2001.[47]	52%	11,410,499
	Nested Duplex RT-PCR	CCL-220 cell spiking, Immunohistochemistry, Western blotting	–	10 mL	68 patients, 11controls/Blood	A–D Dukes	Tien, 2004.[48]	58.8%	15,192,312
BMP4, CycD, FAM3D, GPA33, ZPX2, LGALS4, TACSTD1, hTERT, TFF3, TM4SF3, UGT1A9, VIL1, FLJ20127.	RT-PCR	–	B2M	10–15 mL	16 pooled patients, 16 controls/Blood	I–IV	Solmi, 2004.[49]	-, 100%, 100%, -, 100%, 100%, 100%, 100%, 100%, 37.5%, 83%, -, 36.3%	15,375,555
CK-20, CEA, CK-19, REG4, uPA, TIAM1.	RT-PCR	–	–	–	80 patients, 98 controls/Blood	I–II	Yeh, 2006.[50]	82.5%, 78.8%, 82.5%, 80.0%, 78.8%, 80.0%.	16,391,796
TMEM69, RANBP3, PRSS22.	Microarray screening,	QRT-PCR	–	10–15 mL	2 patients, 4 controls/ Blood	TNM stage	Solmi, 2006.[51]	~3-fold	17,054,783

(*Continued*)

Table 2 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/ year	CTCs positive rate	PMID
LOC644844, FABP1, CEACAM5, MUC13, GUCA2A, ABP1, SLC26A3	Digital Gene Expression Displayer (DGED), RT-PCR	–	–	5 mL	8 patients, 9 controls/ Blood	–	Lauriola, 2010.[52]	–	20,596,680
SERPINB5	qRT-PCR	SW480 and T84 cell spiking	VSNL1, DPEP1, STC1.	5 mL	818 patients, 4 IBD, 8 controls, 36 control without malignant disease/Blood	TNM stage	Findeisen, 2008.[57]	36%	18,949,363
CK20, CK19, EGFR	Multiplex-PCR	–	–	6 mL	81 patients, 38 controls/Blood	0–IV	Vaiopoulos, 2014.[58]		24,922,677
CK20,CEA, EGFR,	Nested RT-PCR	–	–	–	36 patients, 18 controls/Blood	I–IV	Teama, 2010.[59]	41.7, 61.1%, 66.7%	10.1016/j. ejmhg.2009.10.001
EGFR	AdnaTest Colon Cancer Select, AdnaTest Colon Cancer detect	COLO 205, HCC-2998, HCT-116, LoVo, WiDr, CACO-2, HT-29, SW-480, T84, DLD-1, SW-948, SW-1116 cell spiking, IHC, Multiplex RT-PCR.	EPCAM, CEA.	15 mL	20 patients, 22 controls/Blood	TNM stage	Lankiewicz, 2008.[60]	18%	18,936,523
ECT2	Nested qPCR	–	CEA	4 mL	90 patients, 151controls/blood	I–IV	Chen, 2017.[61]	–	28,362,321
FAM172A	Filtration	In situ hybridization	EpCAM, CK8, CK18, CK19, Vimentin, Twist, $CD45^{-}$.	5mL	45/Blood	I–IV	Cui, 2017.[62]	75.6%	28,618,931
A3 adenosine receptors	Real-time RT-PCR	Immunocytochemistry	–	40 mL	30/Blood	I–IV	Gessi, 2003.[63]	–	15,355,922

(*Continued*)

Table 2 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/ year	CTCs positive rate	PMID
TGFβ1, APP, CD9, CLU, ITGB5, LIMS1, RSU 1, TIMP1, TLN1, VCL, BMP6.	CELLection™, Agilent expression arrays	Real-time RT-PCR	EPCAM	7.5 mL	28 patients, 10 controls/Blood	Primary and metastasis	Barbazan, 2012.[53]		22,811,761
VIL1, TBX20, GPA33, FAM132A	CELLection™	Real-time RT-PCR, HT29 and HCT116 cell spiking	CD45⁻, EPCAM	7.5 mL	44 patients, 22 controls/Blood	IV	Barbazan, 2012.[54]	77.2%	22,304,365
TSPAN8, LGALS4.	qRT-PCR	TRAM based data set meta-analysis	EPCAM, SPINK1, COL3A1, CEACAM5, COL1A2, CDH1, CKT18, SLC26A3, REG1A, FN1, LUM, CEACAM6, CK20	5 mL	67 patients, 67 controls/Blood	I–III	Rodia, 2016.[64]	–	26,993,598
LOXL3, ZEB2, VIL1, TIMP1, CLU, TLN1	AdnaTest colon cancer	–	CD45-, EPCAM, CK 8, 18, and/or 19	7.5 mL	50 patients/Blood	Advanced	Alonso-Alconada 2017.[55]	–	29,058,262
VIL1, CLU, TIMP1, LOXL3 and ZEB2	CELLection™	qRT-PCR	EPCAM	7.5 mL	50 patients/Blood		Barbazan 2012.[56]	–	24,752,533

Abbreviations: RT-PCR, real-time polymerase chain reaction; Controls, healthy volunteer/donors; I–IV, TNM classification of malignant tumors (TNM); A–D Dukes, Dukes staging system is a classification system for colorectal cancer.

Table 3 The biomarkers which worked for diagnostic of CRC in Exosome

Biomarker	Technique of exosome isolation	Technique of exosome validation	Technique of markers detection or validation	Related marker	Patients (number/type)	Patient stage	Author/year:	PMID
KRTAP5-4, MAGEA3	Centrifugation syringe filter	TEM, NTA, light microscope	Bioinformatic Analysis, RT-PCR	lncRNA	30 patients, 30 control/Blood	I–IV	Dong, 2016.[79]	27,197,301
GPC1	ExoCapTM	TEM, Flow cytometry, Western blotting	Flow cytometry, Western blot analysis	miR-96-5p, miR-149, miR-182-5p	102 patients, 89 control/tissue and Blood, Cell line (HT-29 & HCT-116), Mouse	I–II	Li, 2017.[78]	28,233,416
EPCAM	PEG	ELISA, SEM	qRT-PCR, SEM, DLS, ELISA	–	HCT-116 Cell line	–	Manri, 2016.[77]	27,917,441
UBC, H3F3A, HIST2H2AA3, AKT3, HSPA1B			GSE100206, GSE100063, GSE32323 (Bioinformatic Analysis)	–	29 patients, 49 control/tissue and Blood	–	Huang, 2018.[81]	doi: 10.21037/tcr.2018.05.32
OATP1B3	Exosome Isolation kit (Thermo Fisher Scientific), PVDF filter and Differential centrifugation	TEM, Western blotting	qRT-PCR, Western blotting	–	HCT116, HT-29, and SW480 cell line, Blood of Mouse	–	Morio, 2018.[80]	29,491,222

Abbreviations: TEM, transmission electron microscopy; NTA, nanoparticle tracking analyzer; PEG, polyethylene glycol polymer; ELISA, enzyme-linked immunosorbent assay; SEM, scanning electron microscope; DLS, dynamic light-scattering.

CD44 variant exon 9 (CD44v9)[136] were proposed as markers in a smaller number of articles. The heterogeneity of CTC markers led some researchers to focus on multi-marker panels in data mining as listed in Table 4.[101,105,109,110,114,125,129,137–139]

Exosomes

Some prognosis markers have nearly the same functional patterns as molecular markers related to CRC. Studies have reported on colorectal exosome prognostic markers such as ALIX (ALG 2-interacting protein X),[140,141] Hsp60,[142] Hsp70,[141] CEA,[143] ATP-binding cassette transporter G1 (ABCG1),[144] copine III (CPNE3),[145] and ΔNp73[70] in cancer patients.

Tauro et al, used multiple isolation methods to detect known exosome markers such as ALIX, TSG101, HSP70, and other specific and novel markers listed in Table 5.[141] Chen et al, applied bioinformatic analysis for introduction of two panels and validated them.[146] Chiba et al, reported that exosomes derived from CRC cell lines contain mRNA, microRNA, and natural antisense RNA as listed in Table 5.[71]

Risk of bias (quality) assessment

All articles related to CTC (39 diagnosis-related and 57 prognosis-related) were assessed by NOS case-control guidelines as reported in Table S1. Of the diagnosis-related articles (40% of the total), 43%, 43%, and 14% scored 7, 6, and 5, respectively. Of the prognosis-related articles (60% of the total), 49%, 31.5%, 14%, and 1.5% scored 7, 6, 5, and 4, respectively; and 4% could not to be scored.

All articles related to exosomes (Five diagnosis-related and nine prognosis-related) were assessed by the NOS case-control guidelines in Table S2. Of the diagnosis-related articles (36% of total), 20%, 40%, and 40% scored 7, 6, and 5, respectively. Of the prognosis-related articles (64% of total), 67%, 22%, and 11% scored 7, 6, and 5, respectively. The 0–3 and 8–9 scores were not given out in these studies, so the NOS number varied from 4 to 7. About 99.3% of systematically imported articles scored over 5, 20% ofthe articless scored 5, and 79.7% scored 6 or 7.

Bioinformatics approach to systematic results

This systematic search identified 66 CTC gene markers for the diagnosis of CRC, 65 CTC gene markers for prognosis with repetition, 10 exosome gene markers for diagnosis of CRC, and 35 exosome gene markers for prognosis as shown in Tables 2–5.

Protein–protein interaction network via STRING analysis

In the gene network, biochemical functions and identified pathways were obtained from gene expression data, and the results are shown in Figures 3 and 4 and supplementary Table S3 (online resources). Surprisingly, the cellular components of exosomes and CTC highlight extracellular space, region and exosome, plasma membrane, and cell junction. Their molecular function highlights cell adhesion molecule binding and protein binding. Biological processes included regulation of cellular component movement, assembly, localization, organization, and response to external stimuli.

Gene ontology

The results of EnrichR web tools in supplementary Table S4 (online resources) can be used to accurately understand the molecular pathways. The common pathways in biomarkers such as proteoglycans in cancer, focal adhesion pathways in cancer, integrin, Rap1, MAPK signaling pathways, angiogenesis, p53 pathways, and viral processes were similar and related to cancer.

Discussion

CRC is a common malignancy that often has a poor prognosis.[147] The tumor microenvironment contributes to its progression[148] and cross-talk between cancer cells and exosomes play a critical role in this dynamic network.[149] Their identification and characterization are important steps to improve understanding of cellular and molecular cancer metastasis. Tracking of tumor-associated molecular markers in the blood can be used to assess the presence of residual disease, recurrence, and resistance.[150] This systematic review highlights new trends and approaches in CRC biomarker discovery using CTC and exosomes.

Evidence related to diagnosis of CRC by means of CTC markers was addressed in 38 articles (Table 2) and 54 articles discussed prognosis of CRC using CTC markers (Table 4). Only 14 articles examined exosomes, five about diagnosis and nine about prognosis (Tables 3 and 5). Our results show that the most common markers introduced in CTCs were CEA (35 of 94 studies) and CK20 (33 of 94 studies), especially using quantitative real-time polymerase chain reaction. Most markers investigated for

Table 4 The biomarkers which worked for prognostic of CRC in circulating tumor cells

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/year	CTCs positive rate	PMID
CK20	RT-PCR	Colo205 cell spiking	–	2 mL	8 patients, 3 controls/Blood	III–IV	Funaki, 1997.[84]	36%	9,048,967
	RT-PCR	HT29 cell spiking	–	10 cells/2 mL	26 patients, 12 controls/Blood	B, C Dukes stage	Wyld, 1998.[82]	48%	9,645,353
	RT-PCR	–	–	10 mL	108 patients, 38 controls/Blood	I–IV	Hinz, 2012.[83]	25%	22,395,998
	qRT-PCR	–	–	5 mL	95 patients, 23 controls/Blood	I–IV	Samija, 2013[85]	–	23,558,939
	RT-PCR	–	–	5 mL	95 patients, 23 controls/Blood	I–IV	Kust, 2016.[86]	–	27,144,776
CEA	RT-PCR	Southern blot hybridization	–	7 mL	69 patients, 16 controls/Blood	I–IV	Piva, 2000.[87]	34%	11,096,345
	qRT-PCR	COLM-2 cell spiking	–	5–7 mL	99 patients, 20 controls/Blood	I–III	Ito, 2002.[88]	44.4%	12,065,095
	RT-PCR			5 mL	108 patients, 76 controls/ Blood	III–IV	Kanellos, 2006.[89]	11.1%	16,788,936
	Membrane arrays	RT-PCR	–	4 mL	141 patients/ Blood	II–III	Lu, 2011.[90]	33.3%	21,343,933
	CellSearch (EPCAM)	CellTracks® Analyzer II	$CD45^{-}$	7.5 mL	20 patients/ Blood	I–III	Thorsteinsson, 2011.[91]	5%	21,378,346

(*Continued*)

Table 4 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/year	CTCs positive rate	PMID
CK20, CEA.	RT-PCR	Colo320 cell spiking	–	10 mL	52 patients, 10 controls/Blood	I–IV	Yamaguchi, 2000.[92]	38.4%, 36.5%	10,862,196
	RT-PCR	HT29 or HT115 cell spiking	–	14 mL	33 patients, 70 controls/Blood	I–IV	Mathur, 2001.[93]	85%	11,417,979
	RT-PCR	LS 180 and C205; ATCC CL-187 and CCL-222 cell spiking	–	12 mL	39 patients, 13 controls (abnormal)/ Blood	I–III	Guller, 2002.[94]	28%	12,454,515
	qRT-PCR	–	–	–	167 patients, 25 controls/ Blood	I–IV	Iinuma, 2006.[95]	22%	16,391,782
	qRT-PCR	HT29 cell spiking	CA19-9	10 mL	46 patients, 23 controls/Blood	I–IV	Liu,2012.[96]	65.21%, 36.95%	22,414,974
CK20, CK19	RT-PCR	Cell Spiking	K-ras, p53	20 mL	35 patients, 23 controls/Blood	I–IV	Nakamori, 1997.[98]	26%	9,378,009
CK20, CEA, CK19.	Nested RT-PCR	–	–	–	62 patients, 12 controls/Blood	I–IV	Huang, 2003.[97]	35.5%, 48.4%, 51.6%	12,684,893
CK20, GCC.	RT-PCR	–	CEA, CA199	5 mL	100 patients, 5 controls/Blood	I–III	Liu, 2017.[103]	–	28,418,917
	qRT-PCR	–	CEA	5 mL	69 patients, 23 controls// Blood	I–III	Liu, 2013.[104]		23,150,200
CK, CEA, CD133.	qRT-PCR	–	CK19, CK20	10 mL	735 patients/ Blood	B–C Dukes	Iinuma, 2011[106]	24.52%	21,422,427

(*Continued*)

Table 4 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/year	CTCs positive rate	PMID
CK20, CEA, CK19, CD133	qRT-PCR	–	–	–	197 patients, 20 controls (benign diseases)/ Blood	B–C Dukes	Shimada, 2012.[100]	63%	22,267,181
CEA, EPCAM.	CellSearch, TRC method	DLD1 cell spiking	–	7.5 mL	67 patients/ Blood	Metastatic	Sato, 2012.[108]	9.0 ±23.4%, 64.3%	21,732,137
CK20, CEA, Survivin.	CD45 immuno magnetic beads + Ber-EP4 immuno magnetic beads	Lovo cell spiking, Real-time RT-PCR	–	10 mL	156 patients, 40 benign patients, 40 healthy/Blood	A–D Dukes	Shen, 2008.[111]	47.4%, 39.1%, 57.7%.	18,845,519
CK20,CEA, ProtM,	Real-time RT-PCR	COLO 205, LS-174-T, CX 2, CX 94, HCT 116, HT 29, CaCo2 cell spiking	PBGD	10 mL	129 patients, 47 controls/ Blood	0–IV	Schuster, 2004.[112]	88%, 86%; 17%	14,639,606
CK19, CK20, MUC1, MUC2.	Immunobead RT-PCR	SW48, SW480, HT29, LIM-2412, LIM-1215, LIM-2099, LIM-2405, LIM-1899, LIM-2463 and LIM-1863 cell spiking	–	20 mL	94 patients, 20 controls/Blood	A–D Dukes	Hardingham, 2000.[99]	20%	10,719,724
CK-19, CK-20, CEA, hTERT.	Membrane arrays	RT-PCR	–	4 mL	72 patients, 30 controls/Blood	I–IV	Wang, 2006.[113]	66.7%, 52.8%, 72.2%, 69.4%	16,736,329
CGM2 (CEACAM7)	RT-PCR	CACO-2 and HT-29 cell spiking	–	20 mL	78 patients, 115 controls/ Blood	A–D Dukes	Douard, 2001.[116]	59%	11,331,451

(*Continued*)

Table 4 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/year	CTCs positive rate	PMID
CEACAM5, CEACAM7	Immuno bead multiplex RT-PCR	–	HBB	20 mL	84 patients, 41 controls, 32 non CRC patients/Blood	I–IV	Douard, 2005.[115]	55%, 45%	15,843,204
EPCAM, EGFR.	Immuno magnetic selection (IMS), multiplex RT-PCR	T84, HT29, SW948 and SW1116 cell spiking	CEA	5 mL	76 patients, 106 controls/ Blood	I–IV	Zieglschmid, 2007.[107]	88%, 12%.	17,649,779
EGFR	RT-PCR	Immunohistochemistry (IHC)	CEA (45%), CK-19 (27%)	5 mL	38 patients, 38 controls/Blood	B, C Dukes	De luca, 2000.[118]	(73%)	10,778,975
	RT-PCR	–	–	3 mL	16 patients, 23 controls/Blood	Advanced-stage	Clarke, 2003.[119]	12.5%	12,527,944
EGFR, HLM	RT-PCR	Northern blotting, HT11C cell spiking	–	3 mL	1 patients, 9 controls/Blood	Metastatic	Fournier, 1999.[120]	100%	10,446,991
EGFR, Tenascin C.		–	–	5 mL	41 patients, 40 controls/Blood	I–IV	Gazzaniga, 2005.[121]	49%	16,211,285
PLS3	RT-PCR	Fluorescent immunocytochemistry	CEA	–	711 patients, 25 controls/ Blood	Dukes A, B, C, and D	Yokobori, 2013.[122]	25%	23,378,342
PLS3, AQP5	RT-PCR	Fluorescent Immuno cytochemistry	CD45 (−)	10 mL	177 patients, 25 controls/ Blood	Dukes A, B, C, and D	Sugimachi, 2014.[123]	-	24,217,791
	CD45 magnetic bead depletion	FISH Immunofluorescent	CEP8≥3 and	7.5 mL	45 patients, 25 controls/Blood	I–IV	Shan, 2014[131]	55%	25,109,507
PKP3, AGR2.	Bioinformatic analysis and RT-PCR	Gp5d, LoVo, DLD1, LS513, HT29, OJC4, OJC5, OJC6 cell spiking	S100A16, S100A6, LGALS4, CLDN3.	10 mL	21 patients and controls/Blood	III–IV	Valladares-Ayerbes, 2008.[124]	40%, 81.8%	18,801,625

(*Continued*)

Table 4 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/year	CTCs positive rate	PMID
AGR2, LGR5.	qRT-PCR	–	–	10 mL	54 patients, 19 controls/Blood	I–IV	Valladares-Ayerbes, 2012.[126]	84.9%, 90.5%	22,605,983
DCLK1, LGR5	qRT-PCR	–	–	10 mL	58 patients, 58 controls/Blood	I–IV	Mirzaei, 2015.[127]	63.7%	25,631,749
LGR5	mRNA ISH	–	EpCAM, CK8, CK18, CK19 Twist1, Vimentin, AKT2, SNAI1, CD45 (–)	5 mL	66 patients,/ Blood	I–IV	Wang, 2018.[128]	86.4%	29,949,050
CK20, Tyrosinase, PSA.	RT-PCR, Nucleic acid sequence-based amplification (NASBA assay)	HT-29 cell spiking, In vitro cell assay	–	2 mL	12 patients, 8 controls/Blood	–	Burchill, 2002.[132]	–	11,857,020
MAGE-A	Electrochemiluminescence (ECL), RT-PCR	Sequencing analysis	uMAGE-A, M-A1, M-A3, M-A12	10 mL	12 patients, 20 controls/Blood	I–IV	Miyashiro, 2001.[133]	29%	11,238,304
DVL1	Microarray and enzymatic chip array (WEnCA)	IHC	PSG2, TMPO, CD55, ELAVL4, PDX1, CTHRC1, CA9, TK1, UBE2C, FOXM1, PDE6D, PSAT1, CHRNB1,CEA, BMI CAP2, MMP13, OLFM4, PTTG1, MYC, MET, ENO2, MUC1, KRT19, BIRC5, HMGB1, KRT20, hTERT, GCNT1, NPM1	4 mL	214 patients/ Blood	I–III	Huang, 2013.[134]	55%	24,129,181

(*Continued*)

Table 4 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/year	CTCs positive rate	PMID
CD47	Cellsearch	–	EPCAM, CD45 (–)	20–30 mL	72 patients/ Blood	I–IV	Steinert, 2014.[135]	14%	24,599,131
CD44v9	OncoQuick	qRT-PCR	–	20 mL	150 patients, 15 controls/ Blood	I–IV	Katoh, 2015.[136]	40%	25,550,556
CK19, AGR2, CK8, CK9.	CellSearchn	–	TSPAN8, LAD1, CK20, IGFBP5, GPX2, FABP1, S100A1,6 CK8, PRSS8, CDX1, CEACA,M5, AKR1C3, RARRES2, REG1A, IGFBP4, CD44, TRIM2, CXCL1, SATB2, NQO1, CK19, MAPT, IGFBP3, COL4A1, FCGBP, SLC6A8, CDH5, CDH17, EGFR, S100P, HOXB9, CDH1, MACROD1,	30 mL	142 patients, 30 controls/ Blood	Metastatic colorectal cancer	Mostert, 2015.[125]	66%	25,655,581
CK20, CEA, AGR2, MGB2, DLL4, EphA2, Her3, PDGFRα	qRT-PCR	–	–	7.5 mL	24 patients/ Blood	III–IV	Bao, 2013.[137]	59%	23,990,866
CK-20, CEA, CK-19, hTERT. TM4SF3, CK19.	Membrane arrays	RT-PCR	–	4 mL	157 patients, 80 controls/ Blood	I–IV	Wang, 2007.[114]	50%	17,406,027
	RT-PCR	–	CEA, CK20, TACSTD1,	10 mL	28 patients, 19 controls/Blood	I–IV	Xi, 2007.[101]	96.4%	17,525,108

(*Continued*)

Table 4 (Continued).

Biomarker	Technique of isolation/ detection of CTC	Technique of validation/related one	Related marker	Cutoff	Patients (number/ type)	Patient stage	Author/year	CTCs positive rate	PMID
DCLK1, LGR5, EpCAM, CK8, CK9, CK19, Vimentin, Twist	qRT-PCR, IHC	–	–	10 mL	78 patients and controls/Blood	I–IV	Mirzaei, 2016.[109]		26,383,518
	CanPatrol CTC enrichment	(ISH) assay	–	5 mL	38 patients, 27 controls/Blood	I–IV	Wu, 2015.[110]	67%	25,909,322
PSG2, ELAVL4, TK1, UBE2C, PDE6D, PSAT1, CHRNB1, BMI1, CAP2, MMP13, OLFM4, PTTG1, MYC, MET, MUC1, HMGB1, hTERT, BIRC5,	Enzyme immunoassay test kit	–	CEA	3 mL	298 patients/ Blood	I–III	Chang, 2016.[138]	-	27,701,415
PI3Kα, Akt-2, Twist1 ALDH1	antiCD45 specific antibodies (Dynabeads, Invitrogen)	qRT-PCR and multiplex-PCR	_	8 mL	78 patients, 20 controls/Blood	I–IV	Ning, 2018.[129]	55%	27,503,579
CK19, MUC1, CD44, CD133, ALDH1	CD45 Human MicroBeads (Miltenyi Biotec), enrichment of cytokeratin (Miltenyi Biotec)	Flowcytometry, CellSearch, qRT-PCR, Cytomorphology, PC3, MDA-MB-231 and SKBR3 cell spiking	_	7.5 mL	63 patients, 40 controls/Blood	I–III	Bahnassy, 2019.[139]	(55.6%), (46.0%), (44.4%), (41.3%) (41.3%)	30,578,762
CEACAM5, CK19, AGR2, LGR5	Inertial microfluidics combined with droplet digital PCR	qRT-PCR, HT-29 and LoVo cell spiking	–	9 mL	Patients and controls/Blood	Advanced	Methai, 2019.[164]	-	31,304,099

Table 5 The biomarkers which worked for prognostic of CRC in exosome

Biomarker	Technique of exosome isolation	Technique of exosome validation	Technique of markers detection or validation	Related marker	Patients (number/ type)	Patient stage	Author/ year:	PMID
Alix	–	–	GSE37364, GSE10714, GSE4183, GSE18105, GSE4107, GSE9348, GSE8671, IHC	PGK1, PKM, ANXA5, ENO1, HSP90AB1, MSN	72 patients, 27 controls, and 98 sample (literature bioinformatic)	I–IV	Valcz G, 2016.[140]	27,150,162
ΔNp73	UC-Exo* centrifugation 120,000 and PVDF filter	Acetylcholinesterase activity, flow cytometry quantification, transmission electron microscopy, Western blot analysis	qRT-PCR, Cell culture and transfection	CEA	69 patients and control tissues, HCT116 cell lines.	I–IV	Soldevilla, 2013.[70]	24,067,531
Hsp60	UC-Exo	TEM AChEase: acetylcholinesterase assay, Western blot	IHC, ELISA, immunogold electron microscopy	Hsc70, Alix, CD57, CD68	57 patients and control tissues, 2 blood sample	I–III	Campanella, 2015.[143]	26,060,090
RPL13A, HMBS, TBP	UC-Exo	BCA, Western blotting	qRT-PCR	miR-21, miR-34, miR-143, miR-192, miR-215, miR-22	WiDr, HCT-15, SW480 cell lines	–	Chiba, 2012.[71]	22,895,844
TSAP6, CEA	UC-Exo	Flow Cytometry, Western blotting	qRT-PCR, IHC, levels of circulating exosomes in plasma		91 patients, 12 controls/tissue and blood	I–IV	Silva, 2012.[142]	22,420,032
Alix, TSG101, HSP70, CD9, CD81, ESCRT-III, VPS32C/CHMP4C, VAMP2, EFNB1, EFNB2, EPHA2–8, EPHB1–4, CTNNB1, TNIK, CRK, GRB2	UC-Exo, DG-Exo: OptiPrep™ density gradient exosome, IAC-Exo: EpCAM immunoaffinity capture	Western blotting, EM: Electron microscopy	GeLC–MS/MS (protein profiling)	–	LIM1863 cell line	–	Tauro, 2012.[141]	22,285,593

(*Continued*)

Table 5 (Continued).

Biomarker	Technique of exosome isolation	Technique of exosome validation	Technique of markers detection or validation	Related marker	Patients (number/type)	Patient stage	Author/year:	PMID
BCL7C, EEF1G, RAB13, RSP3, TPT1, SCARB1, SCD	UC-Exo	A33-Exos and EpCAM-Exos (Dynabeads™), TEM, Western blot	SRP02205476, SRP029880 (Microarray)	–	LIM1863 cell line	–	Chen, 2016.[146]	27,917,920
CPNE3	UC-Exo	TEM, NTA, Western blotting		CEA	92 patients, 32 controls/Blood	Sensitivity of 67.5% and a specificity of 84.4%	Sun, 2019.[145]	30,078,189
ABCG1	Polymer-based precipitation method	TEM, Zetasizer Nano ZSP, Western blotting,	qRT-PCR, IHC, GSE1753749	–	Murine cell line		Namba, 2018.[144]	30,364,132

Abbreviation: UC-Exo, ultracentrifugation exosome.

exosomes, in addition to CD9, CD81, ALIX, and TSG101, were including EPCAM and HSP, especially using ultracentrifugation. Comparison of 131 CTC markers and 45 exosomes markers showed only three common markers (CEA, CD9, and EPCAM) on the gene list as diagnostic and prognostic biomarkers.

A half-century-old investigation of CEA in CRC was the first step in the identification of a much larger family of 12 CEACAMs.[151,152] Gene encoding CEA is a member of the immunoglobulin supergene family[153] that plays a role in cell adhesion and tumor progression,[154] even in protecting the colon from microbial infection.[155] CEA is involved in the metastatic cascade process through positive regulation of cell migration and invasion;[156–158] thus, the monitoring of CEA as a cost-effective and frequent indicator of recurrence of CRC has been investigated for years.[159]

Integrin on tumor exosomes may play an important role in modulating organ-specific metastasis in cancer progression. CD9 is a member of the tetraspanin superfamily commonly detected in all types of exosomes involved in pathophysiologic processes such as cellular adhesion, growth, motility, cell–cell fusion, signal transduction, and tumor metastasis.[160]

EPCAM is a membranous glycoprotein that is a CSC marker in tumor cells in the basolateral surface of most normal epithelial tissue and its role is to connect cells by means of calcium. The expression of this marker increases in benign and malignant tumors that arise from epithelial tissue.[161] The first step in metastasis is the separation of cancerous cells from primary tumors. CEA, CD9, and EPCAM are closely correlated with tumor progression as a poor prognostic factor and is required for the survival of CTCs in some cancers.[162] Taken together, it appears that the signature of the CTC and exosome biomarkers are similar and follow common pathways; thus, exosomes can be applied as alternative tools for guiding better molecular pathology in the fight against cancer.

Precision medicine is changing clinical practice by tailoring treatment based on an individual's genetic makeup. Recent studies have shown that CTC and circulating tumor DNA provide complementary information and the use of both approaches to study tumor metastasis is warranted.[163] CTC and exosomes can pave a path as diagnostic and prognostic procedures using the heterogeneity of tumor sites as they are released into the blood from live origins and can be

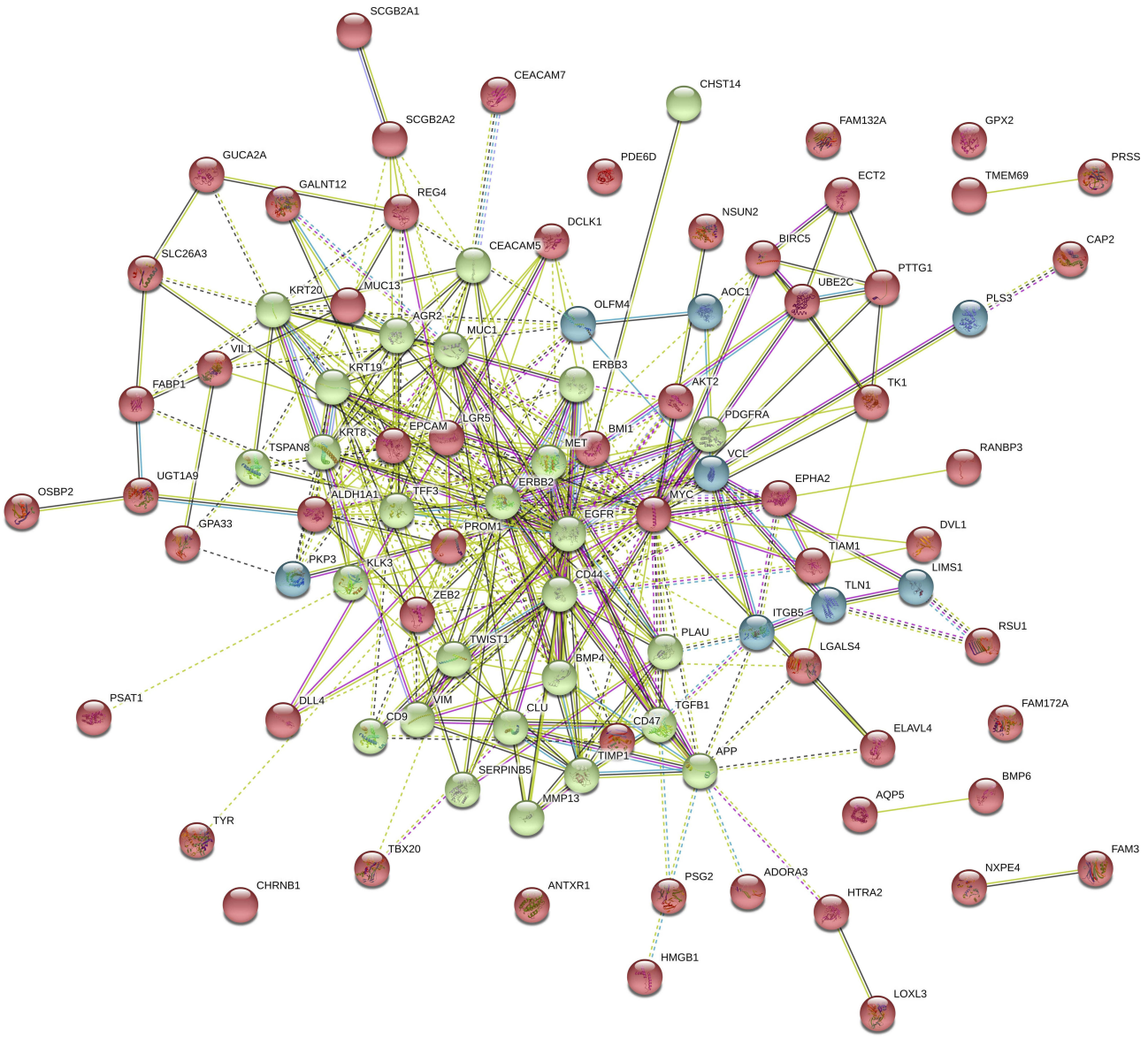

Figure 3 Network and enrichment analysis visualization. Combined screenshots from the STRING website, showing results obtained upon entering a set of 131 proteins suspected to be involved in circulating tumor cell markers. According on kmeans clustering has been selected, the corresponding protein nodes in three categories automatically highlighted in colors.

analyzed at the DNA, RNA, and protein levels. It is undeniable that more investigation is needed to compare them, especially for cancer patients.

Various CTC isolating techniques each have its own advantages and disadvantages as to their CTC capture capacity and subgrouping of CTCs based on various markers. Similar problems also exist for exosomes, with a lack of a proven rapid and high-yield approach for extracting exosomes for downstream analysis.[164] Microfluidic devices and bioinformatics analysis might play an important role in solving the current shortcomings of the liquid biopsy concept. Microfluidics, by using inertial focusing/hydrodynamics (laminar flow in microchannels) and applying spiral, acoustic, electrophoretic, and electromagnetic features passively separate CTCs and exosomes from the other background calls.[165] Immobilizing specific antibodies either on micro-posts or in a herringbone design against their marker might be useful; it is easy to explore and yields quantitative readouts with high sensitivity, low cost, and minimal sample handling. Finally, although the potential clinical utility of these techniques is clear, more effort is needed to use the full potential of liquid biopsy in clinical settings.[166]

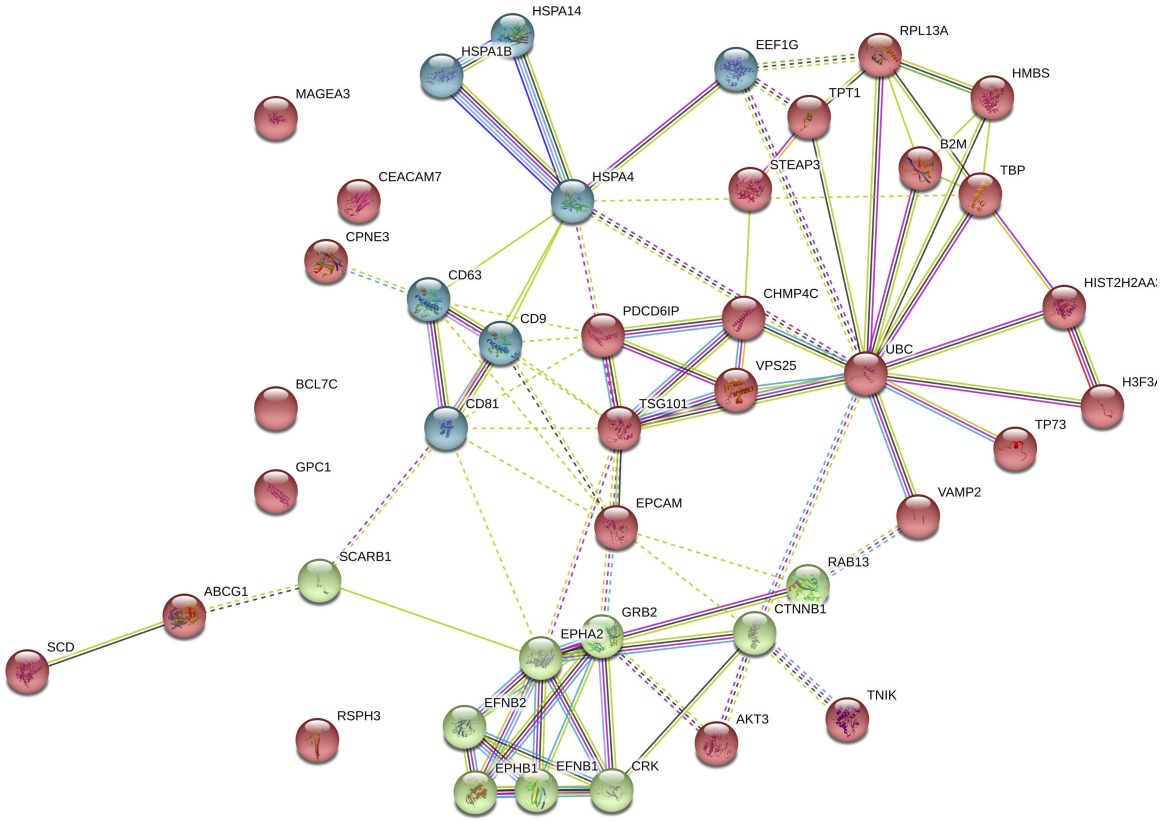

Figure 4 Network and enrichment analysis visualization. Combined screenshots from the STRING website, showing results obtained upon entering a set of 45 proteins suspected to be involved in Exosome markers. According on kmeans clustering has been selected, the corresponding protein nodes in three categories automatically highlighted in colors.

Future perspectives

Currently, isolation and purification of tumor-derived exosome in a worm bag of EVs is technically cumbersome and also isolation of CTCs has its own limitations. Therefore, combined use of these two biomarkers together as a liquid biopsy requires large-scale clinical trials. Microfluidic devices and bioinformatics analysis might play an important role in solving the current shortcomings of the liquid biopsy. Additionally, cross talking of CTCs and tumor-derived exosomes in a tumor microenvironment should become a heated question in exploring the premetastatic niche. As such, more research is needed on CTCs and exosome's overlapping molecular pathways to determine more effective biomarker signatures of CRC, especially in the metastatic form.

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Disclosure

The authors report no conflicts of interest in this work.

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