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Assets of Circulating Extracellular Vesicles for Early Diagnosis and Prognosis of Gastric Cancer by Liquid Biopsy

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Exosomes; non-coding RNAs; microRNAs; long noncoding RNAs; circular RNAs; *Dictyostelium*

Abbreviations:

GC: gastric cancer; EGC: early stage gastric cancer; GCCs: gastric cancer cells; CAG: chronic atrophic gastris; CRC: colorectal cancer; PC: peritoneal carcinomatosis; PLF: peritoneal lavage fluid; MA malignant ascites; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; CA72-4: carbohydrate antigen 72-4; CTCs: circulating tumor cells; cfDNAs: cell-free DNAs; ccfNAs; circulating cell-free nucleic acids; mRNAs: messenger RNAs; miRNAs: microRNAs; ncRNAs: non-coding RNAs; lncRNAs: long non-coding RNAs; lncUEGC1 & lncUEGC2: lncRNAs up-regulated in EGC exosomes; lncHOTAIR: HOX antisense intergenic RNA; circRNAs: circular non-coding RNAs; EVs: extracellular vesicles; sEVs: small EVs; cEVs: Circulating extracellular vesicles; Exos: exosomes; MVs: microvesicles; APBs: apoptotic bodies; ROC curve: receiver open characteristic curve; AUC: Area under the curve; TRI3: tripartite motif protein 3; GKN1: gastrokin protein 1; BMSCs: bone marrow stromal cells; DYNLT1: dynein light chain T-type 1; PODN: podocan, hub gene protein; ZNF521: zinc finger protein 521; CFI: complement factor I; CDH1: cadherin-1; MT1-MMP: 1-matrix metalloproteinase; FZD-10: frizzled protein 10; HSP90: heat shock protein 90; HUR: HU-antigen R; EGFR: epithelial growth factor receptor; VGEF: vascular endothelial growth factor; PD-L1: programmed death ligand 1; CM: conditioned medium; *Dd*: *Dictyostelium discoideum*

1. Abstract

Gastric cancer (GC) is an important human cancer, which needs new biomarkers for early diagnosis in order to improve the patients outcome. The aim of this review is to stress the huge interest of extracellular vesicles circulating into blood and other body fluids, as potential candidates for early diagnosis of gastric cancer. After briefly recalling the previous blood-based liquid biopsies as a non-invasive help to GC diagnosis and the main properties of extracellular vesicles, the review will be focused on the recent progressive emergence of exosomes as important partners in GC liquid biopsy. Faced with the always increasing number of exosomal biomarker candidates, the necessity of two independent searches is suggested. The biologists need to solve some pending biological problems about extracellular vesicles, and the eukaryotic microorganism *Dictyostelium discoideum* might be an appealing both "in vitro and in vivo" model for that purpose. On the other hand, the protocols for isolation and characterization of extracellular vesicles and their cargoes have to be strictly standardized for a further validation at a large scale, prerequisite for an efficient clinical translation of some promising biomarker (s) for gastric cancer diagnosis and prognosis.

2. Introduction

From the GLOBOCAN 2020 worldwide estimate of cancer incidence and mortality for 36 cancers in 185 countries [1], stomach cancer belongs to the top 10 most common cancers and is responsible for over one million new cases in 2020 and an estimated 769,000 deaths, ranking fifth for incidence and fourth for mortality. Incidence rates are highest in Eastern Asia and Eastern Europe. Stomach cancer can be classified into the cardia (upper stomach) and noncardia (lower stomach). Chronic *Helicobacter pylori* infection is considered the principal cause of noncardia gastric cancer (GC). For cardia cancer, emerging evidence suggests that some cancers are linked to *H. pylori* infection, whereas most are linked to environmental and other risk factors. For Gastric Cancer, like many others, an early diagnosis is a prerequisite for improving the prognosis of patients with a satisfying outcome. However, the hidden growing tumor might remain asymptomatic before being detectable by fibroscopy and tissue biopsy. The-

refore liquid biopsy mediated by circulating tumoral components, first in blood (serum /plasma), then in many other body fluids, brought a great hope for achieving earlier diagnosis for many cancers. The worldwide quest for efficient cancer biomarkers began long ago, first focused on a few tumoral circulating cells released from the tumor into blood vessels (CTCs), then searching for tumoral cell-free circulating nucleic acids (ccfNAs) including DNAs, mRNAs, miRNAs and other non-coding RNAs. The state of the art for many previous liquid biopsies applied to gastric cancer until 2015 has been nicely summarised [2, 3]. Nowadays, the more recently explored treasure chest of circulating extracellular vesicles (cEV) is under increasing investigation for finding an early cancer signature within the rich EV cargoes. The different steps of this current EV-mediated search have been recently reviewed for six human cancers (lung, breast, prostate, colorectal, ovary and pancreatic) [4]. The aim of the present review is to focus such an EV-mediated literature search for gastric cancer.

3. Previous Blood-Based Liquid Biopsies as a Non-Invasive Help to GC Diagnosis

For a few cancers, some blood specific antigens, like prostate-specific antigen (PSA) for Prostate cancer or carcino embryonic–antigen (CEA) for other cancers are used for helping diagnosis, but their sensitivity and specificity are not satisfactory and can therefore induce false negative or positive diagnoses. Liquid Biopsy is a rather old promising concept devoted to the search of blood circulating specific tumor components able to replace the previous tissue invasive biopsies, which are used to corroborate the imagery tumor suspicion. Thus, liquid biopsy mediated by circulating tumoral components was aimed to non-invasively inform about the presence of a still hidden silently growing tumor. Over time the focus has been successively centered about rare tumor cells released into the blood vessels, then about circulating cell-free tumor-specific DNAs. Now, different circulating cell-free non–coding RNAs are on the stage for checking their validity as biomarkers for early cancer diagnosis. This biomarker search pathway is also at work for Gastric Cancer.

The most studied non-coding RNAs are microRNAs (20-22 nucleotides (nt)). MiRNA was first identified in 1993 in *C. elegans* and in human body in 2001; in 2012 > 1400 miRNAs were described in humans. MiRNAs are highly conserved endogeneous RNA molecules, that regulate gene expression at the post-transcriptional level. Increasing evidence has demonstrated the importance of miRNAs in regulating biological characteristics common to various tumors. From 2008 to 2012 tumor-specific miRNAs became a hot spot for cancer resarch, and specifically for gastric cancer [5]. The new methods of measurements of non-coding blood-circulating RNAs boosted the search for efficient GC diagnosis biomarkers. Huang and Yu [6] reviewed an impressive number of studies from 2008 to 2015, suggesting specific miRNas or panels of a few miRNAs, circulating in whole blood, serum or plasma, as potential candidates for early GC diagnosis and prognosis after surgery. Some mechanisms of post-transcriptional gene expression of these miRNAs on GC tumor cells were also elucidated. From 2012 to 2015 the same approach was performed with some long non-coding RNAs (lncRNAs). More recently, a combination of plasma H19 and MBG3 lncRNAs with miR-675-5p was claimed to discriminate controls and GC subjects with 88,87% sensitivity and 85% specificity [7]. In most studies, the receiver open characteristic (ROC) curves were used to estimate the corresponding diagnosis efficiency of the new suggested GC biomarkers, through the measured sensitivity and specificity, to be compared with those of the previously used tumor markers. The new ncRNA biomarkers exhibited higher diagnostic values relative to the conventional GC biomarkers. However, reliable methodologies and strategies for the quantification of circulating miRNAs and lncRNAs are urgently needed for clinical utility [6]. Necula et al. [8] extensively reviewed the recent advances in GC early diagnosis. After taking into account the circulating proteomic biomarkers, the GC oncogenes/tumor suppressors, the GC methylation pattern, the CTCs and cfDNAs, they summarized the many recently discovered circulating molecules in body fluids, such as miRNAs, lncRNAs and circular RNAs (circRNAs), which hold the promise to develop new strategies for early diagnosis of GC. It is noticeable that among the many studies involved, only one was concerned with exosomal miRNAs. This was also the case for another review stressing the power of miRNAs as diagnostic and prognostic biomarkers in liquid biopsies applied to solid tumors, including GC [9]. However, the overall protection of the blood-cicrculating non-coding RNAs (miRNAs and lncRNAs) through circulating extracellular vesicles (cEVs) appeared already as an interesting complementary concept in 2015 [3], which should further give rise to an appealing new cEV-mediated liquid biopsy.

4. Summary of the Main Properties of Extracellular Vesicles

EV cell-release is a newly discovered cell property which is common to all living cells from Archeabacteria to Procaryotes and Eucaryotes (plants, animals and human) cells. First considered as mere garbage cell "dusts", EVs are now recognised since about one decade as important mediators of intercellular communications, which are involved in many physiological processes during health, as well as in many human diseases, including cancers. They harbor many specific macromolecular components (proteins, lipids, nucleic acids (RNAs and DNAs), and metabolites), which are specifically targeted into different types of EVs, mainly exosomes (Exos), microvesicles (MVs) and apoptotic bodies (APBs) (for details, see [10-12]), by means of mechanisms not yet elucidated. Most importantly, EVs represent epigenetic messengers able to target specific recipient cells for modifying gene expression by a novel mechanism of genetic exchange between cells [13]. EVs are involved in important biologic functions such as cell growth, immunologic properties, and tumoral conditioning such as angiogenesis and metastasis "niche" formation. EV concentrations are generally greatly increased when released from tumor cells compared with the ones from control cells. Tumor circulating EVs (cEVs) can be devoted with either oncogenic or tumor suppressor properties and they can also confer antitumoral resistance to sensitive cells. Moreover, EVs are also circulating in many human

body fluids (blood (serum /plasma), urine, saliva, etc.), then acting as potential informative biomarkers when originating from tumor cells. Altogether, tumor cell-derived cEVs open new perspectives for cancer research [14].

5. cEV-mediated Liquid Biopsy for Early Diagnosis of Gastric Cancer and Prognosis

5.1. First involvments of circulating exosomes for GC diagnosis and prognosis

As reviewed up to 2015 [3], there is only one study in 2010 regarding the study of circulating EVs in platelet-depleted plasma samples from 37 GC patients compared to those from 10 healthy controls. With regard to gastrointestinal malignancies, including GC, peritoneal carcinomatosis (PC) is a late stage manifestation suffering from late diagnosis and a consequent poor prognosis. Tumor EVs/Exos being progessively revealed as potent new biomarkers in liquid biopsy, protecting circulating tumor-specific components from enzymatic degradation in many cancers, PC was one of the first GC type to focus the medical interest [15]. Tokuhisa et al. [16] investigated for the first time the miRNA content of exosomes isolated from malignant ascites (MA) and Peritoneal Lavage Fluid (PLF) of GC patients. In each group of 6 MA and 6 PLF samples, the expression of exosomal miRNA microarrays were tested. For the 6 MA and the 6 PLF samples, the mean number of miRNAs were 490 and 367, respectively. The number of miRNAs common among the samples in the MA and PLF groups were 327 and 263, respectively. In the six MA fluids, miR-21 showed the highest signal intensity. The authors identified five miRNAs (miR-1225-5p, miR-320c, miR-1202, miR-1207-5p, and miR-4270) with high expression in MA samples, the PLF of serosa-invasive GC, and the conditioned medium of a highly metastatic GC cell line. These candidate miRNA species appeared related to peritoneal dissemination. This was further confirmed for miR-21 and miR-1225-5p, shown to be associated with serosal invasion in GC, which might provide a novel approach to early diagnosis of peritoneal dissemination of GC after curative GC resection. An analogous approach, although less concerned with exosomes, was used by Huang et al. [17], who identified six serum-based miRNAs (miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR20a-3p, and miR296-5p) as potential diagnostic biomarkers for GC. The expression levels of the six identified serum miRNAs were also measured in 188 GC tissue specimens and 28 normal gastric mucosa tissues. Only the expression of miR10b-5p and miR296-5p were significantly higher in tumor samples than in normal tissues and their high expression levels were associated with poor survival in patients without adjuvant chemotherapy. Furthermore, only the expression levels of miR10b-5p, miR20a-3p, and miR296-5p were significantly elevated in exosomes from GC serum samples (n=30). It is to be noticed that no common miRNA biomarkers are pointed out in both studies [16, 17] and this inconsistency of the claimed miRNAs as GC biomarkers will also appear later, possibly explained by the different used methodologies and the complete lack of standardization, which are greatly detrimental for further clinical use.

5.2. Emergence of exosomes as important partners in GC liquid biopsy

For GC, exosomes emerged as important mediators in oncobiology in 2018, through the review devoted to diagnosis and therapy of hepatic carcinoma [18], showing that exosomes serve as crucial regulator of the tumor microenvironment, and explaining the propensity of GC for liver metastasis. When exosomes began to attract attention for GC liquid biopsy, the first studies focused, as for other cancers, on exosomal proteins, Thus, the exosomal tripartite motif protein (TRIM3) was claimed as a potential biomarker for GC growth and metastasis, and even as a novel GC therapy through exosomal delivery of the TRIM3 protein, acting as a tumor suppressor in vivo [19]. In the same way, the exosomal stomach-specific protein Gastrokin-1 (GKN1) was suggested as a potential biomarker for GC diagnosis and treatment [20]. Exosomal GKN1 was studied both in vitro and in vivo, followed by measurements of GKN1 concentrations after heating at 70°C for 10 min, in whole sera from 100 heathy subjects and 245 patients with gastric, colorectal and hepatocellular carcinoma. An important observation of this study is that GKN1 was secreted to extracellular space as an exosomal cargo protein and was internalized through clathrin–mediated endocytosis. Exosomal GKN1 inhibited cell proliferation and induced apoptosis in gastric cancer cells and xenograft tumors. In addition, GKN1 concentrations in the sera of patients with gastric cancer were significantly lower than those in healthy subjects and patients with hepatocellular and colorectal carcinomas. Thus was provided the first evidence that human gastric epithelial cells naturally secrete and internalize GKN1 as an exosomal protein and that GKN1 may be a potential diagnostic and therapeutic target for achieving gastric cancer remission.

Beside proteins, other exosomal components began to attract attention for GC liquid biopsy, such as mRNAs [21], miRNAs [22], and lncRNAs [23]. The prognostic roles of mRNAs of the exosomes derived from bone marrow stromal cells (BMSCs) in common malignancies were elucidated by using bioinformatic tools. A total of 386 genes originating from the BMSC-derived exosomes were identified as statistically significant, which consisted of 150 upregulated genes and 236 downregulated genes. 32 pathways were also identified as significant. The protein-protein interaction network included 100 protein nodes, with three hub proteins, PODN, ZNF521, and CFI, which interacted with ten or more other proteins. For GC, the downregulation of PODN and ZNF521 indicated a good outcome, while the upregulation of CFI indicated a good survival. These findings about the prognostic roles of exosomal mRNAs require large samples and experimental verification [21]. Circulating miRNAs in whole blood (serum/plasma) were earlier already suggested as GC biomarkers [5, 6, 8]. With circulating exosomes as new players of liquid biopsy, exosomal miRNAs were also investigated as GC bio-

markers [22]. In this study, the expression of 9 already selected blood GC miRNAs were detected in 67 GC patients' plasma circulating exosomes. The exosomal level of 3 of them (miR-125b, miR-375 and miR-30a) was reduced when compared to the one of paired healthy controls. MiR-217 was increased both in plasma GC exosomes and in GC tissue samples, and negatively correlated with expression of the calcium-dependent cell-cell adhesion glycoprotein Cadherin-1 (CDH1), reported to be a tumor suppressor. In this study, CDH1 was identified as a direct target of miR-217, and its overexpression enhanced GC cells proliferation, and reduced the CDH1 level, which can be delivered into the microenvironment.

Tumor-originated exosomal lncUEGC1 was also investigated as a circulating biomarker for early-stage gastric cancer (EGC) [23]. In this study, exosomes from the plasma of five healthy individuals and ten stage I GC patients and from culture media of four human primary stomach epithelial cells and four gastric cancer cells (GCCs) were isolated. A total of 79 and 285 exosomal RNAs were expressed at significant higher levels in stage I GC patients and GCCs, respectively, than in normal controls. Two EGC-specific exosomal lncRNAs, lncUEGC1 and lncUEGC2, were further confirmed to be remarkably up-regulated in exosomes derived from EGC patients and GCCs. Almost all the plasma lncUEGC1 was encapsulated in exosomes and thus protected from RNAse degradation rather than circulating freely in the plasma. The diagnostic accuracy of exosomal lncUEGC1 was evaluated and lncUEGC1 exhibited AUC values of 0.8760 and 0.8406 in discriminating EGC patients from healthy individuals and those with premalignant chronic atrophic gastritis (CAG) respectively, which was higher than the diagnostic accuracy of carcinoembryonic antigen (CEA). LncUEG1 may therefore be promising in the development of a sensitive non-invasive biomarker for EGC diagnosis.

In 2019, the roles of extracellular vesicles, and more specifically exosomes, in gastric cancer development were more deeply reviewed [24, 25]. Huang et al. [24] gave a very clear state of the EV-mediated intercellular communications, summarized in their four figures : release of EVs and their content (fig. 1); functions of cancer derived EVs in GC progression and metastasis (fig. 2); the functional network of cancer drived EVs in GC microenvironment (fig.3); the regulation network of microenvironment–derived EVs as well as *H. pilory*-derived EVs (fig. 4). They perfectly illustrated the current state of knowledge about the EV-mediated roles in oncology and their complexity. They discussed how the bidirectional communication between tumor and microenvironment affects GC growth, metastatic behavior and drug resistance. At last, they prospected the clinical application viewpoint of EVs in GC theranostics. They also stressed the many challenging problems, that remain to be elucidated before reaching a better EV clinical translational potential for GC theranostics. Fu et al. [25] focused more specifically on the biological roles of exosomes in GC, and their potential as biomarkers for GC diagnosis and as targets for GC therapy. They summarized, in their table 1, the studies dealing with the discovery of new GC biomarkers among the many tumoral exosomal components and their functions on recipient cells. In their table 2, they focused on exosomes extracted from different body fluids and some of their specific cargo components as diagnostic and prognostic GC biomarkers, with estimation of their clinical value in GC. They also stressed the need for more efforts concerning the knowledge about the mechanims of action of exosomes in GC and the development of their reliable clinical applications. Li et al. [26] questioned the clinical significance of exosomal miRNAs and proteins in three human cancers (lung, liver and gastric) with high mortality in China. For the three cancers, many exosomal miRNAs and a few proteins can be used as diagnostic or prognostic biomarkers, promote tumor progression and metastasis and simultaneously regulate immune response and tumor cells sensitivity to chemotherapy drugs. It is to be noticed that only miR-21 is common to the three cancers, whereas EGFR appears only for lung and gastric cancers. This stresses that each cancer type develops its own very specific pathways, and, therefore, each cancer type needs a specific appropriate theranostic treatment.

Beside the above reviews comforting the interest of exosomal biomarkers for GC diagnosis and prognosis, other studies extended the knowledge about different exosomal miRNAs [27-30]. Thus, Liu et al. [27], aiming to later suppress invasive tissue biopsy, investigated serum circulating exosomal miRNAs for 30 patients with chronic atrophic gastritis (CAG), which is defined as precancerous lesions of GC. A group of 30 chronic non-atrophic gastritis (CNAG), corresponding to healthy donors or patients with moderate gastritis, was used as a control. Six differentially expressed serum exosomal miRNAs were identified in the CAG group, but the most promising biomarker for CAG diagnosis was hsa-miR-122-5p in serum exosomes, and its expression might be correlated with the existence of atrophic and intestinal metaplasia. Another miRNA, miR-129-3p, was described as down-regulated both in GC tumor and in plasma circulating exosomes. Overexpression of miR-129-3p induced more cell apoptosis and inhibited GC cell proliferation, migration and invasion, indicating this mi-RNA component as a powerful anti-tumor miRNA with potential for GC treatment [28]. For advanced GC ending in metastatic PC with poor prognosis, early diagnosis is also an urgency. Through a first large scale evaluation of exosomal miRNAs isolated from malignant ascites in GC, four exosomal miRNAs were significantly downregulated, when compared to those from benign liver-associated ascites. MiR-181-5p showed the best diagnosis performance, even improved when associated with the most commonly used tumor marker CEA [29]. However, the controversial functions of miR-181-5p with either oncogenic or tumor suppressor properties in different cancers need to be further elucidated. MiRNAs were also shown as important regulators of chemoresistance. The expression of miR-374-5p was first found to be upregulated in the serum of GC patients, without any questioning about its possible exosomal protection, and a high level of miR-374-5p was associated with a

poor prognosis [30]. Beside this diagnosis potential, in vitro cell and molecular studies were performed to determine the roles of miR-374-5p in GC chemoresistance. In vivo studies were further used to evaluate the GC therapeutic efficacy of miR-374-5p inhibitor. Exosome-mediated delivery of anti miR-374-5p could re-sensitize GC cells to oxaliplatin by decreasing the expression of multidrug resistance proteins and increasing apoptosis.

Other components already studied in blood (plasma/serum) as potential biomarkers for liquid biopsy were reconsidered at the light of the new knowledge about circulating tumor exosomes/ EVs, such as lncRNAs [31] and mRNAs [32]. Thus, Cai et al. [31] suggested serum exosomal lncRNA PCSK2-2:1, with a length of 465 bp, located on chromosome 20, as a potential novel GC diagnostic biomarker. The expression of this exosomal lncRNA was detected in serum exosomes of 29 healthy people and 63 GC patients with a significative up-regulation. Moreover, the expression level of exo-lnc RNA PCSK2-2:1 was correlated with tumor size, tumor stage and venous invasion. From ROC analysis, the area under the curve (AUC) was 0.896, with the diagnostic sensitivity and specificity of 84% and 86.5% respectively. Compared to the traditional diagnostic markers (CEA, CA724 and CA199), the studied exo-lnc RNA showed significant advantages. Dong et al. [32] detected the expression of exosomal membrane type 1-matrix metalloproteinase (MT1-MMP) mRNA in serum of patients with GC, chronic gastritis or atypical hyperplasia, and healthy controls. Their study enrolled 216 patients, including 33 (17 GC and 16 healthy controls) in training phase and 183 ((119 GC, 33 atypical hyperplasia, 31 chronic gastritis) and 31 healthy controls) in validation phase. Exosomal levels of MT1-MMP mRNA in patients with GC were much higher than in healthy controls, and in patients with chronic gastritis or atypical hyperplasia. The AUC of exosomal MT1-MMP mRNA was 0.788 with 63.9% sensitivity and 87.1%, and was higher than that of CEA (0.655). Higher expression of exosomal MT1-MMP mRNA was statistically correlated with tumor diameter, differentiation, Bormann type, invasion depth, lymphatic metastasis, distal metastasis, and TNM stage, revealing its potential as a reliable GC diagnosis and prognosis biomarker. Lastly, Scavo et al. [33] performed a pioneering study focused on small EVs (sEVs) protein content in colorectal- (CRC) and gastric cancer. The expression of one of the ten human frizzled (FZD) proteins, FZD-10, was investigated in the sEVs extracted from plasma of cancer patients and healthy controls. This FZD-10 protein level was carefully evaluated against the levels of three EVs specific markers, Hsp70, CD63 and Alix proteins. For the first time, the FZD-10 protein, already proven to be involved in tumor development and cancer cell remodeling through the Wnt signaling pathway, was found in plasma as uniquely carried by EVs, rather than present in the whole plasma. Moreover, the FZD-10 protein expression barely detectable in heathy controls was up-regulated in each cancer samples. Interestingly, after surgery and chemotherapy of metastatic tumors, the FZD-10 protein expression depicted a quite different monitoring profile for CRC and GC, stressing the high epigenetic specificity of each cancer. This interesting training study should be comforted by greatly extending the cohort of patients for validating the FZD-10 protein as a biomarker for both GC diagnosis and status monitoring of patients at different treatment stages. Even, a simple, fast and noninvasive diagnostic test might possibly be developed from whole plasma, without waiting for the yet challenging standardization of methods for EVs isolation.

Table 1: Some recent involvment of exosomal biomarkers in liquid biopsy for GC diagnosis and prognosis

Circulating Exosomal Biomarkers	**Studies**	**Liquid samples[a]**	***Functions / Comments***	**AUC**	**Sensitivity (%)**	**Specificity (%)**	**References**
exo (miR-92b-3p+let-7g-5p+miR-146b-5p+miR-9-5p) *up-regulated in GC serum* idem **(4 exo-miRNAs) + CEA**	*Preclinical screening & validation*	serum 36 EGC patients (I, II) 50 EGC patients / 50 healthy controls	*1st study with EGC patients (stages I, II)*	0.773 0.786	60 58	84 90	[35][2020]
exo-miR-15b-3p *up-regulated in GC serum*	*In vitro & In vivo Preclinical*	GC / control cell lines conditioned medium serum 108 GC p. / 108 c.	*new regulation pathway exo-miR-15b-3p / DYNLT1/ Caspase-3 / Caspase-9*	0.820	74.1	80.6	[36][2020]
exo-lnc-GNAQ- 6:1 *down-regulated in GC serum*	*Preclinical*	serum 43 GC p. / 27 c.	*compared with traditional serum biomarkers* (CA72-4, CEA, CA19-9)	0.736	83.7	55.6	[37][2020]
exo-lnc RNA CEBPA-AS1 *up-regulated in GC plasma*	*In vitro Preclinical*	(GC/control cells) CM plasma 281 GC p. / 80 c.	*associated with GC proliferation and apoptosis inhibition*	0.824	87.9	78.8	[38][2020]
exo-lnc-SLC2A12-10:1 *up-regulated in GC plasma*	*In vitro Preclinical*	(GC/ control cells) CM plasma 60 GC p. / 60 c.	*correlated with tumor size, TNM stage, and lymph node metastasis*	0. 776	78.3	75.0	[39][2020]
exo-lnc RNA H19 *up-regulated in preoperative and postoperative, but less in postoperative GC serum*	*Preclinical*	serum 81 GC p. / 79 c.	*the role of serum exo-lncRNA H19 is studied in several cancers, but poorly in GC, correlated with TNM stage*	0.849	74.36	83.95	[40][2020]

[a] p. patients; c. volunteer healthy controls; CM conditioned medium

5.3. Follow-up of some recent involvements of exosomes in GC liquid biopsy

Up to now, the aim of the present review, focused on noninvasive early diagnosis of gastric cancer, has shown the progressive advance of liquid biopsy first through CTCs, then through cf-DNAs and nowadays more and more centered on the appealing new third component of circulating Exos/EVs with their rich protected macromolecular cargo components.

Gao et al. [34] summarized the advances in the role of exosomal non-coding RNAs (miRNAs, lncRNAs and circRNAs) in the development, diagnosis, and treatment of gastric cancer. The putative exosomal miRNAs biomarkers originating from GC patients (GC cells and serum) were enumerated mainly from 2014 to 2019 studies, and the various exosomes-mediated mechanisms of ncRNAs on GC cells were discussed. A follow-up of some more recent involvements of new exosomal biomarkers [35-40] is somewhat disapointing as it might appear as a mere enumeration of already described specific EV components, such as miRNAs, lncRNAs, circRNAs, with a compared estimation of their sensitivity and specificity through ROC curve and AUC calculation (Table 1).

However, some other recent studies [41-46] dipping into the mechanisms of GC progression deserve attention. Zhang et al. [41] analyzed the relationship between miRNAs in plasma exosomes and lncRNAs in GC tissue samples from 87 GC patients. This study showed that the HOX antisense intergenic RNA (HOTAIR), a 2158 nucleotides lncRNA, functions as an onco-lncRNA contributing to GC carcinogenesis via modulating cellular and exosomal miRNAs levels. Strong negative correlations were identified for the first time between the HOTAIR level in GC tissue samples and the miR-30a or-b in plasma exosomes. Moreover, a 10mer target site of miR-30a or-b was identified in the HOTAIR sequence, and HOTAIR regulation of both cellular and exosomal miRNAs expression by direct interaction was confirmed. In vitro, by HOTAIR knockdown, GC cells exhibited decreased migration, invasion, proliferation, and upregulated apoptosis, which released more miR-30a and-b into exososomes. Xie et al. [42] showed that circSHKBP1 was overexpressed in both tumors and serum exosomes of 224 patients with primary GC, and it was correlated with advanced pathological staging and poor survival. The level of circSHKBP1 significantly decreased after gastrectomy. By further in vitro- and in vivo experiments, they demonstrated that exosomal cirSHKBP1 serves as a sponge of miR-582-3p, and promotes GC progression, via regulating the miR-582-3p/HUR/VGEF axis and suppressing HSP90 degradation. Stasevich et al. [43] used an original approach to review the role of ncRNAs (miRNAs, lncRNAs and circRNAs) in the regulation of the proto-oncogene MYC, involved in different types of cancer, including GC. This gives a nice insight of the complexity of the nc-RNAs-mediated regulation of the expression of the MYC gene at the transcriptional and translational levels, together with the stability of the MYC protein.

On the other hand, Liu et al. [44] studied the impact of exosomes derived from GC cell lines (MKN-28, MKN-45, and SGC-7901) on (CD8+) T lymphocytes, which are implied in the immune function in the GC microenvironment. These exosomes changed the gene expression and cytokine secretion levels of on (CD8+) T cells, and both blocked their cell cycle progression and induced apoptosis. In vivo injection of fluorescent labeled exosomes from the three cell lines into C57BL/6 mice showed a favored localization to the lungs. Moreover, these exosomes were mainly taken up by natural killer cells and macrophages in the lung. After long term exposure to injected exosomes (especially from MKN-45 and MKN-28 cells) mice developed an immunosuppressive tumor microenvironment in the lung and promoted lung tumor metastasis. This study provides new insights, into how GC cells-derived exosomes modulate the immune response to create a lung metastatic niche and initiate a mechanism by which GC tumor escapes from the host immune system. On the other hand, programmed death ligand-1 (PD-L1) is an immune checkpoint protein, expressed in multiple cell types, and that interacts with its receptor PD-1 on T cells, triggering inhibitory signals, that prevent T cell activation and proliferation. Miliotis and Slack [45] recently performed a correlation analysis between PD-L1 expression and all host miRNAs in 368 stomach cancer patients. Among 24 significant miRNAs, only a single one, miR-105-5p, was predicted to have a binding site on PD-L1. By complementary in vitro co-culture experiments, they demonstrated that overexpression of miR-105-5p can promote immune surveillance in GC, through down-regulation of PD-L1. Moreover, their study established a regulatory network that connects DNA methylation-controlled upregulation of miR-105-5p with decreased PD-L1 expression and increased immunogenicity in cancer cells. Although not taken into account in this study, the possibility that the secretion of miR-105-5p in the GC microenvironment and in blood, might occur through packaging in exosomes was mentioned, and remains to be checked. On line with these two studies [44, 45], related to GC and immunity, and with the two decades achievement of cancer immunotherapy, Abu and Rus Bakarurraini [46] deciphered the interweaving relationship between EVs and T cells in cancer.

At last, two recent reviews, respectively stressed the emerging role of liquid biopsy in GC [47] and claimed EVs as a promising biomarker resource in liquid biopsy for cancers [48]. Lengyel et al. [47] summarized the current knowledge and explored future possibilities of liquid biopsy in the management of metastatic GC. They recalled the ongoing clinical trials using liquid biopsy approaches for GC, but none yet involving exosomes. By contrast, Tamura et al. [48] stressed not only exosomes, but all extracellular vesicles, as a promising biomarker resource in liquid biopsy for cancer. They discussed the feasibility and practicality of EV-based liquid biopsy in clinical settings. They first argued the advantages and challenges of EV-based liquid biopsy for clinical application. Then, they summarized recent notable studies investigating specific EV- associated biomarkers (main-

ly proteins and RNAs, but also DNAs) for many different human cancers, of which a few related to gastric cancer. In conclusion, they asserted that the development of EV-based liquid biopsy will lead to early diagnosis of fatal cancers and tailor-made treatments for individual patients.

6. Discussion

Instead of always gathering some new biomarkers among the now recognised so rich EVs cargoes (4), it seems more fruitful to discuss about the necessity of urgently performing a more efficiently directed worldwide search to solve some precise pending biological problems about extracellular vesicles. EVs are now recognised as important intercellular communication messengers, but their functional influence during health and disease is far from being understood. Many fundamental issues about EVs remain to be solved, such as the specific influence of each main kind of EVs, giving rise to a wide yet uncontrolled EVs heterogeneity beside the most studied exosomes. Moreover, the specific targeting of a given EVs cargo component into a given EV type is also unexplained, as well as the specificity of different EVs for targeting specific recipient cells, near or distant from the primary tumor.

The micro-organism *Dictyostelium discoideum (Dd)* (http://dictybase.org) as a unique simple both "in vitro and in vivo" eukaryotic cell model offers a very interesting possibility for further deciphering the biological influence of EVs [12]. Briefly, the main *Dd* asset for this purpose is its fully sequenced 6 chromosomes-genomic (3.4 x 10^7 bp) DNA, with a 90% efficient transcription into about 12,500 genes. By comparison, the human (about 10^9 bp) genome is only10% transcribed into about only twice as many genes as *Dictyostelium*. It means that the non-coding genomic DNA, which is now recognized as the important source of the non-coding RNAs regulating gene expression are repectively only about 3.4 x 10^6 bp for *Dd* and 9 x 10^8 bp for human genomic DNA. To my knowledge, the study of the *Dd* non-coding RNAs using the modern technologies is not yet a matter of research and the study of *Dictyostelium* EVs, which we initiated in 1998, only began to nicely attract attention two decades later by being involved in the important cAMP-mediated chemotactic signaling [49]. Moreover, for *Dd* cells growth and starvation-induced differentiation are well separated physiological processes, giving rise to release of different EVs [50], whose respective cargoes remain to be analysed. Lastly, *Dd* is an eukaryotic amoeba at the border of the vegetal and animal kingdoms, which appeared in evolution about one billion years ago and has been extensively studied since its discovery in 1935. Its possible growth in axenic conditions, without any fetal calf serum, and even in defined medium, and an available *Dicty* Stock Center, are two other assets for programing conditioned media (CM) experiments, with the aim of deciphering the still mysterious EVs biological roles.

However, physicians cannot wait for such a long time-consuming biological approach and wish a quick access to the clinical use of the claimed potentially interesting cancer biomarkers. Therefore, they have to solve another recurrent problem, which is that the preclinical trials always suffer from a too restricted number of enrolled patients (Table 1). Therefore, it is urgent to elaborate a strict standardization protocol for EVs isolation and characterisation, taking into account ISEV recommandations for applying EV-based therapeutics to clinical trials [51]. They should, then, submit some promising biomarker(s) to a large scale worldwide medical validation for diagnosis of a well-chosen specific human cancer, such as GC f. ex., in great need for early diagnosis, as a prerequisite for a further optimized patient outcome after treatment.

7. Conclusion

In the sixties, the cancer model included two main steps, i.e. initiation and promotion, with three main types of cancer origin, i.e. genetic, viral or chemical. Nowadays, cancer is becoming a major epigenetic disease and, despite some common hallmarks, each type of cancer seems to be specific, mainly due to the specificity of its many regulation processes during its progression. On the other hand, cEVs are now qualified as being an interesting third component for cancer liquid biopsy. However, one of the issues is the rich EVs cargoes, where many biomolecular compounds might be potential candidates as biomarkers for cancer early diagnosis. Beside the fast growing knowledge about EVs composition and functions in intercellular communications between a tumor and its near or distant microenvironment, what is most challenging is the lack of standardization in EVs isolation and characterisation. Defining a well-standardized protocol is an urgent prerequisite for a further large scale validation of some of the most promising biomarkers for GC early diagnosis and prognosis after tumor resection and treatment. Although cEVs offer many advantages upon the previous liquid biopsies based either on rare CTCs or on cfDNAs, efficient clinical translation into EV-mediated liquid biopsies is still a distant goal. Beside following preclinical trials to find the most promising exosomal biomarkers inside the rich cargoes of circulating EVs, it is probably time to explore in parallel the new cell-released EVs research field, extending the cell properties well beyond the plasma membrane, from both a biological- and a medical viewpoint. Now, the necessary time scale for achieving the goal of reaching an efficient noninvasive EV-mediated liquid biopsy for GC early diagnosis and prognosis cannot be predicted, but the crucial roles of exosomes in cancer, vaccine development and therapeutics are already stressed [52].

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