Extracellular Vesicles in Hepatocellular Carcinoma

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Extracellular vesicles (EVs) are defined as lipid bilayer particles naturally released from cells into the extracellular space. EVs have attracted interest as mediators of intercellular communication following the discovery that EVs contain RNA molecules, including non-coding RNA (ncRNA).
Growing evidence for the enrichment of peculiar RNA species in specific EV subtypes has been demonstrated. ncRNAs, transferred from donor cells to recipient cells, confer to EVs the feature to regulate the expression of genes involved in differentiation, proliferation, apoptosis, and other biological processes. These multiple actions require accuracy in the isolation of RNA content from EVs and the methodologies used play a relevant role. In the liver, EVs play a crucial role in regulating cell-cell communications and several pathophysiological events in the heterogeneous liver class of cells via horizontal transfer of their cargo.

Keywords: extracellular vesicles ; RNA ; non-coding RNA ; liquid biopsy ; biomarker ; hepatocellular carcinoma

1. Extracellular Vesicles as Carriers of RNA Molecules

The discovery that EVs can carry nucleic acids revealed their crucial role in horizontal genetic transfer [1][2].

The circulating RNAs associated with EVs can reach cells other than the originating, both in neighboring cells and in cells located elsewhere in the body, and, once inside, can influence gene expression.

EVs can contain messenger RNAs (mRNAs) [3] and non-coding RNAs (ncRNAs) of different length, including long non-coding RNAs (lncRNAs) [4], microRNAs (miRNAs), and circular RNAs (circRNAs) [5].

The subcellular localization of RNAs and the EV subtypes positively influences their loading.

Interestingly, a selective sorting process was identified for specific RNAs, sharing a short sequence called hEXO motif during the hepatocytes' EV formation. The main component of this loading machinery belongs to the synaptotagmin-binding, cytoplasmic RNA-interacting protein (SYNCRIP) complex, which directly binds to some miRNA enriched in EVs [6].

Different studies demonstrated that EVs produced by different cell types presented different RNA content [7], depending on EV subcellular source and cell physio-pathological conditions [8]. An increasing amount of RNA molecules have been found to be aberrantly expressed in human cancers [9].

Based on this information, it is clear that ncRNAs' biological message is related to tumor cell spreading and oncogenic onset. On the other hand, it is not easy to identify a single ncRNA as a specific disease marker because they act with the principle of cooperation. For example, a single gene targets several miRNAs, just as the same miRNA can act on several genes [10]. Thus, it is more likely to identify a pattern of ncRNAs whose expression is related to a specific alteration.

RNA molecules can be isolated from biological samples (i.e., cell culture medium or blood/plasma) in two ways: RNAs can be obtained by extracting the total RNA from both EV-associated RNA or free and protein-bound RNA. Alternatively, more accurately, EVs can be isolated from biological samples using a differential centrifugation approach, ultracentrifugation, or other methods, such as size exclusion chromatography, and only EV RNA can be isolated [11].

Currently, there is no gold standard technique for EV isolation and, thus, the method should be chosen based on both the type and amount of EVs.

Conventional methodologies for EV isolation suffer from limitations in separation technology. In particular, the detection of EVs is vulnerable to artefacts partly induced by sample collection and the huge heterogeneity of EV populations. Furthermore, the main approaches are based on EVs' physical properties (density, solubility, or size), and are not able to separate the tumor-derived EVs from total EVs [12].

In particular, Sun and colleagues (2020) developed an EV purification system using a multiple marker cocktail to recognize, enrich, and recover HCC EVs secreted from highly heterogeneous HCC [13]. Several biotechnology companies are currently working to develop a quick and easy assay based on precipitation to isolate EVs. These kits often require polyethylene glycol I (PEG1) solutions, that once mixed with samples allow EVs to precipitate at low speed. Nevertheless, this method suffers from co-isolation of non-EV particles and protein complexes and must be further improved [14].

The analysis of RNA molecules is allowed by different approaches, which include microarrays, quantitative real-time polymerase chain reaction (qRT-PCR), digital PCR (dPCR), NanoString's nCounter technology, and next-generation sequencing (NGS) [15][16][17]. The main difference between these methods is the sensitivity of the RNA transcript detection.

The most common RNA detection is the microarray analysis because it can detect simultaneously different nucleic acids and can be customized [18][19].

Digital PCR (dPCR) can be considered an alternative to the qPCR approach and provides more accurate data of the nucleic acid target molecule without a standard curve and dependence on amplification efficiency. The hypersensitivity of dPCR allows detecting RNA molecule targets of low abundance below the qPCR's sensitivity limit [20][21]. This system can easily reveal and quantify the low amount, like EV content, but it is a long procedure and relatively expensive.

The system, formed on a multiplexed probe library, contains two types of probes (capture probe and reporter probe) specific for each nucleic acid molecule to detect. The capture probes are tagged with biotin at the 3′-end, whereas the reporter probes carry a barcode signal at the 5'-end [22]. This technology works without amplification or reverse transcription. In a recent study, the RNA content from EVs was analyzed by the nCounter platform, demonstrating this method's efficacy to detect plasma EV mRNA transcripts [23].

Finally, it was demonstrated that the Nanostring nCounter is a more accurate system than microarrays and comparable in susceptibility to real-time PCR [24].

NGS consists of sequencing technology and is supported by different platforms. It offers the advantage to generate a huge amount of sequence data sets, ranging from megabases to gigabases [25].

2. Extracellular Vesicle-Derived RNAs Correlated with Hepatocellular Carcinoma

A body of evidence highlights the growing interest in the investigation of EV involvement in liver cancer.

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer, which generally arises as a direct progression and evolution of chronic liver diseases (CLD), including liver cirrhosis (LC), and is an overly aggressive carcinoma with a poor prognosis [26][27].

EVs can provide a consistent form of the liver intercellular network between hepatocytes, intrahepatic cholangiocytes, Kupfer cells which are liver-resident macrophages, hepatic stellate cells (HSCs), endothelial cells, fibroblasts, infiltrating immune cells, and recruited mesenchymal stem cells (MSCs), given the multicellular nature of liver [28].

HCC-derived EVs can mediate cell growth, modulation of epithelial–mesenchymal transition, migration, invasion of HCC cells, and the angiogenesis process. EVs shuttle biologically active RNAs that may alter the tumor microenvironment, resulting in HCC progression and metastasis [29].

The analysis of tumor-associated RNA within EVs could allow the identification of novel biomarker candidates.

Many studies have shown aberrantly expressed tumor-associated protein-coding mRNAs and the expression of specific non-coding RNAs, including miRNAs and lncRNAs selectively enriched in EVs released from different HCC cell lines [30].

EVs derived from HKCI-C3, HKCI-8, and MHCC97L cell lines increased RNAs of lengths ranging between 500 and 4000 nucleotides, compared to their parental cells. The RNA analysis identified mainly mRNA and lncRNA molecules. A limited quantity of ribosomal RNA (18S and 28S rRNA) and mRNA can be translated into proteins in the recipient cell [30].

In Hep3B-derived-EVs, 11 miRNAs (miR-133b, miR-142-5p miR-215, miR-367, miR-376, miR-378, miR-451 miR-517c, miR-518d, miR-520f, and miR-584) were the only ones detected. Likewise, the expression of 20 miRNAs was explicitly discovered in PLC/PRF/5- derived EVs [31].

The results obtained in both cell lines suggest a mechanism driving the hepatic tumor cells to sort a specific set of miRNAs into HCC-derived EVs.

In an exciting study, HCC cells were treated with a neutral sphingomyelinase 2 (nSMase) inhibitor (GW4869) and the expression of miR-16, a miRNA expressed in both originating cells and small EVs, was evaluated. The intracellular expression of miR-16 was unchanged, whereas the extracellular expression of miR-16 in small EVs decreased after incubation with GW4869, compared to controls. This result demonstrates that specific miRNAs from HCC cells could be released into EVs in a ceramide-dependent manner [32].

Recent findings highlighted that some miRNAs, called oncogenic miRNAs or oncomiRs (e.g., miR-21), are able to activate the cell proliferation and inhibit the apoptotic processes, thus regulating HCC growth and development [33].

The main oncomiR, which is highly expressed in almost all solid cancers, including HCC, is miR-21, which is also enriched in tumor-derived EVs [34][35][36][37]. Generally, miR-21 has an anti-apoptotic, pro-survival function in tumor cells. The analysis of miRNAs in HCC-derived EVs showed that miR-21 expression level in EVs was positively associated with the intracellular one in cells and negatively associated with its target genes PTEN, PTENp1, and TETs. Therefore, the EV-miR-21 might modulate the expression of the tumor suppressor genes PTEN and PTENp1, affecting HCC cells' growth [38].

One target of the EV-derived miRNA was identified in the transforming growth factor-β activated kinase-1 (TAK1) pathway in HCC cells [31].

TAK1 is a kinase involved in hepatic cellular homeostasis and liver pathology, including HCC tumorigenesis [39][40]. Both cytokines and stress stimuli, such as transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interleukin (IL)-1β, can trigger TAK1 [41].

Some evidence demonstrated that HCC development and metastasis are triggered when the suppression of the constitutive expression of TAK1 is induced in hepatocytes [42].

TAK1 modulation can be mediated by EV-derived miRNAs in recipient cells, enhancing tumoral cell growth. Hep3B-derived EVs were incubated with Hep3B cells, and after 24 h, the cell viability and apoptosis were analyzed. The content of EVs derived from Hep3B cells induced a decrease in cell viability of recipient cells and an increase in caspase-3/7. Hence, it was clear that the EVs with their cargo have a potent effect on tumoral cell behavior.

EV-derived miR-210 released by hepatic tumor cells is transferred into endothelial cells and leads to tumor angiogenesis, inhibiting SMAD4 (mothers against decapentaplegic homolog 4) and signal transducer and activator of transcription 6 (STAT6) expression [43].

EV-derived miR-103 released by HCC cells enhanced vascular permeability and promoted tumor metastasis by directly targeting endothelial junction proteins, including VE-cadherin (VE-Cad), p120-catenin (p120), and zonula occludens 1 (ZO-1) [44].

EV-derived miR-1247-3p released by highly metastatic HCC cells triggered the activation of β1-integrin–NF-κB signaling in fibroblasts and induced cancer-associated fibroblast activation, promoting tumor metastasis [45].

The selective enrichment of lncRNA in HCC-derived EVs has been demonstrated. TUC339, linc-RNA-RoR (long intergenic non-protein-coding RNA, regulator of reprogramming) and linc-RNA-VLDLR (very low-density lipoprotein receptor) are the prominent lncRNA family members detected and implicated in tumor cell behavior [31][46][47].

EVs isolated from HepG2 cells contain a higher amount of one of the ultraconserved lncRNAs, named TUC339, compared to EVs from non-malignant hepatocytes [48].

The same result has been obtained in EVs isolated from PLC/PRF/5 cells, demonstrating that the most highly expressed lncRNA was TUC339 in HCC cell-derived EVs [49].

The inhibition of TUC339 with short interfering RNA (siRNA) decreased the proliferation and adhesion ability of hepatic tumor cells. Accordingly, the delivery of lncRNA-TUC339 via EVs can be considered a novel signaling mechanism for developing HCC growth and metastasis [31].

Healthy hepatocytes display a low level of lncRNAs, including the long intergenic noncoding RNA, regulator of reprogramming (linc-RoR) [46], which can prevent tumorigenesis and cell proliferation by directly regulating the stability of the c-Myc mRNA [50]. EV-derived linc-RoR released by HCC cells was highly expressed during hypoxia conditions. The increase in EV-derived linc-RoR level in HCC cells decreased the expression of miR-145, a linc-RoR target, resulting in an increase in hypoxia-inducible factor-1α (HIF-1α) and pyruvate dehydrogenase kinase isozyme 1 (PDK1) protein expression [46].

Hence, the EV-derived linc-RoR could promote HCC progression by increasing HCC resistance against adverse environmental conditions, including hypoxia [46].

Most recently, HCC-derived circRNAs were found to display an aberrant expression associated with tumoral characteristics and recent studies reported circRNAs enrichment in EVs released from HCC cells [51].

circPTGR1 is a circRNA with three isoforms enriched in EVs isolated by HCC cell-lines, and its expression level was correlated with tumor differentiation stage, indicating its prognostic potential in HCC patients [52]. The study included the analysis of circRNA expression of EVs derived from three different HCC cell lines: non-metastatic (HepG2), low-metastatic (97L), and high-metastatic (LM3) cells. EVs derived from LM3 cells and containing circPTGR1 enhanced the cell migration and invasion attitude of HepG2 and 97 L cells and, on the other hand, knockdown of circPTGR1 expression in LM3 cells inhibited the migration and invasion of HepG2 and 97L cells [52]. Therefore, circPTGR1 was highly abundant and aberrantly expressed in malignant cells and in cells from patients with metastases, thus showing its contribution to HCC progression and metastasis.

A large class of miRNAs acts as tumor suppressors, such as miR-122, indicating that HCC-derived EVs are a system that allows the modulation of HCC growth and progress [53]. MiR-122 is a liver-specific anti-proliferative miRNA and is involved in regulating fatty acid and cholesterol pathway as well as normal cell homeostasis and growth, to maintain tumor growth under control [54]. The hepatic decrease in miR-122 expression level could favor the development of steatohepatitis, such as nonalcoholic fatty liver disease (NAFLD) Studies in NAFLD animal models highlighted the increase in circulating EV-associated miR-122 [55][56][57].

The delivery of EV-miR-122 from normal hepatocytes suppressed tumor progression. However, this effect is inhibited when tumor-initiating cells (T-ICs) start to secrete insulin-like growth factor 1 (IGF-1), arresting miR-122 release from neighboring healthy hepatocytes, thus resulting in a reduction in its anti-proliferative activity and in hepatic tumor development and metastasis [58]. Thus, the expression of miR-122 correlated in the early NAFLD progression with HCC development [59].

Vps4A (vacuole protein sorting 4), a member of the AAA-ATPases (ATPases associated with a variety of cellular activities), was recognized as a tumor suppressor in HCC cells by regulating the release and uptake of EV-derived microRNAs [60].

Wei and colleagues showed that Vps4A inhibited EV function by selectively packaging oncogenic miR-27b-3p and miR-92a-3p into EVs and accumulating tumor-suppressive miR-193a-3p, miR-320a, and miR-132-3p in HCC cells [60].

Furthermore, they found that Vps4A reduced the recipient HCC cell response to EVs via selective uptake of exosomal tumor-suppressive miR-122-5p, miR-33a-5p, miR-34a-5p, miR-193a-3p, miR-16-5p, and miR-29b-3p [60].

3. Conclusion

Research of nucleic acids within EVs to identify a panel of biomarkers has the ability
to provide new biological knowledge and support diagnosis and therapeutic monitoring
in HCC. Despite the exponential interest in the EV field and the recent advances in isolation/ characterization methods, the challenge of acquiring EV samples with high yield and purity and standardizing RNA processing is still open.
It is now well known that RNA molecules are stable within EVs, since the lipid
bilayer conserves them from the enzymatic activity of RNases. Therefore, to evaluate
EV-derived RNAs as potential biomarkers in HCC diagnosis and prognosis, blood samples are appropriate.
Further advancement in the isolation and detection of EV-derived RNAs is required,
defining the different sources from which EV-derived RNAs are obtained.
An important step forward will be taken when tumor properties, such as tumor differentiation stage or the post-surgery tumor relapse, including the presence of microvascular invasion, are associated with the expression level of specific EV-derived RNA molecules.

Finally, for a more relevant clinical use, a pattern of biomarkers associated with EVs
in the progression of HCC may be considered. Correlation panels among RNA content and proteins and lipids associated with EVs could be set up. We are increasingly convinced that EVs contain rich information; therefore, to consider only a part of their content would be reductive from a diagnostic and prognostic perspective.

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