Reviewer #1

1. Would be interesting to know which kind of vesicles are more powerful among RNAs; MicroRNAs; circRNAs; piRNAs; Exosomes; Extracellular vesicles.

(Part of our answer has been included in: Manuscript section **#INTRODUCTION** #paragraph 4) The subtypes, amount and composition of EVs varies and mainly depends on cellular sources and pathophysiological conditions. Furthermore, many studies have shown that there is a different expression of exosomal ncRNAs in cancer patients compared to healthy subjects and often mirrors the type of cancer. The mechanisms of EV packaging remain unclear; however, several factors that affect the composition of EVs have been described so far. These include a) the upregulation of a distinct RNA type in parental cells and b) the existence of sorting processes that may be biotype-specific. {**Abels ER,** Breakefield XO. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell Mol Neurobiol* 2016; **36:** 301-12 [PMID: 27053351 DOI: 10.1007/s10571-016-0366-z]} For instance, it has been reported that the composition of ncRNAs in $CD47^+$ EVs differs from that of $CD47^-$ EVs, despite that they were isolated from the same cells {**Kaur S,** Elkahloun AG, Arakelyan A, Young L, Myers TG, Otaizo-Carrasquero F, Wu W, Margolis L, Roberts DD. CD63, MHC class 1, and CD47 identify subsets of extracellular vesicles containing distinct populations of noncoding RNAs. *Sci Rep* 2018; **8:** 2577 [PMID: 29416092 DOI: 10.1038/s41598-018-20936-7]} However, Chevillet *et al*, found that the number of exosomes and miRNAs in samples of different origin (including normal and cancer cells) was less than a copy of a given miRNA per exosome, regardless of the source {**Chevillet JR,** Kang Q, Ruf IK, Briggs HA, Vojtech LN, Hughes SM, Cheng HH, Arroyo JD, Meredith EK, Gallichotte EN, Pogosova-Agadjanyan EL, Morrissey C, Stirewalt DL, Hladik F, Yu EY, Higano CS, Tewari M. Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc Natl Acad Sci U S A* 2014; **111:** 14888-93 [PMID: 25267620 DOI: 10.1073/pnas.1408301111]} Based on this observation, Lee *et al*, suggested that a subpopulation of "miRNA-rich" EVs can be detected and isolated using density-gradient fractionation. More importantly, they identified caveolin-1, as a possible

biomarker of "miRNA-rich" EVs. {**Lee H,** Groot M, Pinilla-Vera M, Fredenburgh LE, Jin Y. Identification of miRNA-rich vesicles in bronchoalveolar lavage fluid: Insights into the function and heterogeneity of extracellular vesicles. *J Control Release* 2019; **294:** 43-52 [PMID: 30529727 DOI: 10.1016/j.jconrel.2018.12.008]} Indeed, some lncRNAs are found in abundance whereas others are hardly detectable in EVs. {[**Gezer U,** Özgür E, Cetinkaya M, Isin M, Dalay N. Long non-coding RNAs with low expression levels in cells are enriched in secreted exosomes. *Cell Biol Int.* 2014;**38**:1076-9 [PMID: 24798520 DOI: 10.1002/cbin.10301]} Finally, it should be noted that direct comparisons between ncRNA subtypes, are not always available. {**Spinelli C,** Adnani L, Choi D, Rak J. Extracellular Vesicles as Conduits of Non-Coding RNA Emission and Intercellular Transfer in Brain Tumors. *Noncoding RNA* 2018; **5:** 1 [PMID: 30585246 DOI: 10.3390/ncrna5010001]} In conclusion current evidence supports that the different EV subsets may differ in their content of bioactive RNA biotypes, and consequently in their regulatory functions.

2. What is the half life of each vesicle type in circulation (among RNAs; MicroRNAs; circRNAs; piRNAs; Exosomes; Extracellular vesicles.)

A paragraph has been added in-text. (Please refer to: Manuscript section **#EXOSOMAL NON-CODING RNAs IN CCA: POTENTIAL DIAGNOSTIC AND THERAPEUTIC APPLICATIONS. FUTURE EXPECTATIONS** #paragraph 5)

3. How exosome would target cancer cells?

A paragraph has been added in-text. (Please refer to: Manuscript section **#EXOSOMAL NON-CODING RNAs IN CCA: POTENTIAL DIAGNOSTIC AND THERAPEUTIC APPLICATIONS. FUTURE EXPECTATIONS** #paragraph 6)

4. Which stage of cell cycle is more suitable for maximum exosomes release in the fluids and why?

One of the main mechanisms that orchestrate ILV formation and packaging of bioactive exosomal cargo depends on the endosomal-sorting complexes required for transport system (ESCRT). {**Zhang Y,** Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci* 2019; **9**: 19 [PMID: 30815248 DOI:

10.1186/s13578-019-0282-2]} The latest functions during the abscission phase of cytokinesis (M phase of cell cycle). {**Stoten CL,** Carlton JG. ESCRT-dependent control of membrane remodelling during cell division. *Semin Cell Dev Biol* 2018; **74:** 50-65 [PMID: 28843980 DOI: 10.1016/j.semcdb.2017.08.035]} Moreover, it has been suggested that exosome secretion is also coordinated by Rab GTPases, which recently emerged as regulators of abscission. **{Rodriguez-Boulan E,** Kreitzer G, Müsch A. Organization of vesicular trafficking in epithelia. *Nat Rev Mol Cell Biol* 2005; **6:** 233-47 [PMID: 15738988 DOI: 10.1038/nrm1593]} Thus, we could assume that phase M (precisely cytokinesis) is the more suitable cell cycle phase for exosome formation and release, so that MVBs escape from excessive degradation by lysosomes.

5. Table for clinical application provides one study for exosomes and more studies for EVs. While title is about exosomes. If possible inclusion of more studies about exosomes would be good.

As we have already mentioned in our manuscript, the term "EVs" often coincides with the term "exosomes" in the literature. (Please refer to: Manuscript section **#INTRODUCTION** #paragraph 5)**.** It should be noted that the most common method for EV isolation has been differential ultracentrifugation which is not able to accurately separate each type of EVs, due to their heterogeneity. For instance, in their work, Li *et al*, refer to EVs; however, the purification protocol that they used supports exosome isolation. {**Li L,** Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, Georgiades C, Singh VK, Khashab M, Amateau S, Li Z, Okolo P, Lennon AM, Saxena P, Geschwind JF, Schlachter T, Hong K, Pawlik TM, Canto M, Law J, Sharaiha R, Weiss CR, Thuluvath P, Goggins M, Shin EJ, Peng H, Kumbhari V, Hutfless S, Zhou L, Mezey E, Meltzer SJ, Karchin R, Selaru FM. Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. *Hepatology*. 2014;60(3):896-907. Erratum in: Hepatology 2014; **60**: 2135 [PMID: 24497320 DOI: 10.1002/hep.27050]} We conducted an additional search in pubmed (as of 14Aug2020), using the terms "exosomes and microRNAs and cholangiocarcinoma", "exosomes and long non coding RNAs and

cholangiocarcinoma", "exosomes and circular RNAs and cholangiocarcinoma", "exosomes and piwi RNAs and cholangiocarcinoma", "extracellular vesicles and microRNAs and cholangiocarcinoma", "extracellular vesicles and long non coding RNAs and cholangiocarcinoma", "extracellular vesicles and circular RNAs and cholangiocarcinoma", "extracellular vesicles and piwi RNAs and cholangiocarcinoma". The identified studies were included in our review.

Reviewer #2:

1. "CCA is the second most common primary liver cancer after hepatocellular carcinoma" was mentioned in the introduction, but distal cholangiocarcinoma also belongs to liver cancer?

A clarification has been provided in-text. "Intrahepatic CCA is the second most common primary liver cancer after hepatocellular carcinoma and its prognosis is very poor, mainly depending on the potential and extent of surgical resection[2,3];" (Please refer to: Manuscript section **#INTRODUCTION** #paragraph 1)

2. Exosomes are crucial mediators of intercellular communication since they can transfer miRNA/lncRNA/circRNA to tumor microenvironment and alter biological behavior of other cells. The authors should exhibit visually the procedure of exosomes function by figure.

Figure 2 has been added.

3. Noncoding RNA can regulate gene expression at epigenetic, transcriptional and post-transcriptional levels by diverse mechanisms, such as sponge, scaffold, guide, decoy and signal, etc. The authors should discuss the mechanisms of exosomal miRNA/lncRNA/circRNA in CCA by separate sections, as well as recent reports.

A section has been added in-text. (Please refer to: Manuscript section **#DEREGULATION OF NON-CODING RNAs IN CCA: MOLECULAR MECHANISMS**)