Dear Prof. Yuan Qi, Science Editor, Editorial Office

World Journal of Gastroenterology

April 11, 2017

Thank you for your kind letter concerning our manuscript entitled **"Liquid biopsy in patients with hepatocellular carcinoma: Circulating tumor cells and cell-free nucleic acids"** by Okajima and Komatsu et al. We have revised the manuscript using a red color font to highlight the modifications according to reviewer's comments and also enclosed a letter outlining responses to your questions.

We believe that our revised manuscript has been improved by these revisions and satisfy your concerns. We sincerely appreciate your work in reviewing our manuscript. We hope that the revised manuscript is now acceptable for publication in **World Journal of Gastroenterology**.

Sincerely yours

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Comments from Science Editor:

First of all, thank you very much for your long-standing support and continued trust in the World Journal of Gastroenterology!

The review of your manuscript, which you submitted to the World Journal of Gastroenterology, is now completed and the first decision for publication is available. We request that you submit your revision.

Comments to Science Editor:

We thank you for your kind comments and valuable suggestions for our manuscript. We have carefully revised it accordingly. Explanations have been provided point by point. We believe that our revised manuscript has been improved by these revisions, and satisfy your concerns.

We cordially appreciate your contribution regarding our manuscript. We hope that the revised manuscript is now acceptable for publication in the "**World Journal of Gastroenterology**".

Comments from Reviewer #1

This is a well-integrated review paper from Okajima and Komatsu et al describing the role of "liquid biopsy" in patients with hepatocellular carcinoma (HCC). This is a topic of increasing interest hence the timing and the article is of interest. There are some areas where I would like to mention.

Responses to Reviewer #1's comments

Thank you for your kind comments. We found your advice very helpful and have revised our manuscript accordingly. In the summary below, the numbering of our responses corresponds to the numbering of your comments we added.

Major comments

1.

Query:

Theoretically, circulating tumor cells (CTCs) are useful markers for early diagnosis; however, the problem is that the earlier the stage is, the less the cells are (tends to be proportional to tumor volume). In addition, less than 0.01% of CTCs introduced into the circulation survive to produce metastases. Therefore, the performance (sensitivity and specificity) of detection technique should be achieved to a proper level. This should be clearly mentioned in the manuscript.

Reply:

Thank you for your comments. We completely agree with you. Therefore, we revised our manuscript as follows by citing your valuable comments. Thank you very much.

Revised:

Biology and detection of circulating tumor cells

CTCs are generally recognized as the "seeds" of tumors, which are shed into peripheral blood from a tumor in situ and eventually establish metastatic tumors in other organs [18]. Therefore, theoretically, circulating tumor cells (CTCs) are useful markers for early diagnosis. In 1869, Ashworth [19] identified the presence of CTCs for the first time in the blood of a metastatic breast cancer patient in whom cells similar to those in the primary

tumors were found in the blood at autopsy. Since then, many groups have challenged and demonstrated the identification and characterization of CTCs in the peripheral blood of patients with several types of cancers.

However, the effort has been hampered by some difficulties. The problem is that the earlier the stage is, the less the cells are. Namely, the cell tends to be proportional to tumor volume. Moreover, CTCs have estimated infrequencies of approximately 1–10 CTCs in a background of millions of blood cells in patients with metastatic diseases [20]. In addition, less than 0.01% of CTCs introduced into the circulation survive to produce metastases. Furthermore, CTCs are thought to be quite heterogeneous in both phenotype and genotype, and only 2.5% of CTCs develop micrometastases and only 0.01% develop macrometastases [21-23]. Although present in various metastatic tumors with a wide range of frequencies, it is known that CTCs originate from the primary tumor and/or metastatic lesions and are, therefore, extremely rare in healthy subjects and patients with nonmalignant diseases [24]. Therefore, the performance such as sensitivity and specificity of detection technique should be achieved to a proper level, accurate isolation and detection of CTCs with sufficient sensitivity and specificity has been a major technical challenge for researchers.

2.

Query

To divide "CTC detection in patients with HCC and its clinical relevance" into two section: 1) biological method and 2) physical method would make the manuscript more readable.

Reply:

Thank you for your valuable comments. We divided "**CTC detection in patients with HCC and its clinical relevance**" into two section as you indicated. Furthermore, to maintain consistency, we also divided "**Techniques for isolation, enrichment, and identification of CTCs**" section in the same manner.

Revised:

Techniques for isolation, enrichment, and identification of CTCs

Accurate isolation and detection of CTCs with sufficient sensitivity and specificity has been a major technical challenge for researchers. In recent years, various CTC isolation and enrichment technologies have emerged, their approaches can be mainly categorized into two groups: 1) physical methods and 2) biological methods.

1) Physical methods

Physical methods mainly depend on the physical properties of CTCs, such as size, density, electric charge, migratory capacity, and deformability [25]. These methods include density gradient centrifugation, filtration, and dielectrophoresis. Several filtration-based approaches have been developed based on the concept that most CTCs derived from epithelial cancers are generally larger in diameter than other blood cells [26, 27]. However, significant variations in cell size within an individual patient as well as within different types of tumor cells have been reported [28-30]. Therefore, new approaches using multiple filters have been investigated to resolve those issues and achieve an accurate enrichment of CTCs [31, 32]. These micro device could isolate cancer cells from whole blood via their distinctively different physical properties such as deformability and size continuously. For example, Mohamed *et al* designed a micro-machined device, which had arrays of four successively narrower channels, each consisting of a two-dimensional array of columns, were able to fractionate cancer cells without interference from the blood cells. While those new approaches are likely to possess great promise in isolating CTCs, further validation studies should be conducted to verify their significance.

2) Biological methods

Another popular approach is biological methods, which mainly rely on

antigen-antibody binding and antibodies against tumor-specific biomarkers including epithelial cell adhesion molecule (EpCAM), human epidermal growth factor receptor2 (Her2), and prostate-specific antigen (PSA) that are typically used in CTCs purification [33]. Currently, EpCAM-based Cell-Search™ (Veridex LLC, NJ, USA) is the most widely used CTC platform. In this platform, immunomagnetic beads coated with anti-epithelial cellular adhesion molecule (EpCAM) antibodies capture CTCs, followed by immunostaining with two positive markers (cytokeratins (CKs) 8/18/19 for cytoplasmic epithelium and 4′,6′-diamidino-2-phenylindole hydrochloride for nucleic acids) and a negative marker, leukocyte-specific CD45. This system is the only CTC platform to gain the approval of the United States Food and Drug Administration, and its clinical utility has been demonstrated as a diagnostic and prognostic indicator in patients with metastatic breast, prostate, and colon cancers [34-39]. However, it could fail to capture CTCs that have undergone EMT, thereby increasing the malignant potential. Several challenges remain regarding the detection and isolation capability and, thus, the clinical utility of CTCs. To improve sensitivity and specificity despite the heterogeneity of CTCs, new technologies for the isolation and enrichment of CTCs have been developed. "CTC-chip" is the representative thechnology. It is a unique microfluidic platform, capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated microposts under precisely controlled laminar flow conditions [40]. Most recently, CTC-Chip was demonstrated to increase the detection of CTCs by using tumor-specific markers, such as PSA in prostate cancer or HER2 in breast cancer, in addition to epithelial markers [40, 41]. Furthermore, Saucedo-Zeni *et al*. [42] reported a new technology that enables the capture and enrichment of CTCs *in vivo* using a medical Seldinger guidewire inserted through a standard venous

cannula into the cubital veins. However, despite these advances, the isolation and enrichment of CTCs remains at the development stage.

After the isolation and enrichment of CTCs, identification procedures must be conducted to investigate their genetic and biological features. Various methodologies for this process, such as immunocytochemistry and molecular techniques, have been commonly adopted for identifying CTCs. Conventionally, immunostaining using 4′,6′-diamidino-2-phenylindole hydrochloride as a nuclear stain, CK as an epithelial marker, and CD45 as a hematopoietic marker has been widely used [43]. Among molecular approaches, quantitative reverse transcription–polymerase chain reaction (RT–PCR) has been generally employed to investigate the molecular characteristics of CKs, CEA, and other driver markers [44].

CTC detection in patients with HCC and its clinical relevance

To date, many researchers have tried to detect CTCs in patients with HCC and have demonstrated their clinical utility using various approaches. **Table 1** summarizes previous reports of CTCs in patients with HCC with time trends. As described in the last paragraph, these approaches can be mainly categorized into two groups: 1) physical methods and 2) biological methods.

1) Physical methods

Vona *et al.* first reported the isolation by size of epithelial tumor cell (ISET) method to detect CTCs in HCC patients [25]. By cytomorphologic analysis, they demonstrated that the spontaneous circulation of CTCs in peripheral blood is a sign of tumor progression and tumor spread in patients with HCC. Compared with expensive and cumbersome molecular techniques, ISET is simple, inexpensive, and allows applying the cytopathological

diagnosis of tumor cells, already widely used in clinical oncology, to peripheral blood samples. It also affords to specifically identify and count circulating tumor microemboli (CTM), and to perform immunocytological and molecular studies [26]. However, ISET device is still hard to release CTCs from the membrane. This may limit the application of downstream genetic analysis.

2) Biological methods

The presence and clinical utility of CTCs in HCC was first reported by Matsumura *et al*. using RT-PCR [45]. They demonstrated the following. 1) The presence of alpha-fetoprotein (AFP) messenger RNA (mRNA) in peripheral blood could be a marker of circulating HCC cells. 2) The status of AFP mRNA in blood were investigated at entry, extrahepatic metastasis developed more frequently among the AFP mRNA-positive patients than among the AFP mRNA-negative patients. 3) After treatment, AFP mRNA was investigated, and cumulative metastasis-free survival and overall survival were significantly better in patients whose AFP mRNA became negative after treatment than in patients with persistently positive AFP mRNA. In summary, they demonstrated that the presence or absence of AFP mRNA in blood (CTCs' positivity) could be a predictor of outcome in patients with HCC.

Following this study, the clinical utility of peripheral AFP mRNA was validated by other groups [46, 47]; however, the association of peripheral AFP mRNA with prognosis remains controversial [48, 49]. Thus, other tumor-specific molecules in the bloodstream, such as MAGE-1, MAGE3 [50], glypican-3 (GPC-3) [51], keratin 19 (K19), cluster of differentiation 44 (CD44) [52], and hTERT [53] mRNA, have been investigated for markers of circulating HCC cells. For example, MAGE gene transcripts have been regarded as HCC-specific markers [54]. Mou *et al.* demonstrated that detection of MAGE transcripts in blood with a

follow-up survey could predict the prognosis and monitor the response to therapy [50]. GPC-3 is a membrane-anchored heparin sulfate proteoglycan, known to be a reliable biomarker for HCC [55]. Yao *et al.* demonstrated that GPC-3 mRNA abnormality could be used as markers for the diagnosis of HCC and monitoring its metastasis [51]. Furthermore, K19 and CD44 have been shown to be cancer stem cell markers in HCC [56-59], their significance of prognostic factor in peripheral blood were also demonstrated by Choi *et al.* [52]. However, HCC associated genes were not always candidates for the markers of CTCs. Although serum human telomerase reverse transcriptase protein (hTERT) mRNA expression has been suggested as a potential candidate diagnostic marker for HCC [60], there was no relationship between overall survival and hTERT mRNA expression [53].

Liu e*t al*. [61] and Bahnassy *et al.* [62] used flow cytometry analysis to investigate intercellular adhesion molecule 1 (ICAM-1) expression, cytokeratin 19,CD133, and CD90 in HCC blood samples and demonstrated their prognostic value. Among various techniques, EpCAM-based Cell-Search™ is currently the most widely used CTC platform [63-73]. Using this method, Sun *et al.* collected blood samples from 123 HCC patients who underwent curative resection and suggested that EpCAM$^+$ CTCs could serve as a real-time parameter for monitoring treatment response and as a therapeutic target in HCC recurrence [67]. Guo *et al.* collected blood samples from 299 HCC patients with various kinds of treatment and 120 control subjects, and demonstrated that this method could be useful in early decision-making to tailor the most effective antitumor strategies [69]. Most recently, Wang et al. [74] suggested that novel CTC-Chip platform might be a new method for a simple and efficient detection of CTCs in HCC patients. They applied biocompatible and transparent HA/CTS (Hydroxyapatite/chitosan) nanofilm to achieve enhanced topographic interactions with nanoscale cellular surface components, and used sLex-AP (aptamer for

carbohydrate sialyl Lewis X) to coat onto HA/CTS nanofilm. It is potentially clinically useful in monitoring HCC prognosis and guiding individualized treatments in the future.

Overall, the usefulness of CTCs as biomarkers in HCC may be practically guaranteed; however, there are several challenges that must be overcome before CTCs can be considered useful in a clinical setting. Firstly, it is possible that etiological differences of patients and controls, such as background liver disease, haptic status, and race, could be responsible for the heterogeneity of the results. Secondly, the detection of CTCs must overcome the challenge of the rarity and heterogeneity of CTCs, and because the methodology for the detection of CTCs remains in a developmental stage, the approach to CTCs and the results of studies have varied widely. Consequently, it is difficult to assess, compare, and interpret the results of multiple studies and establish the clinical relevance of CTCs. Development of a unified methodology and large-scale validation using patients with homogeneous backgrounds is required for future applications.

3

Query:

Epithelial-to-mesenchymal transition (EMT) need not be explained in detail in the Introduction (page 6). It is sufficient to mention EMT in the paragraph that describes biological method; because EMT alone is not a good marker for CTC detection considering the heterogeneity of tumor.

Reply:

Thank you for your valuable comments. We deleted the description of EMT in the Introduction section ("**Biology and detection of circulating tumor cells"** section).

4

Query:

Please provide more explanation about multiple filters (page 7) and CTC-Chip platform (page 12).

Reply:

Thank you for your valuable comments. We are sorry to lack sufficient description. We added more explanations for them in the two sections such as "**Techniques for isolation, enrichment, and identification of CTCs**" and "**CTC detection in patients with HCC and its clinical relevance**"

Revised:

Same as revised for 2nd Query.

5

Query:

Relevant references are missing in the Introduction section.

Reply:

Thank you for your kind comments. We are sorry to lack sufficient references in the introduction section. We added proper references as listed below.

Revised:

Introduction (Third paragraph)

Numerous genetic and epigenetic aberrations contribute to oncogenesis and cancer progression, and the utility of these alterations for diagnostic, prognostic, and therapeutic purposes in various cancers has been investigated [14-18]. Conventionally, these cancer-related alterations are investigated using tissue samples from surgical or biopsy specimens. These procedures for tissue acquisition from HCC cannot always be performed and repeated due to their invasive nature, anatomical reasons, and/or the patient's poor hepatic status [19, 20]. Thus, conventional examinations may fail to reflect current tumor dynamics and drug sensitivities, which may change during the therapeutic process. Detecting circulating tumor cells (CTCs) and/or cell-free nucleic acids (cfNAs) in the bloodstream by performing a so-called "liquid biopsy" allows repeated sampling and

makes it possible to track the status of a tumor and its heterogeneous characteristics, which single sampling may fail to capture [21-24].

In past decades, numerous studies have shown the potential utility of novel blood-based biomarkers, such as CTCs and cfNAs, for various cancers including HCC [21-24]. These markers are considered to possess great potential and could facilitate therapeutic strategies for cancer. In this article, we review the histological backgrounds, characteristics, and developments of CTCs and cfNAs in cancer research and discuss future perspectives, with a specific focus on HCC.

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Comments from Reviewer #2

Authors have reviewed the available literature on CTCs and cfNAs in patients with HCC, and discuss future perspectives in this field in detail, including the histological backgrounds, characteristics, and developments of CTCs and cfNAs in cancer research and discuss future perspectives, with a specific focus on HCC.

Responses to Reviewer #2's comments

Thank you for your kind comment. We cordially appreciate your comments. If you have further queries, we are willing to reply them. Thank you so much for your helpful comments.