Response to reviewers' comments

Tittle: Targeting head and neck tumoral stem cells: from biological aspects to therapeutic perspectives

Dear reviewer,

Thank you for your insight and your constructive criticism on the above mentioned manuscript. Your comments and remarks have been taken into account in the finalization of our article. Responses to the referee comments are listed below, and appear in red color in the new version of the manuscript.

Minor comments:

- *CD44 and ALDH1 are described as main markers of CSC. However, this are not the only one. Other markers as CD133 and "side population" should be mentioned.*

  We do agree with you opening remark. In order to clarify this point, a 4th paragraph entitled "Other markers and role of side population cells" has been added in the first part of the article.

  "d. Other markers and role of side population cells
  In human oral squamous cell carcinoma, CD133+ stem-like cells possess higher clonogenicity, invasiveness, and tumorigenesis as compared with CD133- cells. CD133+ cells are resistant to standard chemotherapy with paclitaxel [24]. Human CD133 is a glycosylated protein with five transmembrane domains and two large extracellular loops. It was initially characterized as a marker for hematopoietic stem cells [25]. It has been identified then as a marker of cancer stem cells in the human laryngeal tumor Hep-2 cell line. In an in vivo study, CD133+ cells sorted from the Hep-2 cell line showed higher tumorigenic potential than CD133- cells [26]. CD44+ cancer stem-like cells express higher CD133 levels than CD44- cells in HNSCC. CD133 is a useful cancer stem cell marker in HNSCC, and might serve as a putative biomarker to identify head and neck cancer patients that are resistant to conventional chemotherapy [27]. Furthermore, side population cells have been characterized in HNSCC as highly tumorigenic cells with stem-like phenotype: identifying them is technically simple and do not rely on the relative binding efficiencies of antibodies. These cells are isolated thanks to their ability to efflux a fluorescent dye that binds to DNA [28-29]. The fraction of side population cells tends to be high in metastatic and aggressive HNSCC cells [30]. Side population cells exhibit a more aggressive pattern of tumour growth (in vitro) and therapeutic methods targeting these cells need to be designed. Further work on the exact role of side population cells and their implication in tumourigenesis is on-going and remains to be completely elucidated."

- *In the section: CSC-induced immune responses: "In addition to ALDH1, other cancer antigens were found to be preferentially expressed in CSCs." Please detail.*

We thank the reviewer for this suggestion. We gave details of other antigens in acute myeloid leukemia and renal cancer.
": Cyclin A1 was reported in leukemic stem cells of acute myeloid leukemia whereas DNAJB8 was identified as novel cancer antigen in renal CSCs [57-58]."

- *From the text, it can't be understand exactly what is the "immune-suppressive role of CSCs". Please, specify and include some strategies of CSC-driven immune suppression.*

This point is now clarified in the section entitled "the immune suppressive role of CSCs" thanks to details concerning the interaction between the tumor microenvironment and tregs. Furthermore, the role of tumor associated macrophages has been highlighted. Strategies of CSC-driven immune suppression include the ability to target tumor associated macrophages for increasing antitumor T cell responses, with the example of pancreatic adenocarcinoma.

"c. The immune suppressive role of CSCs

Immunotherapeutic approaches for HNSCC are complicated due to the deep immune suppression induced by this disease. Mechanisms such as increased apoptosis of tumor-specific CD8+ T-cells and increased tumor-infiltrating T regulatory cells in peripheral blood and at the tumor site have been demonstrated [59]. Krishnamurthy et al. showed that CSCs are located in close proximity to blood vessels and that endothelial cell-initiated signaling could enhance survival and self-renewal of HNSCC-CSCs [15]. Clinically, patients with recurrent HNSCC showed an increased concentration of IL-6 in serum in comparison with patients with primary HNSCC [45]. Elevated IL-6 levels could independently predict tumor recurrence, poor survival, and tumor metastasis [46]. Yu and al. demonstrated that secretion levels of IL-6 from CSCs were crucial to maintain the self-renewal and tumorigenic properties of CSCs in HNSCC [45]. On the one hand, CSCs can be recognized and inhibited in their outgrowth by the immune system and on the other hand, CSCs can promote tumor progression either by immunoediting for CSCs that are more suitable to survive in an immunocompetent host or by establishing conditions that facilitate tumor outgrowth within the tumor immune-microenvironment. Tumor associated macrophages may play a critical role in tumor progression by interacting with the tumor microenvironment and tregs are thought to promote tumor progression [60]. In a study concerning primary human gliomas, the distribution of TAM at the invasive tumor front was correlated with the presence of CD133+ glioma CSCs. Tumor associated macrophages could significantly enhance the invasive capability of glioma stem cells through paracrine production of TGF-B1 [61]. The role of tumor associated macrophages in the regulation of CSCs drug resistance has been identified by Jinsuhi et al. They found a large amount of tumor associated macrophages in CD44+ ALDH+ colon tumor and CD133+ ALDH+ lung cancer cells: those macrophages allow activating Sonic Hedgehog pathways in CSCs in cooperation with IL-6 [62]. Targeting

tumor associated macrophages by inhibiting either the myeloid cell receptors colony-stimulating factor-1 receptor or chemokine receptor improves chemotherapeutic efficacy, inhibits metastasis and increases antitumor T cell responses in pancreatic ductal adenocarcinoma [63]. All these findings validate the interplay between CSCs and the tumor immune microenvironment. Therefore, specific targeting of head and neck tumoral stem cells by immunotherapeutic approaches may lead to more efficacious and lasting therapeutic results in the future. Nonetheless, it seems necessary to address several points before immunotherapeutic approaches targeting CSCs can be brought into clinical trials. These include the effective isolation of CSCs from bulk tumor mass to measure potential immunotherapeutic effects on CSC, to determine the antigen-profile presented on CSCs specifically to identify specific CSC targets as well as the induction and enhancement of antigen processing and presentation of CSC epitopes. A lot of work remains to be done to get a better understanding of the immune suppressive role of CSCs in HNSCC."

- *Include some data on targeting the self-renewal controlling pathways, as stated in the abstract.*

We thank the reviewer for these comments. The third paragraph of the second part entitled "EMT and molecular pathways" has been enhanced with information on Wnt/beta-catenin, JAK/STAT, Notch and Hedgehog signaling pathways. We hope the modifications will suit you.

"Indeed, a number of small molecules targeting the Wnt pathway are either in the discovery stage or early phase 1 trials directed variously against Wnt/Receptor interactions and cytosolic and nuclear signaling [51-52]. Furthermore, others implicated molecular pathways are still under investigation in HNSCC, including the promising JAK/STAT pathway. Xenotransplant experiments revealed that cucurbitacin I, a STAT3 inhibitor, combined with radiotherapy significantly suppressed tumorigenesis and lung metastasis and further improved the survival rate in HNSCC-CD44+ALDH1+ transplanted immunodeficient mice [53]. Pharmaceutical companies have formulated drugs to target other pathways in CSC formation such as Notch or Hedgehog but the ability of these drugs to selectively target cancer stem cells while sparing normal stem cells remains questionable."

- *Please check the English form, because there are minor typographical errors that should be corrected*

We thank the reviewer for this advice. The entire article has been revised and all the typographical errors have been corrected.

- *The Conclusion section could be restricted and more focused.*

We do agree with your remarks. Then, we focused back on the fundamental issue of targeting head and neck tumoral stem cells. We suppressed details about ongoing trials.

Please, find enclosed the article modified according to your requirements. We hope that the changes that have been made will satisfy you.

Kind regards.